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EFECTOS GENÓMICOS DE LA TESTOSTERONA SOBRE LOS MECANISMOS DE RELAJACIÓN INDUCIDOS POR SALBUTAMOL EN EL MÚSCULO LISO TRAQUEAL DE COBAYO

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ÍNDICE

GLOSARIO

Abreviatura	Significado
°C	Grados Celsius
µg	Microgramos
µl	Microlitros
µM	Micromolar
17β-HSD3	17β-hidroxiesteroide deshidrogenasa tipo 3
3β-HSD2	3β-hidroxiesteroide deshidrogenasa tipo 2
4-AP	4-aminopiridina
5α-DHT	5α-dihidrotestosterona
5β-DHT	5β-dihidrotestosterona
Actino D	Actinomicina D
ADY6	Adenilato ciclasa 6
AMPc	Adenosín monofosfato cíclico
BK_{Ca}	Canales de K^+ activados por Ca^{2+} de alta conductancia
CAM	Calmodulina
COMT	Catecol-O-metil transferasa
CI_{50}	Concentración inhibitoria 50
Cyclohex	Cicloheximida
CYP11A1	Enzima de escisión de la cadena lateral P450
CYP17A1	Citocromo P450 17α-hidroxilasa
DHEA	Dehidroepiandrosterona
ERK 1/2	Quinasas reguladas por señales extracelulares 1/2
ESM	Error estándar de la media
Flu	Flutamida
FPVD	Fluoruro de polivinilideno
Fw	Primer en sentido
GTP	Guanosina-5’ trifosfato
His	Histamina
IBTX	Iberiotoxina

IK^+	Corrientes de K^+
IK_{Ca}	Canales de K^+ activados por Ca^{2+} de conductancia intermedia
IL	Interleucina
ILC2	Células linfoides innatas del grupo 2
IP_3	Inositol 1,4,5-trifosfato
K_{Ca}	Canales de K^+ activados por Ca^{2+}
K_V	Canales de K^+ dependientes de voltaje rectificadores tardíos
L15	Solución de Leibovitz
L-VDCC	Canales de Ca^{2+} dependientes de voltaje tipo L
mg	Miligramo
ml	Mililitro
MLCK	Cinasa de la cadena ligera de la miosina
MLVA	Músculo liso de la vía aérea
mM	Milimolar
mV	Milivoltios
nA	Nanoamperios
nM	Nanomolar
P450aro	Aromatasa P450
PBS	Solución amortiguadora de fosfatos
PKA	Proteína quinasa A
PLB	Fosfolambán
PLC_β	Fosfolipasa C_β
PPIB	Peptidil-prolil cis-trans isomerasa B
Pr	Primer
RA	Receptor androgénico
$RA\beta_2$	Receptor adrenérgico β_2
RS	Retículo sarcoplásmico
Rv	Primer reverso
s	Segundo

Sal	Salbutamol
SERCA	ATPasa de Ca^{2+} del retículo sarcoplásmico
SK_{Ca}	Canal de K^+ activado por Ca^{2+} de baja conductancia
SOCC	Canal de Ca^{2+} operado por el almacén
TES	Testosterona
TES-BSA	Testosterona conjugada con albúmina de suero bovino
Th	Linfocitos T cooperadores
TNF-α	Factor de necrosis tumoral α
TRPC3	Receptor de potencial transitorio canónico 3

LISTA DE FIGURAS

	cobayo no modifica las corrientes de K^+ (IK^+) inducidas por salbutamol (Sal), corroborando que el fenómeno de la TES es a través de un efecto genómico.
11	Los canales de K^+ rectificadores tardíos y los canales de K^+ activados por Ca^{2+} de alta conductancia son las principales proteínas involucradas en el efecto de potenciación de la testosterona (TES, 40 nM durante 48 h) sobre las IK^+ inducidas por pulsos despolarizantes y por salbutamol (Sal) en miocitos traqueales de cobayo.
12	La testosterona (TES) regula a la alta el receptor adrenérgico β_2 en el MLVA.
13	La testosterona (TES, 40 nM durante 48 horas) aumenta el receptor adrenérgico β_2 ($RA\beta_2$) en el músculo liso traqueal de cobayo.
14	El efecto de la testosterona (TES) sobre el incremento en la respuesta a salbutamol no está relacionado con la adenilato ciclasa 6 (ADY6) en el MLVA de cobayo.
15	Representación esquemática del efecto genómico de la testosterona (TES) sobre la relajación inducida por salbutamol en el músculo liso de las vías aéreas.

RESUMEN

Introducción: En pacientes asmáticos varones, los andrógenos han sido relacionados con la severidad de esta enfermedad. Durante la infancia, el asma afecta más a niños que a niñas, y esta proporción se revierte durante la pubertad. En esta etapa de la vida, las concentraciones plasmáticas de andrógenos aumentan en jóvenes varones y esto pudiera estar relacionado con la disminución en la presentación de síntomas de asma. Los efectos no genómicos de los andrógenos ocurren en periodos cortos y son independientes del receptor androgénico (RA), mientras que los efectos genómicos dependen del RA, tardan de horas a días y son suprimidos por inhibidores de la transcripción o de la síntesis proteica. **Pregunta de investigación:** ¿La exposición crónica de testosterona (TES), a concentraciones fisiológicas, potencia la relajación inducida por salbutamol a través de la regulación positiva del receptor adrenérgico β_2 (RAβ_2) y favorece el incremento en las corrientes de K^+ (IK^+) en el músculo liso de la vía aérea (MLVA) de cobayo? **Hipótesis:** La TES a concentraciones fisiológicas aumenta la expresión del RAβ_2 e incrementa las IK^+ inducidas por salbutamol a través de un efecto genómico en el MLVA de cobayo. Este aumento en la expresión del RAβ_2 por la incubación crónica de TES potencia la relajación inducida por salbutamol. **Métodos:** Se utilizaron cobayos machos de la cepa Hartley de 350 a 400 gramos. A través de experimentos en órganos aislados se midió la relajación del MLVA. En experimentos de electrofisiología se midieron las IK^+ en miocitos traqueales de cobayo. Mediante inmunofluorescencia, PCR cuantitativo y Western blot se midió la expresión del RAβ_2. **Resultados:** La incubación crónica de tráqueas de cobayo con TES (40 nM, 48 hrs) potenció la relajación inducida por salbutamol, un efecto revertido por flutamida, no observado en tejidos preincubados con TES conjugada con albúmina de suero bovino (TES-BSA) ni cuando los tejidos fueron preincubados con TES durante 15 o 60 minutos. En miocitos traqueales, la incubación crónica (48 horas) de TES 40 nM incrementó las IK^+ inducidas por salbutamol, un efecto que fue abolido por flutamida, actinomicina D y cicloheximida, y no se observó con TES-BSA. El incremento en las IK^+ fue bloqueado por 4-aminopiridina e iberiotoxina, indicando que el canal de K^+ rectificador tardío (K_V) y canal de K^+ activado por Ca^{2+} (K_{Ca}) de alta conductancia (BK_{Ca}) están involucrados en el efecto de potenciación de la TES. Estudios del RAβ_2 por inmunofluorescencia y Western blot demostraron que la exposición crónica de TES aumentó la expresión génica de esta proteína en el MLVA. **Conclusión:** La exposición crónica de TES a concentraciones fisiológicas en el MLVA de cobayo promueve la regulación positiva del RAβ_2, favoreciendo la respuesta adrenérgica y probablemente limitando la severidad de las exacerbaciones asmáticas en adolescentes y adultos hombres.

ABSTRACT

Introduction: Androgens in asthmatic men may be linked to asthma severity, acting via nongenomic and genomic effects. This ailment affects boys more than girls during infancy, and this proportion reverses in puberty. Plasmatic androgen concentration in young men increases at this age and might be related to lower asthma symptoms. Nongenomic actions occur in a brief period and are independent of the androgen receptor (AR), while genomic effects depend on AR, take hours-days and are modified by transcription or protein synthesis inhibitors. **Research question:** Does chronic testosterone (TES) exposure, at physiological concentrations, enhance salbutamol-induced relaxation by upregulating the β_2 adrenergic receptor (β_2-AR) and promote the increase in K^+ currents (IK^+) in guinea pig airway smooth muscle (ASM)? **Hypothesis:** TES at physiological concentrations increases β_2-AR expression and salbutamol-induced IK^+ through a genomic effect in guinea pig ASM. This increase in β_2-AR expression by chronic TES incubation potentiates salbutamol-induced relaxation. **Methods:** Healthy male Hartley guinea pigs weighing between 350 and 400 g were used. Relaxation of ASM was measured by experiments in organ baths. Patch-clamp studies measured IK^+ in guinea pig tracheal myocytes. Expression of β_2-AR was determined by immunofluorescence, quantitative PCR and Western blot. **Results:** Guinea pig tracheas chronic incubation with testosterone (TES, 40 nM, 48 h) potentiates salbutamol-induced relaxation, an effect that was reversed by flutamide, not observed when tissues were pre-incubated with TES-bovine serum albumin (TES-BSA) nor when tissues were preincubated with TES for 15-60 min. In tracheal myocytes, TES chronic incubation increases salbutamol-induced IK^+, an effect that was also reversed by flutamide, actinomycin D and cycloheximide and not seen with TES-BSA. The increment in IK^+ was blocked by 4-aminopyridine and iberiotoxin, indicating that delayed rectifier K^+ (Kv) and high-conductance Ca^{2+} activated K^+ channels (BK_{Ca}) are involved in the TES potentiation effect. Immunofluorescence studies showed that chronic TES augmented the β_2-AR expression in ASM and this finding was corroborated by q-PCR and Western blot assays. **Conclusion:** Chronic exposure to physiological TES concentration of the guinea pig ASM promotes β_2-AR upregulation favoring β_2 adrenergic responses and probably limiting the severity of the asthmatic exacerbations in teenage boys and men.

INTRODUCCIÓN

Asma y la regulación del tono muscular en la vía aérea

El asma es una enfermedad crónica inflamatoria de las vías aéreas que afecta alrededor de 339 millones de personas en el mundo, de acuerdo al informe de "The Global Asthma Report, 2018." Esta enfermedad se caracteriza por presentar una respuesta exagerada del músculo liso de la vía aérea (MLVA) ante estímulos físicos o químicos, fenómeno conocido como hiperreactividad. Como resultado a esta respuesta se presenta broncoespasmo de las vías aéreas y en consecuencia, favorece la presencia de síntomas como disnea, sensación de opresión torácica, tos y sibilancias (Shah & Newcomb, 2018). La obstrucción del flujo aéreo es definida por parámetros espirométricos que indican disminución en la capacidad vital forzada y en el volumen espiratorio expulsado en el primer segundo (Chhabra, 2015). El manejo de esta enfermedad, se ha centrado en el uso de broncodilatadores inhalados y corticoesteroides para disminuir la inflamación y la presentación de síntomas (Langley et al., 2019). La etiología del asma ha sido relacionada con heredabilidad, exposiciones ambientales y sensibilización a alérgenos (DeChristopher & Tucker, 2020; Ober & Yao, 2011; Smit et al., 2015). En la mayoría de los pacientes asmáticos, la exposición a alérgenos (como el polen, el ácaro del polvo doméstico, los virus y antígenos de cucarachas) produce inflamación eosinofílica mediada por linfocitos T cooperadores (Th)-2 (Fahy, 2015; Lambrecht & Hammad, 2015; Nagata et al., 2020). Inicialmente, las células dendríticas presentan péptidos alergénicos a los linfocitos T no diferenciados y estimulan la producción de células T específicas (Upham & Xi, 2017). Posteriormente, alarminas epiteliales reclutan células Th2 CD4+ y células linfoides innatas del grupo 2 (ILC2) que secretan interleucina (IL)-5, 4 y 13 (Scanlon & McKenzie, 2012). La IL-5 se caracteriza por regular la generación, migración y reclutamiento de eosinófilos (Rosenberg et al., 2013). La IL-4 e IL-13 favorecen la síntesis de inmunoglobulina E por los linfocitos B, lo que promueve la degranulación de mastocitos (Lambrecht & Hammad, 2015; Taskar & Coultas, 2006). En las vías aéreas, la infiltración de mastocitos ha sido asociada con hiperreactividad mediada por la liberación de agonistas broncoconstrictores como la histamina (Brightling et al., 2002; Galli & Tsai, 2012). Asimismo, se ha mostrado que los pacientes asmáticos con síntomas graves y resistentes al

tratamiento con corticoesteroides, desarrollan una respuesta inflamatoria mediada por IL-17 (de Marco et al., 2000; Yung et al., 2018).

Además de la inflamación, el desarrollo de hiperreactividad (respuesta broncoconstrictora exacerbada que no presentan sujetos sanos) favorece la obstrucción del flujo aéreo a través de la contracción del MLVA (Delmotte et al., 2010; Lam et al., 2019; Ressmeyer et al., 2010). En este tejido muscular, el incremento de la concentración del Ca^{2+} intracelular por la entrada de iones extracelulares o salida del almacén intracelular (retículo sarcoplásmico), favorece la contracción. La entrada de Ca^{2+} extracelular es mediada por el receptor de potencial transitorio canónico 3 (TRPC3), canales de Ca^{2+} dependientes de voltaje tipo L (L-VDCC), canales de Ca^{2+} operados por receptores y canales de Ca^{2+} operados por el almacén (SOCCs) activados por la disminución de Ca^{2+} en el retículo sarcoplásmico (RS) (Flores-Soto et al., 2017; Reyes-Garcia et al., 2018). En principio, agonistas broncoconstrictores como la acetilcolina, leucotrienos y la histamina, actúan a través de receptores acoplados a proteínas Gq, favoreciendo la vía de la fosfolipasa C_β (PLC_β) que cataliza la formación de inositol 1,4,5-trifosfato (IP_3) (Berridge, 1993; Song et al., 2015). Éste segundo mensajero, induce la liberación de Ca^{2+} del RS a través de la unión a su receptor. El incremento de Ca^{2+} en el citosol promueve la liberación de más Ca^{2+} a través de receptores de rianodina, localizados en el RS, en un proceso denominado liberación de Ca^{2+} inducida por Ca^{2+} (ZhuGe et al., 1998). Posteriormente, la salida de Ca^{2+} del RS activa los canales SOCCs promoviendo el influjo de Ca^{2+} y Na^{+} al citoplasma. Este último ion induce la despolarización de la membrana plásmatica y la posterior apertura de los L-VDCCs. La acumulación masiva de Ca^{2+} en el citosol estimula la proteína calmodulina (CAM) formando el complejo Ca^{2+}-CAM. Este complejo activa la cinasa de la cadena ligera de la miosina (MLCK) que fosforila a la miosina permitiendo el entrecruzamiento con los filamentos de actina generando en última instancia la contracción muscular (Horowitz et al., 1996).

Agonistas adrenérgicos β como el salbutamol inducen la relajación del MLVA a través de procesos de fosforilación que conducen a la hiperpolarización de la membrana plasmática y a la disminución de los niveles de Ca^{2+} intracelulares (Hirsh et al., 1996). En condiciones fisiológicas, el sistema adrenérgico controla la relajación del MLVA a través de las

catecolaminas circulantes liberadas desde la médula adrenal, las cuales activan a los receptores adrenérgicos presentes en el MLVA (Proskocil & Fryer, 2005). Estos receptores están compuestos por siete hélices transmembranales y se clasifican en tres subtipos: β_1, β_2 y β_3. Los tres subtipos se expresan en el MLVA, siendo el receptor adrenérgico β_2 (RAβ_2) el de mayor importancia en este tejido (Tanaka et al., 2005). En su estado activo, los RAβ_2 se encuentran asociados a una subunidad α de la proteína Gs y median la producción de adenosín monofosfato cíclico (AMPc) (Sun et al., 2018; Zhou et al., 1997). Los agonistas adrenérgicos β_2 como el salbutamol, salmeterol y formoterol, son los broncodilatadores más eficaces utilizados en el tratamiento del asma para revertir el broncoespasmo (Brambilla et al., 2003; Manoharan et al., 2016; Taylor et al., 1998). Cuando un agonista como el salbutamol se une al RAβ_2, activa la enzima adenilato ciclasa (AC). En el MLVA, la isoforma 6 de la AC es la más abundante (Birrell et al., 2015). La AC cataliza la formación de AMPc. El AMPc estimula a la proteína cinasa A (PKA) que se encarga de fosforilar a los canales de K^+, aumentando su probabilidad de apertura. En el MLVA, los principales canales de K^+ son los canales de K^+ activados por Ca^{2+} (K_{Ca}) y los dependientes de voltaje rectificadores tardíos (K_V) (Adda et al., 1996; Boyle et al., 1992; Montaño et al., 2011). Los Kv se activan a voltajes entre –6 y –15 milivoltios (mV) (Sanguinetti & Jurkiewicz, 1990; Volk et al., 1991). En este tejido, se han caracterizado tres subtipos de K_V: K_V1.2, K_V1.5 y K_V7.5 (Adda et al., 1996; Brueggemann et al., 2018). Por otro lado, los K_{Ca} son activados principalmente por incrementos en la concentración de Ca^{2+} intracelular y a través de la vía de señalización del AMPc-PKA (Wang & Kotlikoff, 1996). Estos canales se clasifican en tres subfamilias: de alta conductancia (BK_{Ca}, MaxiK, K_{Ca}1.1), intermedia (IK_{Ca}, K_{Ca}3.1), y baja conductancia (SK_{Ca}, K_{Ca}2.1, 2.2 and 2.3) (Feletou, 2009). El K^+ saliente promueve la hiperpolarización de la membrana celular e impide que aumenten las concentraciones de Ca^{2+} intracelular vía el cierre de los L-VDCC, favoreciendo la relajación del MLVA (Billington & Penn, 2003). Además, la disminución del Ca^{2+} intracelular reduce la activación de la MLCK provocando así la desfosforilación de la cadena ligera de miosina lo que también contribuye la relajación. En este mismo tejido, la vía del AMPc-PKA favorece la despolimerización de actina, promoviendo el acortamiento de la longitud de los filamentos de actina (Hirshman et al., 2001). Todos estos mecanismos en conjunto fomentan la relajación del MLVA inducida por agonistas adrenérgicos β_2.

Asma y andrógenos

Existe una relación entre los andrógenos y el asma. Hay evidencia epidemiológica de diferencias en la prevalencia del asma entre hombres y mujeres relacionadas con cambios hormonales en las diversas etapas de la vida (Townsend et al., 2012). Durante la infancia, los síntomas de esta enfermedad son más frecuentes en niños que en niñas, tendencia que se revierte en la pubertad siendo más severa en mujeres (Holgate et al., 2015; Melgert et al., 2007; Townsend et al., 2012; Vink et al., 2010). Posteriormente, a partir de los 50 años de vida, la prevalencia del asma vuelve a incrementarse en hombres siendo parecida al de las mujeres de la misma edad, posiblemente por la disminución de los niveles séricos de testosterona (TES) (Canguven & Albayrak, 2011; de Marco et al., 2000; Postma, 2007; Zannolli & Morgese, 1997). Se ha propuesto que los niveles elevados de TES en hombres, en comparación con los encontrados en mujeres, provocan un mayor calibre de las vías respiratorias y una mayor capacidad pulmonar (Hoffstein, 1986; Pagtakhan et al., 1984; Townsend et al., 2012). Adicionalmente, se ha reportado que pacientes hombres con asma moderada o severa presentan niveles bajos de TES comparados con aquellos que presentan síntomas leves (Mileva & Maleeva, 1988). En este sentido, se ha propuesto que, durante la pubertad en varones, cuando los niveles plasmáticos de TES aumentan de 6-50 nanomolar (nM), este andrógeno actúa promoviendo un mayor calibre de las vías aéreas, regulando negativamente la inflamación y disminuyendo los síntomas del asma (Becklake & Kauffmann, 1999; Laffont et al., 2017; Muller et al., 2011; Ripoll et al., 2020; Sathish et al., 2015; Townsend et al., 2012).

La TES, es el principal andrógeno en hombres, producido en un 95% en las células de Leydig de los testículos (Stocco & Clark, 1996). Asimismo, cantidades pequeñas de TES son producidas en la corteza suprarrenal (Pon & Orme-Johnson, 1986). La síntesis y secreción de este andrógeno están reguladas por la hormona luteinizante. En las células de Leydig, la hormona luteinizante actúa a través de su receptor acoplado a una proteína de tipo Gs, lo que resulta en la formación de AMPc. En consecuencia, se estimula la movilización de colesterol hacia las mitocondrias mediante la activación de la señalización de la proteína quinasa A (PKA) (Dufau & Catt, 1978). En la Figura 1, se ejemplifica como el colesterol, precursor de las hormonas sexuales, se convierte en pregnenolona por la enzima de escisión de la cadena

lateral del colesterol (CYP11A1 / P450scc) ubicada en la membrana mitocondrial (Miller, 1988). La pregnenolona se puede convertir en progesterona a través de 3β-hidroxiesteroide deshidrogenasa tipo 2 (3β-HSD2) o puede ser hidroxilada a 17α-hidroxipregnenolona. Mediante el citocromo P450 17α-hidroxilasa (CYP17A1 / P450c17), la 17α-hidroxipregnenolona es biotransformada a dehidroepiandrosterona (DHEA) (Miller, 1998). Posteriormente, la DHEA es transformada en androstenediol a través de la 17β-hidroxiesteroide deshidrogenasa tipo 3 (17β-HSD3), o en androstenodiona por la 3β-HSD2. Finalmente, androstenediol y androstenediona se biotransforman a TES por la acción de 3β-HSD2 y 17β-HSD3, respectivamente (Hall, 1989). Adicionalmente, la TES se puede reducir a 5α-dihidrotestosterona (5α-DHT) por la 5α-reductasa y a 5β-dihidrotestosterona (5β-DHT) por la 5β-reductasa. Asimismo, la TES se puede convertir en 17β-estradiol (un estrógeno) a través de la acción de la aromatasa P450 (Miller & Auchus, 2011).

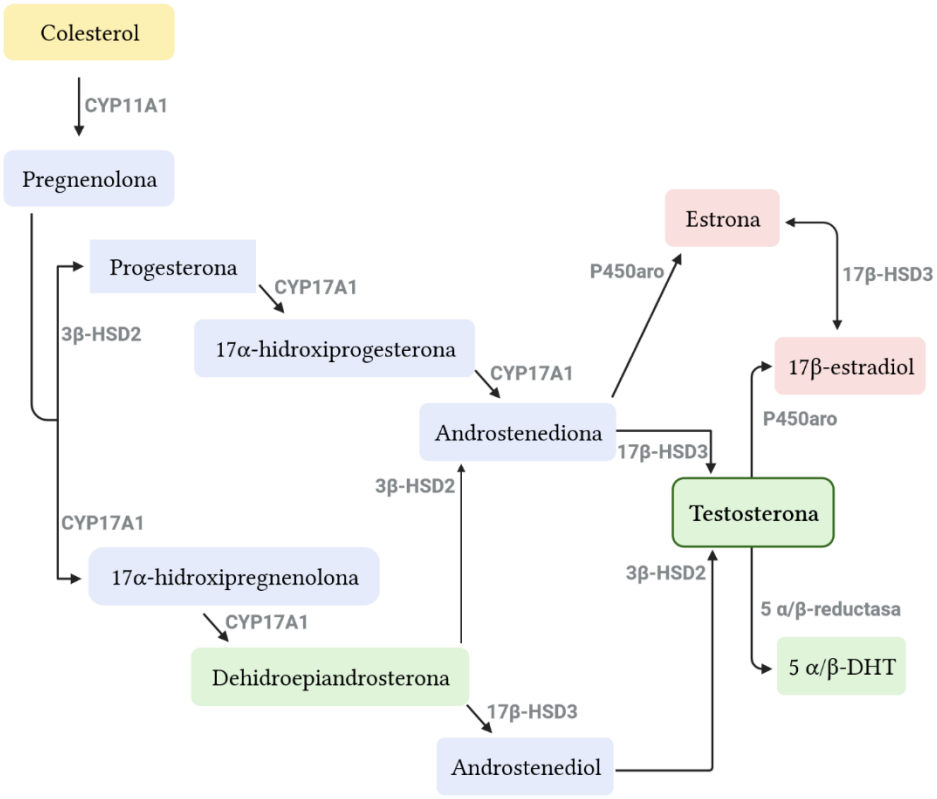

Figura 1. Esteroidogénesis. El colesterol es el precursor de todas las hormonas esteroideas y su conversión en pregnenolona está mediada por la enzima de escisión de la cadena lateral del colesterol (CYP11A1). La pregnenolona se convierte en progesterona por la 3β-hidroxiesteroide deshidrogenasa (3β-HSD2) o puede ser hidroxilada a 17α-hidroxipregnenolona mediante la 17α-hidroxilasa / 17,20 liasa (CYP17A1). La CYP17A1, al eliminar un grupo acetilo, bioconvierte 17α-hidroxipregnenolona en dehidroepiandrosterona (DHEA). Este último producto puede convertirse en androstenediona

mediante 3β-HSD2 o en androstenediol mediante 17β-hidroxiesteroide deshidrogenasa (17β-HSD3). La androstenediona y el androstenediol se biotransforman en testosterona por la 17β-HSD3 y la 3β-HSD2, respectivamente. Asimismo, la testosterona se puede reducir a 5α- o 5β-dihidrotestosterona (5 α/β-DHT) mediante 5 α/β-reductasas. Además, la aromatasa P450 (P450aro) puede convertir testosterona en 17β-estradiol y la androstenediona en estrona.

Los hombres tienen concentraciones séricas más altas de TES que las mujeres. Los valores de TES séricos se encuentran entre 6 y 50 nM, en hombres de 13 a 80 años (Kelsey et al., 2014; Sartorius et al., 2012; Townsend et al., 2012). La 5α-DHT, andrógeno más potente que la TES, representa aproximadamte del 9 al 10% de los niveles séricos de TES en hombres (Bruchovsky & Wilson, 1999; Sartorius et al., 2012). En las mujeres, los valores séricos de TES se mantienen estables (0,7-2,5 nM), excepto durante el embarazo cuando las concentraciones de TES aumentan (3,5-5 nM) (Townsend et al., 2012).

Efectos genómicos y no genómicos de la testosterona en el músculo liso de la vía aérea

Los andrógenos inducen sus efectos fisiológicos a través de vías genómicas y no genómicas. Los efectos genómicos ocurren en un lapso de horas a días e involucran la entrada de los andrógenos a través de la membrana citoplasmática para unirse a su receptor androgénico (RA) en el citosol (Lucas-Herald et al., 2017). Este receptor es un miembro de la familia de receptores nucleares y se encuentra presente en el MLVA (Heinlein & Chang, 2002; Zarazua et al., 2016). La estructura proteica del RA comprende de cuatro dominios funcionales: un dominio N-terminal, un dominio de unión al ADN, una región bisagra y un dominio de unión al ligando (andrógenos) (Heemers & Tindall, 2007). En éste ultimo se une la TES provocando la disociación de proteínas chaperonas y la formación de un complejo que se transfiere al núcleo donde se dimeriza y se une al elemento de respuesta androgénica (Grino et al., 1990; Lucas-Herald et al., 2017). Posteriormente, se reclutan enzimas histona acetiltransferasas y una serie de co-reguladores esenciales que facilitan la unión de la proteína de unión a TATA seguida de factores para comenzar la transcripción de genes regulados por andrógenos (Bennett et al., 2010; Li & Al-Azzawi, 2009). Los antagonistas del RA como la flutamida, se unen al sitio de unión a andrógenos e impiden la activación del receptor (Tran et al., 2009). En contraste, los efectos no genómicos ocurren en periodos cortos (segundos a minutos) y son independientes de la unión del andrógeno a su receptor citoplasmático, como se ejemplifica en la Figura 2. Los efectos no genómicos no son alterados por inhibidores de la

transcripción o traducción y se observan incluso cuando se utiliza testosterona conjugada con albúmina de suero bovino (TES-BSA), una molécula que previene la entrada del andrógeno al citoplasma (Espinoza et al., 2013; Foradori et al., 2008; Lucas-Herald et al., 2017; Montaño et al., 2018).

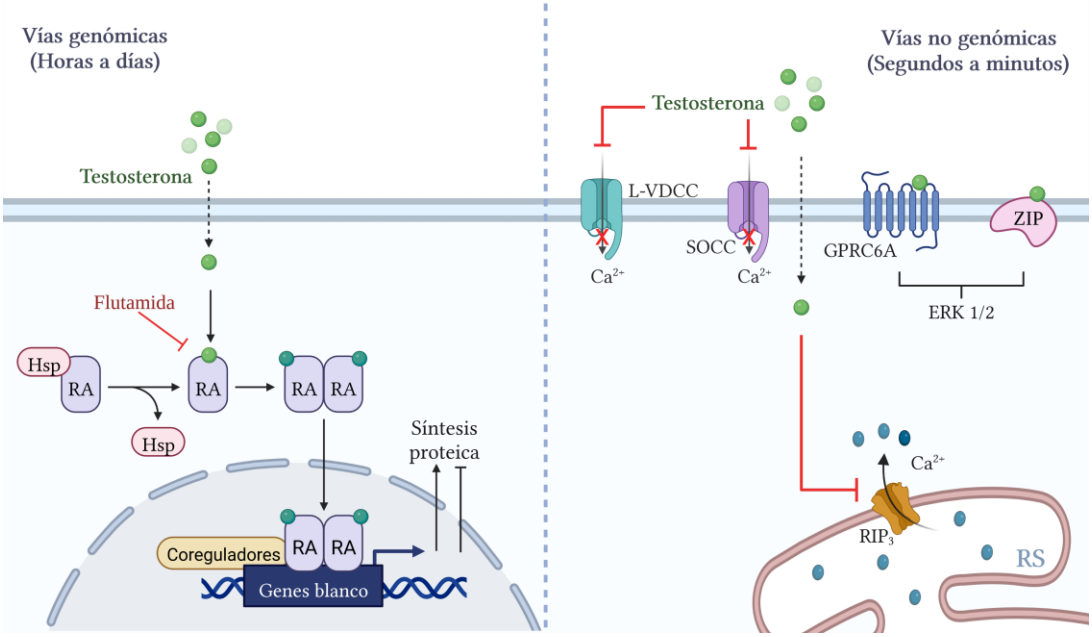

Figura 2. La testosterona induce sus efectos fisiológicos a través de vías genómicas y no genómicas. Los efectos genómicos ocurren en un lapso de horas a días e involucran la entrada del andrógeno a través de la membrana citoplasmática para unirse a su receptor androgénico (RA) en el citosol. Esto provoca la disociación de proteínas chaperonas (Hsp) y la formación de un complejo que se transfiere al núcleo donde se dimeriza y se une al elemento de respuesta androgénica. Posteriormente, se reclutan una serie de coreguladores y factores que regularán la transcripción de genes. Los antagonistas del RA como la flutamida, se unen al sitio de unión a andrógenos e impiden la activación del receptor. En contraste, los efectos no genómicos ocurren en periodos cortos (segundos a minutos) y son independientes de la unión del andrógeno al RA. A través de efectos no genómicos, la testosterona puede bloquear el canal de entrada capacitativa (SOCC), que se activa mediante el vaciamiento de Ca^{2+} del retículo sarcoplásmico (RS), al canal de Ca^{2+} dependiente de voltaje tipo L (L-VDCC) y al receptor de inositol 1,4,5-trifosfato (RIP_3). Asimismo, este andrógeno puede activar proteínas de membrana como GPRC6A, un receptor acoplado a proteínas G y a ZIP9, un transportador de zinc de la familia ZIP. Estos receptores estimulan a las cinasas reguladas por señales extracelulares 1/2 (ERK 1/2), implicadas en diferentes procesos de fosforilación y sus acciones dependen del tipo celular.

El MLVA es el órgano blanco en la enfermedad del asma. Los efectos no genómicos de la TES en el MLVA han sido bien caracterizados. Inicialmente, se reportó que en el músculo liso traqueal de conejo pre-contraído con agonistas colinérgicos, la TES induce relajación del tejido mediante un proceso dependiente del óxido nítrico epitelial (Kouloumenta et al., 2006). Además, en este trabajo, se comprobó que el efecto de la TES era ejercido a través de un mecanismo no genómico ya que la flutamida, un antagonista del RA y la actinomicina D, un

inhibidor de la transcripción, no lo modificaron. Posteriormente, se demostró en el MLVA de bovino y de cobayo, que los andrógenos (TES, 5α-DHT y 5β-DHT) relajaban el tejido pre-contraído con un agonista colinérgico como el carbacol y que su efecto era a través del bloqueo de los L-VDCC e independiente del óxido nítrico (Bordallo et al., 2008). En los últimos años, el grupo de investigación del Dr. Montaño, ha demostrado que los andrógenos (DHEA y TES) a concentraciones suprafisiológicas (del orden micromolar), bloquean el canal de entrada capacitativa, también conocido como SOCC, que se activa mediante el vaciamiento de Ca^{2+} del retículo sarcoplásmico (Flores-Soto et al., 2017). Además, inducen la síntesis de prostaglandina E_2, disminuyen la tensión basal del MLVA en reposo, al igual que la concentración basal de Ca^{2+} mediante el bloqueo del L-VDCC y del TRPC3 (Flores-Soto et al., 2017; Perusquia et al., 2015). A concentraciones fisiológicas (del orden nanomolar), los andrógenos bloquean al L-VDCC y al receptor de IP_3 ocasionando una disminución en los incrementos de Ca^{2+} intracelulares inducidos por agonistas como el carbacol, ATP e histamina (Montaño et al., 2014; Montaño et al., 2018; Perusquia et al., 2015).

Por otro lado, los efectos genómicos de la TES sobre el MLVA han sido poco estudiados y actualmente, las investigaciones se centran en la inflamación de la vía aérea. La inflamación tipo 2, característica del asma alérgica, es mediada por la secreción de diversas citocinas como IL-4, IL-5 e IL-13 por parte de células T CD4+, ILC2 y otras células (Monticelli et al., 2012; Zhu, 2015). En este contexto, se reportó que la TES a concentraciones nanomolares, regula negativamente la producción de citocinas proinflamatorias tipo 2 al inhibir la diferenciación de las ILC2 y de las células CD4+ (Fuseini et al., 2018; Laffont et al., 2017). Por lo tanto, la regulación negativa de estas citocinas disminuiría la hiperreactividad de las vías respiratorias, característica en el asma. Asimismo, está bien documentado que los pacientes asmáticos graves generalmente cursan con una inflamación neutrofílica mediada por la IL-17 (de Marco et al., 2000; Yung et al., 2018). En este sentido, Fuseini y colaboradores, encontraron que la TES induce una disminución de la expresión IL-17A, a través de un efecto genómico (Fuseini et al., 2018). Recientemente, se descubrió que los andrógenos al activar al RA, pueden intervenir en la regulación del incremento intracelular de Ca^{2+} inducido por citocinas proinflamatorias como el factor de necrosis tumoral-α o la IL-

13 en el MLVA de humano (Kalidhindi et al., 2019). Estos hallazgos sugieren que los andrógenos regulan negativamente la inflamación y los incrementos de Ca^{2+}, aliviando la hiperreactividad de las vías aéreas. Colectivamente, los trabajos hasta ahora descritos proporcionan una posible explicación de la mayor prevalencia del asma en mujeres en comparación con los hombres.

Antecedentes

En 1983, un grupo de investigadores demostró que en bronquio de cerdo, la TES a concentraciones suprafisiológicas (40 μM) potenciaba la relajación inducida por isoprenalina (agonista inespecífico adrenérgico β) (Foster et al., 1983). Ellos propusieron que la TES podría afectar la inactivación o disminución de la acción de las catecolaminas a través de la inhibición de la catecol-O-metil transferasa (COMT) o de la recaptura extraneuronal, sin embargo, no lo demostraron. Posteriormente, Bordallo y colaboradores, utilizaron un metabolito reducido de la TES (100 μM de 5α-dihidrotestosterona) y reportaron que potenciaba la relajación inducida por salbutamol (agonista adrenérgico β_2) en MLVA de bovino (Bordallo et al., 2008). Estos investigadores demostraron que el efecto de potenciación de la TES era a través de un efecto no genómico y que no se asociaba con la vía de señalización adrenérgica β_2. Es importante hacer notar que ambos trabajos utilizaron concentraciones de TES que se encuentran muy por encima de los rangos fisiológicos. Dada la controversia y la falta de claridad en los antecedentes, nos propusimos a investigar si la TES (en concentraciones fisiológicas) podría ejercer un efecto genómico sobre alguno de los mecanismos de relajación activados por el RAβ_2, que incluyen la estimulación de la AC y la formación AMPc, la estimulación de la PKA y la apertura de los canales de K^+. En experimentos preliminares realizados en el laboratorio, encontramos que la incubación de anillos traqueales con TES (20 y 40 nM) durante 48 horas potenció la relajación inducida por salbutamol (Figura 3). Este fenómeno pudiera ser el resultado de una regulación positiva, inducida por la TES sobre proteínas y mecanismos implicados en la relajación del MLVA. Por consiguiente, en este trabajo de tesis, exploramos los efectos genómicos de la TES (en concentraciones nanomolares) sobre la expresión del RAβ_2 y su funcionalidad en el músculo liso de la vía aérea, así como el efecto de la TES sobre las corrientes de K^+ (IK^+) inducidas por salbutamol.

PLANTEAMIENTO DEL PROBLEMA

Los andrógenos han sido asociados con el asma y durante la niñez los síntomas de esta enfermedad parecen ser más frecuente en niños que en niñas, tendencia que se revierte en la pubertad. Este fenómeno pudiera estar asociado a un incremento de las concentraciones plasmáticas de TES durante la pubertad (6-50 nM). En experimentos realizados en el laboratorio encontramos que la TES incubada por 48 horas a concentraciones nanomolares (20 y 40 nM), aumentaba notablemente la respuesta de relajación inducida por salbutamol en anillos traqueales de cobayo previamente contraídos con 10 micromolar (µM) de histamina (Figura 3). Anteriormente, se había reportado que los andrógenos potenciaban la relajación inducida por agonistas adrenérgicos β_2. Por lo tanto, es posible que la TES favorezca los mecanismos de relajación del salbutamol a través de un efecto genómico.

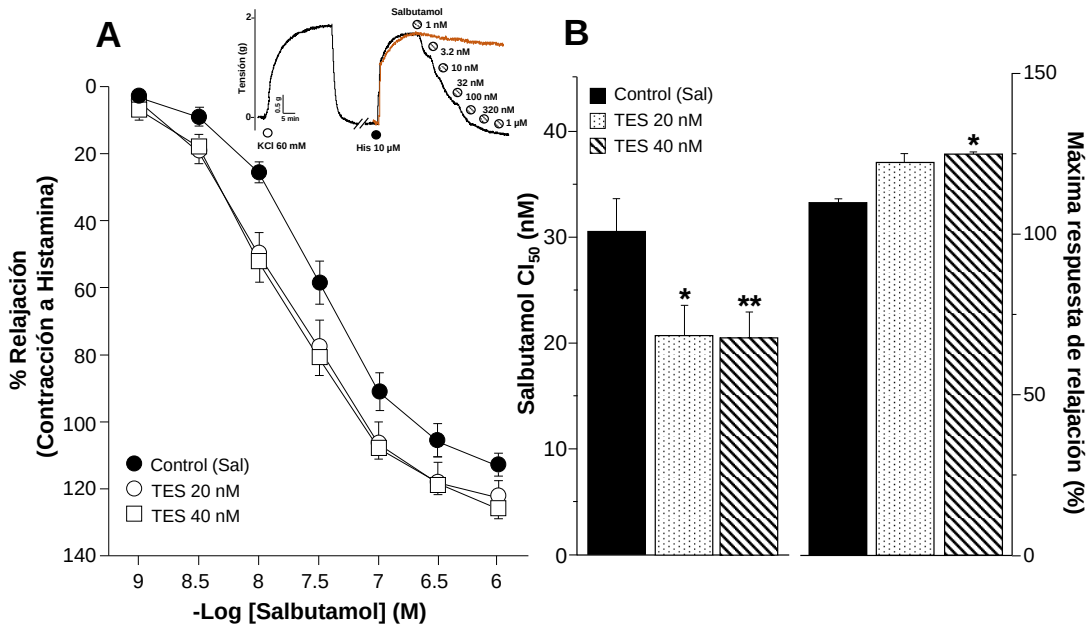

Figura 3. Efecto de la testosterona (TES, 20 y 40 nM) incubada por 48 horas sobre la relajación inducida por salbutamol en anillos traqueales de cobayo. **A)** La curva concentración respuesta de salbutamol (Sal, 1, 3.2, 10, 32, 100, 320, 1000 nM) relajó el tejido precontraído con histamina (His, 10 µM). Los anillos traqueales incubados con TES 20 y 40 nM durante 48 horas, generaron un desplazamiento a la izquierda de la curva concentración respuesta a Sal. El inserto representa un registro original de la relajación a salbutamol. **B)** Las gráficas de barras muestran los análisis de la concentración inhibitoria 50 (CI_{50}) y de la respuesta máxima de relajación obtenida. Se observa que la TES, en ambas concentraciones, disminuye significativamente la CI_{50} del Sal. La respuesta máxima de relajación fue sólo diferente cuando se utilizó la concentración más alta de TES. Las barras representan la media más el error estándar de la media (ESM). Se utilizó un análisis de varianza seguido de una prueba de Dunnett. *p< 0.05, **p< 0.01. n=5.

JUSTIFICACIÓN

El asma afecta alrededor de 339 millones de personas mundialmente. Este padecimiento es un reto de la salud pública debido a los desafíos en lo laboral, económico y en la calidad de vida a los que se enfrentan las personas asmáticas. El asma es multifactorial y se ha propuesto que los andrógenos regulan la presentación de esta enfermedad. Durante la niñez cuando los valores de TES son bajos en relación a otras edades, los síntomas del asma son más frecuentes en niños que en niñas. Este fenómeno se revierte en la pubertad y pudiera estar relacionado a los incrementos en las concentraciones plasmáticas de TES en varones. Anteriormente, se han propuesto diferentes mecanismos por los cuales la TES favorece la relajación del MLVA a través de vías no genómicas. Sin embargo, los efectos genómicos de las TES han sido poco estudiados y los trabajos que existen se enfocan en el área inmunológica. En este sentido, se ha propuesto que los andrógenos regulan negativamente la inflamación a través de un efecto genómico y disminuyen la hiperreactividad de las vías aéreas característica en el asma.

En condiciones fisiológicas, el sistema adrenérgico se encarga de regular el tono del MLVA a través de catecolaminas que actúan en los RAβ_2. Previamente, se estudió que la TES (a concentraciones suprafisiológicas) potenciaba la relajación del MLVA inducida por agonistas adrenérgicos β a través de un efecto no genómico y que no estaba relacionado con la vía de señalización adrenérgica β_2. De manera que, nos propusimos a explorar si la TES (a concentraciones fisiológicas) podría ejercer un efecto genómico sobre alguno de los mecanismos de relajación involucrados en la vía de señalización adrenérgica β_2, su funcionalidad en el MLVA y explorar otra posible explicación por la que los andrógenos favorecen la relajación en las vías aéreas.

HIPÓTESIS

La testosterona (TES), a concentraciones fisiológicas, favorece la regulación positiva del receptor adrenérgico β_2 e incrementa las corrientes de K^+ inducidas por salbutamol a través de un efecto genómico en el músculo liso de la vía aérea de cobayo. Este aumento en la expresión del receptor adrenérgico β_2 por la incubación crónica de TES potencia la relajación inducida por salbutamol en el músculo liso de la vía aérea de cobayo.

OBJETIVOS

Objetivo General

Caracterizar si la TES, en exposición crónica (48 horas) y a concentraciones fisiológicas, potencia la relajación inducida por salbutamol aumentando la expresión del receptor adrenérgico β_2 y a la adenilato ciclasa 6 e incrementando las corrientes de K^+ a través de un efecto genómico en el músculo liso de la vía aérea de cobayo.

Objetivos Particulares

- Valorar si la TES favorece la relajación inducida por salbutamol a través de un efecto genómico mediante el uso del antagonista del RA (Flutamida, 10 μM) en anillos traqueales de cobayo.

- Descartar si existe un efecto no genómico inducido por la incubación de TES con albúmina de suero bovino (40 nM, 48 horas) sobre la relajación del MLVA inducida por salbutamol (1 nM - 1 μM).

- Determinar si la TES incubada en tiempos cortos (15 - 45 min), favorece la relajación del MLVA inducida por salbutamol (1 nM - 1 μM) a través de un efecto no genómico en experimentos de órganos aislados.

- Evaluar los efectos del salbutamol sobre las corrientes de K^+ en células cultivadas de MLVA incubadas con o sin TES (40 nM por 48 horas).

- Indagar si la incubación con TES (40 nM por 48 horas) favorece el aumento en las corrientes de K^+ a través de un efecto genómico mediante el uso del antagonista de los RA (Flutamida, 3.2 μM), de un inhibidor de la transcripción (actinomicina D, 10 μM) y un inhibidor de la síntesis de proteínas (cicloheximida, 40 μM) en células cultivadas de MLVA.

- Investigar si la incubación del MLVA con TES (40 nM por 48 horas) altera la expresión del receptor adrenérgico β_2 y de la adenilato ciclasa 6 (ADY6), proteínas involucradas en el mecanismo de relajación del salbutamol.

METODOLOGÍA

Animales experimentales

Se utilizaron cobayos machos de la cepa Hartley de 350 a 400 gramos de la Unidad Académica Bioterio de la Facultad de Medicina. Los animales fueron criados en instalaciones institucionales con condiciones estándar: aire acondicionado filtrado, 21 ± 1 grados Celsius (°C), 50-70% de humedad y cama esterilizada. El manejo de animales y su sacrificio se realizó de acuerdo con la norma: NOM-062-Z00-1999. Proyecto avalado por la Comisión de Investigación y Ética de la Facultad de Medicina, UNAM # FM/DI/021/20178.

Órganos aislados

Los cobayos se sacrificaron por medio de una sobredosis de pentobarbital sódico (50 mg/kg de peso), posteriormente, se extrajo la tráquea colocándola en una cámara de disección que contenía solución de Krebs-Ringer con la siguiente composición [mM]: 118 NaCl, 25 $NaHCO_3$, 11 Glucosa, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2 $CaCl_2$. La solución fue burbujeada durante 30 minutos con una mezcla de carbógeno (95% O_2 y 5% CO_2) manteniendo un pH estable en intervalos fisiológicos (7.3 -7.4). A la tráquea se le retiró el tejido graso y el conectivo. Las tráqueas fueron seccionadas en 7 segmentos con 4 anillos de cartílago cada uno. Los tejidos sin y con TES (20, 40 nM) fueron incubados durante 48 horas a 9 ºC en tubos separados tipo Eppendorf con 1 mililitro (ml) de solución Krebs-Ringer previamente burbujeada con la misma mezcla de carbógeno durante 1 hora. Otros tejidos fueron incubados con 40 nM TES-BSA (macromolécula impermeable a la membrana plasmática) o con 10 µM de flutamida (antagonista del receptor androgénico) 30 minutos previos a la incubación con TES 40 nM. Al trascurrir las 48 horas de incubación, los tejidos se colgaron en distintas cámaras que contenían 5 ml de solución de Krebs-Ringer a 37 °C, burbujeada con la misma mezcla de carbógeno utilizada para la disección, a una tensión de 1 gramo. La tensión isométrica se registró con un transductor de tensión modelo FT03 (Grass Instruments, West Warwick, RI, EE. UU.) conectado a un sistema integrador de señales CyberAmp 380 (Axon Instruments, Foster City, CA, EE. UU.) y a una interfase Digidata 1440A, (Axon Instruments). Para obtener los datos y registros de la contracción del músculo

liso traqueal de cobayo se utilizó una computadora con el softwafe Axoscope versión 10.2 (Axon Instruments, Foster City, CA, EE. UU.). Una vez colgados, los tejidos se mantuvieron en reposo durante 1 hora antes de iniciar los diferentes procedimientos experimentales. Con el propósito de normalizar las respuestas de los tejidos y optimizar el aparato contráctil, éstos se estimularon tres veces con cloruro de potasio (KCl, 60 mM) hasta alcanzar la respuesta máxima de contracción. Posteriormente, se añadió histamina (His, 10 µM) y en la respuesta máxima de contracción se realizaron curvas acumulativas con salbutamol (Sal, 1, 3.2, 10, 32, 100, 320, 1000 nM) para inducir y estudiar la relajación del MLVA. En una serie diferente de experimentos se incubó con TES (20, 40 nM) durante 15 minutos y se lavó antes de añadir His; en otro grupo la TES se incubó 15 minutos antes de añadir His y permaneció durante toda la curva de salbutamol alcanzando una incubación de 60 minutos. Estas respuestas se expresaron como porcentaje de relajación de la respuesta de contracción a His.

Técnica de fijación de voltaje

Obtención y cultivo de miocitos traqueales de cobayo

La tráquea de cobayo fue obtenida como se describió en el procedimiento de órganos aislados y se realizó la disección del músculo liso traqueal en una cámara de disección que contenía solución Ringer-Krebs. Al músculo se le eliminó el tejido conectivo y el epitelio bajo microscopio estereoscópico y se sometió a una disgregación enzimática y mecánica, transfiriéndolo a una solución Hanks (GIBCO) que contenía 0.05 unidades de papaína (56 mg/ml, Worthington) y 2 miligramos (mg) de L-cisteína (SIGMA). El pH de la solución fue ajustado a 7.4 con $NaHCO_3$ 1 M; el tejido se mantuvo durante 10 minutos a 37 °C. A continuación, el tejido fue lavado con solución de Leibovitz (L15, GIBCO) para remover el exceso de enzimas y posteriormente, se incubó en una solución Hanks que contenía 1 mg/ml de colagenasa tipo I (Worthington) durante 10 min a 37 °C. En seguida, el tejido fue aspirado y liberado hacia la solución enzimática con una pipeta Pasteur aproximadamente 20 veces para permitir la disociación de los miocitos. Posteriormente, se incubó el tejido nuevamente con la colagenasa durante 10 minutos y se realizó el mismo procedimiento de disociación mecánica. La actividad enzimática fue detenida con la solución L15 y se centrifugó a 600 revoluciones por minuto durante 5 minutos a 20 °C, retirando el sobrenadante y añadiendo

nuevamente L15 y volviendo a centrifugar. El botón celular fue suspendido en 6 ml de medio esencial mínimo (MEM, GIBCO) que contenía suero fetal bovino al 10%, 2 milimolar (mM) L-glutamina, 10 U/ml de penicilina, 10 µM/ml de estreptomicina y 15 mM de glucosa, para posteriormente distribuir el medio con las células en cajas multipozos (Falcon Becton Dickinson), añadiendo 1 ml a cada pozo a los cuales anteriormente se les había colocado un cubreobjetos impregnado con colágena estéril de rata. A algunos cultivos de miocitos se les añadió TES (40 nM, 48 horas) y/o flutamida (antagonista del receptor androgénico Flu, 3.2 µM) 30 minutos antes de añadir TES (40 nM), o TES-BSA (40 nM, 48 horas). Otras células se dejaron crecer durante 24 horas antes de añadirles 40 µM de cicloheximida (inhibidor de la síntesis de proteínas) o 10 µM de actinomicina D (inhibidor de la transcripción) 30 minutos antes de incubar 40 nM de TES durante 48 horas. Los cultivos se colocaron en una incubadora de CO_2 al 5% a 37 °C durante 48 horas para posteriormente realizar la técnica de fijación de voltaje.

Registros de la actividad eléctrica en célula completa

Cada cubreobjetos con los miocitos traqueales adheridos fue trasladado a una cámara de registro que fue constantemente perfundida con un volumen de 0.5-1 ml de solución externa a una velocidad de perfusión de 1.5-2 ml/min y a temperatura ambiente. Para la medición de IK^+, la solución externa tenía la siguiente composición (mM): NaCl 130; $CaCl_2$ 1; Ácido Niflúmico 0.1; glucosa 10; Hepes 10; $MgCl_2$ 0.5; $NaHCO_3$ 3; KH_2PO_4 1.2; KCl 5. La solución fue ajustada a un pH=7.4 con NaOH. La cámara de registro fue adaptada sobre el objetivo de un microscopio de luz invertido (Zeiss modelo IP03). Los microelectrodos que se utilizaron para el registro de fijación de voltaje fueron fabricados con capilares de borosilicato (1B200F-6 vidrio, Word Precision Instruments), utilizando un estirador de pipetas (P-87, Sutter Intruments Co). La resistencia de los electrodos se encontraba entre 3-6 megaohmios (MΩ). La capacitancia de la punta antes y después del acceso a la célula fue compensada, aplicando pulsos despolarizantes mediante un Amplificador Axopatch (modelo 200A, Axon Instruments). Se realizaron mediciones de IK^+ utilizando los filtros del amplificador 1-5 KHz con una frecuencia de 10 KHz. La solución de la pipeta de registro, solución interna, contaba con la siguiente composición (mM): NaCl 5; ATP 5.0; HEPES 5; EGTA 1; guanosina-5’ trifosfato (GTP) 0.1; gluconato de K 140; leupeptina 0.1; pH= 7.3

ajustado con KOH. Las corrientes se registraron por medio de su activación con un protocolo de estimulación previamente cargado en el programa pClamp (versión 10.2), el cual consiste en pulsos despolarizantes de -60 a +50 mV en incrementos de 10 mV durante 100 milisegundos, a 1 Hz con un potencial de fijación de membrana de -60 mV. Después del protocolo control, los miocitos de cada grupo experimental fueron perfundidos con diferentes concentraciones de salbutamol (1, 10, 100 y 1000 nM) y recibieron el mismo protocolo de pulsos despolarizantes. Para caracterizar que canales de K^+ están involucrados en este fenómeno, los miocitos fueron perfundidos con 3 mM de 4-aminopiridina (4-AP, bloqueador de los canales de K^+ rectificador tardío) o 100 nM de iberiotoxina (IBTX, bloqueador específico de los canales de K^+ activados por Ca^{2+} de alta conductancia). Los cambios en las corrientes bajo las condiciones descritas fueron evaluados en el pico máximo de la corriente para cada voltaje.

Detección del receptor adrenérgico β_2 y de la adenilato ciclasa 6 mediante inmunofluorescencia

La tráquea de los cobayos fue extraída y colocada en una cámara de disección donde se eliminó el tejido conectivo adyacente. La tráquea se dividió en segmentos de 4 anillos traqueales cada uno. Dichos segmentos fueron incubados con o sin TES 40 nM durante 48 horas a 9 °C como se describió en la técnica de órganos aislados. Una vez transcurridas las 48 horas, los tejidos fueron fijados durante 4 horas con paraformaldehído al 4% diluido en una solución amortiguadora de fosfatos (PBS). Posteriormente, los tejidos fueron deshidratados con sacarosa al 30% diluida en PBS. Se utilizó el compuesto O. C. T. (Tissue Tek, CA, EE. UU.) para incluir los tejidos; éstos fueron congelados a -60 °C, seccionados en rebanadas de 20 micrómetros (µm) de grosor que se colocaron sobre laminillas sinalizadas. Las criosecciones fueron transferidas a PBS y permeabilizadas con 0.5% de tritón-X 100. Con el propósito de bloquear la unión inespecífica a proteínas, se añadió suero de caballo al 10 % a las rebanadas durante 30 minutos. Posteriormente, las rebanadas se incubaron durante toda la noche a 4 °C con los siguientes anticuerpos primarios de conejo: anti-receptor adrenérgico β_2 (Creative Diagnostics, DPABH-26262, NY, EE. UU.) a una dilución 1:50 y anti-adenilato ciclasa 6 (isoforma más abundante en el MLVA (Birrell et al., 2015), Genetex, GTX47798, CA, EE. UU.), dilución 1:100. Una vez transcurrida la incubación de los

anticuerpos primarios, las rebanadas fueron lavadas tres veces con PBS durante 5 minutos. El anticuerpo secundario Alexa488 elaborado en burro, anti-conejo inmunoglobulina G (1: 200, Life technologies, CA, EE. UU.), se incubó durante 30 minutos. Además, para corroborar la identidad de las células de MLVA se utilizaron el anticuerpo primario, anti-α-actina de ratón (Santa Cruz Biotechnology, Cat. No. Sc-58669, TX, EE. UU.) a una dilución 1:250 y el anticuerpo secundario Alexa fluor 555 hecho en burro, anti-ratón (Life Technologies, CA, EE. UU.), dilución 1:400. El compuesto 4', 6 Diamidino-2-fenilindol (DAPI, Life Technologies) se utilizó para marcar los núcleos. Por último, las laminillas se cubrieron con un medio de montaje acuoso fluoromount (Sigma, MO, EE. UU.). Los controles negativos no fueron incubados con anticuerpos primarios (receptor adrenérgico β_2 y adenilato ciclasa 6) y no se obtuvo señal de fluorescencia. La fluorescencia fue observada con un microscopio confocal A1R + STORM (Nikon, Japón). Para fines de visualización, las imágenes se muestran con el RAβ_2 y la adenilato ciclasa 6 en color verde, la α-actina en rojo y los núcleos en azul.

Extracción de ARNm y PCR cuantitativo para el receptor adrenérgico β_2

A los cobayos se les extrajo la tráquea y se colocó en un tubo Eppendorf libre de ARNasa que contenía 1.5 ml de solución de Krebs previamente burbujeada con carbógeno. A las tráqueas se les añadió (o no en el caso del grupo control) TES 40 nM y se almacenaron a 9 °C durante 48 horas como se describió en los experimentos de órganos aislados. Posterior a las 48 horas, las tráqueas fueron colocadas en una cámara de disección con solución fría de Krebs libre de ARNasa. Ahí, se eliminaron los tejidos adyacentes a la tráquea como el tejido conectivo, epitelio y cartílago para obtener únicamente el músculo liso. El músculo liso traqueal se colocó en 200 microlitros (µl) de TRIzol (Invitrogen) en un tubo Eppendorf de 1.5 ml libre de ARNasa. A partir de estas muestras se purificó el ARN total utilizando el kit Direct-zol RNA Miniprep (Zymo, CA, EE. UU.) y para prevenir una posible contaminación con ADN, la muestra fue sometida a una digestión enzimática de ADN con ADNasa I (Zymo). La concentración total de ARN se midió con un espectrofotómetro NanoDrop 2000 (Thermo Scientific, Barrington, IL, EE. UU.). Para evaluar la pureza del ARN, se midieron los cocientes de absorbancia 260:230 nm y 260:280 nm, donde un cociente de 1.8 fue apropiado para proceder con la síntesis de ADNc. El ARN total fue transcrito de forma

reversa a ADNc usando un kit ADNc SuperScript VILO (Life Technologies). Las reacciones de PCR cuantitativa se realizaron en un sistema StepOne Real Time PCR (Foster City CA, EE. UU.). Para la reacción de PCR, se utilizó un volumen final de 20 µl que contenía 4 µl de agua, 10 µl de 2XTaqMan Gene Expression Master Mix (Life Technologies), 5 µl de ADNc diluido y 1 µl de la mezcla del ensayo de PCR al 20X específica para RAβ_2. Los controles negativos fueron preparados sin el ADN templado. Los ensayos de expresión génica Custom TaqMan (ThermoFisherScientific, CA, EE. UU.) se muestran en la Tabla 1. Las condiciones de amplificación fueron 2 min a 50 °C seguido de 10 min a 95 °C, mientras que para la segunda fase fueron 15 s a 95 °C y 1 min a 60 °C durante 40 ciclos. Se probaron una serie de genes (cofilina, clatrina en forma de cadena pesada 1, peptidil-prolil cis-trans isomerasa B) para determinar si presentaban una expresión estable en el MLVA tratado con TES. Finalmente, la cofilina se utilizó como gen constitutivo para la normalización. El método $2^{-\Delta\Delta CT}$ se empleó para analizar la expresión del gen RAβ_2 (Livak & Schmittgen, 2001).

Tabla 1. Ensayos de expresión génica Custom TaqMan

Gen	Secuencia de cebadores y sonda
Cofilina 1	Fw: GCCAGACGGTGGACGAC Rv: GTCCTTGTCTGGTAGCATCTTGA Pr: CCCTACGCCACCTTTG
PPIB	Fw: CCAGGGCGGAGACTTCAC Rv: CGCTCGCCATAAATGCTCTTG Pr: CCTGTGCCATCTCCTC
Clatrina	Fw: ACTGTTCCCCATGAACCTACCT Rv: TGTTTTCTTCTTCAACACAGACTGACA Pr: TTGACGCCAATAATCC
RAβ_2	Fw: CAAAAAGCAGCTCCAGAAGATTGAC Rv: CTGCTCCACCTGGCTGAG Pr: ATCGGCCCTCAGATCT

PPIB: peptidil-prolil cis-trans isomerasa B. RAβ_2: receptor adrenérgico β_2. Fw: Primer en sentido. Rv: Primer reverso. Pr: sonda.

Western blot para el receptor adrenérgico β_2

La expresión relativa del receptor adrenérgico β_2 y β-actina como control de carga se evaluó mediante Western blot y quimiolumiscencia. El MLVA de cobayo fue tratado con y sin TES 40 nM durante 48 horas o flutamida 10 µM 30 minutos antes de la incubación con TES. En seguida, el MLVA fue finamente cortado con micro tijeras para posteriormente llevar a cabo la lisis celular y la obtención de proteínas mediante un sonicador utilizando un buffer de lisis RIPA que contenía (mM): 50 Tris (pH 7.5), 0.5 EDTA, 1% Nonidet ™ P40, 1 PMSF, 0.5 ortovanodato de sodio; inhibidores de proteasa 20 microgramos (µg)/ml: aprotinina, leupeptina y pepstatina. La concentración de proteína total se cuantificó por el método de Lowry. Las proteínas totales (10 µg) se separaron mediante electroforesis unidimensional con geles de poliacrilamida (12%) y se transfirieron a membranas de fluoruro de polivinilideno (FPVD). De manera siguiente, las membranas fueron bloqueadas con la solución Odyssey Blocking Buffer (TBS-LI-COR, NE, EE. UU.) durante 1 hora. Con el propósito de verificar la transferencia, se utilizó azul de Coomassie (geles) y rojo de Ponceau (membranas). Posteriormente, las membranas fueron incubadas durante la noche con un anticuerpo policlonal primario de conejo (dilución 1:100; Creative Diagnostics) y luego, durante 1.5 horas con un anticuerpo secundario anti-conejo de burro acoplado a peroxidasa (dilución 1:15000; Jackson). La señal quimioluminiscente se detectó con el sistema de imágenes (ChemiDoc ™ MP, BIO-RAD, CA, EE. UU.) y las imágenes se adquirieron con el software Image Lab (versión 5.2.1; BIO-RAD). Después, las membranas de FPVD se lavaron con la solución Re-blot plus-mild (Millipore, MA, EE. UU.) para eliminar el anticuerpo para el receptor adrenérgico β_2 y de este modo continuar con la incubación del anticuerpo primario monoclonal para β-actina (clon AC-40) generado en ratón (1: 500; Sigma, St. Louis, MO, EE. UU.). Posteriormente, se añadió el anticuerpo secundario anti-ratón de conejo acoplado a peroxidasa (1: 10000; Jackson, PA). La reacción de quimioluminiscencia y la obtención de las imágenes se realizaron de la misma forma que en el procedimiento anterior. El control de especificidad se realizó omitiendo la incubación con el anticuerpo anti-adrenérgico-β_2. En todos los casos, la densidad óptica se normalizó al control de carga para cuantificar la relación del receptor adrenérgico β_2/β-actina.

FÁRMACOS UTILIZADOS

Testosterona (17β-hidroxi-4-androsten-3-ona), testosterona 3-(O-carboximetil) oxima-BSA, histamina, salbutamol, actinomicina D, cicloheximida y flutamida se obtuvieron de Sigma Chem. Co. (St. Louis, MO, EE. UU.). La iberiotoxina fue adquirida a Enzo Life Sciences (Farmingdale, NY, EE. UU.) y la 4-aminopiridina a Research Chemical LTD (Word Hill, MA, EE. UU.). TES, cicloheximida y flutamida fueron diluidas en etanol y el porcentaje más alto utilizado fue 0.1 porcentaje volumen-volumen (% v/v) del vehículo. La actinomicina se diluyó en dimetilsulfóxido (DMSO).

ANÁLISIS ESTADÍSTICO

En el músculo liso traqueal, las curvas concentración-respuesta a salbutamol se evaluaron mediante la concentración inhibitoria 50 (CI_{50}) y la respuesta máxima de relajación. Cada curva acumulativa de concentración-respuesta se utilizó para evaluar la CI_{50} que se calculó mediante regresión lineal como –log [M] utilizando el software ED50plus v1.0. Estos datos se analizaron mediante un análisis de varianza unidireccional seguido de la prueba de comparación múltiple de Dunnett. En experimentos de registro de la actividad eléctrica de célula completa, las IK^+ se analizaron mediante un análisis de varianza unidireccional seguido de pruebas de Dunnett, Student-Newman-Keuls o prueba t de Student para datos no pareados. Los experimentos de Western blot también se analizaron mediante un análisis de varianza unidireccional seguido de una prueba de Dunnett. En todos los experimentos, cada valor de "n" corresponde a un animal diferente. Los datos obtenidos en todos los experimentos se representaron como la media más el error estándar de la media (ESM). La significancia estadística se estableció cuando el valor de p fue menor a 0.05.

RESULTADOS

La exposición crónica a testosterona aumenta la relajación del músculo liso traqueal inducida por salbutamol.

Los tejidos que fueron preincubados con TES 20 o 40 nM durante 48 horas mostraron un desplazamiento hacia la izquierda de la curva concentración respuesta a salbutamol (1, 3.2, 10, 32, 100, 320, 1000 nM) en comparación al grupo control (anillos traqueales sin TES) (Figura 4A, n=5). Con el propósito de expresar y evaluar las diferencias entre las curvas de relajación generadas en tejidos controles y tratados con TES se hicieron análisis de concentración inhibitoria 50 (CI_{50}) y de respuesta máxima de relajación. La CI_{50} en los dos grupos de TES (20 y 40 nM) fue significativamente menor que la CI_{50} del grupo control, es decir se necesitaron menores concentraciones de salbutamol para relajar el tejido cuando éste fue expuesto a TES. La respuesta máxima sólo fue significativamente diferente para el grupo de TES 40 nM (Figura 4B). El uso de TES-BSA, un complejo macromolecular impermeable, no mostró modificación en la relajación inducida por salbutamol a comparación del grupo control (Figura 4C, D, n=4), lo que sugiere que el fenómeno de potenciación de la relajación inducido por TES es a través de un efecto genómico. Asimismo, la exposición a 20 o 40 nM de TES durante 15 o 60 min no modificó la curva concentración respuesta de salbutamol (Figura 5A, B, n=7), lo que apunta también a un efecto genómico. Además, el uso de flutamida (Flu 10 µM, antagonista del receptor androgénico) anuló el aumento en la respuesta de relajación inducida por salbutamol al incubar los anillos con TES (Figura 6, n=6). Estos resultados corroboran que la potenciación en la respuesta de relajación inducida por el salbutamol está mediada por una vía genómica ya que involucra tiempos largos de incubación y la unión de TES con el RA. De manera que, probablemente la TES favorece la regulación positiva del receptor adrenérgico β_2 o de algún componente de esta vía de señalización.

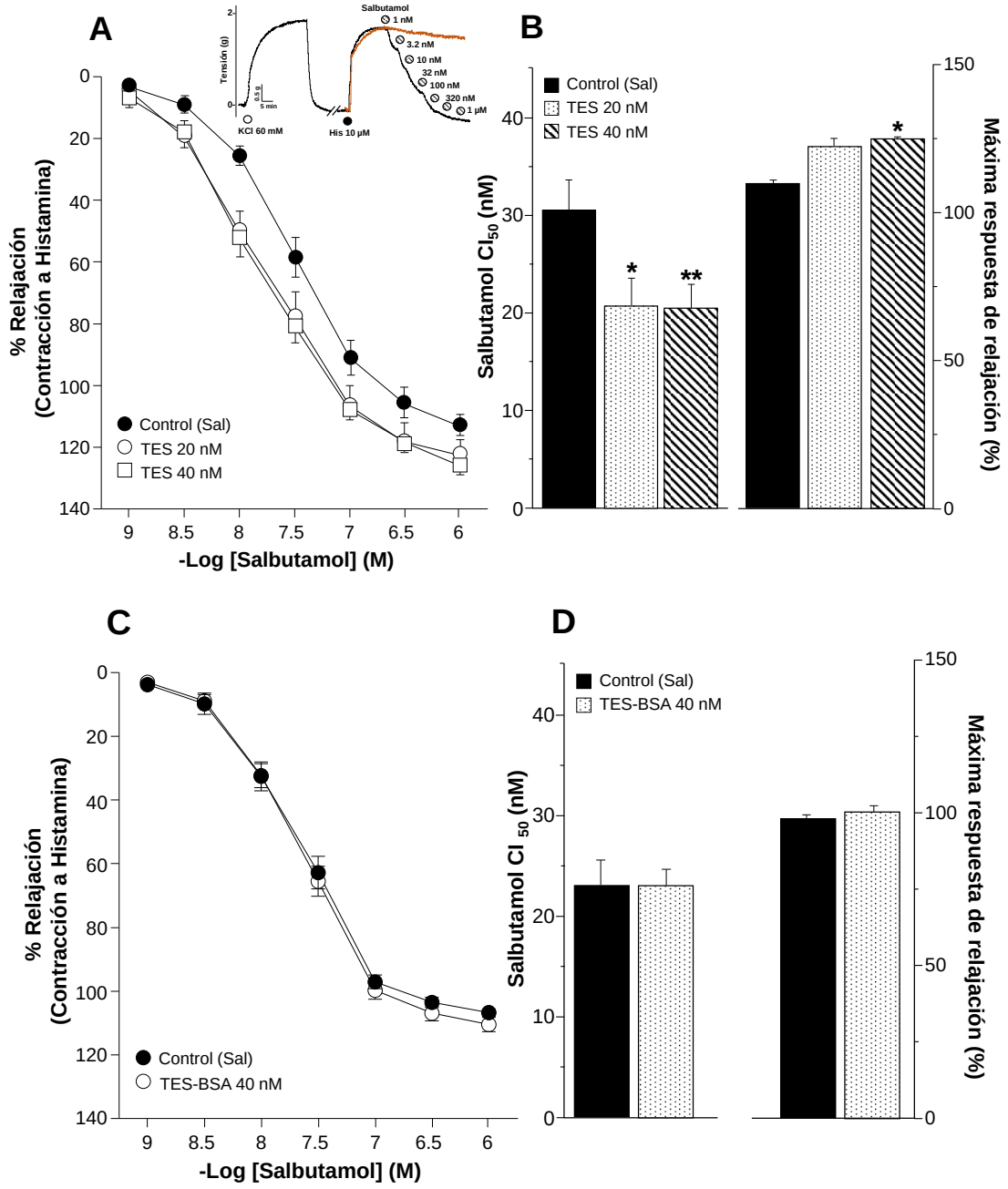

Figura 4. Efecto de la testosterona (TES, 20 y 40 nM) y de la testosterona conjugada con albúmina de suero bovino (TES-BSA, 40 nM) incubadas por 48 horas sobre la relajación inducida por salbutamol en anillos traqueales de cobayo. **A)** La curva concentración respuesta de salbutamol (Sal, 1, 3.2, 10, 32, 100, 320, 1000 nM) relajó el tejido precontraído con histamina (His, 10 µM). Los anillos traqueales incubados con TES 20 y 40 nM durante 48 horas, generaron un desplazamiento a la izquierda de la curva concentración respuesta a Sal. El inserto representa un registro original de la relajación a salbutamol. **B)** Las gráficas de barras muestran que la TES, en ambas concentraciones, disminuye significativamente la concentración inhibitoria 50 (CI_{50}) del Sal. La respuesta máxima de relajación fue sólo diferente cuando se utilizó la concentración más alta de TES. Las barras representan la media más el error estándar de la media (ESM). Se utilizó un análisis de varianza seguido de una prueba de Dunnett. *p< 0.05, **p< 0.01. n=5. **C)** La incubación de TES-BSA 40 nM durante 48 horas no modifica la curva concentración respuesta a Sal. **D)** Análisis estadístico de la CI_{50}

y la respuesta máxima de relajación. Las barras representan la media más el ESM. Se puede observar que no hay diferencias en la respuesta de Sal entre el grupo control y el de TES-BSA. Se utilizó una prueba t de Student, n=4.

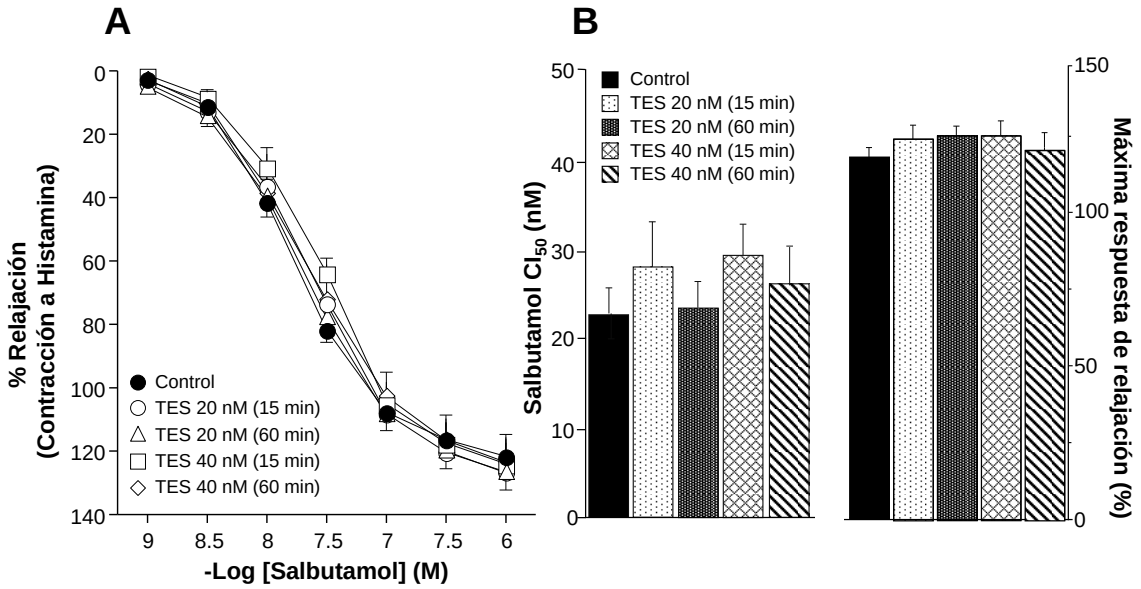

Figura 5. La incubación por tiempos cortos de testosterona (TES, 15 y 60 minutos) no modifica la relajación inducida por salbutamol (Sal) en anillos traqueales de cobayo. **A)** La Testosterona 20 y 40 nM incubada en tiempos cortos no modifica la curva concentración respuesta a salbutamol. **B)** En el análisis estadístico de la CI_{50} del salbutamol y la respuesta máxima de relajación no se encontraron diferencias. Las barras representan la media más el ESM. Se utilizó un análisis de varianza seguido de una prueba de Dunnett, n=7.

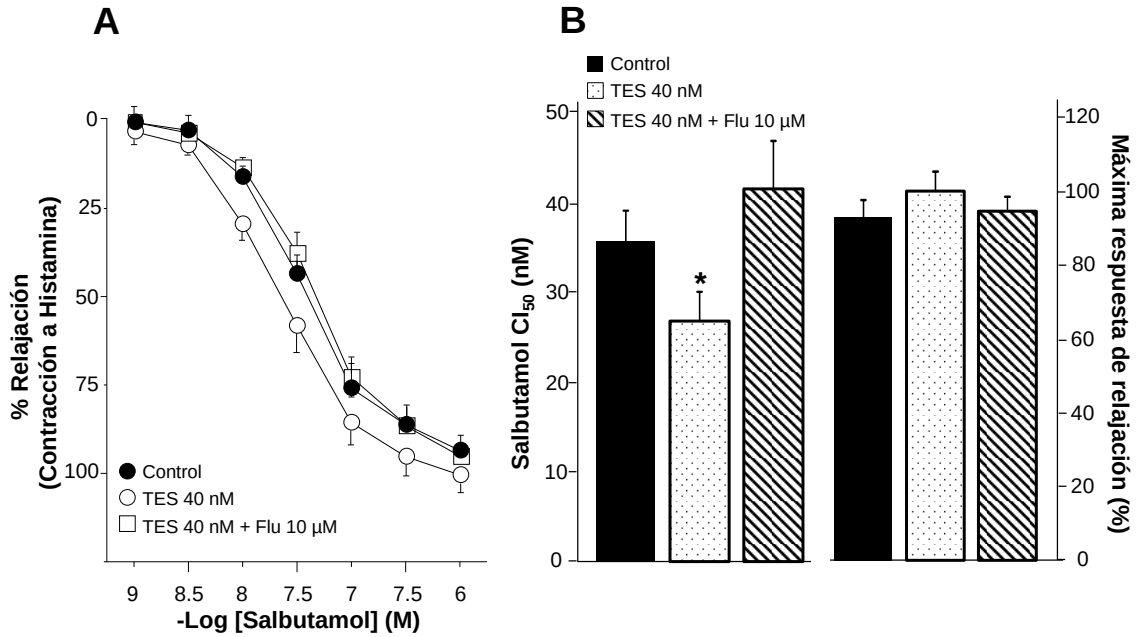

Figura 6. La flutamida anula el efecto de potenciación de la TES sobre la relajación inducida por salbutamol en anillos traqueales de cobayo. **A)** La incubación de TES 40 nM durante 48 horas genera un desplazamiento a la izquierda y potencia la respuesta de relajación a una curva concentración respuesta a salbutamol (1, 3.2, 10, 32, 100, 320, 1000 nM). La adición de flutamida (Flu 10 µM, antagonista del receptor androgénico) 30 min antes de la incubación con TES durante 48 horas

inhibe el efecto de potenciación de la TES sobre la relajación inducida por salbutamol. **B)** Análisis estadístico de la CI_{50} y la respuesta máxima de relajación. Las barras representan la media más ESM. Se utilizó un análisis de varianza seguido de una prueba Dunnet, *p< 0.05; n=6.

La testosterona aumenta las corrientes de K^+ inducidas por salbutamol en células de músculo liso traqueal de cobayo a través de un efecto genómico.

Es bien conocido que el efecto de relajación inducido por salbutamol está mediado en parte por la activación de IK^+ inducidas por distintos tipos de canales de K^+, por lo que se decidió explorar si la TES pudiera ejercer un efecto positivo sobre estas corrientes. En este sentido, se realizaron experimentos donde se evocaron IK^+ por una serie de pulsos despolarizantes (-60 a 50 mV). Esto produjo corrientes salientes de K^+ que en condiciones control (células no expuestas a TES) alcanzaron en su punto máximo ~0.7 nanoamperios (nA) (Figura 7A, círculo cerrado). La adición de salbutamol (1, 10, 100, 1000 nM) además del protocolo de pulsos despolarizantes promovió la apertura de los canales de K^+ de manera concentración dependiente. La máxima concentración de salbutamol utilizada (1000 nM) generó una IK^+ que alcanzó su punto máximo de ~3 nA (Figura 7A). Por otro lado, las células registradas que fueron incubadas con TES 40 nM durante 48 horas presentaron un mayor aumento en las IK^+ inducidas por el protocolo de pulsos despolarizantes y por el salbutamol en cada una de las concentraciones utilizadas (Figura 7B, D-H). Sin embargo, este aumento en las corrientes por la incubación de TES fue anulado con la adición de flutamida (Flu, 3.2 μM), apuntando que la acción de la TES es a través de una vía genómica que involucra al receptor androgénico (Figura 7C). Para efectos de visualización y un análisis adecuado del efecto de la TES y la Flu, el incremento en las IK^+ fue comparado de acuerdo a la concentración de salbutamol utilizada, como se describe de manera siguiente y como se muestra en la Figura 7D-H.

El primer trazo fue generado por la aplicación de pulsos despolarizantes (-60 a +50 mV, Figura 7D, círculo cerrado). Cuando las células fueron incubadas con TES 40 nM (Figura 7D, triángulo abierto) se observó un incremento en la IK^+ y este efecto fue inhibido con la incubación de Flu 3.2 μM (Figura 7D, cuadrado abierto). En los siguientes experimentos, además del protocolo de pulsos despolarizantes se añadieron diferentes concentraciones de salbutamol (1, 10, 100 1000 nM). De manera similar al trazo sin salbutamol, la TES generó un aumento significativo de las IK^+ inducidas por las distintas concentraciones de salbutamol

utilizadas y este efecto fue anulado al incubar con el antagonista del receptor androgénico (Figura 7D-H).

Como se describió anteriormente, las células registradas que fueron incubadas con TES 40 nM durante 48 horas presentaron un mayor aumento en las IK^+ inducidas por salbutamol y este aumento fue abolido por flutamida. Con el propósito de corroborar la naturaleza genómica del efecto ejercido por la TES, se realizaron experimentos con actinomicina D (Actino D, 10 μM, inhibidor de la transcripción) y cicloheximida (Cyclohex, 40 μM, inhibidor de la síntesis de proteínas). En estos experimentos encontramos que el aumento en la IK^+ inducido por la incubación de TES en el protocolo de pulsos despolarizantes y en el protocolo de pulsos despolarizantes más salbutamol (1, 10, 100, 1000 nM) fue bloqueado con la adición de Actino D o con el uso de Cyclohex, sugiriendo que la acción de la TES para incrementar la IK^+ es a través de un efecto genómico que involucra la unión del andrógeno a su receptor con la consecuente activación de la transcripción y la síntesis proteica (Figura 8C, 9C). Para analizar de forma adecuada el efecto de la TES, Actino D y Cyclohex, las corrientes fueron comparadas de acuerdo a la concentración de salbutamol utilizada como se mencionó antes en el texto (Figura 8, 9 D-H).

Por último, con la finalidad de descartar un posible efecto no genómico (mediado por la unión del andrógeno a proteínas de la membrana plasmática) de la TES sobre el incremento en las IK^+, se realizaron experimentos en miocitos traqueales incubados con TES-BSA 40 nM durante 48 horas, los cuales no mostraron diferencias en las IK^+ inducidas por los pulsos despolarizantes o por el protocolo de pulsos despolarizantes más salbutamol en comparación con el grupo control correspondiente (Figura 10 A-G).

Anteriormente, se mencionó que la incubación de TES incrementó las IK^+ inducidas por el protocolo de pulsos despolarizantes (Figura 7D), lo que sugiere que la TES está modificando de alguna forma la actividad de los canales de K^+ per se. Para caracterizar que canales de K^+ estuvieran participando en este fenómeno, se utilizó un bloqueador de los canales K_V (4-AP) y éste anuló desde 0 mV hacia adelante el efecto de la TES (Figura 11A). Estos resultados apuntan a que la TES está aumentando la actividad y/o la expresión génica de estos canales.

Además, encontramos que el efecto de los andrógenos sobre las IK^+ inducido por salbutamol más el protocolo de pulsos despolarizantes fue bloqueado por 4-AP y por IBTX, (bloqueador específico de los canales BK_{Ca}). Estos descubrimientos indican que ambos canales de K^+ están involucrados en el efecto de potenciación de la TES sobre la vía de señalización del receptor adrenérgico (Figura 11B). El incremento de las IK^+ por la incubación de TES nos indica que la vía de señalización adrenérgica β_2 está regulada positivamente por este andrógeno a través de un efecto genómico. Esto explicaría por una parte porque se potencia la relajación inducida por salbutamol en el MLVA. Asimismo, es posible que la expresión génica de los K_V y BK_{Ca} sea regulada a la alta por la incubación crónica de TES.

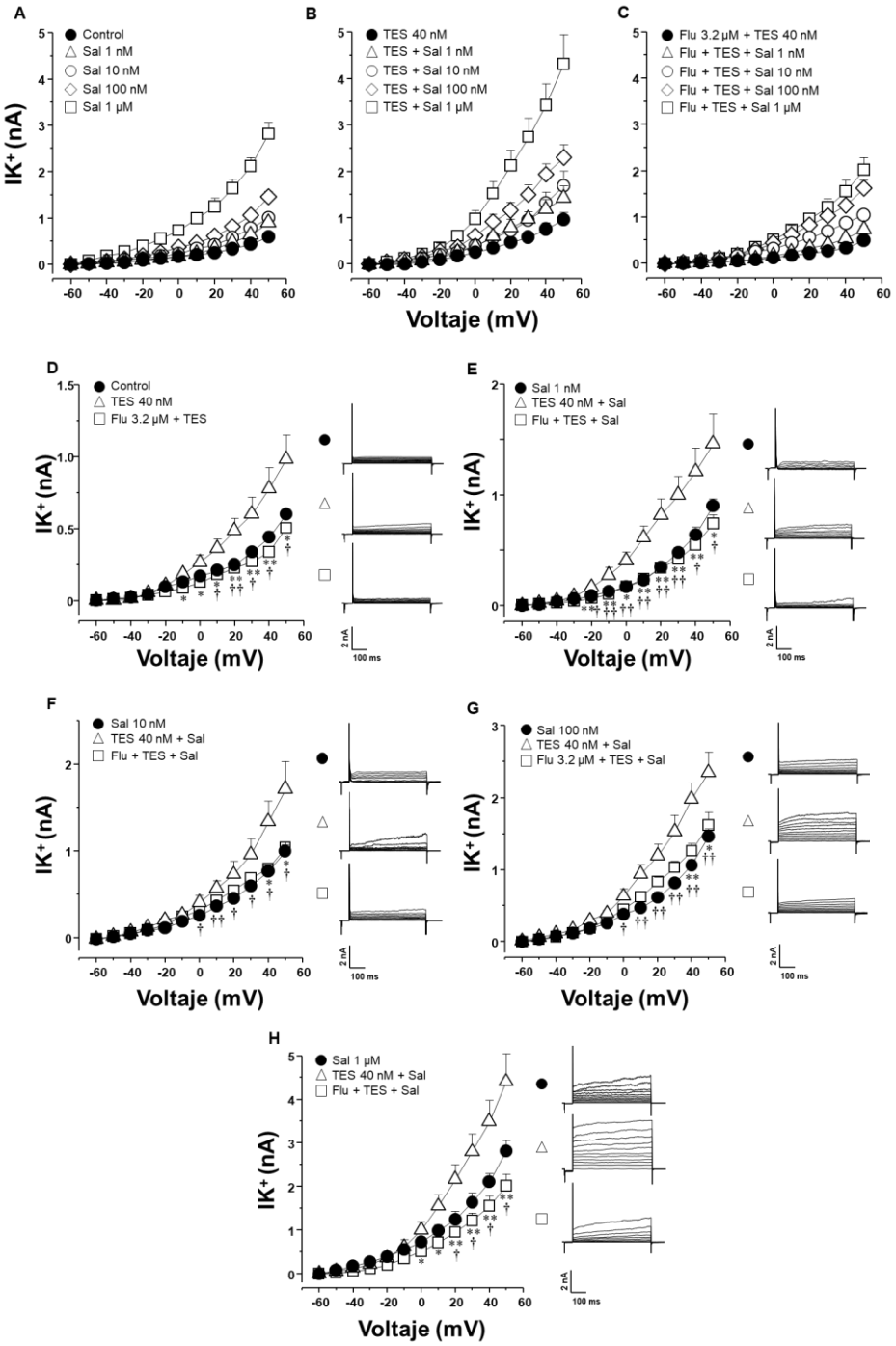

Figura 7. La testosterona (TES, 40 nM) incubada por 48 horas en células cultivadas de MLVA de cobayo incrementa las corrientes de K^+ (IK^+) a través de un efecto genómico. Las IK^+ fueron evocadas por una despolarización seriada de -60 a 50 mV con incrementos de 10 mV. **A)** Miocitos perfundidos con concentraciones acumulativas de salbutamol (Sal, 1 nM, 10 nM, 100 nM y 1000 nM) muestran un aumento en las IK^+ concentración dependiente (n=9). **B)** Células incubadas durante 48 horas con TES 40 nM muestran un aumento en las IK^+ (n=8). **C)** La flutamida (Flu 3.2 µM) anula el efecto de la TES (n=7). **D, E, F, G, H)** Ilustran el análisis estadístico de cada concentración de Sal utilizada en los diferentes grupos experimentales, incluyendo el de flutamida. En las figuras se puede observar que las células que fueron expuestas a TES crónicamente presentaron un mayor aumento de las IK^+ inducidas por los pulsos despolarizantes y/o salbutamol; este aumento fue abolido por la Flu. Los insertos que se muestran en las figuras representan registros originales de electrofisiología. En **D**-**H** se utilizaron diferentes escalas en el eje de las Y, con el propósito de mostrar claramente el análisis

estadístico. Se utilizó un análisis de varianza seguido de una prueba de Dunnett. Los símbolos representan la media ± ESM. *p≤ 0.05, **p≤ 0.01, †p≤ 0.05, ††p≤ 0.01 cuando los grupos son comparados contra TES 40 nM (**D**) o TES + Sal (**E-H**). n=7-9.

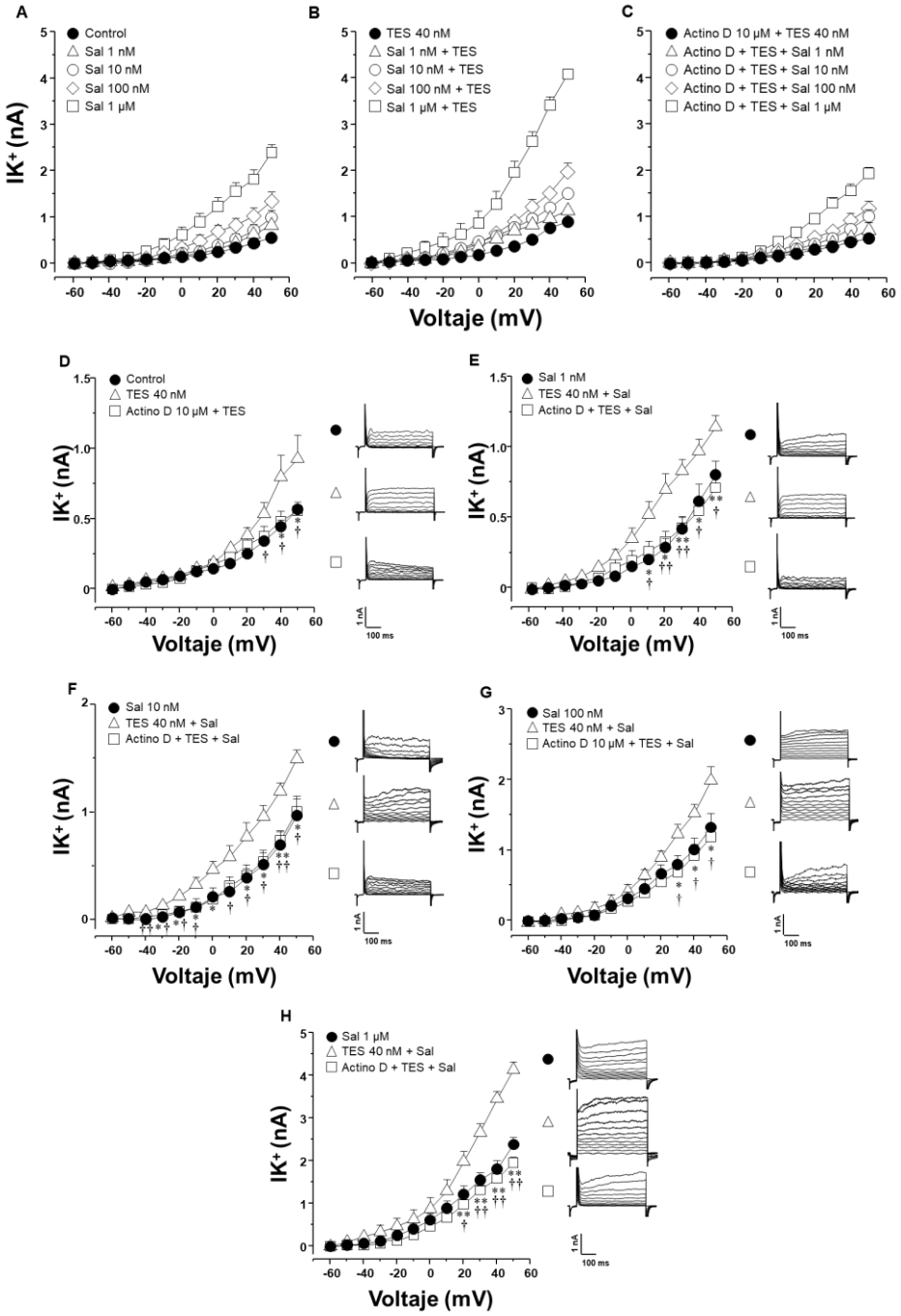

Figura 8. La actinomicina D (Actino D, 10 µM) anula el efecto de la testosterona (TES, 40 nM por 48 horas) sobre el aumento de las corrientes de K^+ (IK^+) inducido por salbutamol a través de un efecto genómico. Las IK^+ fueron evocadas por una despolarización seriada de -60 a 50 mV. **A)** Registro de una curva acumulativa con salbutamol (Sal, 1 nM-1 µM) en células de MLVA en condiciones control. **B)** Representación gráfica de la curva acumulativa con Sal en células incubadas durante 48 horas con TES 40 nM, donde se observa un aumento de las corrientes. **C)** Se muestran curvas acumulativas con Sal en células cultivadas con Atino D 10 µM 30 minutos previos a la incubación con TES; anula el efecto de la TES. **D, E, F, G, H)** Ilustran el análisis estadístico de cada concentración de Sal en los diferentes grupos experimentales, incluyendo

los experimentos con Actino D. Los insertos que se muestran en las figuras representan registros originales de electrofisiología. En **D-H** se utilizaron diferentes escalas en el eje de las Y, con el propósito de mostrar claramente el análisis estadístico. Los símbolos representan la media ± ESM. Se utilizó un análisis de varianza seguido de una prueba de Dunnett. *p<0.05, **p<0.01, †p<0.05, ††p<0.01 cuando son comparados los grupos contra TES 40 nM (**D**) o contra TES + Sal (**E-H**) n=6

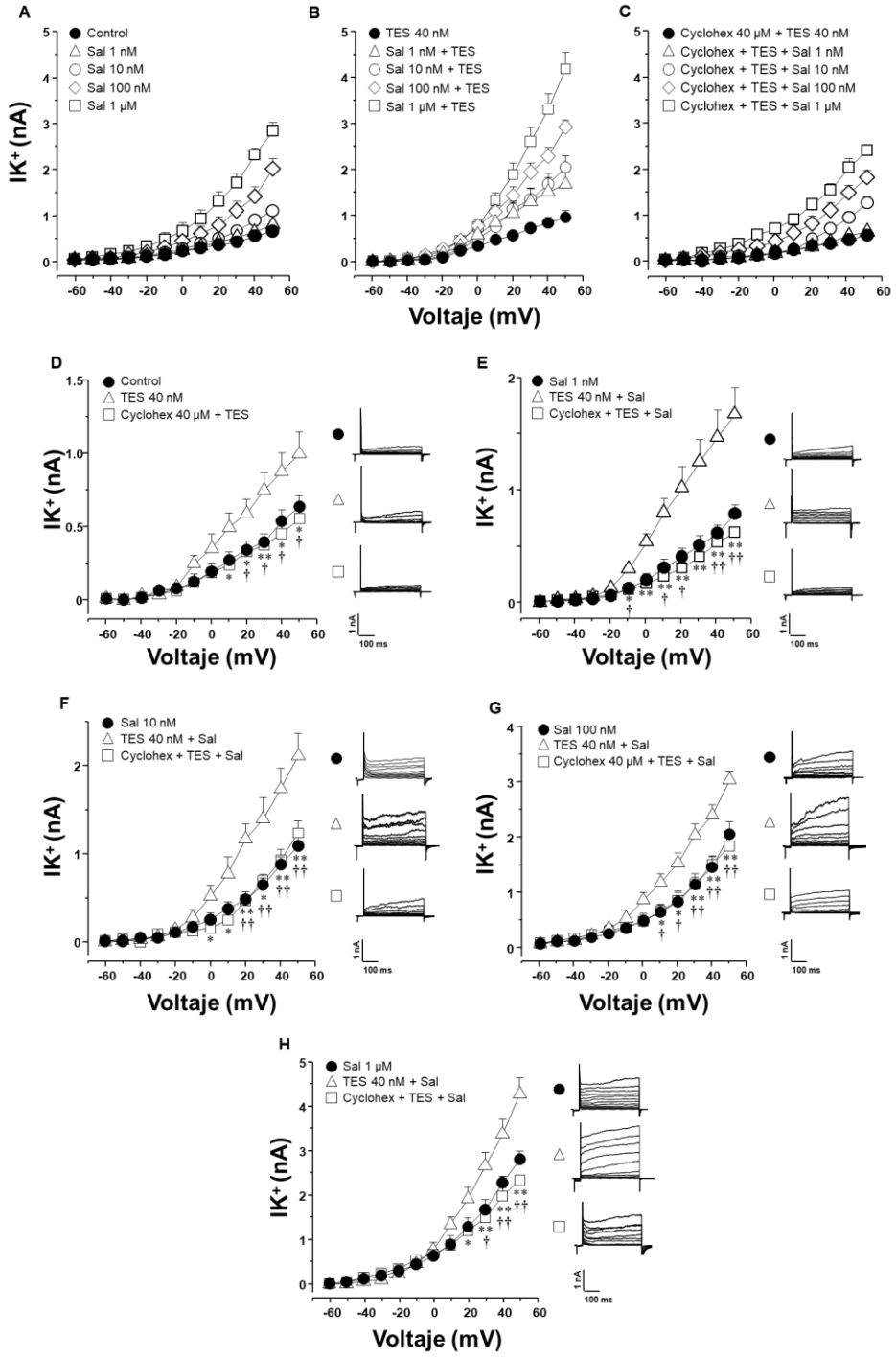

Figura 9. La cicloheximida (Cyclohex, 40 µM, inhibidor de la síntesis de proteínas) bloquea el efecto de potenciación de la TES sobre las corrientes de K^+ (IK^+) inducidas por salbutamol. Las IK^+ fueron evocadas por una despolarización seriada

de -60 a 50 mV. **A)** Registro de una curva acumulativa con salbutamol (Sal, 1 nM-1 µM) en células de MLVA en condiciones control. **B)** Representación gráfica de la curva acumulativa con Sal en células incubadas durante 48 horas con TES 40 nM. **C)** Se muestran curvas acumulativas con Sal en células cultivadas con Cyclohex 40 µM 30 minutos previos a la incubación con TES. **D, E, F, G, H)** Ilustran el análisis estadístico de cada concentración de Sal en los diferentes grupos experimentales, incluyendo los experimentos con Cyclohex. En las figuras se observa como la TES aumenta las IK^+ en MLVA y la Cyclohex anula este efecto. Los insertos que se muestran en las figuras representan registros originales de electrofisiología. En **D**-**H** se utilizaron diferentes escalas en el eje de las Y, con el propósito de mostrar claramente el análisis estadístico. Los símbolos representan la media ± ESM. Se utilizó un análisis de varianza seguido de una prueba de Dunnett. *p<0.05, **p<0.01, †p<0.05, ††p<0.01 cuando son comparados los grupos contra TES 40 nM (**D**) o contra TES + Sal (**E**-**H**). n=6

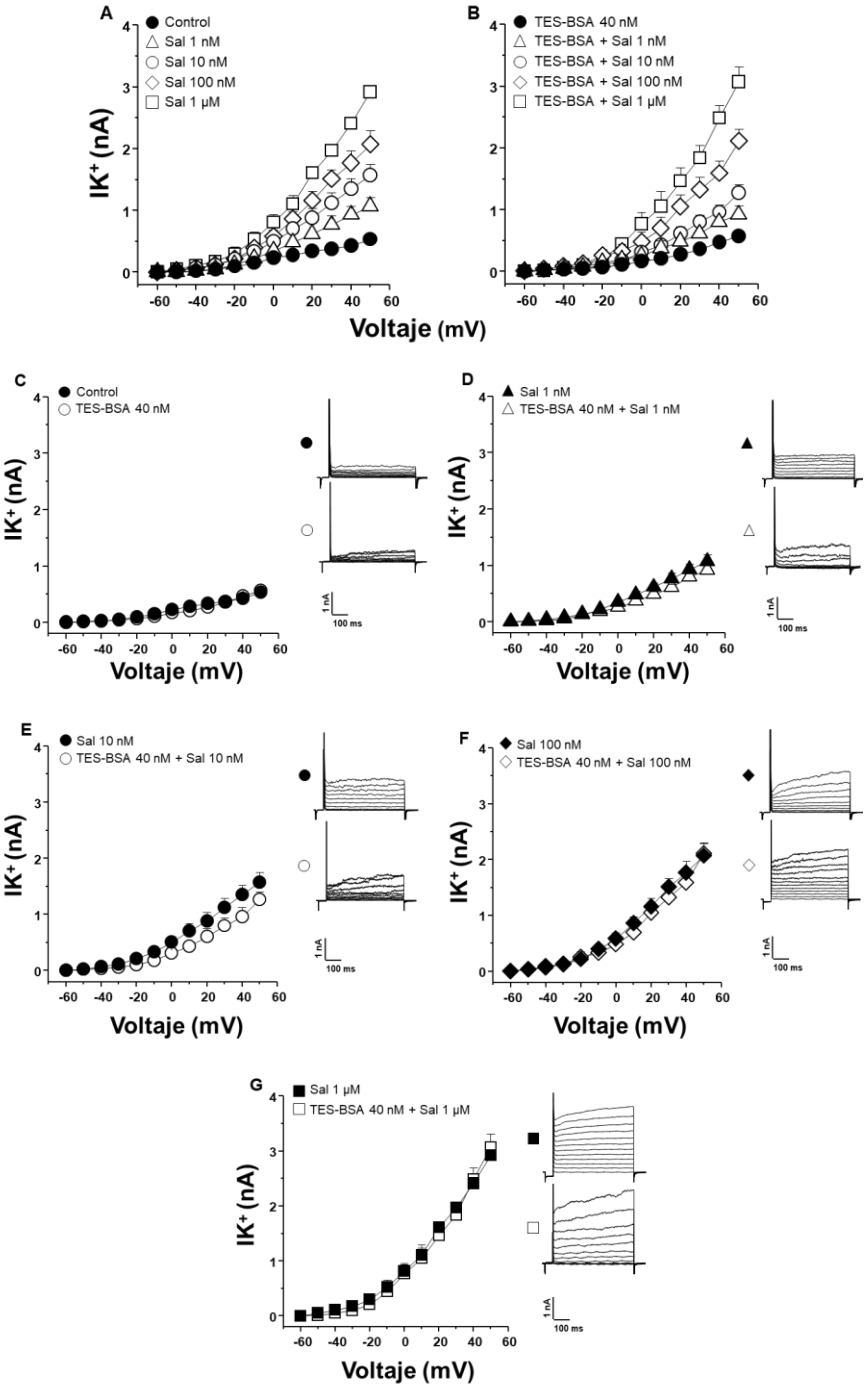

Figura 10. La testosterona conjugada con albúmina sérica bovina (TES-BSA, 40 nM) incubada por 48 horas en células cultivadas de MLVA de cobayo no modifica las corrientes de K^+ (IK^+) inducidas por salbutamol (Sal), corroborando que el fenómeno de la TES es a través de un efecto genómico. Las IK^+ fueron evocadas por una despolarización seriada de -60 a 50 mV. **A)** Registro de una curva acumulativa con salbutamol (Sal, 1 nM-1 µM) en células de MLVA en condiciones control. (n=6) **B)** Representación gráfica de la curva acumulativa con salbutamol (Sal, 1 nM-1 µM) en células incubadas durante 48 horas con TES-BSA 40 nM. Los símbolos representan la media ± ESM. **C, D, E, F, G)** Ilustran el análisis estadístico de cada concentración de Sal utilizada en el grupo control y en el expuesto a TES-BSA. Se observa en las figuras que la incubación de TES asociada a albúmina de suero bovino crónicamente no ejerce cambios sobre las IK^+ inducidas por pulsos despolarizantes y por el salbutamol. Los insertos que se muestran en las figuras representan registros originales de electrofisiología. Para el análisis estadístico se realizó una prueba t de Student. n=5.

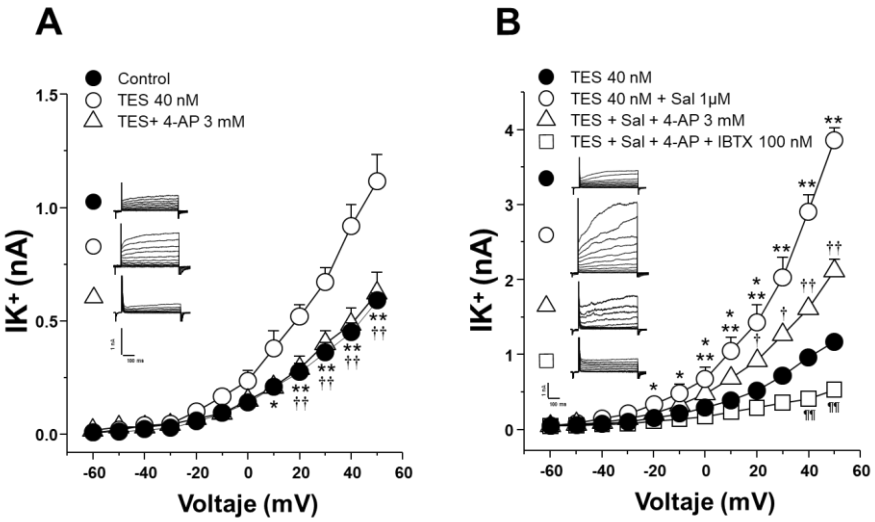

Figura 11. Los canales de K^+ (rectificador tardío) y los canales de K^+ activados por Ca^{2+} de alta conductancia son las principales proteínas involucradas en el efecto de potenciación de la testosterona (TES, 40 nM durante 48 h) sobre las IK^+ inducidas por pulsos despolarizantes y por salbutamol (Sal) en miocitos traqueales de cobayo. **A)** La TES incrementa las IK^+ inducidas por pulsos despolarizantes y este efecto fue abolido por 4-aminopiridina (4-AP, n=6), bloqueador de canales de K^+ del rectificador tardío. **B)** La incubación crónica del andrógeno incrementó las IK^+ inducidas por Sal y esto fue anulado por 4-AP e iberiotoxina (IBTX, n=8), un bloqueador específico de los canales de K^+ activados por Ca^{2+} de alta conductancia. Estos resultados sugieren que la TES está aumentando la expresión de ambos canales. Los símbolos representan la media ± ESM. En la figura A, * p <0.05, ** p <0.01 cuando son comparados los grupos TES + 4-AP (Δ) vs TES (o). †† p <0,01, comparando el grupo Control (●) vs TES (o). En la Figura B, en -20 mV, * p <0,05 al comparar el grupo TES + Sal (o) frente a TES + 4-AP + IBTX + Sal (□). En -10 mV, * p <0,05 cuando se comparan los grupos TES + Sal (o) frente a TES + 4-AP + IBTX + Sal (□) y TES (●). En 0mV, * p <0,05 al comparar el grupo TES + Sal (o) frente a TES (●) y ** p <0,01 al comparar TES + Sal (o) frente a TES + 4-AP + IBTX + Sal (□). En 10mV, * p <0.05 comparando TES + Sal (o) vs TES + 4-AP + Sal (Δ) y ** p <0.01 al comparar TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□) y grupos de TES (●). En 20mV, * p <0.05 cuando se comparan TES + Sal (o) vs TES + 4-AP + Sal (Δ) y ** p <0.01 comparando TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□) y el grupo de TES (●). † p <0.05, comparando el grupo TES + 4-AP + Sal (Δ) vs TES (●). En 30mV, ** p <0.01 al comparar el grupo de TES + Sal (o) vs TES + 4-AP + Sal (Δ), TES (●) y el grupo de TES + 4-AP + IBTX + Sal (□). † p <0.05, comparando el grupo TES + 4-AP + Sal (Δ) vs TES (●). En 40mV, ** p <0.01 cuando se compara TES + Sal (o) vs TES + 4-AP + Sal (Δ), TES (●) y TES + 4-AP + IBTX + Sal (□). †† p <0.01, al comparar TES + 4-AP + Sal (Δ) vs TES (●) y ¶¶ p <0.01 entre el grupo TES (●) vs TES + 4-AP + IBTX + Sal (□). En 50 mV, ** p <0,01 comparando los grupos TES + Sal (o) frente a TES + 4-AP + Sal (Δ), TES (●) y TES + 4-AP + IBTX + Sal (□). †† p <0,01, al comparar TES + 4-AP + Sal (Δ) vs TES (●) y ¶¶ p <0,01 entre el grupo TES (●) vs TES + 4-AP + IBTX + Sal (□). En la Figura A, se utilizó el análisis de varianza unidireccional seguido de la prueba de comparación múltiple Dunnett y en la Figura B se realizó un análisis de varianza unidireccional seguido de la prueba Student-Newman-Keuls de comparación múltiple.

La incubación crónica de testosterona en el músculo liso de la vía aérea aumenta la expresión del receptor adrenérgico β_2.

Está muy bien reportado que la vía de señalización del salbutamol es a través de la activación de su receptor adrenérgico β_2 que estimula la cascada de señalización de la adenilato ciclasa-AMPc. De acuerdo a los resultados anteriores, la incubación crónica de TES favoreció la relajación y potenció las IK^+ inducidas por salbutamol. Estos efectos fueron abolidos al utilizar un antagonista del RA, sugiriendo que el efecto de la TES es a través de una vía genómica. De manera que, para determinar la regulación positiva de la vía de señalización adrenérgica β_2, se realizaron experimentos para confirmar la presencia y el posible aumento en la expresión de las proteínas $RA\beta_2$ y adenilato ciclasa (AC) derivado de la incubación de TES. En el MLVA, se encontró presente el $RA\beta_2$ al realizarse inmunofluorescencia y el control negativo no mostró marca corroborando su especificidad contra esta proteína (Figura 12A, C). Se observó un incremento en la fluorescencia cuando los tejidos fueron incubados con TES 40 nM por 48 horas. (Figura 12B). En la Figura 12 A-C se muestra al $RA\beta_2$ en color verde, la α-actina en color rojo y los núcleos en azul. Al realizar el traslape de los distintos paneles se observó la localización conjunta del $RA\beta_2$ y la α-actina (en color amarillo), corroborando que el receptor adrenérgico está presente en el músculo liso (Figura 12B). Además, la expresión de ARNm del $RA\beta_2$, así como la expresión de la proteína se incrementaron mediante la incubación de TES (Figura 13A, B), lo cual fue revertido mediante flutamida (Figura 13B). Esto confirma que la exposición crónica a TES promueve la regulación positiva del $RA\beta_2$ a través de una acción genómica. En otro grupo de experimentos, se realizó inmunofluorescencia en tejidos incubados con y si TES para adenilato ciclasa 6 (la isoforma más prevalente en el MLVA). Las imágenes mostraron que no existe un aumento en la fluorescencia para esta enzima en anillos traqueales incubados con TES cuando se comparan con el grupo control (Figura 14). Estos resultados sugieren que el aumento en la relajación y las corrientes de K^+ inducidas por salbutamol están mediados por un aumento en la expresión del $RA\beta_2$ y no de la AC.

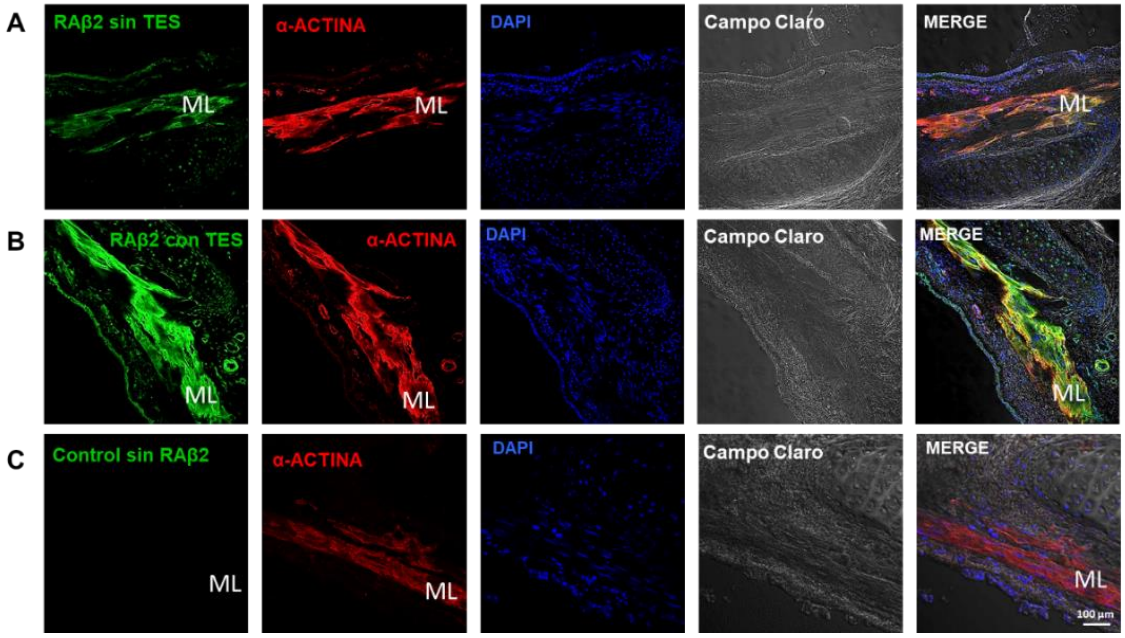

Figura 12. La testosterona (TES) regula positivamente al receptor adrenérgico β_2 en el MLVA. Inmunofluorescencia del receptor adrenérgico β_2 ($RA\beta_2$) incubado sin TES (**A**), con TES 40 nM 48 horas (**B**) y el control sin el anticuerpo del $RA\beta_2$ (**C**) en tráqueas de cobayo. La primera columna muestra la inmunoreactividad para el $RA\beta_2$ (verde) en tejidos controles **(A)** e incubados con TES **(B).** La fluorescencia no se observa en ausencia del anticuerpo para $RA\beta_2$ (control negativo) **(C).** La segunda y tercera columna ilustran la α-actina del MLVA (en rojo) y el núcleo (DAPI, en azul), la cuarta columna es el campo claro y la quinta es el traslape (MERGE), en donde se superponen las 4 imágenes. ML = músculo liso.

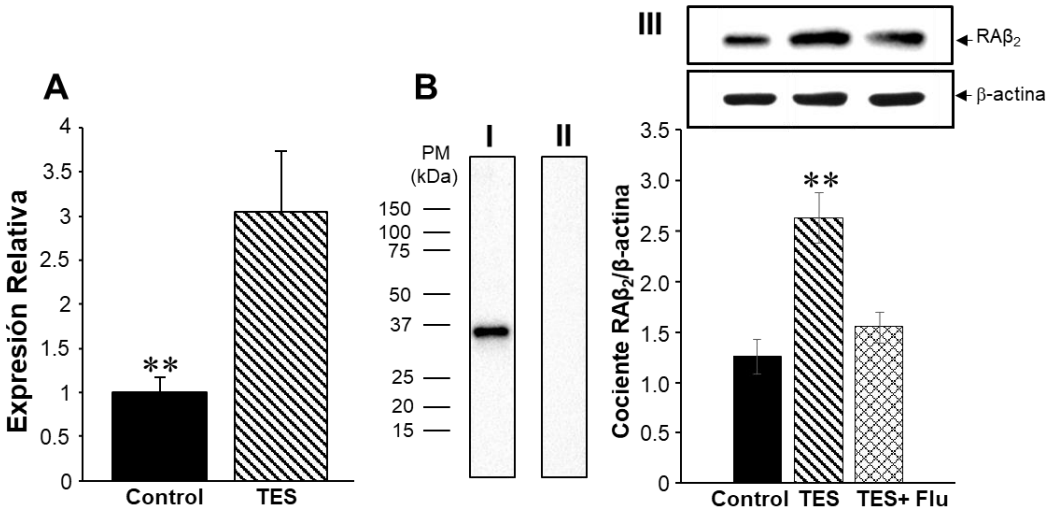

Figura 13. La testosterona (TES, 40 nM durante 48 horas) aumenta la expresión del receptor adrenérgico β_2 ($RA\beta_2$) en el músculo liso traqueal de cobayo. **A)** Gráfico de barras que ilustra la expresión relativa del ARNm del $RA\beta_2$ detectado por PCR cuantitativo analizado con el método $2^{-\Delta\Delta CT}$ y su aumento significativo inducido por TES, n = 4. **B)** Análisis del Western blot sobre la expresión del $RA\beta_2$; **(I)** se ilustra una banda de alrededor de 37 kDa correspondiente al peso molecular del $RA\beta_2$ y **(II)** el control negativo sin anticuerpo. **(III)** Gráfica de barras de la densitometría que ilustra el aumento en la expresión del $RA\beta_2$ cuando los tejidos fueron tratados con TES y la abolición de este fenómeno con el tratamiento con flutamida 30 minutos antes de la incubación del tejido con TES. ** $p < 0.01$, n = 3. Las barras representan la media ± ESM.

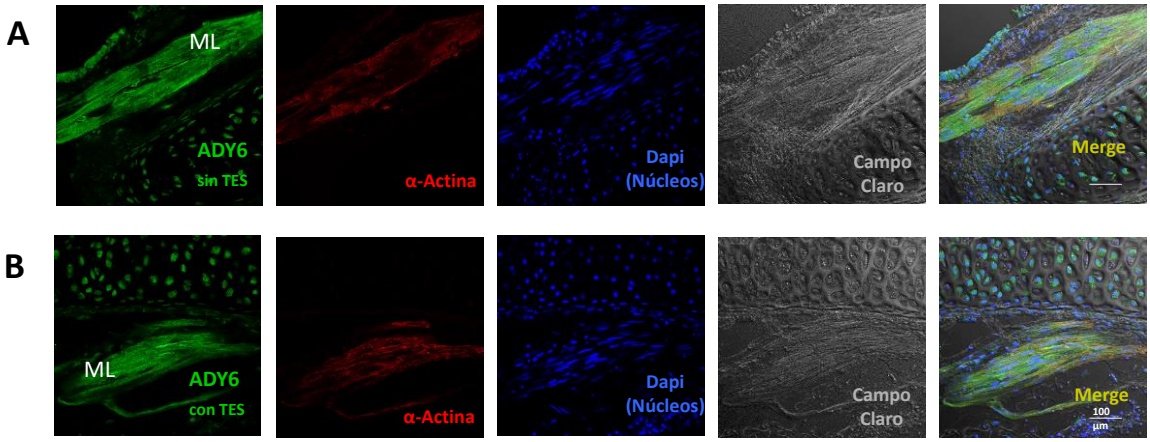

Figura 14. El efecto de la testosterona (TES) sobre el incremento en la respuesta a salbutamol no está relacionado con la adenilato ciclasa 6 (ADY6) en el MLVA de cobayo. La primera columna muestra la inmunoreactividad para adenilato ciclasa 6 (ACY6, verde) en tráqueas de cobayo sin TES **(A)** e incubados con TES **(B).** La segunda y tercera columna ilustran la α-actina del MLVA (en rojo) y los núcleos (DAPI, en azul), la cuarta columna es el campo claro y la quinta es el traslape (MERGE), en donde se superponen las 4 imágenes. Se observa que no hay un aumento evidente en la fluorescencia cuando los tejidos fueron tratados con TES. ML = músculo liso.

DISCUSIÓN

La prevalencia del asma en la infancia es más frecuentemente en niños que en niñas, tendencia que se revierte durante la pubertad siendo más severa en mujeres jóvenes (Holgate et al., 2015; Melgert et al., 2007; Townsend et al., 2012; Vink et al., 2010). Distintos autores han propuesto que las diferencias en la presentación del asma entre sexos es en parte por el aumento de los niveles séricos de testosterona (TES, 6-50 nM) durante la pubertad en varones (Canguven & Albayrak, 2011; de Marco et al., 2000; Postma, 2007; Zannolli & Morgese, 1997). Diversos trabajos de investigación han explorado los efectos de los andrógenos sobre el músculo liso de la vía aérea. En cuanto a los efectos no genómicos de la TES (a concentraciones fisiológicas) sobre el MLVA, se ha reportado que favorece la relajación al bloquear a los L-VDCCs y al receptor de IP_3 provocando la disminución del Ca^{2+} intracelular (Montaño et al., 2014; Montaño et al., 2018; Perusquia et al., 2015). Por otro lado, los efectos genómicos explorados de la TES en MLVA se han enfocado en la inflamación de la vía aérea, rasgo característico del asma. En este sentido, se reportó que la TES regula negativamente la producción de citocinas proinflamatorias tipo 2 e induce la disminución de la expresión de IL-17A (Fuseini et al., 2018; Laffont et al., 2017).

Anteriormente se reportó que los andrógenos, a través de efectos no genómicos y a concentraciones suprafisiológicas, potenciaban la relajación inducida por agonistas adrenérgicos β (Bordallo et al., 2008; Foster et al., 1983). En este sentido, Bordallo et al demostraron que el uso de concentraciones altas de 5α-DHT (100 μM) potenciaba la relajación inducida por salbutamol (Bordallo et al., 2008). Sin embargo, la caracterización farmacológica de los mecanismos implicados en este fenómeno, mostró que la potenciación no fue modificada por un antagonista no selectivo de los RAβ (propranolol) o por el antagonista selectivo del $RA\beta_2$, ICI-118,551. Por lo tanto, ellos excluyeron la posibilidad de que el andrógeno estuviera ejerciendo su efecto a través de la vía adrenérgica β. Por otro lado, se postuló que la TES potencia la relajación inducida por isoprenalina (agonista adrenérgico β inespecífico) en bronquio de cerdo, y esto pudiera estar relacionado a la inhibición de la COMT o a la recaptura extraneuronal (Foster et al., 1983). Estos trabajos, nos dieron la pauta para investigar si la TES, a concentraciones fisiológicas, potencia la

relajación inducida por salbutamol (agonista adrenérgico β_2) a través de un efecto genómico en el MLVA.

Uno de los criterios para definir un efecto genómico es el tiempo en el que el andrógeno permanece en el tejido. En este contexto, para evaluar el efecto genómico de la TES sobre la relajación inducida por salbutamol y la potenciación de las corrientes de K^+, tejidos y miocitos traqueales fueron incubados con TES durante 48 horas; lo que representa un tiempo suficiente para inducir la síntesis proteica (Ma et al., 2005). Los resultados indican que la exposición crónica con TES, a concentraciones fisiológicas, favorece la relajación inducida por salbutamol en anillos traqueales de cobayo (Figura 4A). Debido al tiempo de exposición con TES, es probable que el fenómeno de potenciación sobre la relajación inducida por salbutamol sea a través de un efecto genómico. Además, se encontró que la concentración más alta utilizada de TES (40 nM) presentaba una respuesta máxima de relajación mayor en comparación a los tejidos incubados con TES 20 nM. Por este motivo, decidimos utilizar 40 nM de TES en los experimentos posteriores. Por otro lado, se ha propuesto que los efectos no genómicos de la TES se caracterizan por ser rápidos (segundo a minutos) y por llevarse a cabo sobre la membrana plasmática. En este sentido, se ha documentado que la TES puede activar diversas proteínas de membrana como la GPRC6A, un miembro de la familia de receptores acoplados a proteínas G y a ZIP9, un transportador de zinc de la familia ZIP (Berg et al., 2014; Pi et al., 2015). Asimismo, se demostró que estos receptores pueden desencadenar distintas vías de señalización a través de proteínas Gs, Gi y/o Gq11, pudiendo en algún punto de la cascada activar la trascripción génica. Además, estos receptores estimulan a las cinasas reguladas por señales extracelulares 1⁄2 (ERK 1/2), implicadas en diferentes procesos de fosforilación y sus acciones dependen del tipo celular (Clemmensen et al., 2014; Thomas et al., 2018). Los experimentos realizados con incubaciones cortas de TES descartan que el efecto observado de potenciación en la relajación del MLVA se deba a un efecto no genómico (un efecto rápido que no involucra al receptor androgénico) ya que las exposiciones breves a TES durante 15 o 60 minutos no modificaron las respuestas de relajación de salbutamol (Figura 5). Además, encontramos que la exposición crónica del MLVA a TES-BSA (una molécula impermeable) no alteró la relajación inducida por salbutamol, descartando la posibilidad de que la TES actúe sobre alguno de los receptores

membranales mencionados anteriormente (Figura 4C). Siguiendo la línea de investigación y con el propósito de definir, si el efecto observado de la TES efectivamente fuera mediante un efecto genómico, se realizaron experimentos con flutamida (antagonista del receptor androgénico; 10 µM). Los experimentos revelaron que este antagonista anula la potenciación en la relajación del MLVA inducida por el salbutamol a través de la incubación con TES 40 nM durante 48 horas (Figura 6). Estos resultados apuntan fuertemente a que el fenómeno que observamos es de carácter genómico ya que involucra tiempos largos de acción y la unión del andrógeno a su receptor, lo que favorecería la transcripción y la síntesis proteica. Por ende, nos propusimos estudiar si la TES regula positivamente la vía de señalización adrenérgica β_2 a través de un efecto genómico.

Es conocido que la activación de los RAβ_2 induce la relajación del MLVA mediante la vía de señalización de AMPc-PKA. En el MLVA, esta vía promueve la despolimerización de actina, promoviendo el acortamiento de la longitud de los filamentos de actina y la desfosforilación de la cadena ligera de miosina lo que produce relajación (Hirshman et al., 2001). En el RS, el fosfolambán (PLB) inhibe a la ATPasa de Ca^{2+} (SERCA) del retículo sarcoplásmico, encargada de bombear Ca^{2+} del citosol al interior de este almacén celular (McGraw et al., 2006; Sathish et al., 2008). La PKA al fosforilar a PLB, anula sus efectos inhibidores sobre SERCA y conlleva a la disminución de la concentración de Ca^{2+} intracelular (Masterson et al., 2011). Otro efecto de la PKA, es inhibir a la PLC$_\beta$ y por ende disminuir la síntesis de IP_3 (Billington & Penn, 2003). Además, bloquea la unión del IP_3 con su receptor en el RS (Schramm et al., 1995). También, la PKA puede fosforilar a los canales de K^+, aumentando su probabilidad de apertura y conduciendo a la hiperpolarización de la membrana (Campos-Bedolla et al., 2006; Jones et al., 1990). Es bien conocido que los canales de K^+ contribuyen de forma muy importante a la relajación del MLVA tras la activación del RAβ_2 (Campos-Bedolla et al., 2006; Huang et al., 1993; Jones et al., 1990). En este sentido, se realizaron experimentos de electrofisiología para evaluar si la TES estuviera ejerciendo un efecto de potenciación sobre las IK^+ inducidas por salbutamol. La exposición crónica de TES en las células de MLVA, aumentó las IK^+ inducidas por el protocolo de pulsos despolarizantes y por el protocolo de pulsos más salbutamol. De manera interesante este efecto de potenciación se anuló con flutamida (Figura 7), actinomicina D (Figura 8) y cicloheximida (Figura 9). De la misma manera que en los experimentos de relajación y con

la finalidad de descartar un posible efecto no genómico de la TES, se realizaron experimentos con TES-BSA. Las corrientes de K^+ inducidas por salbutamol no se vieron modificadas con el uso de esta molécula impermeable, lo que descarta la posibilidad de un efecto no genómico (Figura 10). Estos resultados sugieren que la TES aumenta las IK^+ inducidas por salbutamol a través de un efecto genómico. Además, el hecho que la TES potencie las corrientes de K^+ sin el estímulo de salbutamol sugiere un aumento no sólo en el receptor adrenérgico sino también un aumento en la expresión y/o actividad de los canales de K^+. Los principales canales de K^+ presentes en el MLVA son los K_{Ca} y los K_V (Adda et al., 1996; Boyle et al., 1992; Montaño et al., 2011). Nuestros resultados mostraron que 4-AP, un bloqueador de los K_V, anuló desde 0 mV hacia adelante el efecto de la TES (Figura 11A), lo que sugiere que la TES está aumentando la expresión génica de estos canales. Por otro lado, en el laboratorio se ha demostrado que en el MLVA de cobayo, los BK_{Ca} participan en la bronco-relajación inducida por serotonina y ATP (Campos-Bedolla et al., 2008; Montaño et al., 2011). En los experimentos en miocitos incubados con TES, el uso de 4-AP e IBTX (bloqueador de los BK_{Ca}) bloqueó el aumento en las IK^+ inducidas por salbutamol (Figura 11B). Estos resultados indican que ambos canales de K^+ están involucrados en el efecto de potenciación de la TES sobre la vía de señalización del salbutamol. Además, el efecto de la TES sobre las IK^+ fue revertido por el antagonista del receptor androgénico (flutamida). Por ende, es posible que la expresión génica de alguno de estos canales se encuentre aumentada debido a la exposición crónica de TES.

En un trabajo del 2011 realizado por Sun J. y colaboradores, demostraron que la TES mejoraba el rendimiento del miocardio al aumentar la expresión del receptor adrenérgico β_2 en músculo cardiaco y este efecto fue anulado por flutamida (Sun et al., 2011). Esta referencia nos dio la pauta para pensar en que el efecto genómico relacionado con los aumentos en la relajación del MLVA y las corrientes de K^+ inducidas por salbutamol, pudiera deberse al aumento del receptor adrenérgico β_2. En este contexto, encontramos que la expresión del receptor aumentaba cuando el MLVA fue incubado con TES y fue confirmado por imágenes de inmunofluorescencia (Figura 12) y experimentos de western blot y PCR cuantitativa (Figura 13). Por otro lado, en un trabajo del 2018 realizado por López-Canales y colaboradores, demostraron que, en el músculo liso vascular de la rata, la TES redujo la

expresión de adenilato ciclasa y por consiguiente disminuyó la vasorelajación mediada por el $RA\beta_2$ (Lopez-Canales et al., 2018). De forma interesante, los experimentos de inmunofluorescencia para adenilato ciclasa 6 (la isoforma más prevalente en el MLVA), no mostró diferencias cuando los tejidos fueron expuestos a TES (Figura 14). Estos resultados confirman que el aumento en la relajación del MLVA y en las IK^+ inducidas por salbutamol se debe al aumento de la expresión del $RA\beta_2$ y descartan la participación de otros componentes o mecanismos activados por el salbutamol como el de la adenilato ciclasa.

Estudios anteriores revelan que la TES favorece un mayor calibre de las vías aéreas, regula negativamente la inflamación y disminuye los síntomas del asma (Becklake & Kauffmann, 1999; Laffont et al., 2017; Muller et al., 2011; Ripoll et al., 2020; Sathish et al., 2015; Townsend et al., 2012). La regulación positiva del receptor adrenérgico β_2, de los K_V y los K_{Ca} (Figura 15), pueden añadirse con los efectos genómicos y no genómicos de la TES estudiados con anterioridad y servir como otra posible explicación sobre el estado favorable sobre la relajación del MLVA. Por todo lo anterior, los niveles plasmáticos de TES durante la pubertad pueden ser relevantes sobre la función respiratoria de los hombres. No obstante se necesita mayor investigación sobre la regulación positiva de los canales de K^+ y corroborar si existe un aumento en la expresión génica de estas proteínas. Además, resultaría interesante investigar si los efectos no genómicos de la TES sobre el MLVA como el bloqueo del L-VDCC, del receptor de IP_3 y los SOCC, pudieran tener además un componente genómico donde la TES regule su expresión a la baja favoreciendo la relajación del MLVA y la disminución de la sintomatología asmática. Todos estos supuestos forman parte de las perspectivas de este trabajo de tesis y representan una continuidad coherente en esta línea de investigación.

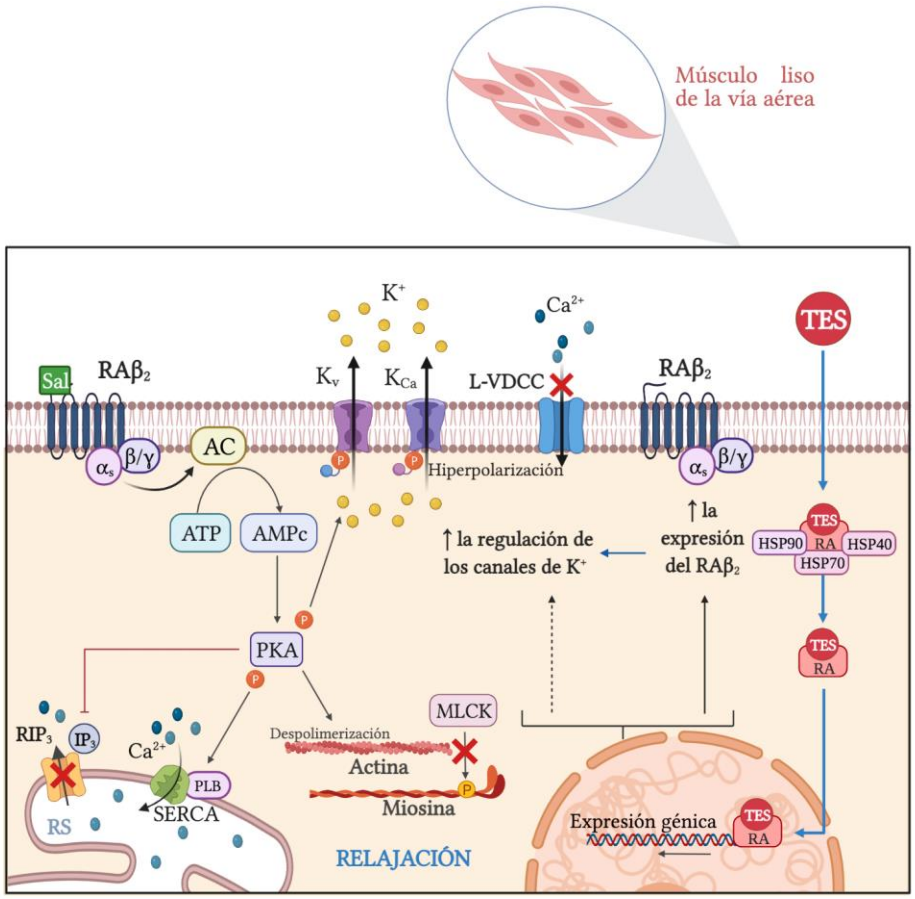

Figura 15. Representación esquemática del efecto genómico de la testosterona (TES) sobre la relajación inducida por salbutamol en el músculo liso de las vías aéreas. Los agonistas adrenérgicos β_2, como el salbutamol (Sal), actúan sobre los receptores adrenérgicos β_2 ($RA\beta_2$) que están acoplados a una proteína Gs compuesta por 3 subunidades: α, β y γ. La subunidad α_s activa a la adenilato ciclasa (AC) y cataliza la formación adenosín monofosfato cíclico (AMPc) a través de adenosín trifosfato (ATP). El AMPc activa a la proteína cinasa A (PKA) que fosforila múltiples sustratos para disminuir la concentración de calcio celular. La PKA fosforila a los canales de K^+ y aumenta su probabilidad de apertura (Campos-Bedolla et al., 2006; Jones et al., 1990; Wang & Kotlikoff, 1996). El K^+ saliente promueve la hiperpolarización de la membrana celular que impide el aumento de las concentraciones de Ca^{2+} intracelular a través del cierre de los canales de Ca^{2+} dependientes de voltaje tipo L (L-VDCC), favoreciendo la relajación del MLVA. La disminución del Ca^{2+} reduce la activación de la cinasa de cadena ligera de miosina (MLCK) favoreciendo así la desfosforilación de la cadena ligera de miosina lo que produce la relajación. Además, la vía de AMPc/PKA favorece la despolimerización de actina, promoviendo el acortamiento de la longitud de los filamentos de actina (Hirshman et al., 2001). En el retículo sarcoplásmico (RS), fosfolambán (PLB) inhibe a la ATPasa de Ca^{2+} del retículo sarcoplásmico (SERCA), encargada de bombear Ca^{2+} del citosol al interior de este almacén celular (McGraw et al., 2006; Sathish et al., 2008). La PKA al fosforilar a PLB, reduce sus efectos inhibidores sobre SERCA y favorece la disminución de la concentración de Ca^{2+} intracelular (Masterson et al., 2011). Asimismo, la PKA inhibe la vía de señalización de la fosfolipasa C_β (PLC_β)-inositol trifosfato (IP_3) y por consiguiente, disminuye la salida de Ca^{2+} del RS. Todas las acciones de la PKA favorecen la relajación del MLVA a través de la disminución de las concentraciones de Ca^{2+} intracelular. La vía genómica de la testosterona (TES) implica que el andrógeno traspase la membrana plasmática, se una con su receptor androgénico (RA) y se disocien las proteínas chaperonas del receptor. El complejo TES-RA se transloca al núcleo donde se dimeriza y une a los elementos de respuesta androgénica (ARE) para modular la transcripción génica y la síntesis de proteínas (Bennett et al., 2010; Li & Al-Azzawi, 2009). La estimulación con TES aumenta la expresión del $RA\beta_2$ en el MLVA de cobayo y regula positivamente a la alta los canales de K^+ activados por Ca^{2+} (K_{Ca}) y los canales de K^+ rectificadores tardíos (K_V). Finalmente, la TES a través de un efecto genómico, favorece la relajación inducida por salbutamol.

CONCLUSIÓN

La exposición crónica del MLVA, a una concentración fisiológica de TES, promueve la regulación positiva del receptor adrenérgico β_2, los K_V, y en consecuencia, favorece la respuesta de relajación mediada por agonistas adrenérgicos β_2.

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ANEXOS

Anexo 1. Artículo requerido para la obtención del grado.

Carbajal-García A, Reyes-García J, Casas-Hernández MF, Flores-Soto E, Díaz-Hernández V, Solís-Chagoyán H, Sommer B, Montaño LM. Testosterone augments β_2 adrenergic receptor genomic transcription increasing salbutamol relaxation in airway smooth muscle. Mol Cell Endocrinol. 2020 Jun 15;510:110801. doi: 10.1016/j.mce.2020.110801. Epub 2020 Apr 8. PMID: 32278021.

Anexo 2. Artículo de revisión.

Carbajal-García A, Reyes-García J, Montaño LM. Androgen Effects on the Adrenergic System of the Vascular, Airway, and Cardiac Myocytes and Their Relevance in Pathological Processes. Int J Endocrinol. 2020 Nov 12;2020:8849641.

Anexo 3. Capítulo de libro.

Reyes-García J, Montaño LM, **Carbajal-García A**, Wang YX. Sex Hormones and Lung Inflammation. Adv Exp Med Biol. 2021;1304:259-321.

Anexo 4. Artículo de revisión.

Reyes-García J, **Carbajal-García A**, Montaño LM. Transient receptor potential cation channel subfamily V (TRPV) and its importance in asthma. Eur J Pharmacol. 2022 Jan 15;915:174692.

Anexo 5. Artículo de revisión.

Reyes-García J, **Carbajal-García A**, Di Mise A, Zheng YM, Wang X, Wang YX. Important Functions and Molecular Mechanisms of Mitochondrial Redox Signaling in Pulmonary Hypertension. Antioxidants (Basel). 2022 Feb 28;11(3):473.

Anexo 1. Artículo requerido para la obtención del grado.

Carbajal-García A, Reyes-García J, Casas-Hernández MF, Flores-Soto E, Díaz-Hernández V, Solís-Chagoyán H, Sommer B, Montaño LM. Testosterone augments β_2 adrenergic receptor genomic transcription increasing salbutamol relaxation in airway smooth muscle. Mol Cell Endocrinol. 2020 Jun 15;510:110801. doi: 10.1016/j.mce.2020.110801. Epub 2020 Apr 8. PMID: 32278021.

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Testosterone augments β_2 adrenergic receptor genomic transcription increasing salbutamol relaxation in airway smooth muscle

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ABSTRACT

Androgens in asthmatic men may be linked to asthma severity, acting via nongenomic and genomic effects. This ailment affects boys more than girls during infancy, and this proportion reverses in puberty. Plasmatic androgen concentration in young men increases at this age and might be related to lower asthma symptoms. Nongenomic actions occur in a brief period and are independent of the androgen receptor (AR), while genomic effects depend on AR, take hours-days and are modified by transcription or protein synthesis inhibitors. Guinea pig tracheas chronic incubation with testosterone (TES, 40 nM, 48 h) potentiates salbutamol-induced relaxation, an effect that was reversed by flutamide, not observed when tissues were pre-incubated with TES-bovine serum albumin (TES-BSA) nor when tissues were preincubated with TES for 15–60 min. In tracheal myocytes, TES chronic incubation increases salbutamol-induced K^+ currents (IK^+), an effect that was also reversed by flutamide, actinomycin D and cycloheximide and not seen with TES-BSA. The increment in IK^+ was blocked by 4-aminopyridine and iberiotoxin, indicating that delayed rectifier K^+ and high-conductance Ca^{2+} activated K^+ channels were involved in the TES potentiation effect. Immunofluorescence studies showed that chronic TES augmented the β_2 adrenergic receptor (β_2-AR) expression in ASM and this finding was corroborated by q-PCR and Western blot assays. β_2-AR affinity for salbutamol after TES incubation was increased. In conclusion, chronic exposure to physiological TES concentration of the guinea pig ASM promotes β_2-AR upregulation favoring β_2 adrenergic responses and probably limiting the severity of the asthmatic exacerbations in teenage boys and men.

1. Introduction

In men, approximately 95% of testosterone (TES), the principal sex hormone, is produced by the testes and secreted by the Leydig cells (Lucas-Herald et al., 2017) to maintain a plasmatic concentration of 5–50 nM (Muller et al., 2011; Townsend et al., 2012); this androgen results in nongenomic and genomic actions. In general, androgen nongenomic actions in airway smooth muscle (ASM) occur in a brief period (seconds to minutes) independently of the androgen receptor (AR) occupancy by these male sex steroids, are not modified by synthesis or transcription inhibitors, and do not depend on a functional nucleus (Espinoza et al., 2013; Flores-Soto et al., 2017; Montaño et al., 2018).

In ASM, ***nongenomic*** actions of androgens were initially studied in precontracted rabbit tracheal smooth muscle that was relaxed by TES in an epithelium-dependent way related to nitric oxide. Furthermore, this androgen effect was characterized as nongenomic because flutamide, an AR antagonist, and actinomycin D, a DNA transcription inhibitor, did not change it. Additionally, non-permeant TES-bovine serum albumin (BSA-TES) also diminished the contraction induced by a cholinergic agonist (Kouloumenta et al., 2006). Shortly thereafter, androgens were found to relax precontracted guinea pig and bovine tracheal smooth muscle in an epithelium-independent way, through blocking membranal L-type voltage dependent Ca^{2+} channels (Bordallo et al., 2008). Recently, our group showed that in guinea pig tracheal smooth muscle, TES-induced relaxation is concentration dependent; TES blocks L-type voltage-dependent Ca^{2+} channels (Montaño et al., 2014; Perusquía et al., 2015) and inositol triphosphate receptors located in

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the sarcoplasmic reticulum (Montaño et al., 2018) at physiological nanomolar concentrations. Meanwhile, at micromolar (supraphysiological) levels, which probably produce non-specific effects, it blocks capacitative Ca^{2+} entry through store-operated Ca^{2+} channels activated by sarcoplasmic reticulum-Ca^{2+} depletion, induces PGE_2 synthesis (Flores-Soto et al., 2017; Perusquía et al., 2015), and decreases ASM tension at rest and intracellular basal Ca^{2+} concentration by blocking L-type voltage-dependent Ca^{2+} channels and transient receptor potential canonical 3 (Flores-Soto et al., 2017). Furthermore, ***nongenomic*** relaxation induced by androgens in ASM does not depend on K^+ channels opening since increasing tetraethylammonium chloride concentrations, a non-selective K^+ channel blocker, did not affect it (Montaño et al., 2014). Moreover, 100 μM of 5α-dihydrotestosterone (a reduced metabolite of TES) at a supraphysiological concentration, was reported to potentiate the relaxation induced by salbutamol in bovine tracheal ASM, and this response was not related to an androgen interaction with the β_2 adrenergic receptor (Bordallo et al., 2008). Although the authors do not define the mechanisms involved in androgen-induced salbutamol potentiation, it may be related to a non-specific effect due to the high androgen concentration used. In a previous study in pig bronchus, it was found that 40 μM of TES caused 6.7-fold potentiation of isoprenaline-induced relaxation. These authors concluded that the TES-induced increment in the potency of catecholamines could be related to the inhibition of catechol-O-methyl transferase (COMT) or extraneuronal uptake (Foster et al., 1983). In this context, a study in the isolated rat heart, reported that TES, at micromolar concentrations, was a potent and selective inhibitor of the extraneuronal $uptake_2$ for catecholamines (Salt, 1972). Therefore, a possible explanation of the potentiation in the salbutamol-induced relaxation produced by 100 μM of 5α-dihydrotestosterone in the bovine tracheal ASM could be related to the inhibition of the $uptake_2$ and COMT in this tissue.

Meanwhile, ***genomic*** actions imply TES permeation through the cell membrane into the cytosol where the AR is located. This receptor has been found in several smooth muscles, including ASM (Zarazua et al., 2016). TES binds to the AR, inducing its dissociation from its chaperone proteins and forming a complex that transfers to the nucleus to begin gene transcription and later protein synthesis, which occurs in hours (Lucas-Herald et al., 2017). At nanomolar concentrations, androgen ***genomic*** actions in airways have been scantly investigated, and research mainly focuses on airway inflammation since TES negatively regulates type 2 inflammation carried out by group 2 innate lymphoid cells (ILC2s) and $CD4^+$ Th2 cells (Th2) (Fuseini et al., 2018; Laffont et al., 2017). In asthmatic patients, these lymphocytes participate in allergic airway inflammation mediated by eosinophils, basophils, mast cells, macrophages, increased atopy (augmented immunoglobulin E, IgE), hyperresponsiveness, and remodeling of the airways (Fahy, 2015; Lambrecht and Hammad, 2015; Yung et al., 2018). Nevertheless, not all asthmatic patients have type 2 airway inflammation. Some have IL-17A mediated neutrophil inflammation with mucus production (de Marco et al., 2000; Evasovic and Singer, 2019; Yung et al., 2018), and this cytokine expression is decreased by nanomolar TES concentration, consequently reducing them (Fuseini and Newcomb, 2017; Fuseini et al., 2018). Recently, it was found that AR activation mediates the regulation of intracellular Ca^{2+} increment induced by pro-inflammatory cytokines in human ASM (Kalidhindi et al., 2019).

Since previous studies using supraphysiological concentrations of androgens evidencing their **nongenomic** actions showed that augmented response to β_2 adrenergic agonists was dependent on inhibition of COMT/or extraneuronal uptake and did not include the β_2 adrenergic participation, herein we aimed to explore **genomic** actions induced by TES at nanomolar concentrations on the expression of β_2 adrenergic receptor and its functionality in ASM. Additionally, it is well known that the activation of the β_2 adrenergic receptor in ASM favors opening probability of different K^+ channels contributing to tissue hyperpolarization and relaxation (Campos-Bedolla et al., 2006; Johnson, 1998; Tanaka et al., 2003). Therefore, we also studied the effect of TES on salbutamol-induced K^+ currents and defined the K^+ channels involved.

2. Methods

2.1. Experimental animals

Healthy male Hartley guinea pigs weighing between 350 and 400 g were used. Animals were bred in our institutional animal facilities with standard conditions: filtered conditioned air, 21 ± 1 °C, 50–70% humidity and sterilized bed. They were fed with Harlan® pellets and sterilized water at libitum. The Scientific and Bioethics committees of the Facultad de Medicina, Universidad Nacional Autónoma de México approved the experimental protocol. The Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training published by the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) were followed during the experimental proceedings as well as the Mexican National Laws on Animal Protection and the General Health Law Related to Health Research (NOM-062-Z00-1999).

2.2. Airway smooth muscle contraction experiments

Animals were deeply anesthetized with pentobarbital sodium (35 mg/kg, i.p.) and exsanguinated afterward. After carefully dissecting the trachea free of connective tissue, eight rings were obtained. Each ring was placed in a 2-ml Eppendorf tube containing 1 ml of Krebs solution (in mM): 118 NaCl, 25 $NaHCO_3$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 2 $CaCl_2$, saturated with 30 min continuous bubbling with 5% CO_2 in oxygen to maintain pH at 7.4. Some tissues were added with 20 or 40 nM of TES, and all tubes were sealed and placed at 9 °C for 48 h. Another set of tissues was incubated with either 40 nM of TES-BSA, an impermeable macromolecular complex or 10 μM of flutamide (an AR antagonist), under the same condition. The purpose of this procedure was to lower tissue metabolism and allow tissue preservation. Interestingly, it has been demonstrated that in Chinese hamster ovary cell monolayers, cold shock (around 0 °C) for 20 min reduces the protein synthetic capacity by 84% and this effect was completely reversed after maintaining the cells at 28 °C for 20 min. Furthermore, transcription and translation processes were not affected by this procedure (Stapulionis et al., 1997). Therefore, our tissues incubated at 9 °C during 48 h, should have a proper protein synthetic rate (higher than 16%). Afterward, each ring was hung in a 5 ml organ bath with Krebs solution at 37 °C, and continuously bubbled with 5% CO_2 in oxygen. Tracheal rings were tied with silk thread to an isometric force transducer (model FT03; Grass Instruments, West Warwick, RI, USA) that was connected to a signal conditioner (CyberAmp 380, Axon Instruments, Foster City, CA, USA) and an analog-to-digital interface (Digidata 1440A; Axon). Preparations were set at a resting tension of 1 g for 60 min at the beginning of the experiment. After this period, tissues were conditioned, and contractile apparatus optimized by three consecutive KCl stimulations of 60 mM; this procedure lasted for approximately 4 h. Tissues not chronically exposed to TES were incubated with this androgen (20 or 40 nM) for 15 min, while others remained in the presence of TES during the whole experiment (60 min). All tissues were pre-contracted with 10 μM of histamine, and once the maximal contraction was reached, a cumulative concentration-response curve with salbutamol (1 nM-1 μM) was made. Some tissues were incubated during 15 min with ICI-118.551, a selective antagonist of β_2 adrenergic receptor (1, 3.2, 10, 32, and 100 nM), before the cumulative concentration-response curve with salbutamol.

2.3. Patch-clamp studies

Guinea pig tracheas free of epithelium and connective tissues, were

placed in 5 ml of Hanks solution containing 2 mg of cysteine and 0.04 U/ml papain to isolate myocytes; pH was adjusted to 7.4 with 1 M of $NaHCO_3$, and preparations were incubated for 10 min at 37 °C. Leibovitz's solution was used to dispose of enzyme excess, and myocytes were then placed in solution with 5 mg/ml of collagenase type I for ~10 min at 37 °C. Tissue was gently dispersed by mechanical agitation until detached cells were observed, and Leibovitz's solution was used to stop enzymatic activity. Cells were then centrifuged at 600 rpm and 20 °C for 5 min, and the supernatant was discarded. This last step was repeated once. After re-suspending the cell pellet in minimum essential medium containing 5% fetal bovine serum, 2 mM of L-glutamine, 10 U/ml of penicillin, 10 μg/ml of streptomycin, and 15 mM of glucose, cells were cultured by plating them on rounded cover slips coated with sterile rat tail collagen placed in a 6 multi-well box, and some wells were added with 40 nM of TES or 3.2 μM of flutamide, an antagonist of the AR, 30 min before 40 nM of TES and grown at 37 °C in a 5% CO_2 in oxygen for 48 h. Other wells were incubated with 40 nM of TES-BSA that contains between 20 and 30 mol of TES per mol of BSA, and solutions were made considering 20 mol of TES. Additionally, some cells were allowed to grow for 24 h before adding 40 μM of cycloheximide (a protein synthesis inhibitor) or 10 μM of actinomycin D (a transcription inhibitor) for 30 min; afterward, 40 nM of TES was added for 48 h.

Cells settled down at the bottom of a 0.7 ml cover glass submerged in a chamber that was perfused by gravity (1.5–2.0 ml/min) with an external solution containing in mM: 130 NaCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 3 $NaHCO_3$, 1.2 KH_2PO_4, 5 KCl, 0.1 niflumic (to block Cl^- currents), 10 glucose, 10 HEPES, and, pH 7.4, adjusted with NaOH. All experiments were performed at room temperature (~21 °C).

Depolarizing voltage steps (voltage clamp) activated K^+ currents (IK^+), which were recorded using the whole cell configuration through an Axopatch 200A amplifier (Axon Instruments). A horizontal micropipette puller (P-87, Sutter Instruments Co., Novato, CA) was used to make patch pipettes with 1B200F-6 glass (Word Precision Instruments, Sarasota, FL) that had a resistance between 2 and 4 MΩ. The internal solution was constituted by (mM): 5 NaCl, 140 K^+ gluconate, 5 ATP, 0.1 GTP, 5 HEPES, 1 EGTA, and 0.1 leupeptine, adjusted to pH 7.3 with KOH. Currents filtered at 1–5 KHz, digitized (Digidata 1440A, Axon) at 10 KHz, were analyzed through specialized software (pClamp v10.2, Axon).

All cells received a step depolarization protocol from −60 to +50 mV in 10-mV increments from a holding potential of −60 mV during 100 ms, 1 Hz. These stimulations produced a voltage-dependent outward IK^+. After the control protocol, myocytes in each experimental group were perfused with different salbutamol concentrations (1, 10, 100, or 1000 nM) and received the same step depolarization protocol. To characterize the K^+ channels involved in these phenomena, myocytes were perfused with 3 mM of 4-amynopyridine (4-AP, a delayed rectifier K^+ channels blocker) or 100 nM of iberiotoxin (IBTX, a specific blocker of the high-conductance Ca^{2+} activated K^+ channels). Changes in the currents were evaluated as maximal current peak to each voltage tested.

2.4. Double immunofluorescence

Guinea pig tracheal tissues incubated with and without 40 nM of TES for 48 h at 9 °C as in organ bath experiments were fixed in 4% paraformaldehyde in sodium phosphate buffer (PBS) for 4 h and dehydrated with 30% sucrose/PBS until their inclusion in O.C.T. compound (Tissue Tek, CA, USA) and frozen at −60 °C. Cryosections transferred into PBS and permeabilized with 0.5% Triton in PBS were 20-μm thick. Nonspecific binding was blocked by adding 10% horse serum on the slices for 30 min. Afterward, slices were incubated with the following primary rabbit antibodies: anti-β_2 adrenergic receptor (β_2-AR, Creative Diagnostics, DPABH-26262, NY, USA), dilution 1:50, and an antibody against the most abundant isoform in ASM (Birrell et al., 2015), anti-adenylyl cyclase 6, (Genetex, GTX47798, CA, USA), dilution 1:100, overnight at 4 °C. The secondary antibody Alexa488 donkey anti-rabbit immunoglobulin G (IgG, Life Technologies, CA, USA) was incubated (1:200) for 30 min. The next primary antibody, mouse anti-α-actin (Santa Cruz Biotechnology, Cat. no. sc-58669, TX, USA), dilution 1:250 was applied. The secondary antibody Alexa Fluor 555 donkey anti-mouse (Life Technologies, CA, USA), dilution 1: 400 was used. 4′,6-Diamidino-2-phenylindole (DAPI, Life Technologies) was used to counterstain the nuclei. Slides were cover-slipped with fluoromount aqueous mounting medium (Sigma, MO, USA). Negative controls received no primary antibodies (β_2-AR and adenylate cyclase 6) and no fluorescence signal was obtained. A confocal microscope A1R + STORM, (Nikon, Japan) was used to observe immunofluorescence. Merged images were constructed for displaying purposes, in which the β_2-AR and adenylate cyclase 6 were green, α-actin was red and nuclei blue.

2.5. mRNA extraction and quantitative PCR for β_2 adrenergic receptor

Guinea pig tracheas were obtained and placed in cold RNAase-free Krebs solution, and the esophagus was carefully removed using a stereoscopic microscope (SMZ-10 Nikon, Japan). Each trachea was placed in a 2-ml Eppendorf tube free of RNAase containing 1.5 ml of Krebs solution. Some tracheas were incubated during 48 h with 40 nM of TES at 9 °C as described in organ bath experiments. Afterward, tracheas placed in cold RNAase-free Krebs solution were dissected free of connective tissue and cartilage to obtain smooth muscle and epithelium. Each tissue was separately placed in 200 μl of Trizol in an RNAase-free 1.5-ml Eppendorf tube on ice. Total RNA was purified through Direct-zol RNA Miniprep Kit (Zymo, CA, USA) and DNA contamination was prevented with DNase I treatment (in column) (Zymo) and eluted with RNAse-free water. Total RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Barrington, IL, USA). To assess RNA purity, the absorbance ratios 260:230 nm and 260:280 nm, in which a 1.8 ratio was appropriate for proceeding with cDNA synthesis, were used.

Total RNA was reverse-transcribed to cDNA using a SuperScript VILO cDNA synthesis kit (Life Technologies) and stored at −20 °C for later use. Quantitative polymerase chain reactions (qPCR) were performed in a StepOne Real-Time PCR system (Foster City CA, USA). A final volume of 20 μl of PCR reaction was employed, containing 4 μl of water, 10 μl of 2X TaqMan Gene Expression Master Mix (Life Technologies), 5 μl of diluted cDNA, and 1 μl of 20X mix PCR assay of specific probe for β_2-AR. Negative controls were prepared without template. Custom TaqMan gene expression assays (ThermoFisher Scientific, CA, USA) are shown in Table 1. Cycling conditions in the first phase were 2 min at 50 °C and 10 min at 95 °C, while for the second phase, these were 15 s at 95 °C and 1 min at 60 °C during 40 cycles. A series of normalizing genes (cofilin, clathrin heavy chain-like 1, peptidyl-prolyl cis-trans isomerase B) was tested on the samples for their

Table 1
Custom TAQMAN gene expression assays.

GENE	Sequence of primers and probe
COFILIN 1	Fw: GCCAGACGGTGGACGAC
	Rv: GTCCTTGTCTGGTAGCATCTTGA
	Pr: CCCTACGCCACCTTTG
PPIB	Fw: CCAGGGCGGAGACTTCAC
	Rv: CGCTCGCCATAAATGCTCTTG
	Pr: CCTGTGCCATCTCCTC
CLATHRIN	Fw: ACTGTTCCCCATGAACCTACCT
	Rv:TGTTTTCTTCTTCAACACAGACTGACA
	Pr: TTGACGCCAATAATCC
β_2-AR	Fw: CAAAAAGCAGCTCCAGAAGATTGAC
	Rv: CTGCTCCACCTGGCTGAG
	Pr: ATCGGCCCTCAGATCT

PPIB: peptidyl-prolyl cis-trans isomerase B. β_2-AR: β_2 adrenergic receptor.

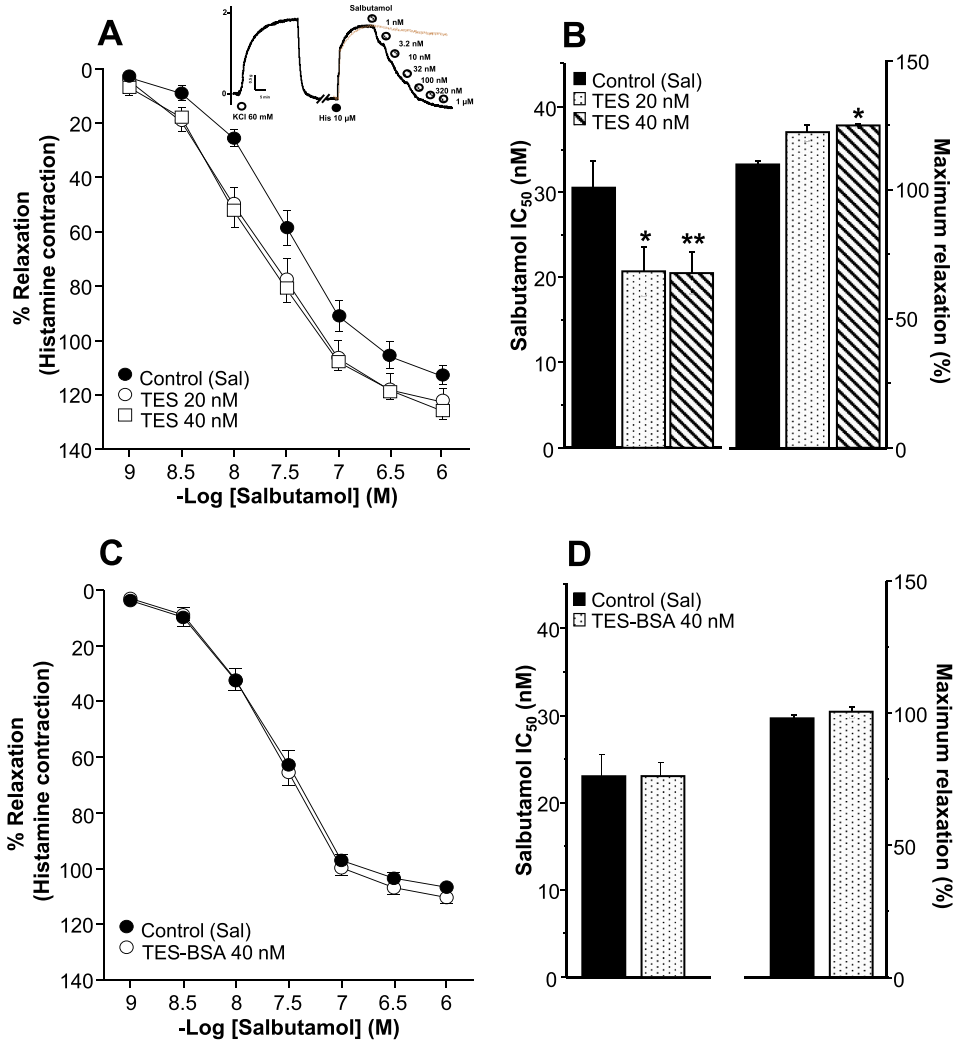

Fig. 1. Testosterone (TES) incubated for 48 h potentiates guinea pig tracheal smooth muscle relaxation induced by salbutamol, while testosterone conjugated with bovine serum albumin (TES-BSA) during the same period does not, suggesting the genomic nature of the phenomenon. **A)** Salbutamol (Sal) cumulative concentration curve relaxes histamine (10 µM) pre-contracted tissues. When pre-incubated with TES (20 or 40 nM, n = 5) for 48 h, the concentration-response curve to salbutamol had a leftward shift for both TES concentrations. Inset in this figure illustrates original traces showing the Sal cumulative concentration curve; orange trace depicts sustained contraction induced by histamine (control). **B)** Bar graphs showing that TES, at both concentrations tested, significantly diminished the Sal inhibitory concentration 50% (IC_{50}, left panel). Maximal response (right panel) to Sal was only significantly different for the highest TES concentration used. **C)** Sal cumulative concentration curve induced relaxation was not modified by TES-BSA, n = 4. **D)** Bar graphs illustrating no differences in Sal responses between control and TES-BSA treated tissues. Symbols and bars depict mean ± S.E.M., *p < 0.05, **p < 0.01. One-way analysis of variance followed by Dunnett's multiple comparison test was used for TES experiments and paired Student *t*-test for TES-BSA experiments.

stable expression in airway smooth muscle, and eventually cofilin was designated as a housekeeping gene for normalization. The $2^{-\Delta\Delta CT}$ method was employed to analyze β_2-AR gene expression (Livak and Schmittgen, 2001).

2.6. Western blot for β_2 adrenergic receptor

The relative expression of the β_2-AR and β-actin as loading control were evaluated by Western blot in guinea pig ASM with and without 40 nM of TES for 48 h or 10 µM of flutamide 30 min before TES incubation. Tissues were finely cut and cell lysis was performed by sonication using RIPA lysis buffer containing (in mM): 50 Tris (pH 7.5), 0.5 EDTA, 1% Nonidet™ P40, 1 PMSF, 0.5 sodium orthovanadate; protease inhibitors (20 µg/ml each): aprotinin, leupeptin, and pepstatin. The total protein concentration was quantified by the Lowry method. Total proteins (10 µg) were separated by one-dimensional electrophoresis, transferred to polyvinylidene diflouride (PVDF) membranes and detected by Western blot and chemiluminescence. All membranes were blocked with the commercial Odyssey Blocking Buffer in tris-buffered saline (TBS, LI-COR, NE, USA) for 1 h. Expression of the β_2-AR was evaluated by incubating the membranes overnight with a polyclonal primary antibody raised in rabbit (concentration 1:100; Creative Diagnostics) and afterward, for 1.5 h with a peroxidase-coupled donkey anti-rabbit secondary antibody (concentration 1:15000; Jackson). After membrane stripping using the Re-blot plus-mild solution (Millipore, MA, USA), β-actin was detected with a monoclonal (clone AC-40) primary antibody raised in mouse (1:500; Sigma, St. Louis, MO, USA) and a peroxidase-coupled rabbit anti-mouse secondary antibody (1:10000; Jackson, PA). The chemiluminescent signal was detected with the imaging system (ChemiDoc™ MP, BIO-RAD, CA, USA) and images were acquired with the Image Lab software (version 5.2.1; BIO-RAD). Antibody specificity control was assessed omitting the incubation with the anti-β_2-AR antibody. In all cases, the optical density was normalized to the loading control to quantify the ratio of β_2-adrenergic receptor/β-actin.

2.7. Drugs and chemicals

Testosterone (17β-hydroxy-4-androsten-3-one, TES), testosterone 3-(O-carboximethyl) oxime: BSA, histamine, salbutamol, ICI-118.551, actinomycin D, cycloheximide, flutamide were all purchased from Sigma Chem. Co. (St. Louis, MO, USA), iberiotoxin was purchased from Enzo Life Sciences (Farmingdale, NY, USA) and 4-aminopyridine from Research Chemical LTD (Word Hill, MA, USA). TES, cycloheximide, and flutamide were diluted in absolute ethanol and the highest percentage used was 0.1% v/v of vehicle. Actinomycin was diluted in dimethyl sulfoxide (DMSO).

2.8. Statistical analysis

In tracheal smooth muscle, salbutamol concentration response curves were evaluated by the inhibitory concentration 50% (IC_{50}) and maximum relaxation. Each cumulative concentration–response curve was used to evaluate the IC_{50} that was calculated by straight-line

regression as –log [M] using the ED_{50} plus v1.0 software. These data were analyzed by a one-way analysis of variance followed by Dunnett's multiple comparison test. By analyzing data in an Excel spreadsheet, Schild's equation was used to calculate dissociation constant (K_B) and pA2 values for ICI-118.551 (Jankovic et al., 1999). In patch clamp experiments, IK^+ at each voltage step was analyzed by a one-way analysis of variance followed by Dunnett's, Student-Newman-Keuls multiple comparison tests or non-paired student t-test. Western blot was also analyzed by one-way analysis of variance followed by Dunnett's comparison test. In all experiments, each "n" value corresponds to a different animal. In the manuscript and figures, data are expressed as mean ± S.E.M. Statistical significance was set at $p < 0.05$ bimarginally.

3. Results

3.1. Chronic exposure to testosterone augments salbutamol-induced airway smooth muscle relaxation

Guinea pig tracheal rings incubated for 48 h at 9 °C had a KCl response (1.9 ± 0.14 g, n = 15) similar to those tissues not subjected to this maneuver (1.7 ± 0.15 g, n = 15), suggesting that this procedure does not affect the contractile apparatus. Tracheal rings (sham control) precontracted with histamine relaxed to salbutamol in a concentration-dependent way. Contrastingly, tissues preincubated with 20 or 40 nM of TES for 48 h, showed a leftward shift of the salbutamol cumulative concentration response curve (Fig. 1A, n = 5). IC_{50} salbutamol values, the concentration required to obtain 50% relaxation, for both TES concentrations used were significantly lower than IC_{50} from the sham group, and maximum response was only significantly different for the TES 40 nM group (Fig. 1B). TES-BSA, an impermeable macromolecular complex, showed no modification in the relaxation induced by salbutamol (Fig. 1C, D, n = 4). This TES-induced increase in the relaxation produced by salbutamol was abolished by incubation with 10 μM of flutamide (Fig. 2, n = 6). On the other hand, acute exposure to 20 or 40 nM of TES for 15 or 60 min did not modify the concentration response curve to salbutamol (Fig. 3A, n = 7), neither IC_{50} nor maximum response were different from sham group (Fig. 3B). These results strongly suggest that increased salbutamol responses are related to chronic exposure to TES, probably involving a genomic pathway.

3.2. Testosterone increases salbutamol-induced K^+ currents in airway myocytes through a genomic effect

In airway myocytes, depolarizing pulses provoke voltage-dependent outward K^+ currents (IK^+). Cells perfused with increasing concentrations of salbutamol (1–1000 nM) showed a concentration-dependent increment in IK^+ (Fig. 4A). Myocytes previously incubated with TES 40 nM for 48 h, displayed even higher IK^+ in response to salbutamol perfusion (Fig. 4B). Flutamide lowered the IK^+ increment produced by TES in the salbutamol-induced K^+ current (Fig. 4C). Fig. 4D illustrates that TES incubation significantly increases IK^+ induced by depolarizing pulses from −10 mV ahead; this effect was ablated by flutamide. The addition of salbutamol at different concentrations augmented the depolarizing step-induced IK^+ that was significantly further increased by TES from −20 or 0 mV ahead and this effect was reverted by flutamide (Fig. 4E–H). The genomic nature of the TES-induced increment of IK^+ was further confirmed because myocytes incubation with TES-BSA, an impermeable macromolecular complex, showed no modification of the IK^+ induced by depolarization pulses or increasing salbutamol concentrations (Fig. 5A–G). Additionally, actinomycin D, a transcription inhibitor, and cycloheximide, a protein synthesis inhibitor, also diminished the IK^+ increment produced by TES in the salbutamol-induced K^+ current (Figs. 6C and 7C). Figs. 6D and 7D show that these inhibitors revert TES-induced increases in IK^+ induced by depolarizing pulses from 30 mV or 10 mV ahead, respectively. Additionally, actinomycin D and cycloheximide inhibit the effect of TES on the salbutamol induced increment of IK^+ (Fig. 6E–H, 7E-H). These results highlight the genomic nature of the TES-induced increment of IK^+.

The TES increase in IK^+ induced by depolarizing pulses was blocked by 4-AP, a delayed rectifier K^+ channel blocker, from 0 mV ahead, indicating that TES is augmenting the expression of these channels (Fig. 8A). In this context, the androgen increment in the IK^+ induced by salbutamol was blocked by 4-AP and by IBTX, a specific blocker of the high-conductance Ca^{2+}-activated K^+ channels, indicating that both K^+ channels were involved in the TES potentiation effect (Fig. 8B). Conceivably, the expression of these channels is also augmented by TES.

3.3. Testosterone chronic incubation induces β_2 adrenergic receptor (β_2-AR) upregulation in airway smooth muscle

Immunofluorescence studies showed that TES 40 nM incubation for 48 h augmented the β_2-AR expression in this tissue (Fig. 9A–B). No fluorescence was detected in negative controls without primary antibody, corroborating its specificity against the β_2-AR (Fig. 9C). Smooth muscle α actin was also detected to confirm the co-localization of the β_2-AR in the airway smooth muscle (Fig. 9A and B, last panel). Additionally, the β_2-AR mRNA expression was significantly increased by TES incubation (Fig. 10A) as well as the protein presence, which was reverted by flutamide (Fig. 10B), confirming that chronic TES exposure promotes β_2-AR upregulation through a genomic action. Moreover, immunofluorescence for adenylyl cyclase 6 in tissues with and without TES incubation did not show any differences (Supplementary Fig. 1).

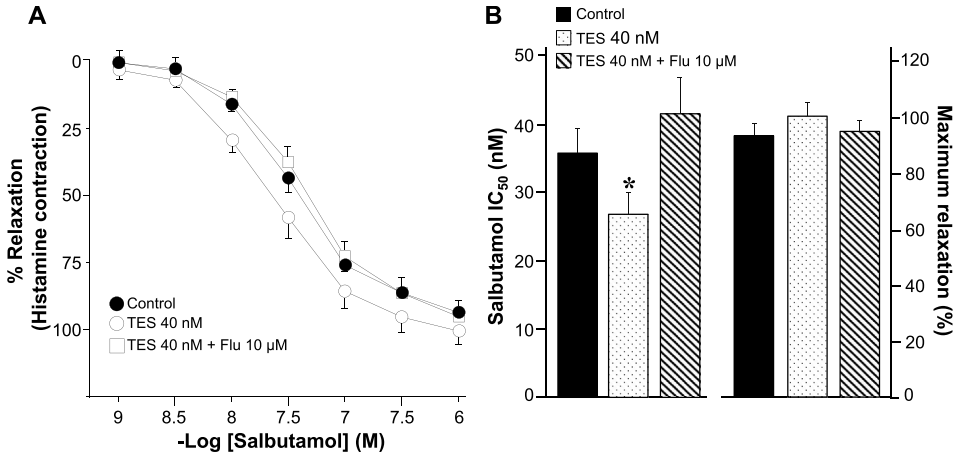

Fig. 2. Testosterone (TES) induced potentiation of the salbutamol relaxing response was abolished by the blockade of the androgen receptor in guinea pig tracheal preparations. **A)** Salbutamol cumulative concentration curve relaxes histamine (10 μM) pre-contracted tissues. When pre-incubated with TES (40 nM, n = 6) for 48 h, the concentration response curve to salbutamol had a leftward shift. This TES effect was abolished by incubation with 10 μM of flutamide (Flu, an antagonist of the AR) before TES. **B)** Bar graphs showing that TES diminishes salbutamol IC_{50} and that this effect is reversed by Flu. Maximum relaxation was not modified by any treatment. Symbols and bars depict mean ± S.E.M., *$p < 0.05$.

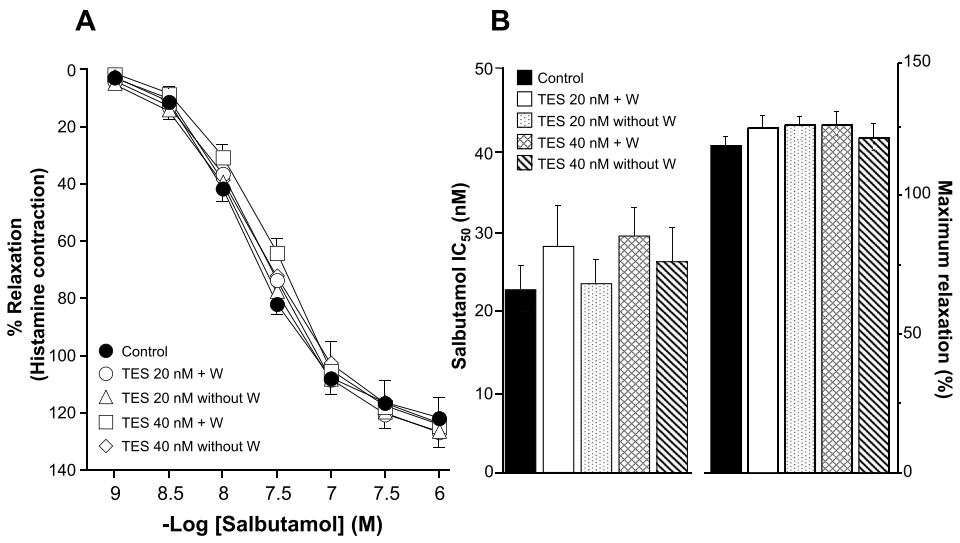

Fig. 3. Testosterone (TES) pre-incubated for 15 or 60 min does not modify the relaxation induced by salbutamol (Sal) in guinea pig tracheal preparations. **A)** Sal cumulative concentration curve was not altered by TES 20 or 40 nM 15 min pre-incubation washed (W) before Sal addition, nor when TES was always present during the curve (without W, 60 min), completing 1h incubation. **B)** Bar graphs illustrating that TES, at both concentrations and incubation times tested, did not modify the Sal inhibitory concentration 50% (IC_{50}, left panel). Maximal response (right panel) to Sal was also not modified in any of the experimental procedures. Symbols and bars depict mean ± S.E.M., n = 7.

3.4. Testosterone induced upregulation of the β_2-AR favors salbutamol response in airway smooth muscle

To explore whether a modified β_2-AR function mediates the TES increment in the salbutamol response, guinea pig tracheal rings received different concentrations of ICI-118.551, a specific β_2-AR antagonist (1, 3.2, 10, 32, and 100 nM). This antagonist concentration-dependently shifted salbutamol response curves to the right (Fig. 11A). TES incubation shifted the salbutamol cumulative curve to the left (IC_{50} = 25.6 ± 5.4 nM, n = 7, Fig. 11B) as compared with the curve without TES (IC_{50} = 39.9 ± 3.1 nM, p = 0.05, n = 6). ICI also shifted this concentration curve to the right in a concentration-dependent fashion. pA2 value obtained with the Schild's equation, was reduced with TES incubation during 48 h (Fig. 11C), i.e., the concentrations required for ICI to occupy 50% of the β_2-AR available at the smooth muscle membrane was lower. This value is an indirect measure of an antagonist's affinity for its receptors and, in this study, provides information about the role of the β_2-AR on the augmented salbutamol response. Therefore, the -log pA2 value transformed to K_B, the dissociation constant, was 10.4 ± 4.1 nM for the control group vs 3.4 ± 0.7 nM for the TES group. The K_B value for TES treated tissue was lower, corroborating an augmented β_2-AR expression in airway smooth muscle.

4. Discussion

Our results indicate that chronic exposure to nanomolar TES concentrations in guinea pig ASM induces augmented β_2-AR expression and therefore an increase in the relaxing responses to salbutamol that was abolished by flutamide. Furthermore, TES-BSA had no effect on the relaxation induced by salbutamol, and acute exposure to TES also did not modify this response. In myocytes, this chronic androgen exposure increases salbutamol-induced IK^+, and this increment was eliminated by flutamide, actinomycin D, and cycloheximide. Contrastingly, TES-BSA did not modify the salbutamol-induced IK^+.

The effect of androgens on β adrenergic receptors in other tissues, such as cardiac and vascular muscles has been explored. In rat cardiac tissues, TES improves the myocardial performance by upregulating the expression of β_2-AR, and this effect was annulled by flutamide, suggesting a genomic action of the androgen (Sun et al., 2011). Meanwhile, in rat aortic smooth muscle, TES impairs the β adrenergic vasorelaxation without modification of the β_2-AR expression. In this case, TES diminished the expression of adenylyl cyclase, which was responsible for the diminished β adrenergic vasorelaxation (Lopez-Canales et al., 2018). In ASM, we did not find any modification of the adenylyl cyclase-6 expression in tissues chronically exposed to TES, ruling out its participation in the augmented salbutamol response induced by TES (Supplementary Fig. 1).

In bovine airway smooth muscle, it has been reported that acute exposure to a high concentration of 5α-dihydrotestosterone (100 μM) increases the salbutamol relaxation without affecting the β_2-AR (Bordallo et al., 2008), and this effect might be due to a non-specific action related to the high androgen concentration tested. In this context, in pig bronchus, 40 μM of TES augmented the isoprenaline-induced relaxation by approximately 6.7-fold, and this response was related to the inhibition of COMT or extraneuronal catecholamine uptake (Foster et al., 1983). Seemingly, this androgen action on ASM should be related to a nongenomic effect.

In this work, we found that chronic exposure to nanomolar TES concentrations favors salbutamol-induced relaxation of the histamine-precontracted ASM. This effect is not due to a nongenomic rapid action, since brief exposures to TES for 15 or 60 min did not modify the relaxing salbutamol responses (Fig. 3).

On the other hand, it has been documented that TES can activate diverse membrane proteins such as GPRC6A, a class C orphan G protein-coupled receptor family member (Pi et al., 2015), and ZIP9, a zinc transporter from the ZIP family (Berg et al., 2014). These receptors can activate G proteins, such as Gs, Gi, and/or Gq11, triggering signaling pathways due to TES stimulation. Furthermore, extracellular-signal-regulated kinases 1 and 2 are also activated, participating in many phosphorylation processes. The final response to androgen stimulation of these receptors depends on the cellular type (Clemmensen et al., 2014; Thomas et al., 2018). In this regard, we found that chronic exposure of ASM to TES-BSA did not alter salbutamol-induced relaxation, discarding the possibility of TES acting on the previously mentioned membrane receptors (Fig. 1C).

The K^+ channels have been reported to be involved in ASM relaxation (Campos-Bedolla et al., 2008; Huang et al., 1993; Jones et al., 1990). Because TES augmented salbutamol-induced ASM relaxation, we explored the effects of TES on these channels. Chronic exposure of tracheal myocytes to TES produced an increase of the IK^+ induced by depolarizing pulses as well as the salbutamol-induced IK^+. Both effects were abolished by flutamide (Fig. 4), actinomycin D (Fig. 6), and cycloheximide (Fig. 7). These results strongly suggest that the augmented salbutamol-induced IK^+ by TES is due to a genomic action. While, it is possible that membrane receptors activated by TES could be involved in this phenomenon, TES-BSA did not modify the salbutamol-induced IK^+ currents, ruling out their participation (Fig. 5).

Meanwhile, β_2-AR activation induces ASM relaxation through adenylate cyclase-cAMP pathways, including diverse K^+ channels, by

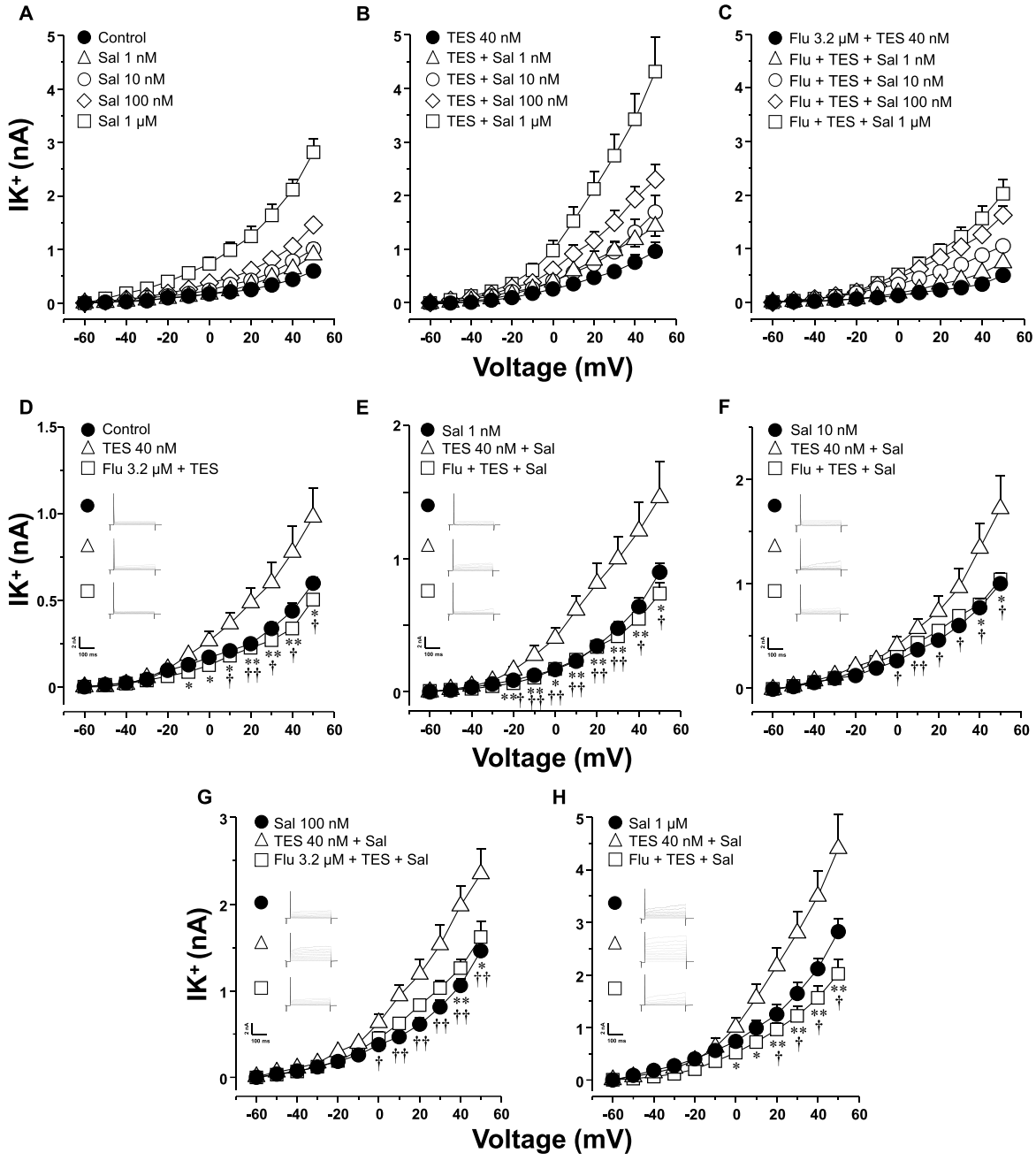

Fig. 4. Testosterone (TES, 40 nM) incubated for 48 h augmented K^+ outward currents (IK^+) induced by salbutamol in guinea pig tracheal myocytes through a genomic action. Cells received a step depolarization protocol from −60 to +50 mV in 10 mV increments from a holding potential of −60 mV during 100 ms, 1 Hz. These stimulations produced a voltage-dependent outward IK^+. **A)** Myocytes perfused with different concentrations of salbutamol (Sal) showed a concentration-dependent increase in IK^+, n = 9. **B)** Myocytes incubated with TES showed a higher IK^+, n = 8, while **C** illustrates that this effect was abolished by flutamide (Flu, n = 7). Figures D, E, F, G and H illustrate statistical analysis of each Sal concentration used, including experiments with Flu. As can be seen in all these figures, Flu reverses TES effect on salbutamol increase of the IK^+. Notice that TES alone induces a significant increment of the IK^+ which was annulated by Flu. These results clearly demonstrate a genomic effect of TES on airway smooth muscle. Insets show original recordings. Please notice that in figures D-H, different Y-axis scales are used in order to clearly show statistical significances. Symbols represent mean ± S.E.M. In figure D, *p < 0.05, **p < 0.01 by comparing Flu + TES (□) vs TES groups (Δ) and †p < 0.05, ††p < 0.01 when comparing Control (●) vs TES (Δ) groups. For the remaining figures (E-H) *p < 0.05, **p < 0.01 comparing Flu + TES + Sal (□) vs TES + Sal (Δ) groups and †p < 0.05, ††p < 0.01 comparing Sal (●) vs TES + Sal (Δ) groups.

producing membrane hyperpolarization (Campos-Bedolla et al., 2006; Chavez et al., 2007; Huang et al., 1993; Johnson, 1998; Jones et al., 1990; Tanaka et al., 2003). In this tissue, the main K^+ channels are the Ca^{2+} activated K^+ channels (K_{Ca}) and the voltage-dependent delayed rectifier K^+ channels (K_V) (Adda et al., 1996; Boyle et al., 1992; Montaño et al., 2011).

The K_{Ca} are mainly activated by increases in intracellular Ca^{2+} concentration and through the cAMP-PKA signaling pathway (Wang and Kotlikoff, 1996) and have been classified in three subfamilies: those of high conductance (BK_{Ca}, MaxiK, $K_{Ca}1.1$), intermediate conductance (IK_{Ca}, $K_{Ca}3.1$), and low conductance (SK_{Ca}, $K_{Ca}2.1$, 2.2 and 2.3) (Feletou, 2009). BK_{Ca} have been characterized as the most important

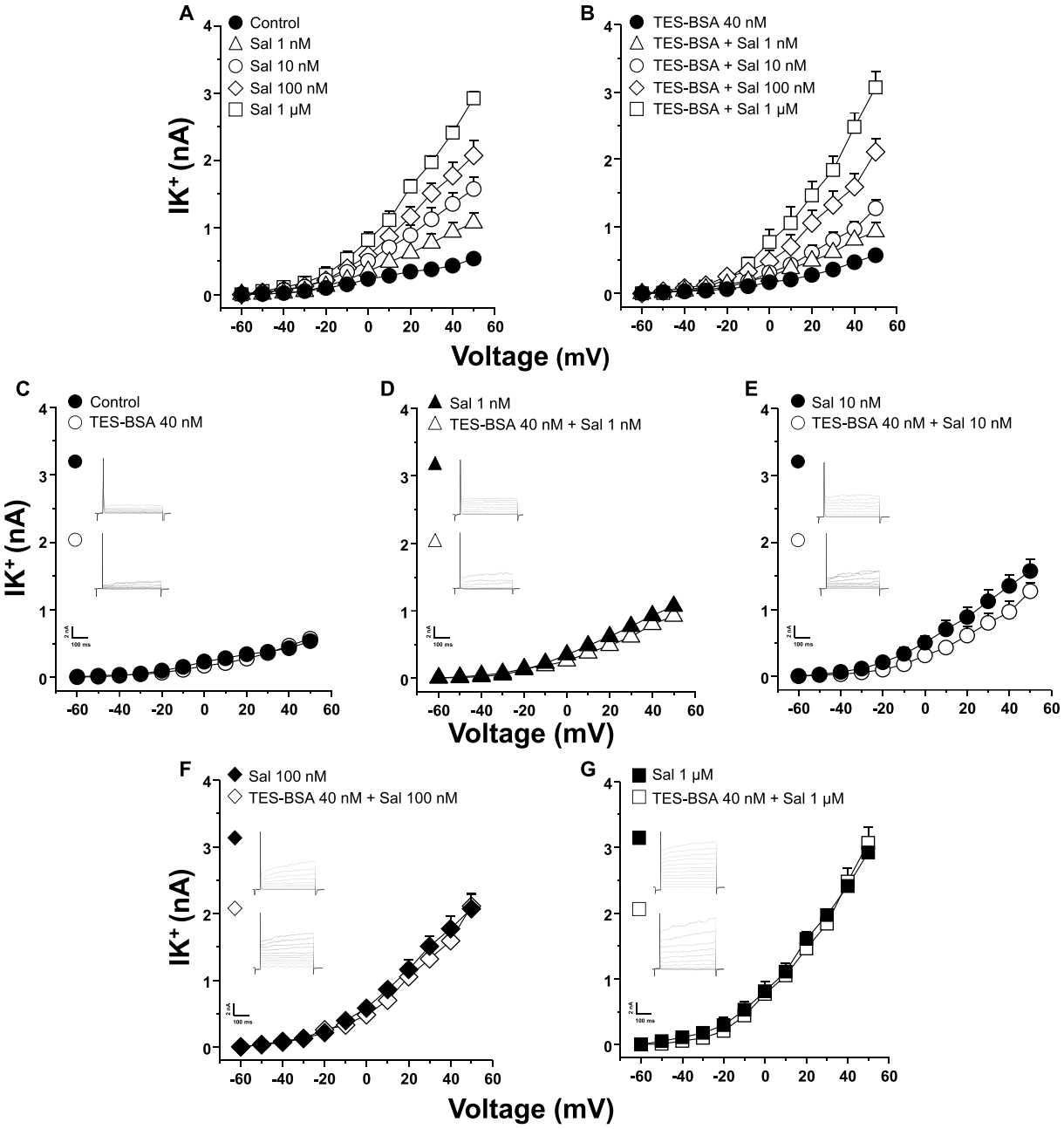

Fig. 5. Tracheal guinea pig myocytes incubation with testosterone conjugated with bovine serum albumin (TES-BSA) during 48 h has no effect on the IK^+ induced by salbutamol (Sal) corroborating the genomic nature of the phenomenon. **A)** Myocytes perfused with increasing concentrations of Sal showed a concentration-dependent augmentation in IK^+, n = 6. **B)** Myocytes incubated with TES-BSA, showed no changes in IK^+ compared with the group without TES, n = 5. Figures C, D, E, F and G illustrate statistical analysis of each Sal concentration used in TES-BSA experiments. Symbols represent mean ± S.E.M.

channels in bronchorelaxation induced by 5-HT and ATP (Campos-Bedolla et al., 2008; Montaño et al., 2011). Since IBTX partially blocked the TES-induced increment in IK^+ induced by salbutamol (Fig. 8B), the role of BK_{Ca} in this phenomenon is demonstrated. Furthermore, because this TES increment was reverted by flutamide (Fig. 4), an augmented expression of these channels is highlighted.

Meanwhile, K_V in ASM have been characterized as $K_V1.2$ and $K_V1.5$ and are blocked by 4-AP (Adda et al., 1996). Recently, the presence of $K_V7.5$ in human ASM was proposed (Brueggemann et al., 2018). Our results showed that this blocker completely reversed TES increase in the IK^+ induced by increasing voltage pulses (Fig. 8A). These results imply that this androgen is upregulating the K_V channels in this tissue. Notwithstanding, in the TES potentiation of the salbutamol-induced IK, K_V are also involved, since 4-AP partially abolished this current (Fig. 8B). However, the role of $K_V7.5$ in both phenomena is questionable since this channel is not affected by 4-AP (Khammy et al., 2018).

Because TES induces genomic actions related to the increases in salbutamol-induced ASM relaxation and IK^+, we explored whether an upregulation of the β_2-AR was involved. In this context, we found that this receptor expression was enhanced in ASM chronically exposed to TES, which was confirmed by immunofluorescence imagen (Fig. 9), Western blot, and qPCR (Fig. 10). Augmented β_2-AR functionality was corroborated because pA2 value was lower after chronic TES exposure (Fig. 11). Nevertheless, this upregulation and functionality of the β_2-AR induced by TES may also be improved if chronic exposure at nanomolar concentrations of this androgen has an inhibitory effect on COMT or extraneuronal catecholamine uptake, although this assumption requires further research. Contrastingly, TES nongenomic effects on the L-type

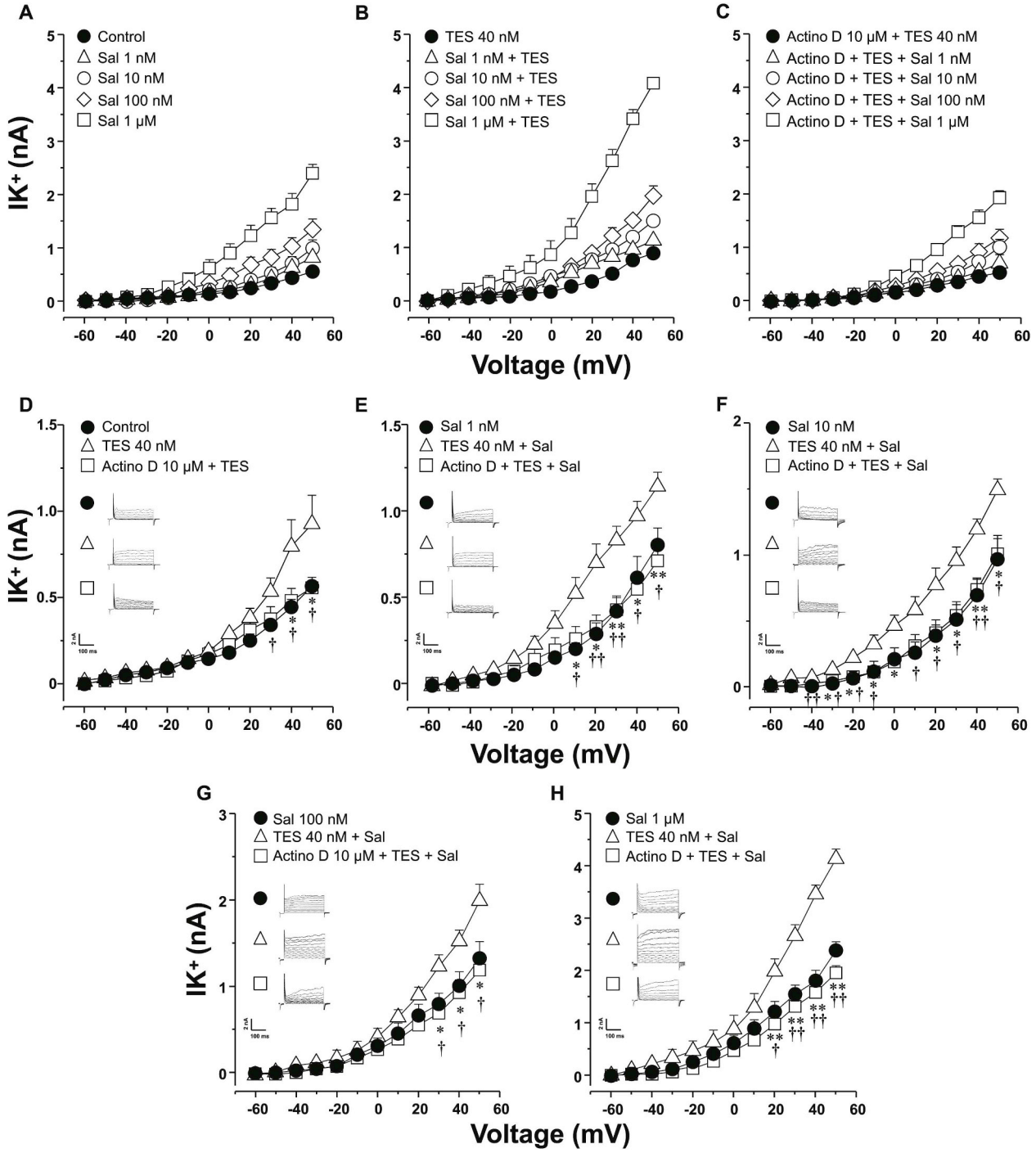

Fig. 6. Actinomycin D annulled testosterone (TES, 40 nM for 48 h) augmented IK^+ induced by salbutamol (Sal) confirming a genomic action. **A)** Different concentrations of Sal perfusion to tracheal myocytes, showed a concentration-dependent rise in IK^+. **B)** Myocytes incubated with TES showed a higher IK^+, while **C** illustrates that this effect was abolished by actinomycin D (Actin D, a transcription inhibitor) preincubation for 30 min before TES. Figures D, E, F, G and H illustrate statistical analysis of each salbutamol concentration used, including experiments with Actin D. As can be seen in all these figures Actin D reverses TES effect on salbutamol increase of the IK^+. Notice in figure D that TES alone induces a significant increment of the IK^+ which was annulated by Actin D. These results further demonstrate a genomic effect of TES on airway smooth muscle. Insets show original recordings. Please notice that in figures D-H, different Y-axis scales are used in order to clearly show statistical significances. Symbols represent mean ± S.E.M. In figure D, *p < 0.05 comparing Actino D + TES (□) vs TES groups (Δ) and †p < 0.05 comparing Control (●) vs TES (Δ) groups. For the remaining figures (E-H) *p < 0.05, **p < 0.01 comparing Actino D + TES + Sal (□) vs TES + Sal (Δ) groups, †p < 0.05, ††p < 0.01 comparing Sal (●) vs TES + Sal (Δ) groups, n = 6.

voltage-dependent Ca^{2+} channels, inositol triphosphate receptors, and store-operated Ca^{2+} channels already found in ASM (Montaño et al., 2014, 2018; Perusquía et al., 2015) might also be a target for the TES chronic effects most probably inducing a downregulation of these proteins and therefore contributing to the decrease of the excitation contraction coupling of this tissue. Nevertheless, this hypothesis warrants more investigation.

It has been extensively documented that plasmatic androgen concentrations, play an important role in asthma symptom severity that decrease when young asthmatic boys reach puberty. Contrastingly,

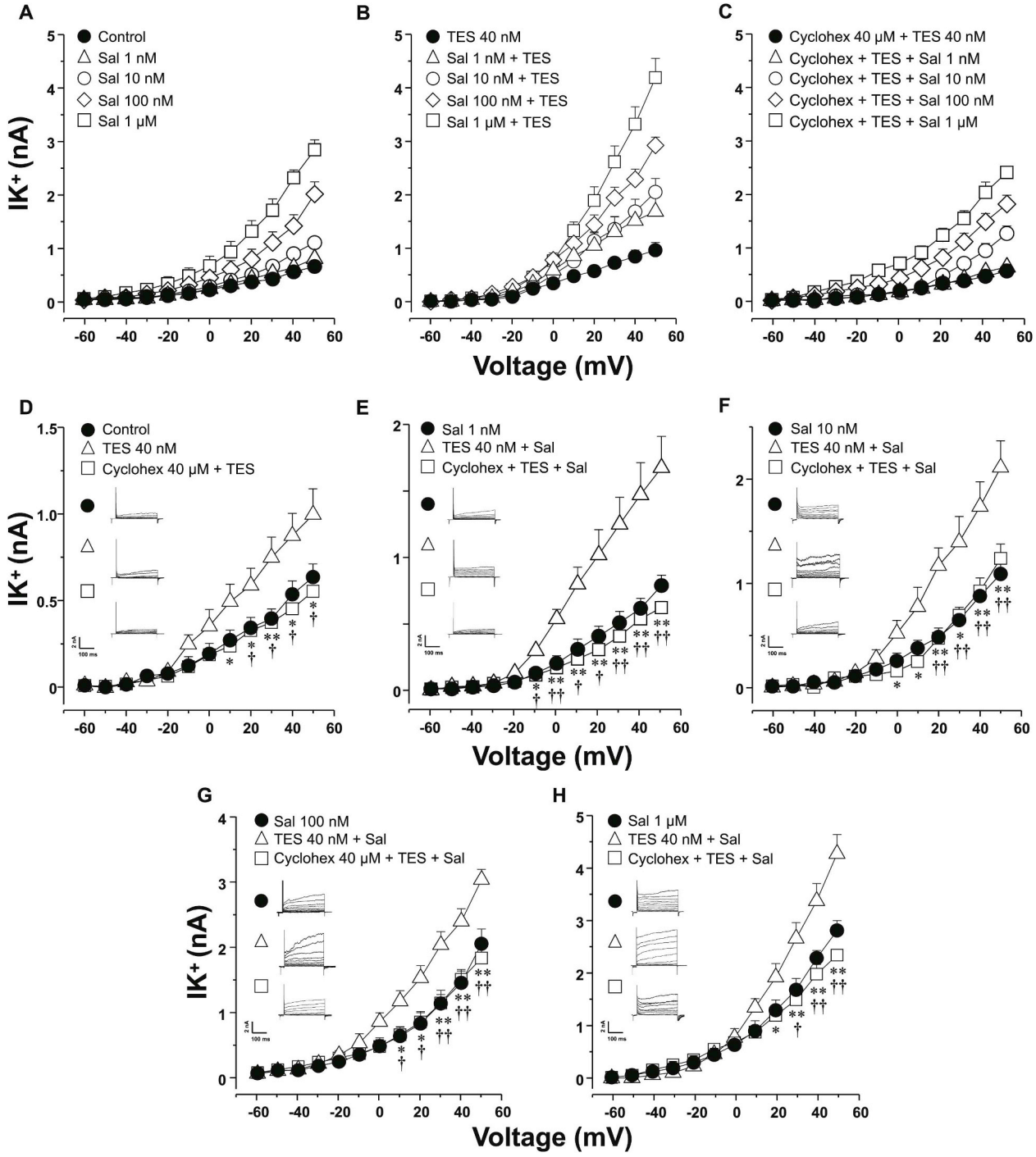

Fig. 7. Cycloheximide abolished testosterone (TES, 40 nM for 48 h) augmented IK^+ induced by salbutamol (Sal) through a genomic action. **A)** Different concentrations of Sal perfusion to tracheal myocytes, showed a concentration-dependent rise in IK^+. **B)** Myocytes incubated with TES showed a higher IK^+, while **C** illustrates that this effect was abolished by cycloheximide (Cyclohex, a protein synthesis inhibitor) preincubation for 30 min before TES. Figures D, E, F, G and H illustrate statistical analysis of each Sal concentration used, including experiments with Cyclohex. As can be seen in all these figures, Cyclohex reverses TES effect on salbutamol increase of the IK^+. Notice that TES alone induces a significant increment of the IK^+ which was annulated by Cyclohex (D). These results also demonstrate a genomic effect of TES on airway smooth muscle. Insets show original recordings. Please notice that in figures D-H, different Y-axis scales are used in order to clearly show statistical significances. Symbols represent mean ± S.E.M., n = 6. In figure D, *p < 0.05, **p < 0.01 comparing Cyclohex + TES (□) vs TES groups (Δ) and †p < 0.05 comparing Control (●) vs TES (Δ) groups. For the remaining figures (E-H) *p < 0.05, **p < 0.01 comparing Cyclohex + TES + Sal (□) vs TES + Sal (Δ) groups, †p < 0.05, ††p < 0.01 comparing Sal (●) vs TES + Sal (Δ) groups.

symptoms worsen in girls of the same age (de Marco et al., 2000; Townsend et al., 2012; Zannolli and Morgese, 1997). Therefore, higher TES plasmatic concentrations (6–50 nM) in teenage boys are probably related to lower asthmatic state (Canguven and Albayrak, 2011; Muller et al., 2011; Townsend et al., 2012). This tendency prevails during the men lifetime and decreases in old age. Thus, the augmented expression of the β_2-AR by TES may contribute to this enhanced health state in males.

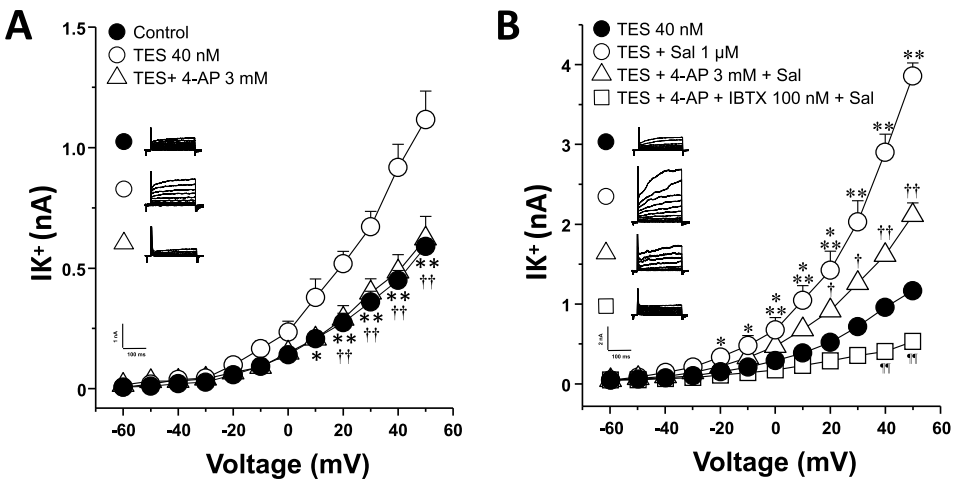

Fig. 8. Voltage dependent delayed rectifier K^+ and Ca^{2+} activated K^+ channels are the main proteins involved in the testosterone (TES, 40 nM for 48 h) augmented IK^+ induced by depolarizing pulses and by salbutamol (Sal) in guinea pig tracheal myocytes. **A)** The TES increase in IK^+ induced by depolarizing pulses was blocked by 4-aminopyridine (4-AP, n = 6), a delayed rectifier K^+ channels blocker. **B)** The androgen increase in the IK^+ induced by Sal was abolished by 4-AP and iberiotoxin (IBTX, n = 8), a specific blocker of the high-conductance Ca^{2+} activated K^+ channels. These results indicate that TES is augmenting the expression of both channels. Symbols represent mean ± S.E.M. In figure A, *p < 0.05, **p < 0.01 comparing TES + 4-AP (Δ) vs TES (o) groups. ††p < 0.01, comparing Control (●) vs TES (o) group. In figure B, at **-20 mV**, *p < 0.05 when comparing TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□) group. At **-10 mV**, *p < 0.05 comparing TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□) and TES (●) groups. At **0 mV**, *p < 0.05 when comparing TES + Sal (o) vs TES (●) group and **p < 0.01 comparing TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□). At **10 mV**, *p < 0.05 comparing TES + Sal (o) vs TES + 4-AP + Sal (Δ) and **p < 0.01 comparing TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□) and TES (●) groups. At **20 mV**, *p < 0.05 comparing TES + Sal (o) vs TES + 4-AP + Sal (Δ) and **p < 0.01 comparing TES + Sal (o) vs TES + 4-AP + IBTX + Sal (□) and TES (●) groups. †p < 0.05, comparing TES + 4-AP + Sal (Δ) vs TES (●) group. At **30 mV**, **p < 0.01 comparing TES + Sal (o) vs TES + 4-AP + Sal (Δ), TES (●) and TES + 4-AP + IBTX + Sal (□) groups. †p < 0.05, comparing TES + 4-AP + Sal (Δ) vs TES (●) group. At **40 mV**, **p < 0.01 comparing TES + Sal (o) vs TES + 4-AP + Sal (Δ), TES (●) and TES + 4-AP + IBTX + Sal (□) groups. ††p < 0.01, comparing TES + 4-AP + Sal (Δ) vs TES (●) group and ¶¶p < 0.01 between TES (●) vs TES + 4-AP + IBTX + Sal (□) group. At 50 mV, **p < 0.01 comparing TES + Sal (o) vs TES + 4-AP + Sal (Δ), TES (●) and TES + 4-AP + IBTX + Sal (□) groups. ††p < 0.01, comparing TES + 4-AP + Sal (Δ) vs TES (●) group and ¶¶p < 0.01 between TES (●) vs TES + 4-AP + IBTX + Sal (□) group. In Figure A, one-way analysis of variance followed by Dunnett's multiple comparison test was used and in B one-way analysis of variance followed by Student-Newman-Keuls multiple comparison test.

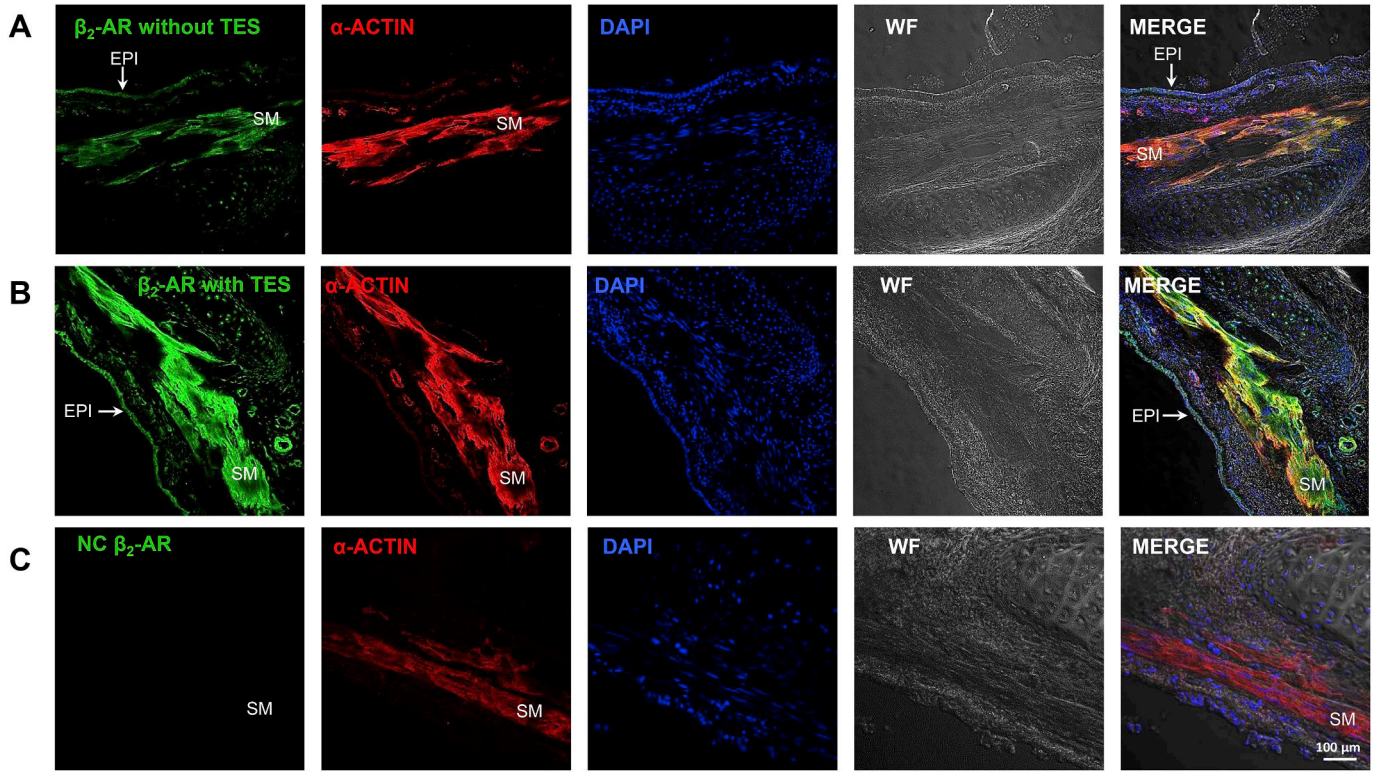

Fig. 9. The testosterone (TES) induced genomic effect on salbutamol response increase is related to β_2 adrenergic receptor (β_2-AR) upregulation in guinea pig airway smooth muscle. The first column illustrates immunofluorescence for the β_2-AR in guinea pig tracheal preparation and is depicted in green on airway smooth muscle in tissues without **(A)** and with **(B)** 40 nM of TES incubation for 48 h. Notice the increment in the fluorescence when tissues were exposed to TES. **C)** The absence of the primary antibody for the β_2-AR showed no fluorescence (NC = negative control). Smooth muscle α-actin (red) is depicted in the second column and the third column illustrates cell nuclei (blue). The fourth column presents white field (WF). Last column shows merged images of the former four panels. Notice that the yellow color implies co-localization of the β_2-AR and α-actin, increased in **B** merged image. EPI = epithelium, SM = smooth muscle.

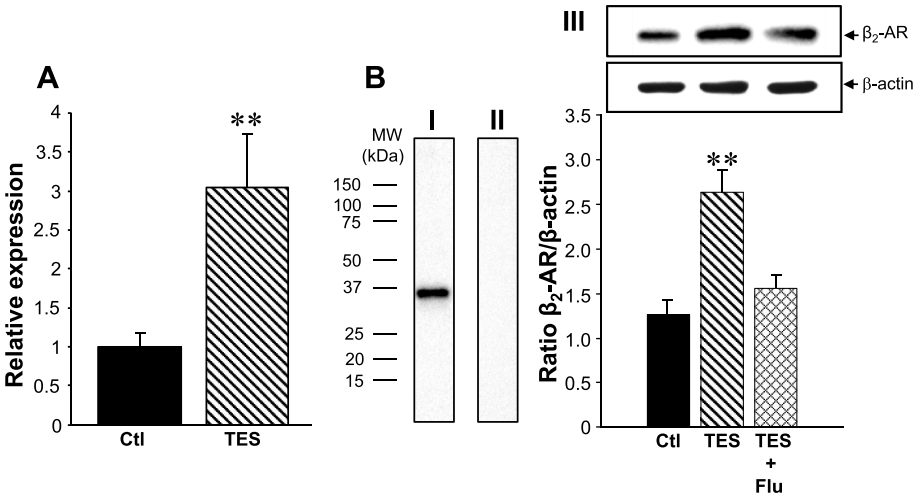

Fig. 10. Testosterone (TES, 40 nM for 48 h) increases β_2-adrenergic receptor (β_2-AR) in guinea pig tracheal smooth muscle. **A)** Bar graph illustrating the relative expression of β_2-AR mRNA as detected by qPCR analyzed with the $2^{-\Delta\Delta CT}$ method and its significant increase induced by TES, n = 4. **B)** Western blot analysis of β_2-AR expression; (**I**) shows the blot illustrating one band around 37 kDa corresponding to β_2-AR and (**II**) the negative control without antibody. (**III**) Densitometry results illustrating that the β_2-AR expression is augmented by TES and reduced with flutamide pre-treatment 30 min before TES. A representative Western blot is included at the top of the bar graph. **p < 0.01, n = 3. Bars represent mean ± S.E.M. Ctl = control.

5. Conclusions

Our results show that chronic ASM exposure to a physiological TES concentration (nanomolar) promotes β_2-AR, K_V, and K_{Ca} upregulation and consequently favors the response to β_2 adrenergic agonists, extensively used as bronchodilators in asthma.

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CRediT authorship contribution statement

Abril Carbajal-García: Methodology, Data curation, Formal analysis. **Jorge Reyes-García:** Methodology, Data curation, Formal analysis. **María F. Casas-Hernández:** Methodology, Data curation, Formal analysis. **Edgar Flores-Soto:** Conceptualization. **Verónica Díaz-Hernández:** Methodology, Data curation, Formal analysis. **Héctor Solís-Chagoyán:** Methodology. **Bettina Sommer:** Writing - original draft. **Luis M. Montaño:** Supervision, Resources, Validation, Formal

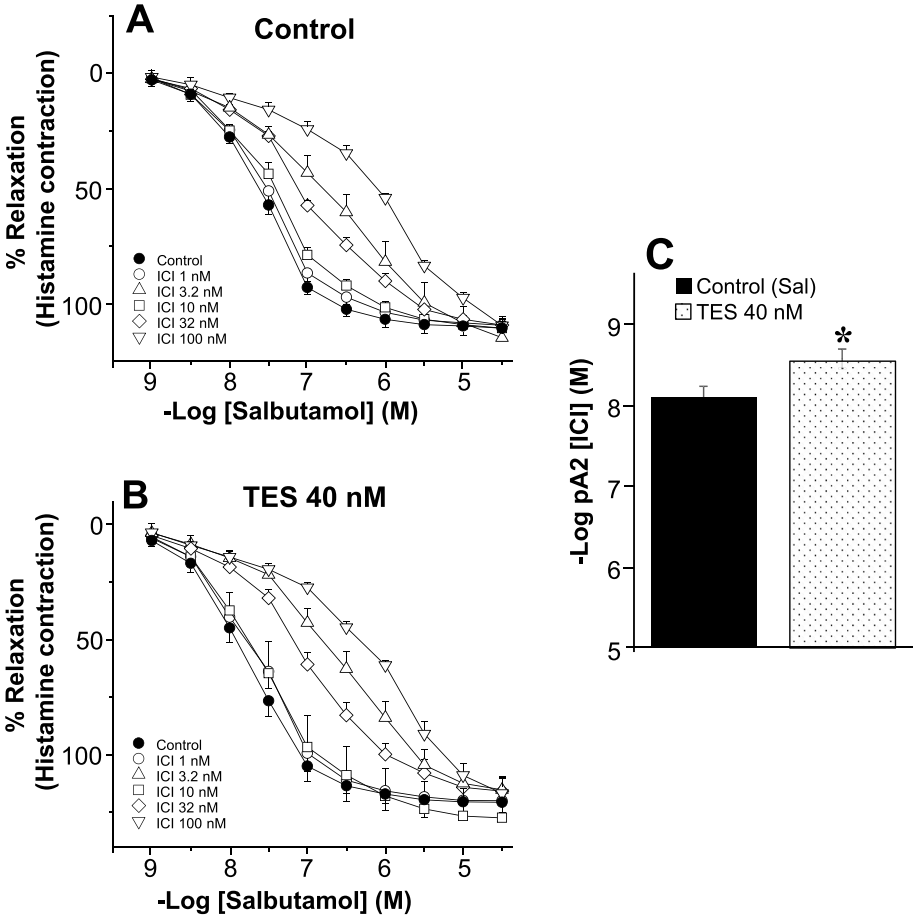

Fig. 11. Testosterone (TES, 40 nM for 48 h) decreases ICI 118.551 pA2 value in guinea pig airway smoot muscle, implying the augmented β_2-AR expression which mediates the increased salbutamol (Sal) relaxation. **A)** Salbutamol cumulative concentration curve showed a rightward shift when ICI 118.551 (ICI), a selective β_2-AR antagonist, was added; this displacement was ICI concentration dependent (1, 3.2, 10, 32 and 100 nM). **B)** TES incubation shifted the Sal cumulative concentration curve to the left (IC_{50} = 25.6 ± 5.4 nM, n = 7) as compared with the curve without TES (IC_{50} = 39.9 ± 3.1 nM, p = 0.05, n = 6). ICI also shifted the Sal concentration curve to the right in a concentration dependent fashion. With the IC_{50} values of each curve in **A** and **B**, the pA2 value was calculated. **C)** pA2 values were obtained with the Schild's equation; this value is an indirect measure of antagonist's affinity for its receptors and in this study, provides information about the role of the β_2-AR on the augmented Sal response. The bar graph illustrates that pA2 value was significantly lower (p < 0.05) for TES treated tissues when compared with the control (Sal) group without androgen. Symbols and bars represent mean ± S.E.M. *p < 0.05.

analysis, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest or financial relationships that may have infiuenced the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110801.

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Anexo 2. Artículo de revisión.

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Review Article

Androgen Effects on the Adrenergic System of the Vascular, Airway, and Cardiac Myocytes and Their Relevance in Pathological Processes

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Introduction. Androgen signaling comprises nongenomic and genomic pathways. Nongenomic actions are not related to the binding of the androgen receptor (AR) and occur rapidly. The genomic effects implicate the binding to a cytosolic AR, leading to protein synthesis. Both events are independent of each other. Genomic effects have been associated with different pathologies such as vascular ischemia, hypertension, asthma, and cardiovascular diseases. Catecholamines play a crucial role in regulating vascular smooth muscle (VSM), airway smooth muscle (ASM), and cardiac muscle (CM) function and tone. *Objective*. The aim of this review is an updated analysis of the role of androgens in the adrenergic system of vascular, airway, and cardiac myocytes. *Body*. Testosterone (T) favors vasoconstriction, and its concentration fluctuation during life stages can affect the vascular tone and might contribute to the development of hypertension. In the VSM, T increases α1-adrenergic receptors (α_1-ARs) and decreases adenylyl cyclase expression, favoring high blood pressure and hypertension. Androgens have also been associated with asthma. During puberty, girls are more susceptible to present asthma symptoms than boys because of the increment in the plasmatic concentrations of T in young men. In the ASM, β_2-ARs are responsible for the bronchodilator effect, and T augments the expression of β_2-ARs evoking an increase in the relaxing response to salbutamol. The levels of T are also associated with an increment in atherosclerosis and cardiovascular risk. In the CM, activation of α_{1A}-ARs and β_2-ARs increases the ionotropic activity, leading to the development of contraction, and T upregulates the expression of both receptors and improves the myocardial performance. *Conclusions*. Androgens play an essential role in the adrenergic system of vascular, airway, and cardiac myocytes, favoring either a state of health or disease. While the use of androgens as a therapeutic tool for treating asthma symptoms or heart disease is proposed, the vascular system is warmly affected.

1. Introduction

1.1. Metabolic Pathways of Steroids. Testosterone (T), the main testicular hormone, is produced by Leydig cells in high concentrations (95%). However, smaller amounts of T are also synthesized by the adrenal cortex [1–4]. The production and secretion of this androgen are regulated through luteinizing hormone (LH) stimulation. Cholesterol is the precursor of T, and the steroidogenesis is carried out through cytochrome P450 enzymes [5]. The conversion of cholesterol to pregnenolone is the first step in producing T and is accomplished by the P450 side-chain cleavage enzyme (P450cc/CYP11A1) [4, 5]. Subsequently, this progestogen is biotransformed either to 17α-hydroxypregnenolone or to progesterone via P450 17α-hydroxylase (P450c17/CYP17A1) and 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2), respectively. Afterward, 17α-hydroxypregnenolone is converted to dehydroepiandrosterone (DHEA) by cytochrome P450c17/CYP17A1 [5–7]. The conversion of DHEA to androstenedione via 3β-HSD2 or to androstenediol via 17β-hydroxysteroid dehydrogenase (17β-HSD3) is followed by the biotransformation to T by 17β-HSD3 or 3β-HSD2, respectively [5]. Furthermore, T is either reduced to 5α-dihydrotestosterone (5α-DHT) by 5α-reductase or to

5β-dihydrotestosterone (5β-DHT) by 5β-reductase [8–10]. Additionally, T can be converted to 17β-estradiol (E2) via the aromatase (P450aro/CYP19A1) action, and 17β-HSD3 catalyzes the formation of E2 from estrone (Figure 1) [5].

In women, T is produced and secreted by the ovarian stroma, particularly by theca and granulosa cells (25%), the adrenal zona fasciculata (25%), and from circulating androstenedione (50%) [11, 12]. Peripheral tissues such as placenta, liver, skin, prostate, and adipose tissue possess the specific enzymes (or the isoforms) required for the novo synthesis of androgens or their activation from circulating precursors [13]. Furthermore, in the vascular smooth muscle (VSM), airway smooth muscle (ASM), and heart (the tissues that this review is focused on), the expression of some steroidogenic enzymes has been demonstrated. For instance, CYP11A1 and 3β-HSD are expressed in cardiac [14, 15], vascular [15, 16], and lung tissue [17]. Nevertheless, CYP17A1, which is required for the conversion of pregnenolone into 17-hydroxypregnenolone, was not found in the heart [14, 15], and it has not been reported in vascular or ASM. Therefore, de novo androgen biosynthesis is unlikely to occur in those tissues. However, the expression of 17β-HSD5 in the fetal lung [18, 19] and 17β-HSD1,2 in the heart [20] can lead to the biotransformation of pre-existing precursors to T. Interestingly, no significant expression of 17β-HSD3 was found in the heart and the lung since this enzyme is considered to be testis-specific [21]. Furthermore, the presence of 5α-reductase in the cardiac tissue allows the formation of 5α-DHT [20]. Additionally, P450aro has been found in vascular tissues [22, 23], heart [20], and lung epithelial cells [24].

Men usually have much higher levels of T serum concentrations than women. In men from 13 to 80 years old, values of serum T are between 6 and 50 nM [25–27]. 5α-DHT (a more potent androgen) represents about 9-10% of the plasma T levels in males of most species [26, 28]. In women, stable serum values of T (0.7–2.5 nM) are maintained except during pregnancy when T concentrations increment (3.5–5 nM) [27]. Also, 5α-DHT is essentially produced in peripheral tissues and circulates in very low concentrations in women plasma (0.069 nM) [29].

1.2. Nongenomic and Genomic Actions of Androgens. The androgen signaling comprises nongenomic and genomic pathways. The nongenomic effects of androgens are independent of the binding to the cytosolic AR and occur in seconds to minutes [30]. Importantly, these effects are not altered by inhibitors of transcription and seem to be carried out by the androgen binding to plasma membrane lipids or ionic channels [2, 31–35]. Recently, two distinct membrane proteins have been suggested as membrane androgen receptors (mARs): G protein-coupled receptor family C group 6-member A (GPRC6A) and zinc-regulated transporter [Zrt]-protein 9 (ZIP9); both of them may stimulate intracellular pathways via G proteins or mitogen-activated protein kinases (MAPKs) [31, 36–38].

GPRC6A is a member of the C family of G protein-coupled receptors (GPCRs) activated by several ligands such as extracellular Ca^{2+}, cations, basic amino acids, osteocalcin, and T [31, 39–41]. Pi et al. in 2010 showed that the stimulation of GPRC6A triggers the inhibitory G protein α-subunit (Gαi), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), proto-oncogene c-Src kinase (Src), and Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways [42]. Most recently, the same authors reported that the activation of GPRC6A by testosterone induces cell proliferation and inhibits autophagy through the mammalian target of the rapamycin complex 1 (mTORC1) signaling cascade in prostate cancer cells [43]. ZIP9 is a protein that possesses seven membrane-spanning domains and was first identified as a member of the SLC39A zinc transporter family in Atlantic croaker ovaries [44]. The stimulation of ZIP9 leads to the activation of the Gq protein α-subunit (Gq11) in spermatogenic cells, the stimulatory G protein α-subunit (Gαs) in ovarian follicle cells, and the inhibitory G protein α-subunit (Gαi) in prostate cancer cells [36, 37, 44]. Moreover, the activity of ZIP9 (dependent on **T** stimulation) has also been explored in a Sertoli cell line, where this receptor modulates the phosphorylation of ERK1/2 [45]. While the MAPKs signaling pathway can lead to transcription modulation [46], the role of the mARs in the physiology of cardiac and smooth muscle cells is still unrevealed.

The genomic effects of T occur from hours to days and involve the binding of the androgen to a cytosolic androgen receptor (AR). This hormone receptor, also known as NR3C4, is a member of the nuclear receptor family [47, 48]. As in other nuclear receptors, the protein structure of the AR comprises the *N*-terminal domain (NTD), the DNA-binding domain (DBD), the hinge domain (HD), and the ligand-binding domain (LBD) [49]. The stimulation of the AR by T or 5α-DHT elicits the dissociation of chaperone proteins and the formation of a complex that is transferred to the nucleus where it modulates gene transcription and protein synthesis [2]. 5β-DHT, the other reduced metabolite of T, possesses minor androgenic activity due to a lower binding affinity than 5α-DHT [50]. The AR is expressed in several mammalian tissues, including vascular and airway smooth muscles and cardiac myocytes [2, 51–56]. Furthermore, the activity of the AR has been implicated in cardiovascular and respiratory ailments such as vascular ischemia [53], hypertension [57, 58], asthma [52], and cardiac hypertrophy [54].

In the last years, numerous AR splice variants have been molecularly identified and characterized in humans. Although the function of these alternative AR transcripts in the human physiology is not completely understood, these variants have been related to pathological conditions such as prostate cancer (PCa) and androgen insensitivity syndrome (AIS) [59–62]. In 2005, Ahrens-Fath et al. reported the existence of an NTD-truncated AR isoform with a molecular weight of 45 kDa (AR45) in the heart, skeletal muscle, uterus, prostate, breast, and lung [63]. However, the expression level of AR45 compared with the wild-type AR in these tissues is arguable since a semiquantitative RT-PCR was performed by Ahrens-Fath et al. Also, this receptor variant is expressed in

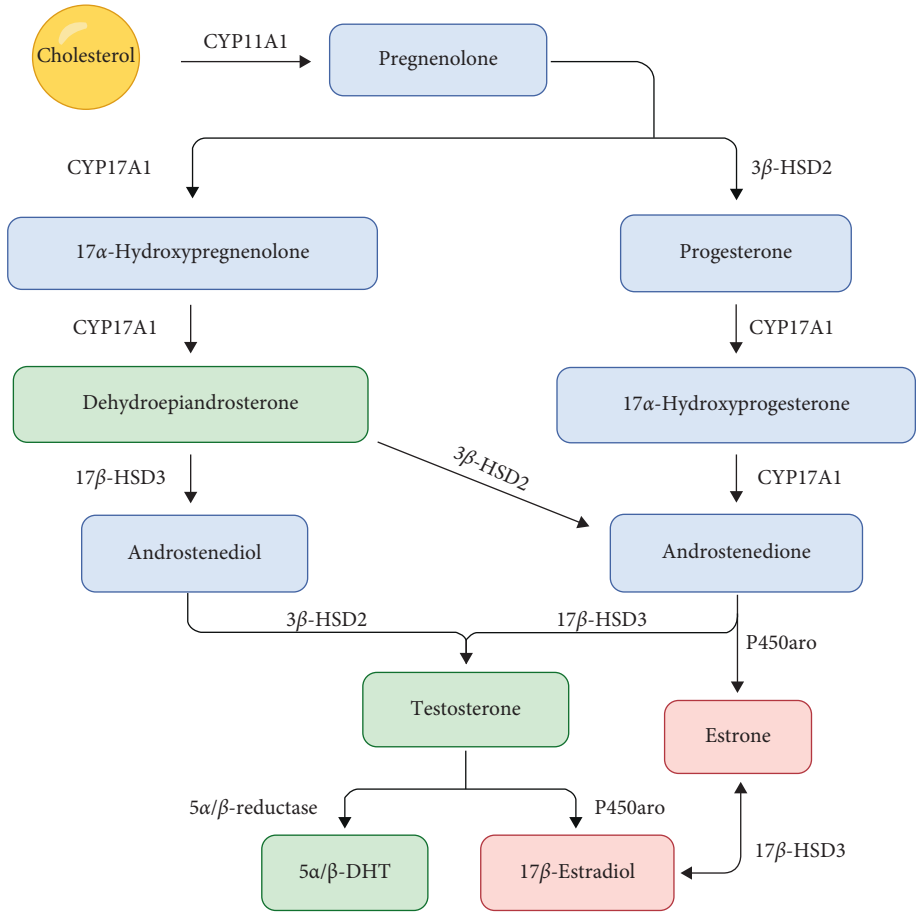

Figure 1: Androgen synthesis from cholesterol. Steroidogenesis in males is carried out mainly by Leydig cells and in females by theca and granulosa cells. Cholesterol is the precursor of all sex steroids, and its conversion to pregnenolone is mediated by the cholesterol side-chain cleavage cytochrome P450 enzyme (CYP11A1/P450scc). Once formed, this progestogen is converted into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD2). Then, 17α-hydroxylase/17,20 lyase (CYP17A1/P450c17) hydroxylates pregnenolone to produce 17α-hydroxypregnenolone and subsequently removes the acetyl group to form dehydroepiandrosterone (DHEA). This last product can be either converted into androstenedione via 3β-HSD2 or into androstenediol by 17β-hydroxysteroid dehydrogenase (17β-HSD3). Androstenedione and androstenediol are further biotransformed to testosterone by 17β-HSD3 and 3β-HSD2, respectively. Furthermore, testosterone can be reduced to 5α- or 5β-dihydrotestosterone (5-α/β-DHT) by 5-α/β-reductases. Furthermore, P450 aromatase (P450aro) may convert testosterone into 17β-estradiol and androstenedione into estrone. Finally, 17β-HSD3 catalyzes the formation of 17β-estradiol from estrone.

the normal prostate tissue and in human prostate adenocarcinoma derived from the left supraclavicular lymph node metastasis (LNCaP) cells [64, 65]. Additionally, it has been shown that AR45 may repress or stimulate wild-type AR activity [63]. Interestingly, 12 AR variants lacking the LBD (ARV1-12) have been identified in PCa cell lines [64–67]. Among all the ARV isoforms, ARV7 (also known as AR3) has gained relevance due to its demonstrated capability of mediating constitutively AR functions, i.e., constitutive gene transcription in the absence of androgen stimuli. Moreover, ARV7 has been suggested as a predictive biomarker in castrate-resistant PCa since it promotes cancer progression and androgen depletion-resistant growth by regulating serine/threonine kinase 1 encoding gene (AKT1) [64–67]. In spite of the emerging evidence about AR splice variants, further studies are imperative in order to elucidate the possible expression and the physiological role of these alternative transcripts in vascular and airway smooth muscles and cardiac muscle.

Noteworthy, it has been proposed that androgen nongenomic and genomic actions may converge. For instance, in the vascular smooth muscle, the regulation of K^+ channels is dependent on nongenomic and genomic effects of androgens [30]; however, cellular mechanisms and signaling pathways displayed in both types of actions are entirely different and carried out by distinct effector proteins.

1.3. Androgens and Vascular, Airway, and Cardiac Muscles. Vascular smooth muscle (VSM), airway smooth muscle (ASM), and cardiac muscle (CM) cells are excitable entities, with the primary function of contracting and relaxing [68]. Several research groups have shown that androgens interact with the contraction and relaxation mechanisms of different

muscular cell types from distinct species through nongenomic and genomic effects.

With respect to nongenomic actions, in the VSM, numerous authors have reported that androgens induced vasorelaxation in different arteries [69–74]. In this regard, in the ASM, our group and others have observed that DHEA, T, 5α-DHT, and 5β-DHT induced-relaxation through nongenomic actions [33, 75–78].

In relation to the genomic actions, it has been reported that T and DHT induced in the VSM, the genic expression of proteins such as adenylyl cyclase (AC), Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}), and L-type voltage-dependent Ca^{2+} channels (L-VDCCs) [73, 79]. Most recently, we found in the ASM that T augmented the expression of β_2-ARs, favoring an increase in the relaxing response to salbutamol [51]. In the CM, it has been described that androgens (via a genomic effect) increased the expression of the voltage-dependent delayed rectifier K^+ channel 1.5 ($K_V1.5$), leading to shortening of the action potential duration in mice ventricular cardiomyocytes [80], and also enhanced the expression of $K_V1.7$ diminishing the QT intervals in rats [81]. Testosterone nongenomic and genomic actions and their association with the adrenergic system of vascular, airway, and cardiac myocytes are discussed in the next sections of this manuscript.

1.4. Adrenergic Receptors in Vascular, Airway, and Cardiac Muscles. Under physiological conditions, the adrenergic system plays a critical role in regulating vascular, airway, and cardiac function. In the VSM and CM, sympathetic innervation modulates contraction [82] and the intrinsic conduction system [83, 84], respectively. The ASM tone is partly regulated through circulating catecholamines such as epinephrine released from the adrenal medulla [85, 86]; this hormone acts as an adrenergic receptor agonist. The adrenergic receptors or adrenoceptors are members of the superfamily of G protein-coupled receptors (GPCRs) and modulate several pathways through effectors such as AC or phospholipase C (PLC) [87]. Adrenergic receptors have been classified into three major categories: alpha-1-adrenergic receptors (α_1-ARs), alpha-2-adrenergic receptors (α_2-ARs), and beta-adrenergic receptors (β-ARs). Moreover, each of these groups has been further subclassified into multiple subtypes defined by the differences in their genetic sequences and their pharmacological action: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, and β_3 [88, 89].

α_1-ARs are coupled to a heterotrimeric Gq protein and PLC signaling pathway. PLC triggers the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), resulting in the increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the activation of protein kinase C (PKC) [87, 90–92]. Also, the stimulation of α_1-ARs promotes an extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs) [93] and triggers extracellular signal-regulated kinases 1 and 2 (ERK1/2) [94, 95]. In humans, α_{1A}, α_{1B}, and α_{1D} adrenergic receptors are encoded by distinct genes located on chromosomes 8, 5, and 10, respectively [87]. The three subtypes of α_1-ARs are present in most blood vessels modulating smooth muscle contraction and vascular tone. α_{1A} is the most prevalent subtype in human arteries; nevertheless, the expression levels of α_1-ARs depend on the vascular bed studied. α_{1D}-AR subtype predominates in large conduction vessels as the aorta and carotid arteries, whereas α_{1A}-AR subtype is involved in regulating vascular tone of mesenteric, splenic, pulmonary, and caudal (in mice and rats) arteries controlling organ blood flow [96–101]. While α_{1A} and α_{1D}-ARs are the main subtypes involved in vascular contractions, α_{1B}-AR subtype is also expressed in several blood vessels, and it was thought that it did not require extracellular Ca^{2+} to activate smooth muscle contraction [99, 100]. Unfortunately, studies related to α_{1B}-AR function in the VSM have been restrained by the lack of selective antagonists. However, this receptor subtype has been proposed to be involved in the regulation of systemic BP [102–104] and coronary blood flow [105].

The evidence of α-ARs in the ASM is also present; nonetheless, these receptors seem not to be relevant in the functionality of this tissue. In this context, norepinephrine-induced contraction has been observed in guinea pig [106, 107], rabbit, cat, and rat [107] tracheal preparations but only after β-AR blockade. Interestingly, Kneussl and Richardson in 1978 found that human and dog ASM did not contract in response to norepinephrine, unless they were previously stimulated with histamine or KCl [108]. These insights confirm the predominance of relaxant β-AR function in the ASM of most mammals. However, in 1985, Montaño et al. revealed that the *Erythrocebus patas* monkey possesses α-AR predominance in this tissue [109].

In cardiomyocytes of species such as mice, rats, and humans, all three α_1-AR mRNAs have been detected with the predominance of α_{1A}- and α_{1B}-AR subtypes [105, 110–112]. Although the stimulation of these receptors in cardiomyocytes can evoke muscle contraction, most works have been focused only on ventricular heart sections. In this context, it has been observed that norepinephrine and epinephrine can induce positive inotropic and chronotropic effects in the right atrium from mice, probably through α_1-AR signaling [113]. Nevertheless, different studies have shown that, in the heart, α_1-ARs are mainly involved in processes such as hypertrophic responses, upregulation of myosin light chain-2, modulation of the atrial natriuretic factor (ANF), and heart failure [114–120].

α_{2A}, α_{2B}, and α_{2C} adrenoceptor genes are located on human chromosomes 10, 4, and 2, respectively. The encoded products share about 50% of amino acid identity and show the same affinity for norepinephrine and epinephrine [121]. All α_2-ARs are coupled to the pertussis toxin-sensitive G proteins such as Gi/Go and to the inhibition of the AC. The consequence of the inhibition of this enzyme is a decrease in the production $3', 5'$-cyclic adenosine monophosphate (cAMP), reducing the activity of protein kinase A (PKA) [121–125]. Additionally, Gi/Go signaling cascade modulates Ca^{2+} [126, 127] and K^+ [128, 129] channels without the involvement of other second messengers. VSM cells express all subtypes of α_2-ARs, and their stimulation is related to contraction and vasopressor effects [124, 130–132]. α_{2A}-Adrenoceptor is the most predominant subtype in this tissue

and participates in the regulation of the muscular tone in the aorta [133] and carotid (possibly controlling cerebral blood flow) [134, 135] and mesenteric arteries [136] and in the peripheral vasoconstriction related to the skin blood flow [137]. α_{2B}-AR is more involved in the vascular tone of smaller arteries [138] but contributes to BP regulation to a greater extent than α_{2A}- and α_{2C}-ARs [139]. Furthermore, α_2-adrenoceptors seem to play a minor role in cardiac contractility compared to β- and α_1-ARs. Recently, it was demonstrated in ventricular cardiomyocytes that the stimulation of α_2-ARs could modify $[Ca^{2+}]_i$ and induce myocardial contraction [140, 141].

β-ARs, like all other adrenergic receptor subtypes, are composed of seven transmembrane spanning helices. The three subtypes (β_1, β_2, and β_3) are found in VSM [87], ASM [142], and CM [143] cells. Their coding genes are located in human chromosomes 10, 5, and 8, respectively [87, 144, 145]. The stimulation of the β-AR mediates the activation of AC and the subsequent increment in the production of cAMP [146, 147]. In its active state, β-AR is associated with the α-subunit of Gs protein. In the VSM, the activation of β-ARs induces the relaxation of the tissue, regulating the peripheral vascular resistance and controlling the organ blood flow and vascular tone [87]. Among all β-ARs, β_2-AR is the predominant expressed subtype in most vascular beds, while a minor proportion of β_1-ARs is also present. Apparently, β_1-adrenoceptors play an essential role in the function of coronary and cerebral arteries [87, 148–151]. β-Adrenoceptors also occur in endothelial cells where they mediate vasodilation through nitric oxide (NO) production [152]. In airways, β_2-agonists are well known as the most effective bronchodilators. The β_2-agonist binding to the β_2-AR in the cell membrane of the ASM triggers the formation of cAMP by the action of the AC [153–155]. Subsequently, the increment of the cAMP levels activates PKA, a phosphorylating protein, which favors K^+ channel opening and bronchorelaxation [156]. In the heart, β_1 and β_2 are the most valuable adrenoceptor subtypes with a predominance of β_1-ARs over β_2-ARs (ratio of ~80/20). The stimulation of these receptors in cardiomyocytes mediates positive chronotropic, inotropic, and lusitropic effects [157–159]. Gs-PKA signaling in cardiomyocytes promotes the phosphorylation of phospholamban (PLB), L-VDCCs, ryanodine receptors (RyRs), and cardiac myosin-binding protein C leading to an increase in $[Ca^{2+}]_i$ and favoring muscle contraction [160]. Interestingly, it has been demonstrated that a sustained activation of β_1-ARs may induce cardiotoxic effects, and β_2-ARs switch their natural Gs coupling to Gi protein coupling, opposing the positive β_1-AR effects [157, 161, 162].

It is well known that adrenoceptors play a key role in maintaining vascular, airway, and cardiac muscular function. In this regard, the modulation by T of the adrenergic receptor signaling pathway has been investigated, and the observed effects appear to be dependent on the studied tissue and the predominance of the adrenergic receptor subtypes either favoring muscle relaxation or contraction [51, 79, 163, 164]. This review focuses on the effects of T on the adrenergic system in the vascular, airway, and cardiac muscles and its relevance in pathological processes related to this system.

2. Vascular Smooth Muscle

The maintenance of the vascular tone is due to the balance between vasoconstriction and vasorelaxation modulated by several neurotransmitters and hormones [165]. The VSM found in the medial layer of the blood vessels is responsible for controlling vascular tone and blood pressure (BP) [166]. The regulation of the VSM membrane potential and the vascular tone is mainly determined by Ca^{2+} and K^+ channels [167, 168]. The main K^+ channels expressed in the VSM are the voltage-dependent delayed rectifier K^+ channels (K_V), Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}), ATP-sensitive K^+ channels (K_{ATP}), and inward-rectifier K^+ channels (K_{IR}) [169, 170]. VSM constriction is caused by increments in $[Ca^{2+}]_i$ [171]. Vasoconstrictor agonists act on GPCRs coupled to the $_q\alpha$ subunit (GPCR-$_q\alpha$) such as α_{1A}-, α_{1B}-, and α_{1D}-ARs, bradykinin, histamine H_1, and thromboxane-A_2 receptors, among others [172–174]. These receptors activate the PLC enzyme and IP_3 signaling pathway, inducing the release of Ca^{2+} from the sarcoplasmic reticulum (SR) and the influx of this ion through VDCCs [175]. In the VSM, two major subtypes of VDCCs with distinct electrophysiological properties are present. L-VDCCs are activated by large depolarizations and inactivated relatively slowly. T-type voltage-dependent Ca^{2+} channels (T-VDCCs) are activated by small depolarizations and inactivated rapidly [176, 177].

Moreover, VDCCs are not the only source of extracellular Ca^{2+}. The influx of this ion is also carried out by nonselective cation channels such as receptor-operated Ca^{2+} channels (ROCCs), store-operated Ca^{2+} channels (SOCCs), and transient receptor potential (TRP) channels. The Ca^{2+} influx exerted by these channels is thought to be triggered by agonists such as norepinephrine, vasopressin, and acetylcholine via GPCRs linked to the phospholipase C_β (PLC_β) signaling pathway and the formation of IP_3 and DAG. This last second messenger regulates the activity of ROCCs, and IP_3 induces depletion of internal Ca^{2+} stores leading to capacitative Ca^{2+} entry through SOCCs [178–181]. Additionally, TRP channels have been classified as ROCC subtypes, and transient receptor potential canonical channels 3, 6, and 7 (TRPC3, TRPC6, and TRPC7) have been shown to be susceptible to DAG stimulation promoting its opening and contributing to Ca^{2+} influx. Afterward, Ca^{2+} complexes with calmodulin to activate myosin light-chain kinase (MLCK) causing vasoconstriction [168, 182]. Conversely, the decrease in cytosolic Ca^{2+} leads to vasorelaxation [183]. Vasodilator agonists that stimulate GPCRs coupled to the $_s\alpha$ subunit (GPCR-$_s\alpha$) such as β_1- and β_2-ARs, histamine H_2, prostaglandin E_2, and adenosine A_2 receptors, among others [184], induce the synthesis of cAMP and $3', 5'$-cyclic guanosine monophosphate (cGMP); therefore, they activate PKA and protein kinase G (PKG), respectively [185], leading to a decrease in the vascular tone [183]. In the last two decades, the evidence about the relationship between androgens and vascular reactivity has increased. The

nongenomic effects of T in the VSM can be due to its action on ion channels resulting in vasorelaxation. In 1996, Perusquía et al. postulated that T, 5β-DHT, and 5α-DHT induced vasorelaxation in the rat aorta [186]. Later on, the same group observed that T was capable of blocking the extracellular Ca^{2+} influx inducing vasorelaxation of the precontracted human umbilical artery [72]. More recently, it was demonstrated that 5β-DHT and T induced vasorelaxation by blocking L-VDCCs in the rat thoracic aorta [70]. In addition to blocking Ca^{2+} entry through L-type Ca^{2+} channels, T is capable of activating K^+ channels. The efflux of K^+ evokes membrane hyperpolarization and closes Ca^{2+} channels leading to vasorelaxation in pig [187] and rabbit [188] coronary arteries. In this regard, different types of K^+ channels have been proposed as targets for T modulation. In the dog coronary artery [189] and rat aorta [190], K_{ATP} channels have been shown to be involved in the T-associated relaxant effect. BK_{Ca} channel activation in the human internal mammary artery [191] and pig coronary artery [187] is also implicated in T-induced vasorelaxation. Moreover, Saldanha et al. demonstrated that T produced relaxant responses in human umbilical artery rings precontracted with serotonin (5-HT), histamine, and KCl, and these effects were dependent on both BK_{Ca} and K_V channel activity (Figure 2(a)). They also studied the long-term effects of androgens in the same model, founding that DHT, through genomic actions, decreased the mRNA expression of the α-subunit of L-VDCC and upregulated the β_1-subunit of BK_{Ca}, favoring relaxation [73].

2.1. The Effects of Testosterone on Adrenergic Receptors in the Vascular Smooth Muscle. Sex differences in cardiovascular diseases, i.e., hypertension, have been broadly studied. Men are more likely to develop hypertension or coronary heart disease (CHD) than women [192–194]. Hypertension is defined as persistent systolic BP $\geq$ 140 mmHg and or diastolic BP $\geq$ 90 mmHg, according to 2018 ESC/ESH guidelines [195]. The World Health Organization has rated hypertension as one of the deadliest causes of premature death worldwide due to its asymptomatic behavior that can result in concomitant diseases after years. In this regard, sex differences in the development of hypertension have been reported. Female sex hormones, such as estrogens, have been widely implicated in the hypertension-related gender differences [57]; however, several authors have pointed out a prohypertensive role for androgens [58]. Studies in humans and castrated rats revealed that androgens exert a prohypertensive effect, while estrogens appear to oppose the increase in BP [196]. In this context, Torres et al. found in castrated male Wistar rats an increment in aortic vasodilation, indicating a sex hormone influence [197]. Another research group observed that gonadectomized hypertensive rats, both males and females, showed a reduced BP, and the administration of T restored it in the castrated male experimental group [198]. Moreover, it has been proposed that the effect of T on VSM does not benefit a state of relaxation but rather favors vasoconstriction. Fluctuations in androgen concentrations throughout life stages can affect the vascular tone, and T may contribute to developing hypertension [58]. In this sense, hyperandrogenism (HA) in pre- and postmenopausal women has been associated with an unfavorable metabolic profile, obesity, and hypertension [199–201]. HA is defined as an excess of androgen production and secretion by adrenal glands or the ovaries [202]. Moreover, the development of HA in females has shown to be associated with ovarian disorders, e.g., ovarian hyperthecosis (OH) [203], virilizing ovarian tumors (VOTs) [204], and polycystic ovary syndrome (PCOS) [199–201]. PCOS is one of the most common endocrine disorders affecting women of reproductive age [205]. The metabolic phenotype in PCOS is characterized by increased LH compared with the follicle-stimulating hormone (FSH) and HA [205, 206]. Furthermore, evidence points out that hyperandrogenemia in women suffering from PCOS is associated with an increased systolic and diastolic BP, and this relation is independent of other risk factors such as obesity and insulin resistance [207].

During aging, the vascular tone is led to vasoconstriction, and β-ARs have been proposed as targets of several drugs related to hypertension disease [208]. Aged animals have a weak vascular response to β-AR agonists, and possibly, mechanisms of the β-AR signaling pathway are altered [209]. Vascular tone is modulated through the action of the sympathetic nervous system (SNS) on β-ARs promoting the increase in cAMP levels [142, 210]. It has been reported that androgens promote vasoconstriction by increasing catecholamine (mainly norepinephrine) levels [57]. In 2005, Martin et al. demonstrated that the adrenergic system (through norepinephrine action) reduced the mean arterial pressure in castrated male spontaneously hypertensive rats (SHR) [211]. In other studies, vascular tone at different stages of rat growth was compared to explore the role of T in β-adrenergic-induced vasodilation [79]. In aortic rings of mature rats, vasorelaxation response induced by isoproterenol (a well-known unspecific β-adrenergic agonist) showed an impairment of this response compared to aortic rings obtained from younger rats. According to the authors, this impaired relaxing response could be related to higher plasma T-levels in older rats. The authors elegantly demonstrated that T reduced the β-AR-elicited vasorelaxation without any alteration in the expression of the β_2-AR but interfering downstream in the signaling cascade. Furthermore, the authors exhibited that T (via a genomic effect) diminished the expression of AC and yielding of cAMP in castrated rats [79]. These findings point out that changes in the levels of T could lead to high BP and hypertension.

Furthermore, the vessel tone is also regulated by α-ARs. These receptors promote vasoconstriction and might contribute to hypertension development [92]. In this context, the modulation of the α_1-AR by T has been reported. Testosterone replacement therapy increased BP in gonadectomized SHR and the number of α_1-ARs in the tail artery [164]. Furthermore, in 1999, it was found that the incubation for 24 hr with T (0.1 nM–1 μM) increased the abundance of α_{1B}-AR mRNA in VSM cells through a genomic action. The same study reported that glucocorticoids, such as dexamethasone, increased catecholamine-mediated

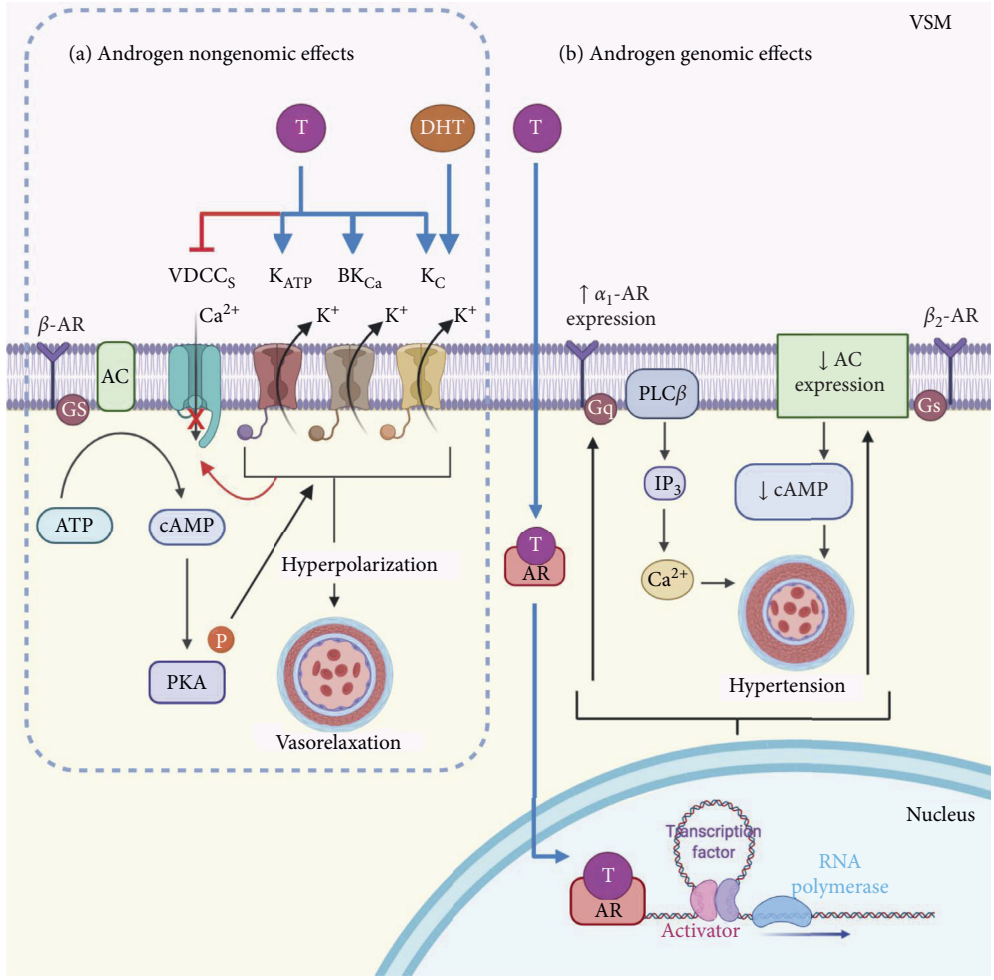

Figure 2: Androgen effects on the adrenergic system in the vascular smooth muscle (VSM). (a) Stimulation of the β-adrenergic receptor (β-AR) leads to an increase in the activity of the K^+ channels and to plasma membrane hyperpolarization. β-AR receptor is coupled to a Gs protein (Gs) that activates adenylyl cyclase (AC), which enhances the synthesis of 3′,5′-cyclic adenosine monophosphate (cAMP) and consequently promotes the protein kinase A- (PKA-) induced phosphorylation of the K^+ channels. K^+ channel phosphorylation increases their open probability and evokes membrane hyperpolarization that closes Ca^{2+} channels, leading to vasorelaxation. Testosterone, via a rapid response (nongenomic), activates ATP-sensitive K^+ channels (K_{ATP}), Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}), and voltage-dependent delayed rectifier K^+ channels (K_V). Dihydrotestosterone (DHT, a reduced metabolite of T) enhances the activity of the K_V channel. T also blocks VDCCs. Androgen-induced vasorelaxation mediated by the activation of K^+ channels and the blockade of VDCCs might improve the response of β-AR signaling. (b) The genomic androgen receptor (AR) signaling involves androgen crossing the plasma membrane, entering the cytoplasm, dissociation of chaperone proteins, and binding to its cytosolic receptor. AR stimulation by T results in a decrement of AC expression and a reduction of cAMP synthesis. Moreover, T increases the α_1-adrenergic receptor (α_1-AR) expression. This receptor is coupled to a Gq protein (Gq), which, through phospholipase C$_\beta$ (PLC$_\beta$), catalyzes the formation of inositol-1, 4, 5-triphosphate (IP_3) and triggers intracellular calcium release from the sarcoplasmic reticulum (SR). The genomic effects of T favor vasoconstriction in the VSM and might lead to hypertension development.

vasoconstriction due to an increased α_{1B}-AR expression [212]. In this context, T is not the only steroid hormone related to vascular physiology. High concentrations of glucocorticoids (such as cortisol) promote the retention of sodium and decrease the activity of prostaglandins leading to a contracted state of the VSM [213].

Although the regulation through norepinephrine of the vessel tone is essential for both females and males, the existence of sex differences in vessel vasoconstriction and vasodilatation has been reported. In 2017, Al-Gburi et al. demonstrated that the α-adrenergic vasoconstriction was weaker in female than male rats. They also found that the stimulation of β_1-, β_2-, and β_3-ARs evoked a greater response of relaxation in females than in males [214]. The diminished vasoconstriction and the enhanced vasorelaxation were due to the upregulated expression of the β_1- and β_3-ARs mainly in an endothelial location in female rats. Later on, Riedel et al. confirmed the overexpression of the β_1- and β_3-ARs in endothelial cells of the blood vessel by the action of the estrogens. The endothelial adrenergic stimulation caused an enhanced NO-dependent vasorelaxation in female rats [215], counteracting the vasoconstrictive outcomes

modulated by the α-ARs [215]. These findings could explain very well the sex and age differences on the role of the adrenergic response in the VSM.

In conclusion, **T** reduces the β-AR-elicited vasorelaxation by interfering downstream in the signaling pathway and upregulates the α-AR expression (Figure 2(b)). These hormonal effects are carried out principally through genomic actions leading to vasoconstriction and might be involved in the development of hypertension. Nevertheless, androgen nongenomic actions have opposite outcomes in the VSM, yielding their effects to vasorelaxation. However, the genomic actions of androgens (long-term effects) seem to be the predominate deleterious effects favoring hypertension. Therefore, the possible use of androgens, due to their nongenomic actions, as a therapeutic tool for the treatment of hypertension could not be appropriated based on their long-term genomic actions.

3. Airway Smooth Muscle

The maintenance of proper air flux through the airways results from the balance between contraction and relaxation of the ASM. The response of the ASM to physiological and pathophysiological stimuli determines the airway caliber in order to regulate the airflow [216]. The basal tone of the ASM is maintained by the influx and efflux of Ca^{2+} across the cell membrane, keeping an intracellular basal Ca^{2+} concentration ($_b[Ca^{2+}]_i$) around 100–150 nM [33, 77, 217–219]. The SR, ion channels, GPCRs, ATPases, and other mechanisms preserve $_b[Ca^{2+}]_i$ in the ASM cells. The mechanisms responsible for the Ca^{2+} influx are carried out by transient receptor potential canonical 3 (TRPC3), L-VDCCs and T-VDCCs, ROCCs, SOCCs, and reverse-mode Na^+/Ca^{2+} exchanger (NCX_{REV}) [77, 219]. Endogenous agonists such as acetylcholine, histamine, and leukotrienes act through the GPCRs-$_q\alpha$ pathway. These receptors activate the PLC_β enzyme, which catalyzes the formation of DAG and IP_3, favoring SR Ca^{2+} release through the IP_3 receptor [220]. Increased Ca^{2+} in the cytosol promotes the release of more Ca^{2+} (Ca^{2+} sparks) through RyRs; this event is known as Ca^{2+}-induced Ca^{2+} release (CIRC) [221, 222]. Increase in $[Ca^{2+}]_i$ is restored by two ATPases: sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plasma membrane Ca^{2+}-ATPase (PMCA) [223, 224]. Airway smooth muscle relaxation is predominantly mediated by the sympathetic system. Circulating epinephrine is more important in mediating relaxation in human airways than norepinephrine. In the ASM, β_2-AR is the main adrenoceptor subtype responsible for the bronchodilator effect [156]. Activation of this receptor triggers the formation of cAMP and, consequently, the activation of PKA [156]. PKA-mediated phosphorylation modulates proteins involved in the control of the airway muscle tone by regulating the Ca^{2+} availability and inactivating myosin light-chain kinase [225]. Furthermore, it is well known that the activation of the β_2-AR favors hyperpolarization and relaxation of the ASM through the opening of different K^+ channels [156, 226]. In the ASM, the main K^+ channels are the Ca^{2+}-activated K^+ channels (K_{Ca}) and K_V [227, 228]. K_{Ca} are activated by increases in $[Ca^{2+}]_i$ and through the cAMP-PKA signaling pathway [229, 230]. There are three subfamilies of K_{Ca}, all of them occurring in airways: high conductance (BK_{Ca}), intermediate conductance (IK_{Ca}), and low conductance (SK_{Ca}) [229]. Moreover, K_V have been characterized as $K_V1.2$, $K_V1.5$, and $K_V7.5$ in the ASM [227, 231]. Several agonists can lead to the bronchodilation of the ASM involving the opening of distinct K^+ channels. In this regard, it has been shown that the most critical channels in bronchodilation induced by 5-HT and ATP are BK_{Ca} [226, 228]. Most recently, our research group demonstrated that both K_{Ca} and K_V are implicated in salbutamol-induced relaxation in guinea pig airways [51].

Sex hormones play a role in the development of lung diseases. Androgens have been associated with asthma. During puberty, girls are more vulnerable to present asthma symptoms than boys, until the fifth life decade, when men become more susceptible than women [232, 233]. It has been reported that the variations in sex hormones during the menstrual cycle, hormone replacement therapy, and pregnancy have an influence in asthma patients [234–236]. Asthma is a chronic and inflammatory disease, characterized by hyperresponsiveness of the airways (AHR). This phenomenon is presented as an increased reactivity of the ASM to different agonists that leads to exaggerated bronchoconstriction. In addition, this disease is conducted by a type 2 immune response through eosinophils, basophils, mast cells, etc. [237]. However, not all asthma patients course with type 2 inflammation; instead, they can display interleukin-17- (IL-17-) mediated neutrophil inflammation [238].

Several studies have exposed that T induces a potential ASM relaxation effect through a nongenomic effect. An early work was conducted in the rabbit tracheal smooth muscle previously contracted with cholinergic agonists. The addition of T relaxed the ASM in an epithelium-dependent way involving NO production [78]. Later on, it was found that T relaxed precontracted guinea pig and bovine tracheal smooth muscles in an epithelium-independent way by blocking L-VDCCs [76]. In this context, our group demonstrated that T blocked L-VDCCs and SOCCs in the guinea pig ASM [34]. Additionally, the same study revealed that T induced the synthesis of prostaglandin E_2 (PGE_2), the main relaxing prostanoid in the airways [34]. Our studies pointed out that the blockade of the L-VDCCs and SOCCs and the production of PGE_2 are the main components of the T-induced relaxation in guinea pig precontracted airways. Then, we observed that T did not only relax the guinea pig ASM but lowered $_b[Ca^{2+}]_i$ and the muscular tone through the inhibition of L-VDCCs and TRPC3 [77, 219]. Most recently, our research group found that T interfered with the IP_3 receptor, decreasing the cholinergic-induced guinea pig ASM contraction [33]. Noteworthy, all the previously mentioned effects of T on ASM were carried out through nongenomic effects. Likewise, T, via a genomic action, negatively regulates type 2 inflammation and the expression of IL-17A [239, 240]. Furthermore, it was found that androgens, via AR activation, mediate the regulation of intracellular Ca^{2+} increment induced by proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interleukin-13 (IL-13) in the human ASM [52]. All these

androgen effects contribute to diminishing the ASM reactivity and favor the absence of asthma symptoms.

3.1. The Effects of Testosterone on Adrenergic Receptors in the Airway Smooth Muscle. Treatment with β-agonists to reverse airway obstruction, as seen in asthma and chronic obstructive pulmonary disease (COPD), has an essential role in controlling exacerbations. Therapeutically, there are two types of β-agonists: long-acting β-agonists to manage asthma together with glucocorticoids and short-acting β-agonists to relieve exacerbations [241]. Physiologically, the circulating catecholamines mediate the relaxation of the airways in humans. The androgen effects on the expression or function of the β-AR in the ASM have been scantly studied. In 1972, Salt and Iverson reported that T, via a nongenomic action, acted as an inhibitor of the extraneuronal uptake for catecholamines in the CM [242]. In this context, it was found that T potentiated the relaxation induced by isoprenaline (a nonselective β-adrenergic agonist) in pig bronchus, also via a nongenomic effect. The authors claimed that the potentiation effect observed was due to the inhibition of catechol-O-methyl transferase (COMT) or abolition of extraneuronal uptake [243]. In 2008, Bordallo et al. showed that 5α-DHT (a reduced metabolite of T) potentiated the relaxation induced by salbutamol, a β_2-adrenergic agonist, in the bovine tracheal ASM [76]. However, the effect of 5α-DHT seemed not to be related to a direct interaction with β_2-AR. Although the authors did not define the cause of the potentiation, it might be related to the inhibition of both the uptake of catecholamines and COMT (Figure 3(a)). Most recently, our group studied the genomic effects of T on β_2-AR. We found that chronic guinea pig ASM exposure to T augmented the expression of β_2-AR and evoked an increase in the relaxing responses to salbutamol (Figure 3(b)). Interestingly, this effect was abolished by flutamide (antagonist of the AR) [51]. We also observed that T potentiated salbutamol-induced potassium currents (I_K) involving the K_V and K_{Ca} upregulation (Figure 3(b)). Contrasting with other studies in the VSM [79], we did not find any modification of the adenylyl cyclase 6 (AC-6, the main isoform in the ASM) expression in tissues chronically exposed to T [51]. In summary, in the ASM, T and its metabolites, through nongenomic and genomic actions, have complementary effects. Consequently, androgens might play an important role as potential physiological modulators of the ASM tone, facilitating relaxation via β_2-AR, and therefore could be a therapeutic alternative for asthma treatment, although further research is needed (Figure 3).

4. Cardiac Muscle

Traditionally in the CM, autonomic control is derived by extrinsic signals or electrical stimulation of peripheral nerves. Moreover, neurocardiac control is maintained by an extensive network of intrinsic cardiac neurons, i.e., the intrinsic cardiac nervous system (ICNS) [244–246]. The ICNS comprises collections of neuronal somas residing on supraventricular tissues and the epicardial surface. This system is also composed by connecting nerve fibers known as ganglionic plexuses (GPs) [222, 246]. GPs are distributed in 5–7 regions comprising the right dorsal atrial, ventral right atrial, left dorsal, ventral left atrial, middle dorsal, right coronary, and left coronary plexuses [247]. Neuronal activity is modified by the activation of sensory nerves [248] and neuroactive chemicals, including acetylcholine, histamine, α- and β-adrenergic agonists, NO, neuropeptide Y (NPY, coreleased alongside norepinephrine), and calcitonin gene-related peptide (CGRP) [246, 249].

Similar to other muscular cells, $[Ca^{2+}]_i$ determines the contractile function of the heart through distinct Ca^{2+}-handling proteins. In the sinoatrial node, the pacemaker cells start depolarization of the cardiac myocytes. This process is regulated by the parasympathetic nervous system (PNS) and the SNS [250]. The self-depolarization produces action potentials along the CM, allowing the influx of Ca^{2+} through L-VDCCs and T-VDCCs. Furthermore, the Ca^{2+} influx elicits calcium release from the SR via RyR isoform 2 (RyR_2) [251]. Cardiac contraction results from a sudden increase in $[Ca^{2+}]_i$ and the formation of the Ca^{2+}-calmodulin complex with the further activation of MLCK. Afterward, Ca^{2+} is sequestered to the SR by SERCA, and the cell takes it out by the Na^+-Ca^{2+} exchanger in its forward mode (NCX). In addition, K_{Ca} channels are activated, leading to membrane hyperpolarization. These are the main mechanisms responsible for CM relaxation [252, 253]. Physiologically, catecholamines, through β-ARs, induce the synthesis of cAMP and the activation of PKA. This kinase promotes cardiac contraction by phosphorylating the L-VDCCs and RyR_2 since they increase their open probability and therefore the increment in $[Ca^{2+}]_i$ [254, 255]. PKA is also capable of evoking the relaxation of the CM by phosphorylating PLB, allowing SERCA to pump Ca^{2+} into the SR more rapidly [256]. In human ventricular cardiomyocytes, β_1- and β_2-ARs enhance cardiac frequency and contractility; meanwhile, β_3-ARs mediate negative inotropic effects [257]. The β_2-ARs essentially trigger the G_s/AC/cAMP/PKA pathway but are also involved in nonclassical G_i signaling displaying adverse effects on PKA activation and the inotropic response mediated by G_s [258].

There is increasing evidence that gender is highly related to cardiovascular states of health and disease. Whether androgens play a significant role in these dissimilarities is still investigated. Moreover, the genomic effects of T on ventricular cardiomyocytes' performance have been demonstrated. In this regard, Golden et al. showed that this androgen increased the mRNA expression of several critical Ca^{2+}-handling proteins. Treatment of rat ventricular cardiomyocytes with T increased the levels of gene expression of L-VDCC, β_1-AR, and NCX with 8 and 24 hours of exposure [259]. The T-induced changes in the mRNA expression levels of the mentioned proteins could be related to the improvement of the function of the cardiomyocytes and also be implicated in the development of hypertrophy and heart failure. These results point out an essential role of T in sex-related differences in the cardiac function.

Besides their electrophysiological properties, VDCCs are also classified using a standard nomenclature based on their

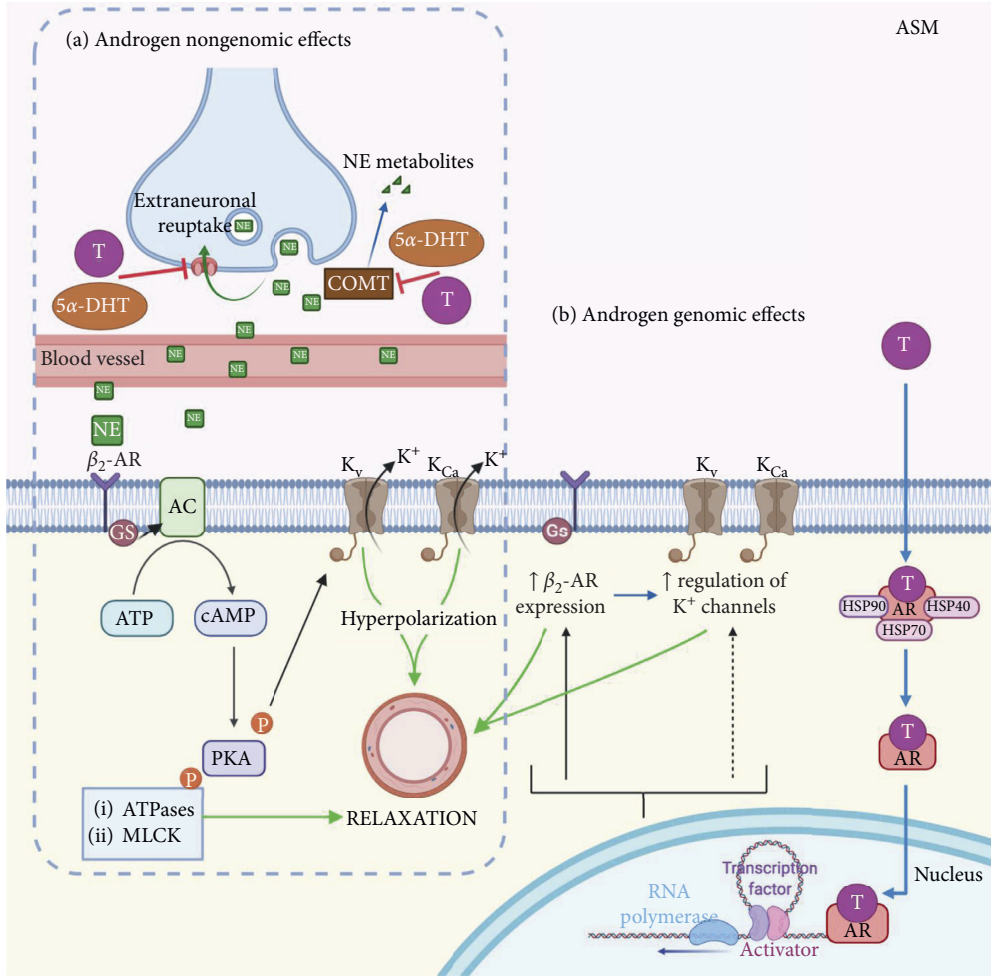

FIGURE 3: Androgen effects on the adrenergic system in the airway smooth muscle (ASM). (a) Testosterone (T), through a nongenomic effect, potentiates the relaxation induced by the β_2-adrenergic agonist through the inhibition of catechol-O-methyl transferase (COMT) or by the abolition of extraneuronal uptake of catecholamines. The inhibition of these mechanisms by T or 5α-dihydrotestosterone (5α-DHT, a reduced metabolite of T) leads to the accumulation of catecholamines such as norepinephrine (NE). The β_2-adrenergic agonist activates adenylyl cyclase (AC), leading to the activation of protein kinase A (PKA), which phosphorylates K^+ channels, ATPases, and myosin light-chain kinase (MLCK). These targets promote the relaxation of the ASM. (b) The androgen receptor (AR) signaling involves androgen crossing the plasma membrane, entering the cytoplasm, dissociation of chaperone proteins, and binding to the AR. Testosterone stimulation increases the β_2-adrenergic receptor (β_2-AR) expression in the guinea pig ASM and upregulates the Ca^{2+}-activated K^+ channels (K_{Ca}) and the voltage-dependent delayed rectifier K^+ channels (K_V). K_{Ca} are activated by increases in intracellular Ca^{2+} and through the cAMP-PKA signaling pathway. In the ASM, the main K^+ channels are the Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}). The K_V channel subtypes characterized in the ASM are $K_V1.2$ and $K_V1.5$. T (nongenomic and genomic effects) favors the relaxation of the ASM and might contribute to decreasing asthma symptoms.

molecular features of the pore-forming α1-subunit. Therefore, VDCCs are named using the chemical symbol of the permeating ion (Ca) with the physiological modulator (voltage) indicated as a subscript (Ca_V). A numerical identifier resembles the channel α1-subunit gene subfamily (1 to 3) and the order of discovery of the α1-subunit within that subfamily (1 through n). According to this nomenclature, the L-VDCCs are represented by $Ca_V1.1$–$Ca_V1.4$ subunits, and T-VDCCs correspond to $Ca_V3.1$–$Ca_V3.3$ [260]. In this regard, it has been reported that chronic administration of T enhanced Ca^{2+} influx through L- and T-VDCCs due to an increased expression of the $Ca_V1.2$, $Ca_V3.1$, and $Ca_V3.2$ subunits in ventricular cardiomyocytes, initially upgrading their performance but subsequently bringing the cell into new ${}_b[Ca^{2+}]_i$ [261, 262]. These studies suggest that augmented ${}_b[Ca^{2+}]_i$, via the upregulation of the Ca^{2+} channels aforementioned, might contribute to chronic cardiac pathogenesis when T levels are elevated.

While the nongenomic effects of T on the CM have been reported, the studies are scarce and seem to be contradictory regarding the Ca^{2+} handling and the cardiac contraction/relaxing outcomes. On the one hand, a group of researchers demonstrated in cultured rat cardiomyocytes that the acute exposure to T rapidly increased $[Ca^{2+}]_i$, and this augment was not abolished by an antagonist of the androgen receptor. Elegantly, the authors confirmed that the mechanism involved in the T-induced increase in $[Ca^{2+}]_i$ was mediated by the activation of a plasma membrane AR associated with a

pertussis toxin- (PTX-) sensitive G protein (Gi/o) and with the activation of the PLC-IP_3 signaling pathway leading to cardiac hypertrophy and failure [263]. The activation of PLC may be mediated through the action of $\beta\gamma$-subunits of the Gi/o proteins [264, 265]. On the other hand, it has been shown that acute exposure of T decreased the L- and T-VDCC activity by reducing their open probability [262, 266].

4.1. The Effects of Testosterone on Adrenergic Receptors in the Cardiac Muscle. Gender-related differences in cardiovascular disease (CVD) seem to be affected by age. It is well documented that the risk of dying for men between ages 45 and 64 from a CVD is higher than for women in the premenopausal period; however, there is a slight increase in the risk of CVD death in women after menopause [267].

The role of sex hormones in CVD is still unclear; particularly, a controversy about the effects of T in cardiovascular (CV) health and disease currently exists in the medical community. It is generally accepted that normal levels of T are beneficial for CV health in men, and a decline in these levels is related to an increase in CV events [268]. Nevertheless, a potential risk of developing CV events in patients receiving testosterone therapy has been reported [269, 270]. In postmenopausal women, endogenous elevated levels of total T (>0.9 nM) have been associated with CVD risk factors, such as high blood pressure and insulin resistance [271–275]. In this context, it has been postulated that estrogen augments the levels of sex hormone-binding globulin (SHBG). After menopause, the loss of ovary function leads to a general decline in sex steroid levels. Moreover, the fall of estrogen may lead to decreased levels of SHBG and higher free androgen levels [276]. Therefore, higher androgen levels and decreased estrogen levels in postmenopausal women have been suggested to be partially responsible for the CDV risk [268, 275]. Contrastingly, a study performed by Kaczmarek et al. demonstrated that low T levels are associated with coronary artery disease (CAD) in postmenopausal females [277]. During the menopausal transition, obesity is closely associated to CVD since it favors the secretion of proinflammatory cytokines, reactive oxygen species (ROS), and prothrombotic mediators [278–282]. Obesity promotes an unfavorable lipid profile which is associated with the development of CVD in elderly women [283, 284]. This profile is characterized by low high-density lipoprotein (HDL), higher total cholesterol (TC), and triacylglycerol (TAG) plasma levels [285]. In this regard, it was proved that oral DHEA therapy increased HDL and reduced TAG and LDL in adrenal-androgen-deficient postmenopausal women [286]. Also, obesity is a common feature of PCOS, exacerbating its symptoms and conferring a greater risk for CVD. The androgenic status, i.e., hyperandrogenism, needs to be considered when evaluating the metabolic and CV risk in PCOS women [200]. The ongoing controversy regarding the role of T in CVD might be moderately explained by the interaction between T and adrenoceptors in cardiac myocytes as discussed in the following.

It has been proposed that low plasmatic levels of T in older men are associated with an increase in atherosclerosis and cardiovascular risk, suggesting that this androgen plays a cardioprotective role against CVDs, such as coronary heart disease (CHD) and chronic heart failure (CHF) [287]. Moreover, it has been reported that an association between younger age at menopause and a greater risk of CDH in women coursing a natural menopause process [288]. Furthermore, T could confer cardiac protection against ischemic injuries by increasing the effects of the α_1-AR signaling pathway. The activation of α_1-AR improves the myocardial performance after an infarction, reducing injury and arrhythmias [289]. CHD is characterized by myocardial ischemia and cardiac injury [290]. In this regard, it has been shown that patients with CHD have lower androgen levels than healthy men and that low doses of T improved ischemic threshold in men suffering from angina [291, 292]. Furthermore, the administration of T enhanced the function recovery of the myocardium after a no-flow ischemia challenge in rats [293]. These observations point out to a reduction induced by T in the susceptibility to present myocardial ischemia and favor dilation of the coronary artery [294].

The SNS (through norepinephrine) activates α- and β-ARs controlling the CM tone. However, during myocardial ischemia, the release of norepinephrine increases the risk and contributes to cardiac injury [295, 296]. In this regard, it has been shown that the T-induced overexpression of the β_1-AR triggered proapoptotic pathways, weakening the cardiac structure and accelerating heart injury and failure progression [259, 297]. This overexpression also led to muscle hypertrophy in mice while producing an initial increase in contractility followed by a progressive dysfunction (Figure 4) [298].

On the contrary, the α_1-ARs may play an important role in cardioprotection, specifically, the α_{1A}-subtype. The overexpression of α_{1A}-AR can improve the outcome after myocardial infarction [299], cardiac contractility, and reduced arrhythmias [119, 300]. In 2008, Tsang et al. demonstrated in rat ventricular myocytes that T replacement therapy (TRT) upregulated the α_1-AR expression and augmented the cardiac responses, leading to a reduction in ischemia and cardiac injury [289]. Later on, in 2009, the same research group demonstrated that T enhanced the contractile function induced by the stimulation of both α_1- and β_1-AR in perfused rat hearts (Figure 4). Also, T treatment accelerated the relaxing response of the cardiac tissue. Interestingly, both phenomena were mediated by the AR [301]. The enhanced contractile response was explained since T augmented the function of RyR, leading to increased Ca^{2+} release from the SR. Otherwise, the augmented relaxing response was due to a more efficient activity of NCX regarding α_1-AR stimulation and a heightened SERCA activity, accompanied with increased phosphorylation of PLB in the case of β_1-AR stimulation [301]. Interestingly, they additionally found that the absence of T downregulated the expression of β_2-AR in rat hearts, indicating that this androgen may also interact with this receptor subtype [289]. Moreover, it has been documented that the activation of β_2-AR reduced apoptosis and increased the contractile mechanisms but did not accelerate relaxation as α_1-AR and β_1-AR

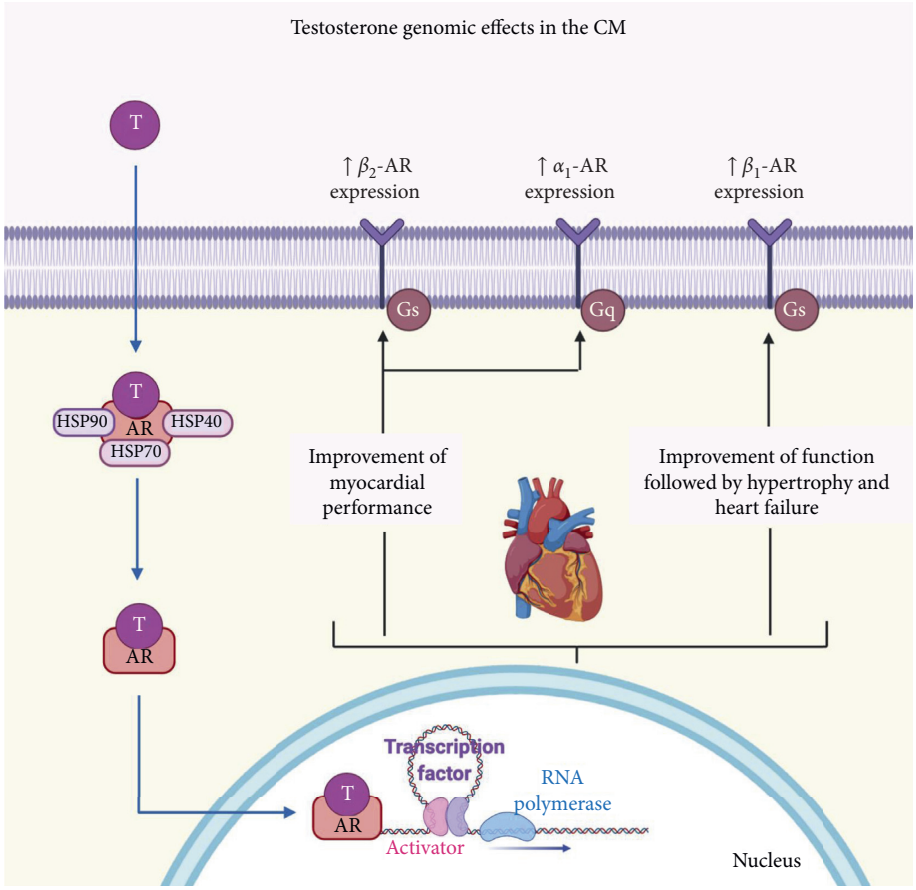

Figure 4: Genomic androgen effects on the adrenergic system in the cardiac muscle (CM). The genomic AR signaling involves androgen crossing the plasma membrane, entering the cytoplasm, dissociation of chaperone proteins, and binding to the AR. Testosterone (T) stimulates the expression of the α_1-adrenergic receptor (α_1-AR) and β_2-adrenergic receptor (β_2-AR), improving ionotropic activity and leading to the development of contraction without cardiotoxic effects. In addition, T increments the expression of the β1 adrenergic receptor (β1-AR), acutely improving the cardiomyocytes' function, but chronically leading to hypertrophy and heart failure.

stimulation did [302]. Although several studies have conducted about the relationship between cardiac function and androgens, more information is required to determine if T might play a key role in CHD.

Testosterone has also been associated with CHF [303]. This disease is a metabolic syndrome characterized by endocrine and inflammatory alterations, including elevated circulating catecholamine levels [304]. Testosterone deficiency (in hypogonadal subjects) has been demonstrated in 26% to 37% of male patients with CHF [305, 306]. Moreover, drugs used in CHF treatment, e.g., spironolactone and β-blockers, may diminish the function of Leydig cells, leading to a decline in the production of T [307, 308]. The low levels of T have been associated with reduced ejection fraction and increased systemic vascular resistance [309]. In this regard, the effects of T on β-AR have been investigated. In 2011, Sun et al. demonstrated that a TRT in a heart failure rat model reversed the damage (decrease in contractility, apoptosis, and fibrosis in cardiomyocytes) through the protection of the cardiac β-adrenergic system. Notably, the stimulation of the AR by T upregulated the expression of β_2-AR, improving the myocardial performance (Figure 4) [163]. Furthermore, it has been proposed that TRT in men with CHF induced an increase in the cardiac output and afterload [310].

The genomic regulation of the β-AR has been associated with cardiac remodeling and heart failure [311, 312]. In this regard, it has been shown that exercise training in rats reverses β-AR dysfunction by reducing the levels of G protein-coupled receptor kinase-2 (GRK2), an enzyme implicated in β_1-AR and β_2-AR dysregulation in CHF [313–315]. Moreover, exercise seems to restore the adrenal GRK2/α_2-AR/catecholamine production axis [313]. Also, exercise augments vascular β-AR responsiveness and diminishes the activity of GRK2 [316]. Interestingly, β_1-AR expression in the heart would be directly influenced by anabolic-androgenic steroids (AAS, synthetic derivatives of T) [317]. The use of AAS in combination with resistance training frequently improves the physical performance and helps athletes gain muscle mass and strength [318, 319]. However, numerous AAS abuse side effects include endocrine (hypogonadism) and detrimental cardiovascular issues [320–322]. For instance, vigorous training, anabolic steroid abuse, and the sympathetic nervous system's stimulation in mice increased cardiac levels of IL-1β and TNF-α and plasmatic levels of total cholesterol [320]. Furthermore, it

has been demonstrated that the use of AAS induced cardiac hypertrophy and increased myocardial susceptibility to ischemia injury [322, 323]. In this context, the administration of nandrolone (AAS) to male rats under an exercise training protocol increased the expression of β_1- and β_2-AR in the cardiac right atrium, provoked the prolongation of the QTc interval, and increased the BP [324]. In addition, the exposure of nandrolone augmented hypertension in SHR rats and β_1-AR protein expression in the left ventricle [317]. These data suggest that myocardial injury may be predisposed by high-performance training, steroid abuse, and the sympathetic nervous system's stimulation. Moreover, these insights may explain cardiac ailments and deaths in athletes under an AAS regimen.

Given the differences between studies showing the protective role of T in CV events and reports pointing out adverse CVD outcomes, it has been remarkably proposed that the use of **T**, as a treatment in CVD, should only be considered for male patients with a diagnosis of hypogonadism. Moreover, due to the increase of T therapy for postmenopausal women, the potential risk of developing CDV events needs further research [268, 275].

5. Conclusions

The adrenergic system plays a pivotal role in the control of vascular, airway, and cardiac physiology. A relationship between androgens with the adrenergic system of these tissues is proposed. This review summarizes that, in the vascular smooth muscle, T, via the androgen receptor, reduces the AC expression and increases the α_1-AR expression, leading to high BP and hypertension. Moreover, in the airway smooth muscle, T, via nongenomic action, potentiates the β-adrenergic-induced relaxation through the inhibition of COMT or by the abolition of extraneuronal uptake. This androgen, via a genomic effect, also augments the expression of β_2-AR and induces an increase in the relaxing responses to salbutamol. In the cardiac muscle, T upregulates the expression of α_{1A}-AR and β_2-AR mediated by the AR signaling, improving the myocardial performance. Moreover, T also increments β1-AR expression, improving the cardiomyocytes' function; however, the enhancement in muscle work during a long period ends up developing hypertrophy and heart failure.

Consequently, we might argue that androgen genomic actions have deleterious effects in the VSM favoring hypertension. Nevertheless, in the ASM, nongenomic and genomic actions of androgens contribute to diminish the hyperresponsiveness of this tissue, favoring the absence of asthma symptoms. Therefore, androgens could be a therapeutic alternative for asthma treatment. However, in heart diseases, further research is required to determine the possible therapeutic use of androgens in these ailments.

Finally, the use of T and DHEA as a therapeutic tool for the treatment of asthma symptoms or some cardiovascular diseases, is questionable. T has virilizing adverse effects, androgenic actions that favor prostate cancer, and its aromatization leads to the production of estrogens. Additionally, DHEA is further biotransformed into various sex steroids, such as T and estrogens, with their subsequent effects. However, 5β-DHT, a well-known T metabolite without genomic effects, could be a prospective therapeutic agent for the treatment of these illnesses.

Abbreviations

AAS: Anabolic-androgenic steroids
AC: Adenylyl cyclase
AC-6: Adenylyl cyclase 6
AHR: Airway hyperresponsiveness
α_1-ARs: Alpha-1-adrenergic receptors
α_2-ARs: Alpha-2-adrenergic receptors
ANF: Atrial natriuretic factor
AR: Androgen receptor
ASM: Airway smooth muscle
Basic bFGF: Fibroblast growth factor
β-ARs: Beta-adrenergic receptors
BK_{Ca}: Ca^{2+}-activated K^+ channels of high conductance
BP: Blood pressure
cAMP: 3′, 5′-Cyclic adenosine monophosphate
cGMP: 3′, 5′-Cyclic guanosine monophosphate
CGRP: Calcitonin gene-related peptide
CHD: Coronary heart disease
CHF: Chronic heart failure
CIRC: Ca^{2+}-induced Ca^{2+} release
CM: Cardiac muscle
COMT: Catechol-O-methyl transferase
COPD: Chronic obstructive pulmonary disease
CV: Cardiovascular
CVD: Cardiovascular disease
DAG: Diacylglycerol
DHEA: Dehydroepiandrosterone
5α-DHT: 5α-Dihydrotestosterone
5β-DHT: 5β-Dihydrotestosterone
EGFR: Epidermal growth factor receptor
ERK1/2: Extracellular signal-regulated kinases 1 and 2
FSH: Follicle-stimulating hormone
GDP: Guanosine diphosphate
GPRC6A: G protein-coupled receptor family C group 6-member A
GPCR-$_q\alpha$: GPCRs coupled to the $_q\alpha$ subunit
GPCRs: G protein-coupled receptors
GPCR-$_s\alpha$: GPCRs coupled to the $_s\alpha$ subunit
GRK2: G protein-coupled receptor kinase-2
GPs: Ganglionic plexuses
GTP: Guanosine-5′-triphosphate
HA: Hyperandrogenism
HDL: High-density lipoprotein
HGFR: Hepatocyte growth factor receptor
3β-HSD: 3β-Hydroxysteroid dehydrogenase
17β-HSD: 17β-Hydroxysteroid dehydrogenase
ICNS: Intrinsic cardiac nervous system
I_K: K^+ currents
IK_{Ca}: Ca^{2+}-activated K^+ channels of intermediate conductance
IL-13: Interleukin-13
IL-17: Interleukin 17

IP_3: Inositol-1, 4, 5-triphosphate
$_b[Ca^{2+}]_i$: Intracellular basal Ca^{2+} concentration
$[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration
K_{ATP}: ATP-sensitive K^+ channels;
K_{Ca}: Ca^{2+}-activated K^+ channels
K_{IR}: Inward-rectifier K^+ channels
K_V: Voltage-dependent delayed rectifier K^+ channels
$K_V1.5$: Voltage-dependent delayed rectifier K^+ channel 1.5
LH: Luteinizing hormone
L-VDCCs: L-type voltage-dependent Ca^{2+} channels
MAPKs: Mitogen-activated protein kinases
MEK: Mitogen-activated protein kinase kinase
MLCK: Myosin light-chain kinase
NCX: Na^+-Ca^{2+} exchanger
NCX_{REV}: Reverse-mode Na^+/Ca^{2+} exchanger
NO: Nitric oxide
NPY: Neuropeptide Y
PCa: Prostate cancer
PCOS: Polycystic ovary syndrome
PDGF: Platelet-derived growth factor
PGE_2: Prostaglandin E_2
PI3K: Phosphatidylinositol 3-kinase
PKA: Protein kinase A
PKC: Protein kinase C
PKG: Protein kinase G
PLB: Phospholamban
PLC: Phospholipase C
PLC_β: Phospholipase C_β
PMCA: Plasma membrane Ca^{2+}-ATPase
PNS: Parasympathetic nervous system
PTX: Pertussis toxin
ROCCs: Receptor-operated Ca^{2+} channels
RyR_2: RyR isoform 2
RyRs: Ryanodine receptors
SERCA: Sarcoplasmic reticulum Ca^{2+}-ATPase
5-HT: Serotonin
SHR: Spontaneously hypertensive rats
SK_{Ca}: Ca^{2+}-activated K^+ channels of low conductance
SNS: Sympathetic nervous system
SOCCs: Store-operated Ca^{2+} channels
SR: Sarcoplasmic reticulum
T: Testosterone
TAG: Triacylglycerol
TC: Total cholesterol
TNF-α: Tumor necrosis factor alpha
TRPC: Transient receptor potential channels
TRPC3: Transient receptor potential canonical 3
TRT: Testosterone replacement therapy
T-VDCCs: T-type voltage-dependent Ca^{2+} channels
VDCCs: Voltage-dependent Ca^{2+} channels
VEGFR-1/2: Vascular endothelial receptors 1/2
VSM: Vascular smooth muscle
ZIP9: Zinc-regulated transporter [Zrt]-protein 9.

Data Availability

No data were used to support this study.

Disclosure

Figures of this review were created with BioRender.com.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Abril Carbajal-García and Jorge Reyes-García contributed equally to this work.

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Anexo 3. Capítulo de libro.

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Sex Hormones and Lung Inflammation

15

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Abstract

Inflammation is a characteristic marker in numerous lung disorders. Several immune cells, such as macrophages, dendritic cells, eosinophils, as well as T and B lymphocytes, synthetize and release cytokines involved in the inflammatory process. Gender differences in the incidence and severity of inflammatory lung ailments including asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis (PF), lung cancer (LC), and infectious related illnesses have been reported. Moreover, the effects of sex hormones on both androgens and estrogens, such as testosterone (TES) and 17β-estradiol (E2), driving characteristic inflammatory patterns in those lung inflammatory diseases have been investigated. In general, androgens seem to display anti-inflammatory actions, whereas estrogens produce pro-inflammatory effects. For instance, androgens regulate negatively inflammation in asthma by targeting type 2 innate lymphoid cells (ILC2s) and T-helper (Th)-2 cells to attenuate interleukin (IL)-17A-mediated responses and leukotriene (LT) biosynthesis pathway. Estrogens may promote neutrophilic inflammation in subjects with asthma and COPD. Moreover, the activation of estrogen receptors might induce tumorigenesis. In this chapter, we summarize the most recent advances in the functional roles and associated signaling pathways of inflammatory cellular responses in asthma, COPD, PF, LC, and newly occurring COVID-19 disease. We also meticulously deliberate the influence of sex steroids on the development and progress of these common and severe lung diseases.

Keywords

Testosterone · 17β-Estradiol · Inflammation · Asthma · COPD · Lung cancer · Pulmonary fibrosis

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Abbreviations

AA	Arachidonic acid
AC	Adenylate cyclase
AECs	Airway epithelial cells
AHR	Airway hyperresponsiveness
AP-1	Activator protein 1
AR	Androgen receptor
ASM	Airway smooth muscle
ASMCs	Airway smooth muscle cells
BALF	Bronchoalveolar lavage fluid
cAMP	Cyclic adenosine monophosphate
CCL C-C	chemokine ligand
CCR C-C	chemokine receptor
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
COPD	Chronic obstructive pulmonary disease
CYP11A1	P450 side chain cleavage enzyme
CYP17A1	P450 17α-hydroxylase
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DHEA	Dehydroepiandrosterone
E2	17β-Estradiol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ET	Endothelin
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDM	House dust mite
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2innate lymphoid cell
IPF	Idiopathic pulmonary fibrosis
JNK	Jun N-terminal kinase
LC	Lung cancer
LTs	Leukotrienes
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
OC	Oral contraceptives
OVA	Ovalbumin
P4	Progesterone
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PEFR	Peak expiratory flow rate
PF	Pulmonary fibrosis
PI3K	Phosphoinositide 3-kinase
PMA	Perimenstrual asthma
PR	Progesterone receptor
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
STAR	Steroidogenic acute regulatory protein
TAM	Tumor-associated macrophages
TES	Testosterone
TGF-β1	Transforming growth factor beta 1
Th	T-helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TSPO	Translocator protein

15.1 Introduction

15.1.1 Lung Inflammation

Inflammation is a complex biological response to harmful stimuli (i.e., bacterial and viral infections, irritants or environmental pollutants, and damaged cells), which is orchestrated by the immune system [1–4]. Two phases of inflammation can be distinguished: acute and chronic inflammation. In the acute phase (hours to days), host cells recognize danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) through the action of pattern recognition receptors (PRRs), expressed predominantly in monocytes, macrophages, neutrophils, and dendritic cells [1, 2, 5, 6]. These cells migrate to the injured site along a

chemotactic gradient mediated by specific cytokines. Cellular stimulation leads to the inflammatory process through the activation of transcription factors like nuclear factor kappa B (NF-κB) and the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, IL-8, IL-12, IL-17 and interferon (IFN)-I and IFN-II [3, 7–11]. IL-8 acts as a neutrophil chemotactic agent, and TNF-α augments the expression of adhesion molecules in the endothelial cells of lung capillaries. Then, antigen-presenting cells (APCs) present the T lymphocytes with the foreign antigen (virial/bacterial or damaged cell components) and evoke either a type 1 T helper (Th)1 lymphocyte- or Th2 lymphocyte-mediated response [10–13]. The persistence of inflammation due to a long-time exposure to inflammatory stimuli, and a failed or incomplete acute response resolution leads to the chronic phase of inflammation that may last for weeks or months and in some circumstances for years. In this phase, the inflammatory response is amplified, and tissue damage may occur. In most cases of chronic lung inflammation, profibrotic and immunoregulatory Th2 cytokines govern [1, 2, 10]. Acute and chronic inflammation are typical markers in numerous lung disorders, including infectious [14, 15], immunological [16, 17], genetic [18, 19], neoplastic [20, 21], and environmental [22, 23] ailment-related.

Commonly, cellular mechanisms of lung inflammation include the expression of adhesion molecules, the release of systemic inflammatory mediators, and the recruitment of distinct leukocytes into the lung vasculature [10, 13, 24]. Neutrophils are the first type of immune cells to be recruited, followed by the resident macrophages, including both alveolar and interstitial and, in some cases, pulmonary intravascular macrophages. The displayed profile of immune cells and cytokines will depend on the developed type of lung disease or injury [10, 16].

15.1.2 Sex Differences in Lung Inflammatory Diseases

Lung disease is a major health issue. According to the Centers for Disease Control and Prevention and to the National Center for Health Statistics in the USA, the number of deaths from chronic lower respiratory diseases reaches ~154,000 every year. Furthermore, it is estimated that this kind of illnesses affects more than 500 million people across the world [25]. It is well known that sex hormones play diverse regulatory effects on the human lung development and physiology [26–30]. Moreover, sex differences are essential predictors in a lot of common diseases, used in diagnosis, prognosis, and treatment recommendations [31, 32].

The influence of sex hormones in the incidence and severity of the inflammatory response in lung disease has been widely studied and recognized for years. For instance, one of the most prevalent lung inflammatory illnesses, chronic obstructive pulmonary disease (COPD), affects both men and women; nevertheless, recent studies point out that females are at a higher risk of developing COPD with lower exposures to tobacco smoke [31, 33–37]. During childhood, asthma symptoms seem to be more prevalent in boys than in girls, and this trend reverts during puberty; however, the incidence in asthma symptoms increases in older men when testosterone (TES) levels decrease, suggesting a potential protective role of the androgens in this ailment [25, 28, 31, 38–42]. Contrariwise, female sex hormones have been related to negative outcomes in asthma [25, 42–44]. Pulmonary fibrosis (PF), an interstitial lung illness, affects more men than women with a higher mortality rate in males [45–47]. Moreover, murine models of bleomycin-induced PF have shown the influence of sex hormones (both males and females) in the decrease of lung function [31, 48, 49]. In cystic fibrosis (CF), a genetic disease, women have been described to display more severe consequences than men, especially in response to bacterial respiratory infections [31, 50, 51]. Pulmonary arterial hypertension (PAH) is another pulmonary disease influenced by gender showing a female predominance [52–55] that has been related to the estrogen receptor alpha (ERα) [56, 57]. Lung cancer (LC) is also an ailment in which the evidence suggests that its incidence and progression are affected by sex hormones differences, particularly by the action of estrogens and their receptors [58, 59]. Furthermore, gender dif-

ferences have been described as well in lung illnesses caused or enhanced by infectious agents [52, 60, 61].

In spite of the marked gender differences in several lung ailments and the great amount of studies related to them, the role of sex hormones in the mechanisms associated with these illnesses has not been fully elucidated. This chapter summarizes the advances in basic and clinical studies of sex hormones (mainly testosterone and estradiol) as modulators of the inflammatory response in lung disease with particular emphasis on asthma and COPD. The information obtained from sex-specific research on lung physiology and pathology would potentially help in the development of sex-specific therapeutics for inflammatory lung diseases regarding the hormonal status of the patient.

15.2 Sex Hormone Steroidogenesis

15.2.1 Classification of Sex Hormones

Sex hormones are involved in processes such as growth, development, reproduction, and systemic homeostasis [62, 63]. This group of hormones possesses a common structure of cyclopentaneperhydro-phenanthrene, a complex of 17-carbon atoms forming a 4-ring system. According to the number of carbon atoms, sex steroids can be divided into three major groups: progestins (21-carbon atoms), androgens (19-carbon atoms), and estrogens (18-carbon atoms) [64]. The same synthesis pathway of steroid hormone production is carried out by different organs in men and women, i.e., testis, adrenal cortex, ovary, brain, placenta, etc.; however, the amount and the main type of synthetized hormone molecule rely on the expression and activity specific to each tissue [64–66].

Cholesterol is the precursor of sex hormones, both male and female, which are synthetized in specialized cells [62, 65, 67]. This precursor is an indispensable element of the cellular plasma membrane contributing to the fluidity, permeability, and regulation of transmembrane signaling pathways [68]. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA or HMGCR) is the enzyme responsible for the metabolic pathway that produces cholesterol from acetyl-CoA [69–71]. Additionally, cholesterol can be taken from low-density lipoprotein (LDL, through the LDL receptor pathway) and high-density lipoprotein (HDL), via the scavenger receptor class B type (SR-BI) pathway or lipid droplets [72–74].

15.2.2 Androgen Biosynthetic Pathway in Leydig Cells

In men, the synthesis of TES (the main androgen) is carried out in a major proportion (95%) by Leydig cells from the adult testis through the action of cytochrome P450 enzymes. The synthesis and secretion of this androgen are tightly regulated by luteinizing hormone (LH) [67]. In addition, smaller amounts of TES are produced in the adrenal cortex [65]. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus and stimulates gonadotropic cells in the anterior pituitary gland to release LH. This hormone stimulates its Gs protein-coupled receptor, resulting in the activation of adenylyl cyclase (AC). AC induces the formation of cyclic adenosine monophosphate (cAMP), which stimulates the mobilization of cholesterol to the mitochondria by activating protein kinase A (PKA) signaling [75]. The importation of cholesterol into mitochondria is carried out by the transduceosome, a protein complex conformed mainly by the steroidogenic acute regulatory protein (STAR) and the translocator protein (TSPO) [73, 74]. After the synthesis of cholesterol, this precursor is converted into pregnenolone by the P450 side chain cleavage enzyme (P450scc/CYP11A1) located in the mitochondrial membrane [63, 65, 74, 76]. Pregnenolone can either be converted to progesterone via 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) or be hydroxylated to 17α-hydroxypregnenolone and then transformed to dehydroepiandrosterone (DHEA, an andro-

gen) by cytochrome P450 17α-hydroxylase (P450c17/CYP17A1/C17-C20 lyase, an enzyme with hydroxylase and lyase activity) [63, 65, 77, 78]. DHEA is further reduced to androstenediol via 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) [79, 80] or converted to androstenedione by 3β-HSD2 [78, 81]. Androstenediol and androstenedione are finally biotransformed to TES by 3β-HSD2 and 17β-HSD3, respectively [65, 78–81]. Furthermore, TES is reduced to 5α-dihydrotestosterone (5α-DHT) by 5α-reductase and to 5β-dihydrotestosterone (5β-DHT) by 5β-reductase [76, 82, 83]. In addition, TES can be converted to 17β-estradiol (E2, an estrogen) through the P450 aromatase (P450aro/CYP19A1 aromatase) action [63] (Fig. 15.1).

Although TES is necessary for estrogen production, men have much higher plasmatic levels of TES than women. The synthesis of TES by Leydig cells in men is seven to eight times greater than that produced in women ovaries. In men, TES plasma concentration reaches values between 6 and 50 nM depending on the person's age. On the other hand, women display stable TES values between 0.6 and 2.4 nM that are maintained along the different life stages, except during pregnancy when TES concentrations increment (3.5–5 nM) [31].

15.2.3 Estrogen Biosynthetic Pathway in Theca and Granulosa Cells

E2 and progesterone (P4) are considered the main female sex hormones. The former is a type of estrogen and the latter is a type of progestogen, both essentially produced in ovaries [36, 64, 84]. In addition to E2, two more estrogen molecules naturally occur in women: estrone (E1) and estriol (E3). Estriol is the predominant estrogen during pregnancy, while estradiol is the prevalent form in non-pregnant premenopausal females. In menopause, estrone is the predominant type of estrogen [62, 85]. Ovaries are the vastest source of estrogens before menopause. Nevertheless, in postmenopausal women and in men, these female hormones are locally produced from circulating testosterone and adrenal cortex steroids in non-reproductive and reproductive tissues [86–88]. Ovarian steroids are synthetized through the interaction between theca (TCs) and granulosa cells (GCs), a process regulated by LH and follicle-stimulating hormone (FSH) [36, 62, 84, 85, 89, 90]. GnRH secreted from the hypothalamus stimulates LH and FSH. LH acts on both TCs and GCs, and FSH exerts its effects mainly on GCs. These hormones stimulate AC activity and cAMP formation. This cyclic nucleotide triggers PKA activation and the further expression of steroidogenic enzymes [91–93] (Fig. 15.2).

P4 is predominantly produced in luteal cells through a system of three cholesterol-modifying enzymes: STAR, P450scc, and 3β-HSD. STAR catalyzes cholesterol transfer within the mitochondria, which is considered the rate-limit step in the production of all steroids [84]. This regulatory protein is mostly expressed in luteal cells; however, STAR can be found in theca and granulosa cells during follicle development or during luteinizing phase, respectively, conferring to these cells the ability to synthetize progesterone [84, 85]. The first step in female steroidogenesis is the initial conversion of cholesterol into pregnenolone by the action of STAR and P450scc [36, 90, 94]. Subsequently, pregnenolone is converted to progesterone by 3β-HSD2 in both theca and granulosa cells [90, 94]. In theca cells, P450c17 hydroxylates pregnenolone to produce 17α-hydroxypregnenolone and subsequently removes the acetyl group in order to form DHEA. This last product can be either converted into androstenedione via 3β-HSD2 or metabolized into androstenediol by 17β-HSD1 [85, 94, 95]. These androgens are further biotransformed to TES via 17β-HSD5 and 3β-HSD2, respectively. TES and androstenedione diffuse across the follicle membrane into the follicular fluid, where they are taken up by granulosa cells [36, 85, 90, 95]. The endoplasmic reticulum of granulosa cells expresses P450aro, which converts TES to E2 and androstenedione to E1 by the addition of an aromatic A ring. Additionally, in granulosa cells, 17β-HSDs regulate the interconversion between E1 and E2. 17β-HSD1 catalyzes the formation of E2 from E1, and

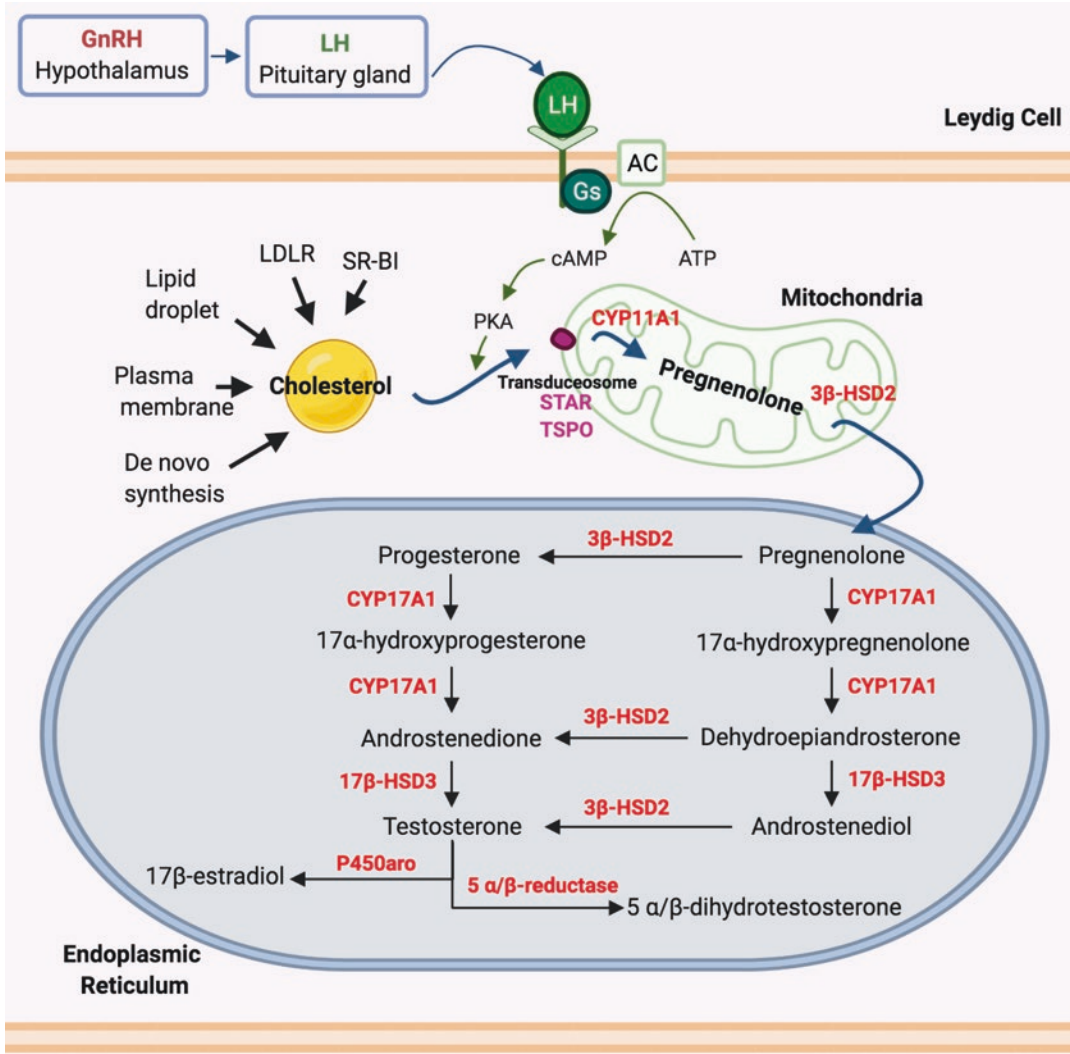

Fig. 15.1 Synthesis of androgens from cholesterol in Leydig cells. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus and stimulates gonadotropic cells in the anterior pituitary gland to release luteinizing hormone (LH), which tightly regulates the synthesis and secretion of androgens in Leydig cells. LH binds to its Gs protein-coupled receptor, resulting in the activation of adenylyl cyclase (AC) and increased intracellular cyclic adenosine monophosphate (cAMP) formation. cAMP stimulates the mobilization of cholesterol to the mitochondria by activating protein kinase A (PKA) signaling. Cholesterol is the precursor of all sex steroids. This lipid precursor can be produced de novo or taken from low-density lipoprotein (LDL) and high-density lipoprotein (HDL) via the scavenger receptor class B type (SR-BI) pathway, plasma membrane, or lipid droplets. Once synthetized, cholesterol is imported into mitochondria through the transduceosome (a protein complex), composed of the steroidogenic acute regulatory protein (STAR), the translocator protein (TSPO), and other proteins. The first step is the conversion of cholesterol to pregnenolone by the C27 cholesterol side chain cleavage cytochrome P450 enzyme (CYP11A1) located on the matrix side of the inner mitochondrial membrane. Then, pregnenolone is converted into testosterone by 3β-hydroxysteroid dehydrogenase (3β-HSD2, located at the mitochondria), 17α-hydroxylase/17,20 lyase (CYP17A1), and type 3 17β-hydroxysteroid dehydrogenase (17b-HSD3) in the endoplasmic reticulum. Furthermore, testosterone can be reduced to 5α- or 5β-dihydrotestosterone by 5α-/β-reductase. Finally, P450 aromatase (P450aro) can convert testosterone to 17β-estradiol in Leydig cells of the adult testis

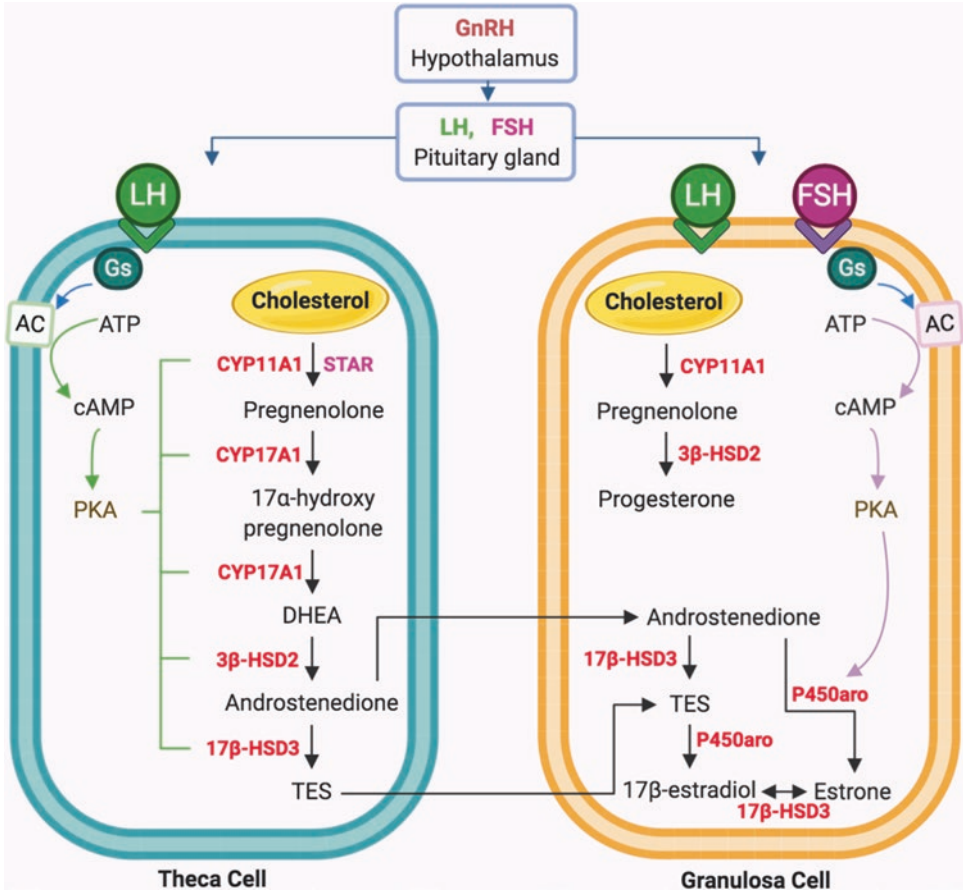

Fig. 15.2 Steroid hormone biosynthesis pathways in the ovary by theca and granulosa cells. Ovarian steroids are synthetized through the interaction between theca and granulosa cells, a process regulated by gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus. GnRH stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH acts on both theca and granulosa cells; FSH acts only on granulosa cells. These hormones stimulate adenylyl cyclase (AC) via Gs protein-coupled receptors. The cyclic adenosine monophosphate (cAMP) generated from adenosine triphosphate (ATP) activates protein kinase A (PKA) to stimulate the expression of steroidogenic enzymes in theca and granulosa cells. The first step in female steroidogenesis is the initial conversion of cholesterol to pregnenolone by the action of steroidogenic acute regulatory protein (STAR) and C27 cholesterol side chain cleavage cytochrome P450 enzyme (CYP11A1). Later, pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD2). In theca cells, 17α-hydroxylase/17,20 lyase (CYP17A1) hydroxylates pregnenolone to produce 17α-hydroxypregnenolone and subsequently removes the acetyl group in order to form dehydroepiandrosterone (DHEA). This last product can be converted into androstenedione via 3β-HSD2 and further biotransformed into testosterone (TES) via 17β-hydroxysteroid dehydrogenase (17β-HSD3). TES and androstenedione diffuse across the follicle membrane into the follicular fluid where they are taken up by granulosa cells. The endoplasmic reticulum of granulosa cells expresses P450 aromatase (P450aro) that converts TES to 17β-estradiol and androstenedione to estrone. Additionally, in granulosa cells, 17β-HSD3 catalyzes the formation of 17β-estradiol from estrone

17β-HSD2 catalyzes the oxidation of E2 to E1 [89, 90, 94] (Fig. 15.2). Moreover, estrogens can be produced in the Leydig cells, Sertoli cells, and mature spermatocytes from the male gonads [96].

Variation of estrogens levels during life is based on different factors such as age, menstrual cycle phase, and pregnancy. Interestingly, androgen levels in women are higher than estrogen levels most of the time. An exception occurs during

the preovulatory and midluteal phases of the menstrual cycle [31, 85, 97]. In non-pregnant women, E2 serum levels fluctuate between 80 and 800 pM. These levels may increase up to 150 nM during pregnancy and highly decrease, oscillating between 40 and 120 pM in menopausal period. Progesterone plasmatic levels also vary between 1 and 60 nM in non-pregnant women and reach values of 1000 nM during pregnancy [31].

15.3 Sex Hormone Receptors

15.3.1 Sex Hormone Binding Globulin

The distribution of sex hormones to different tissues, including the lung, is regulated by sex hormone binding globulin (SHBG), a key steroid hormone binding protein in human plasma. Plasma SHBG is a homodimeric protein largely produced in hepatocytes [36, 98, 99]. Each monomer possesses a steroid binding pocket and a Ca^{2+}-binding site [99, 100]. This globulin binds steroids such as TES, 5α-DHT, and E2 with nanomolar affinities [36, 99–102]. Normally, in humans, between 40% and 65% of circulating TES and between 20% and 40% of circulating E2 are bound to SHBG [101, 103]. Literature suggests that sex hormones bound to SHBG do not display biological activity. Moreover, sex steroid dissociation from this globulin in the circulatory system allow them to bind and activate male or female sex hormone receptors, triggering gene transcription and protein synthesis [103, 104]. Interestingly, SHBG binds TES with a higher affinity than it does for E2, acting as an estrogen amplifier [36, 98, 103, 104].

Sex steroids exert their physiological effects mostly through the binding to their own receptors, e.g., the androgen receptor (AR), estrogen receptors (ERs), and progesterone receptors (PRs) [25, 31, 76, 105–107]. Sex steroid actions comprise genomic and non-genomic effects. Genomic effects occur from hours to days and involve a direct modulation of gene transcription and protein synthesis via the binding and activation of nuclear hormone receptor complexes. Non-genomic effects are mediated by plasma membrane receptors or ion channels that trigger intracellular signaling pathways that mayresult in transcriptional regulation [25, 31, 36, 76, 106, 108].

15.3.2 Androgen Receptor

The AR modulates the activity and effects of male sex hormones and their influence on lung development. Testes from fetuses produce TES after sex differentiation, and this androgen retards the production of surfactant during gestation [109]. Also, it has been suggested that branching morphogenesis of the human lung is regulated by androgens' effects [110]. The AR is found in several lung tissues and immune cells, including airway smooth muscle (ASM) [111], lung parenchyma, bronchial epithelium [112, 113], dendritic cells (DCs) [114], macrophages, neutrophils, and T and B lymphocytes [115–118]. The AR, also known as NR3C4, is a cytoplasmatic/nuclear receptor that is activated by binding to TES or to its more active reduced metabolite, 5α-DHT, in the cytosol. Male sex steroids binding to the AR promote their dissociation from its chaperon proteins, forming a complex that is translocated into the nucleus. The formed complex acts as a DNA-binding transcription factor that modulates gene transcription and protein synthesis [25, 76, 95, 119, 120]. 5β-DHT, the other reduced metabolite of TES, possesses less androgenic activity than TES and 5α-DHT, because it has a lower binding affinity for the AR [121]. In addition, TES is capable of activating plasma membrane receptors such as ZIP9, a zinc transporter from the ZIP family [122–125], and GPRC6A (class C, group 6, subtype A, G protein-coupled receptor family member) [124, 125]. The binding of TES to ZIP9 or GPRC6A triggers signaling pathways that implicate the activation of G proteins, including Gs, Gi, and Gq11, and the stimulation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) [122–128].

15.3.3 Estrogen Receptors

Estrogen and progesterone receptors contribute to sexual and lung development [30, 37, 95]. Two classes of ERs have been described: nuclear ERs, ERα (ESR1) and ERβ (ESR2), and the plasma membrane ER (mER), G protein-coupled receptor 30 (GPER/GPR30) [25, 129–132]. Estrogen receptors exert their physiological actions through either genomic pathways leading to gene transcription or non-genomic signaling pathways (rapid effects that involve phosphorylation processes) [133–137]. ERα and ERβ function as transcription factors. The stimulation of ERs in the cytoplasm causes dimerization, nuclear translocation, and binding to estrogen response elements (EREs) in the promoter region of target genes. Moreover, ERs are able to indirectly modulate gene transcription by forming complexes with proteins such as c-fos and c-jun, essential components of activator protein 1 (AP-1) [136, 137]. Also, the activation of ERs induces rapid effects mediated by protein members of the mitogen-activated protein kinase (MAPK) family, e.g., ERK1/2, p38 MAPK, and the c-Jun N-terminal kinase (JNK) [138–140]. ERα and ERβ occur in numerous lung cells and tissues, e.g., ASM, bronchial epithelial cells, macrophages, DCs, and T and B lymphocytes [25, 141–146]. Some studies have demonstrated the critical role of both ERα and ERβ in fetal lung development. The expression level of these receptors is significantly elevated in fetal lungs compared to postnatal and adult lungs from mice [147]. Furthermore, ERα and ERβ participate in alveolar formation [148, 149].

In addition, two shorter or truncated splice variants of the human ERα (hERα-66/ER66 due to its molecular weight) have been identified as mERs: 46 kDa ER (hERα-46/ER46) and 36 kDa ER (hERα-36/ER36) [150, 151]. It has been demonstrated that ER66 can translocate to the plasma membrane via the interaction with the scaffolding protein of caveolae (caveolin-1), a process dependent on palmitoylation [152–155]. ER66-induced transcription is mediated by two activation domains: the ligand-independent activation function (AF)-1, which is located in the N-terminal domain, and the ligand-dependent AF-2, situated in the C-terminal domain [156, 157]. The truncated ER46 isoform lacks the AF-1 domain; however, this splice variant maintains the corresponding domains for caveolin-1 association and palmitoylation [150, 152]. The absence of the AF-1 domain in the ER46 supposes null influence in its ability to evoke non-genomic actions. In this context, it has been shown that ER46 variant stimulates the phosphorylation of the endothelial nitric oxide synthase (eNOS) in a higher degree than ER66 [158]. ER36, the other truncated isoform of ER66, lacks the AF-1 and AF-2 domains [151]. Non-genomic actions mediated by ER36 have been described. The activation of ER36 by E2 elicits the mobilization of intracellular Ca^{2+} in different breast cancer cell lines. Also, ER36 triggers MAPK pathways leading to cell growth and proliferation in HEK293 cells [159]. In HEK293 cells as well, saturation binding assays show that the equilibrium dissociation constant (K_d) for the binding of E2 to ER66 and ER46 corresponds with the serum levels of this estrogen found in women (68.8 pM and 60.7 pM, respectively), while ER36 exhibits no saturable specific binding [160]. Furthermore, the evidence about the molecular characterization of ERβ splice variants is still unclear. The presence of membranal estrogen receptor insinuates an additional regulation mechanism for estrogens' actions; however, little is known regarding the function of ER-truncated isoforms in the lung [133, 161].

GPR30 was identified as a functional membrane receptor, different from the ERα-truncated splice variants, involved in rapid E2 signaling pathways [131, 132, 162, 163]. GPR30 binds E2 with high affinity (K_d = 6 nM) [164, 165] and triggers numerous intracellular signal transduction pathways such as cyclic adenosine monophosphate (cAMP) production, Ca^{2+} mobilization, and the activation of phosphatidylinositol 3-kinase (PI3K) and ERK1/2 [129, 131, 132]. Moreover, the stimulation of GPR30 has been involved in the activation of the epidermal growth factor receptor (EGFR)-mediated signaling in breast and lung cancer [132, 162, 165, 166]. The expression and function of GPR30 in the lung have not been fully elucidated yet. In this context, Townsend et al. did not find a significant expression of this receptor in human ASM [167].

15.3.4 Progesterone Receptors

Progesterone (P4) plays a crucial role in the maintenance of pregnancy. This hormone modulates the transition of the endometrium from a proliferative stage to a secretory phase and promotes the implantation of the blastocyst [64]. The lipophilic nature of P4 allows it to cross the cell membrane and binding to the two types of progesterone receptors (PRs) identified in mammals: PR-A and PR-B. Once PRs are activated, enter the nucleus, and promote DNA modulation, gene transcription and protein synthesis [64, 168–171]. Both progesterone receptors are encoded by the same gene but display different patterns on progesterone response elements, i.e., distinct transcriptional activity [172, 173]. PR-B is considered a strong promoter of gene transcription, whereas PR-A is associated with repressor responses [172]. The stimulation of the PRs triggers the activation of transcriptional regulatory proteins known as steroid receptor coactivators 1, 2, and 3 (SRC-1, SRC-2, and SRC-3). SRCs contribute to the regulation of DNA transcription by assisting nuclear receptors [174, 175]. Moreover, PR-B activation allows the association with the N-terminal domain of the ER. The association of the PR-B with the ER causes the proto-oncogene tyrosine-protein kinase (Src)/p21-Ras/ERK pathway [176]. Progesterone receptors have been identified in ASM [111], cilia from the airway epithelium [177], and endothelial cells from vascular beds [178, 179]. The expression of PRs in immune cells is still under debate; however, some reports have suggested the occurrence of this receptors in DCs [179, 180].

Non-genomic effects of P4 are mediated by membrane receptors. The existence of non-classical membrane PRs (mPRs) has been pointed out and reviewed in mammalian tissues, including reproductive and non-reproductive systems [181, 182]. The non-classical PRs were first identified in fish where they modulate gamete physiology [183]. mPRs are classified into the progesterone and adipoQ receptor (PAQR) family, which belongs to the superfamily of GPCRs. Until now five different subtypes of mPRs have been characterized: mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5), mPRδ (PAQR6), and mPRε (PAQR9) [181, 182, 184, 185]. These receptors have been described in immune cells, such as peripheral blood mononuclear cells (PBMCs) and T cells [181, 182, 186–190]. The stimulation of mPRs evokes intracellular signal pathways implicating Ca^{2+} mobilization, the increase in cAMP levels, and the activation of p38MAPK and JNK [181, 182, 190].

15.4 Immune Cells

15.4.1 Neutrophils

Neutrophils are essential granulocytes of the innate immune system. These cells are the most abundant circulating leukocytes, comprising around 70% of all white blood cells in healthy humans [191]. Neutrophils are commonly the first cells responding to damage and migrating to the injured tissues, including the lung. The activation of neutrophils and the release of chemokines induce the recruitment of monocytes into the inflamed tissues [10, 191, 192]. The evidence of sex steroid actions on the development and physiology of neutrophils is present. A study shows that the genetic ablation of the AR in mice drastically diminishes (90%) the proliferative activity of neutrophil precursors and retards neutrophil maturation. Also, neutropenia is exhibited in mice with testicular feminization (Tfm). The same study points out that androgens induce the production of neutrophils via the modulation of granulocyte colony-stimulating factor (G-CSF) [193]. Additionally, TES suppresses the production of superoxide and the anti-microbial capacity of human neutrophils [194]. Furthermore, in an ozone-induced lung inflammation mice model, the expression of neutrophil-attracting chemokines (*Ccl20*, *Cxcl5*, and *Cxcl2*) and the number of neutrophils are significantly higher in females compared with males [195].

15.4.2 Macrophages

In humans, the respiratory tract harbors mononuclear phagocytic cells to provide one of the first lines of defense against inhaled allergens, pathogens, particles, and gases. Most of these phagocytic cells are macrophages distributed along the lung, airways, and alveoli [196, 197]. Besides the resident macrophages, two more distinct populations are found in the lung: alveolar and interstitial macrophages [198, 199]. Alveolar macrophages are considered the main leukocyte subtype in the lung. Cell counting analysis of bronchoalveolar lavage fluid (BALF) from healthy adults reveals 72–96% of macrophages, 2–26% of lymphocytes, 0–4% of neutrophils, and 0–1% of eosinophils, basophils, and mast cells [200]. After an injury or damage is produced, monocytes derived from the bone marrow are recruited to the lung and differentiated into alveolar macrophages. The differentiation process and the following nesting into the alveoli are dependent on the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) by alveolar type 2 cells. Signals triggered by alveolar type 1 and 2 cells, such as exposure to surfactant-rich fluid and elevated oxygen tension, may modulate different functional phenotypes of alveolar macrophages [196, 201]. In this context, classically activated macrophages (CAM/M1) and alternatively activated macrophages (AAM/M2) have been described. Macrophages may undergo M1 polarization in response to cytokines produced by Th1 lymphocytes, e.g., interferon gamma (IFN-γ), and by Toll-like receptor (TLR) ligands found in bacterial and viral products. The activation of M2 macrophages is provoked by Th2 cytokines such as IL-4 and IL-13 [202].

Regarding sex hormones' influence in these immune cells, it has been shown that TES decreases the expression of the TLR4 in macrophages from mice [203]. Also, TES reduces the production of IL-1β, IL-6, and TNF-α in human macrophages and human monocytes and increases the expression of IL-10 in macrophages from humans, as well as in a murine macrophage cell line [204, 205]. Moreover, TES is capable of diminishing nitric oxide (NO) production induced by the stimulus of lipopolysaccharide (LPS) [205]. Female sex hormones also exert a regulation in the activity of macrophages. Ovalbumin (OVA)-induced asthmatic mice display increased numbers of M2 macrophages in the lung compared with male mice [206]. Estrogens facilitate the resolution phase of inflammation toward a M2 phenotype dependent on IL-10, contributing to tissue remodeling and shortening the pro-inflammatory state [207]. Progesterone inhibits NO production and the release of microparticles (MPs) with pro-inflammatory and prothrombotic properties. MPs are discharged by macrophages stimulated by ligands of TLRs in a NO-dependent process [208].

15.4.3 Eosinophils

Eosinophils are key modulators in allergic inflammation as occurring in asthma [209]. They are developed from granulocytic precursor populations in the bone marrow and are activated through the action of the IL-5 secreted by Th2 cells. The maturation of eosinophils is also mediated by GM-CSF. Degranulation of activated eosinophils releases pro-inflammatory cytokines, leukotrienes (LTs), platelet-activating factor (PAF), reactive oxygen species (ROS), and the cationic proteins, e.g., basic protein and eosinophil cationic protein [209, 210]. LTs contribute to bronchospasm in asthma [211]. While the expression of sex steroid receptors in eosinophils is unclear, the impact of sex hormones on these immune cells has been investigated. In gonadectomized male mice, the peripheral and bone marrow eosinophils are augmented, and TES abolishes peripheral and bone marrow eosinophil responses at the early phase of infection by *Brugia pahangi* in females [212]. Moreover, TES decreases human eosinophil viability and adhesion properties in vitro [213]. Conversely, female mice display more severe eosinophilia than males, a phenomenon that is reversed after gonadectomy [214], and progesterone treatment enhances the recruitment of eosinophils and induces airway hyperresponsiveness (AHR) in a murine model of allergic asthma [215].

15.4.4 Mast Cells and Dendritic Cells

Mast cell development occurs in the bone marrow, and their maturation is mediated by the interaction between the stem cell factor (SCF) with its own receptor c-kit and by the influence of IL-3, IL-4, IL-9, and IL-10. Activated mast cells participate in the acute and chronic phases of inflammation. For instance, these cells contribute to the infiltration of leukocytes, tissue remodeling, and fibrosis [216, 217]. The expression of the AR has been reported in skin mast cells; nevertheless, the numbers and distribution of these cells seem not to be affected by androgens [218, 219]. Instead, TES interferes with the production of IL-6 [220] and induces the expression of IL-33 [221] by mast cells. IL-33 is known to regulate the formation of innate lymphoid cells (ILCs) and the production of Th2 cytokines [222]. Regarding female sex hormone action on mast cells, serum levels of immunoglobulin E (IgE, an antibody that induces the degranulation of mast cells) seem to fluctuate depending on the menstrual cycle phase [223]. Moreover, estrogen enhances IgE-induced mast cell degranulation and the release of histamine [224–226]. Meanwhile, progesterone diminishes the migration of mast cells and histamine secretion [227, 228].

Dendritic cells are originated from a CD34+ hematopoietic precursor that gives rise to myeloid (MP) and lymphoid (LP) progenitors [229]. The maturation of these cells is associated with the recognition of PAMPs and/or DAMPs. The process of maturation also implicates metabolic changes and gene transcription that lead to the migration of dendritic cells from peripheral tissues to secondary lymphoid organs. Mature dendritic cells express large amounts of C-C chemokine receptor type 7 (CCR7) and secrete IL-12 and IL-23, which promotes T-cell differentiation [230–232]. The effect of sex steroids on dendritic cells and their influence on lung disease have not been well explored. The acute exposure of bone marrow-derived dendritic cells (BMDCs) to 5α-DHT during antigen priming decreases the stimulation of T-cell cytokine production (IL-4, IL-10, and IL-13) in vitro [233]. On the other hand, estrogen promotes the differentiation of DCs from bone marrow precursors [234] and enhances their T-cell stimulatory capacity [233, 235]. Progesterone treatment decreases the production of the pro-inflammatory cytokines TNF-α and IL-1β by BMDCs [114].

15.4.5 T and B Lymphocytes

T and B lymphocytes or T and B cells are critical elements in the adaptive immune response. B cells carry out the antibody response, whereas T cells play a critical role in the cell-mediated response. In the antibody response, B cells secrete specialized proteins called immunoglobulins (Igs) that circulate across the bloodstream where they bind to and inactivate foreign antigens. Cell-mediated response employs T cells that react directly against foreign antigens, which are presented to them and further neutralize the cells that have been infected with distinct pathogens (bacteria or virus) [236–238]. The development of T cells occurs through phenotypic changes that involve the expression of essential membrane markers such as CD3, CD4, and CD8 [239]. Th1, Th2, Th17, and regulatory T (reg) cells conform the different lineages of CD4+ effector T cells [240].

T- and B-cell development is negatively regulated by androgen actions. For instance, castrated male mice show increased numbers of double positive CD4+ CD8+ T cells in the thymus and B cells in the spleen [241]. Furthermore, castration not only increases the number of B cells in the spleen but in the bone marrow, and this phenomenon is not altered by preceding thymectomy [242]. The negative regulation of B cells exerted by androgens has shown to be dependent on the AR activity. A murine model of general AR knockout (ARKO), and B-cell-specific ARKO, displays enhanced B-cell lymphopoiesis defined by increased numbers of B cells in the blood and bone marrow. Interestingly, the B-cell-specific ARKO group shows a lesser effect on B-cell lymphopoiesis compared with the general ARKO group, pointing out that the negative effect of androgens comprises a regulation of B cells and

the stroma [243]. Contrariwise, estrogens restrain the B-cell lymphopoiesis by reducing the precursors, pro-B cell, pre-B cell, and mature B cells of the bone marrow [244]. Also, estrogens enhance the activity and antibody production of mature B cells [245].

Likewise, androgens negatively modulate T lymphocytes and monocytes in the blood. In this context, Yao et al. demonstrate in Sprague-Dawley rats that TES reduces the numbers of monocytes. However, the lymphocyte subpopulations show an increase in CD8+ T cells, while the numbers of CD3+, CD4+, and double positive CD4+ CD8+ T cells remain unaffected after TES treatment. Therefore, the immunosuppressive role of TES may be due to a decline in the number of monocytes, the change in CD4+/CD8+ ratio, and the increase of CD8+ T cells [246]. Moreover, TES causes a shift in the balance of Th1/Th2 cytokines through a reduction in TNF-α secretion in T-cell lines [247] and stimulates the production of IL-10 (an anti-inflammatory cytokine) in CD4+ T cells TES [248]. In addition, in a model of androgen deficiency, rats show decreased levels of IL-2, IL-6, IL-10, IL-12, and IL-13, whereas TES supplementation restores those levels [249].

Furthermore, it has been reported that estrogens modulate the differentiation and function of distinct T-cell phenotypes. In this context, E2 signaling through the ERα impedes the differentiation of Th1 and Th17 cells, conferring a protective mechanism against inflammation in experimental encephalomyelitis [250]. Besides, physiological concentrations of E2 promote the proliferation of T lymphocytes and the production of IFN-γ in vitro [251]. In Th2 cells, the stimulation of the ERα by E2 increases the expression of IL-4 [252].

The role of sex hormones on the function of immune cells in lung ailments has been established (Fig. 15.3). For instance, TES negatively regulates type 2 immune response seen in asthma evoked by Th2 cells [253]. E2 enhances the severity of pneumonia in adult mice with cystic fibrosis by improving the inflammation mediated by Th17 cells [254]. The involvement of immune cells, e.g., neutrophils, macrophages, eosinophils, and T and B lymphocytes, in the pathogenesis of lung diseases, such as asthma and COPD, and the influence of sex hormones on these cells and their mediators are discussed in the next sections.

15.5 Sex Hormones in Inflammatory Lung Pathologies

15.5.1 Asthma and Chronic Obstructive Pulmonary Disease

Although asthma and COPD differ regarding pathogenesis, progression, prognosis, and treatment options, both ailments exhibit similar symptoms and inflammatory mechanisms [255–258]. Asthma is a chronic airway inflammatory disease that affects around 339 million people worldwide, as informed by the Global Asthma Report 2018. This airway disease is characterized by episodes of variable airflow obstruction (limitation of expiratory airflow), hyperresponsiveness, inflammation, and mucus production [41]. The etiology of this ailment has been related to heritability, environmental exposures, and sensitization to inhalant allergens [259–261]. Airflow obstruction is defined by spirometry parameters indicating a reduced forced vital capacity (FVC) and a reduced forced expiratory volume in 1 second (FEV1, less than 80%). Moreover, FEV1/FVC ratio is found minor than 0.7 in asthmatic patients [262]. Inhaled bronchodilators and corticosteroids are the main treatment in order to reduce inflammation and mitigate the symptoms of this illness [263]. Gender differences in the incidence and the severity of the asthma symptoms have been described and associated with hormonal changes through different life stages. During childhood, boys are more susceptible than girls to develop this ailment; however, in puberty, this trend reverses, and women display more severe symptoms [31, 264–266]. Fluctuations in progesterone and estrogens levels, such as those occurring during the menstrual cycle, have been correlated with the aggravation

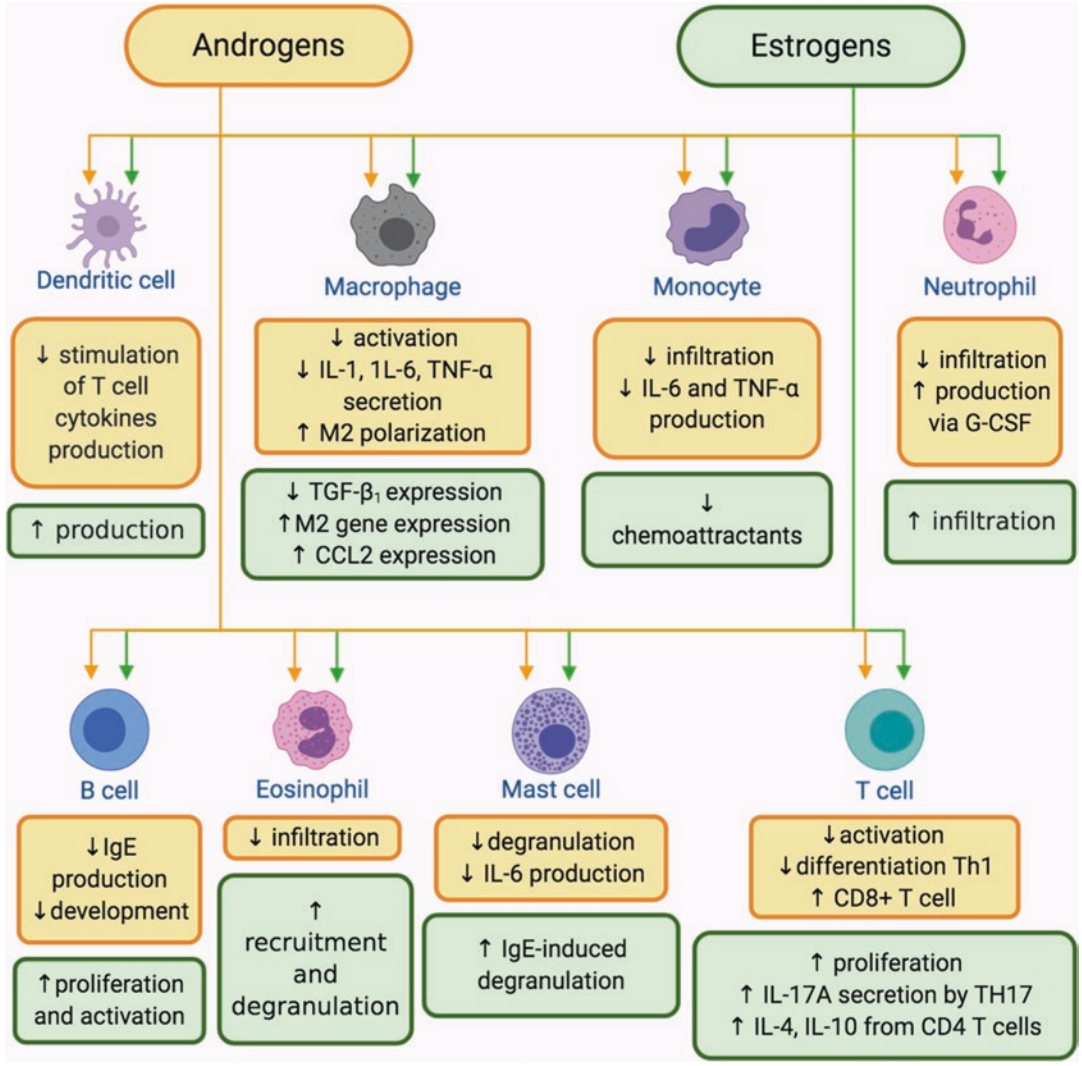

Fig. 15.3 Summary of androgen and estrogen effects on individual inflammatory cell types. Lung disease encompasses a substantial inflammatory component by the action of immune cells playing an essential role in the initial response such as dendritic cells, macrophages, monocytes, and T and B lymphocytes involved in the adaptive immune response. This figure summarizes current knowledge of androgen and estrogen actions on specific types of immune cells that are particularly important in lung ailments. Symbols' meaning and abbreviatures: ↓, inhibits; ↑, enhances. Tumor necrosis factor alpha (TNF-α); interleukin (IL)-1, 4, 6, 10, 17-A; monocyte chemoattractant protein, MCP-1 (CCL2); transforming growth factor beta1 (TGF-β_1); granulocyte colony-stimulating factor (G-CSF); immunoglobulin E (IgE). For details, see the *Immune Cells* section

of this disease [25, 41, 267, 268]. Several studies have shown that 20–40% of premenopausal women experience pre-menstrual or perimenstrual asthma (PMA) [269–276], suffering from an exacerbation of the symptoms with increased bronchial inflammation in the week preceding menstruation [273, 276]. PMA is defined as a cyclical worsening of asthma during the luteal phase and/or during the first days of menstruation [274, 277]. PMA has also been associated with less atopy and poor lung function [273]. Furthermore, the use of oral contraceptives (OC) alleviates perimenstrual exacerbations in women with mild to moderate asthma [278, 279]. In

addition, between 7% and 10% of all pregnant and childbearing-aged women have been reported to present asthma [280–282], and the use of OC does not improve the symptoms in women diagnosed with severe disease [283]. In women from 50 years of age, menopause may correspond with the beginning of asthma or be associated with exacerbations of a preexisting asthma condition, conforming a new phenotype described as menopausal-onset asthma [284, 285]. Around 18% of the total female asthma population suffers from menopausal-onset asthma, which is distinguished by the absence of atopy, aspirin sensitivity, persistent sinusitis, and frequent rate of hospitalizations [285]. In postmenopausal women with asthma, symptoms are commonly severe, and E2 levels have been found higher compared to those in non-asthmatic women [285–287]. Moreover, menopausal hormone therapy (MHT), and particularly the use of estrogen, increases the risk of developing asthma [288, 289]. In addition, progesterone in plasma reaches a peak about 24-fold higher than follicular phases. Interestingly, circulating progesterone is positively correlated with the peak expiratory flow rate (PEFR) during the luteal phase of the menstrual cycle [25].

On the other hand, androgens seem to reduce asthma exacerbations [76]. Higher plasma levels of TES in men, compared with those found in women, are thought to be useful favoring a bigger airway caliber and lung capacity [31, 290, 291]. It has been shown that, following the age of 11 years, the provocative concentration of methacholine necessary to produce a 20% decrement in FEV1 (PC20) increases in teenage boys but not in girls, pointing out an androgen-related improvement in airway responsiveness during puberty [292]. In this regard, non-genomic and genomic effects of androgens on airway smooth muscle [76, 293–297] and the inflammatory response in the asthmatic condition have been extensively explored [38, 42, 253, 298, 299]. Interestingly, the protective role of androgens against asthma in male patients declines after the fifth decade of life, and the symptoms of this ailment appear again. This could be explained by the decrease in plasmatic TES levels during this life stage [31, 300, 301]. Moreover, Mileva and Maleeva [302] reported that male patients with moderate to severe asthma have lower levels of TES compared with those with mild symptoms. In addition, it has been noticed that asthmatic women carrying female fetuses are more susceptible to present symptoms of this illness compared to those with male fetuses [303]. The effects of androgens and estrogens (mainly E2) on the pathogenesis of asthma are discussed below with a special focus in inflammatory cells and their mediators.

COPD is a lung disease characterized by the presence of obstruction ventilator trouble (OVT), i.e., a persistent limitation of airflow that is not fully reversible [304]. This illness is usually caused by exposure to harmful particles or gases that induces emphysematous lung destruction and airway narrowing [305, 306]. Clinical symptoms include cough, sputum production, and progressive dyspnea that is unresponsive to steroids and bronchodilator therapy [307]. Similar to asthma, the diagnosis of COPD relies on clinical evidence and spirometry data, including FEV, FVC, and the ratio FEV1/FVC. A FEV1/FVC ratio less than 0.7, and the lack of full reversibility after the administration of salbutamol (400μg), indicate OVT [304]. It has been estimated that more than 12% of the world population suffers from this illness [308, 309]. The prevalence of COPD is more common in men than in women [308, 310]. This could be explained by the fact that men present higher occupational risks and/or higher rates of smokers [311, 312]. However, data suggests that women have a greater predisposition to this disease. The number of tobacco smoker women with a diagnosis of COPD has notably increased in the last years [33, 313]. The rate of lung function declines faster in female tobacco users than in male smokers. Also, the majority of COPD cases involving non-smokers or never smokers are women [314–317]. Typically, men with COPD have more emphysematous deterioration of the lung, while women tend to have more reactive airways and more pronounced airway narrowing [318, 319]. The apparent increased female susceptibility points out a sex hormone modulation of this illness [37, 320].

Asthma and COPD are characterized to produce chronic inflammation of the airways. Cells and mediators of inflammation implicated in these ailments are even targets for medical treatment [321, 322]. The inflammatory response in both lung diseases involves innate and adaptive immunity. Most asthmatic patients' course with eosinophilic inflammation that is driven by Th2 lymphocytes. This type of inflammation occurs due to exposure to allergens such as pollen, house dust mite (HDM), viruses, cockroach antigens, etc. [323–325]. Dendritic cells present allergenic peptides to uncommitted T lymphocytes and stimulate the production of allergen-specific T cells [326]. Then, epithelial alarmins IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) recruit $CD4^+$ Th2 cells and group 2 innate lymphoid cells (ILC2s) that secrete IL-5 and other interleukins [327]. This interleukin regulates the generation of eosinophils in the bone marrow [325]. Eosinophil migration and recruitment into the lung is regulated by IL-5 as well and by the epithelial-secreted chemokines and C-C motif chemokines (CCL) 11 and 5 [328]. Moreover, type 2 inflammation is also associated with the production of other cytokines, including IL-4 and IL-13, which promote the synthesis of IgE by B lymphocytes [324, 329]. IgE regulates mast cell activity and degranulation. Significantly, mast cell infiltration in the airways has been associated with airway hyperresponsiveness (AHR) [330, 331] mediated by the release of bronchoconstrictor substances such as histamine, LTs, and prostaglandin (PG)-D_2. Mast cells and Th2 cells, additionally secrete IL-4, IL-5, IL-9, IL-13, and TNF-α [332, 333]. TNF-α, a well-known pro-inflammatory cytokine, is elevated in patients with asthma and is involved in the induction of AHR [334]. Experimental evidence has shown that TNF-α potentiates the agonist-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [335] and contractile responses [334–339]. In addition to be involved in switching the isotype of B cells, IL-4 plays a key role in the differentiation of Th2 cells from uncommitted Th0 cells and in the initial sensitization to allergens [333]. IL-5 is deeply associated with the differentiation of eosinophils from their precursor cells in the bone marrow as well as in eosinophil survival [340]. The use of glucocorticoids in asthma treatment decreases airway eosinophilia by inducing eosinophil apoptosis and inhibiting the response to IL-5 [341, 342]. IL-9 participates in mast cell proliferation and activation [343, 344]. IL-13 is another representative Th2 cytokine that not only participates in mucus production, airway inflammation, and remodeling but has been suggested to modulate steroid insensitivity [345–347]. In this regard, it has been shown that the administration of the anti-IL-13 antibody improves airflow obstruction in asthma patients when inhaled corticosteroid treatment seems not to work [348].

Nevertheless, not all asthmatic patients show this inflammatory pattern but develop an IL-17-mediated neutrophil inflammatory response that is mostly described in subjects with the most severe symptoms [42, 105]. Th17 cells are recognized as a distinct population of CD4+ T cells secreting IL-17A, IL-17F, and IL-22 [349]. The differentiation of this group of cells occurs after the stimulation of naive T cells by IL-1β, IL-6, IL-21, IL-22, IL-23, and transforming growth factor beta1 (TGF-β1) produced by macrophages and epithelial and dendritic cells [38, 42, 350, 351]. IL17-A is a key inductor of neutrophilic inflammation by evoking granulopoiesis and neutrophil chemotaxis [352, 353]. Furthermore, the inflammatory response mediated by Th17 cells is known to be highly associated with the resistance to corticosteroid treatment [42, 354].

COPD patients suffer from an accelerated decrease of lung function related to progressive airway obstruction caused by mucus hypersecretion and ciliary dysfunction [355, 356]. The inflammation developed in COPD subjects, located predominantly in the peripheral airways and lung parenchyma [357], is characterized by the presence of alveolar macrophages, neutrophils, T lymphocytes, dendritic cells, and B lymphocytes [358]. Most severe cases of COPD are commonly associated with higher numbers of B lymphocytes and neutrophils [304, 358]. T lymphocytes implicated in this disease are mostly CD8+ T-cytotoxic cells (Tc), but CD4+ Th1 cells are also increased [359]. In the blood of COPD

patients, the proportions of IFN-γ- and TNF-α-producing CD8+ T cells are augmented compared with healthy ones [360]. Moreover, increased numbers of Th17 cells have been reported in COPD patients. IL-17A and IL-22 secreted by these cells modulate the recruitment of neutrophils and cause inflammation [361, 362]. Also, the number of macrophages is increased in the lungs from patients with COPD, and this increment corresponds to the severity of the disease [363]. Several inflammatory mediators are involved in the development of this ailment; for instance, alveolar macrophages and epithelial cells from COPD patients release more chemical mediators than in normal subjects [364, 365], including IL-1β, IL-6, TNF-α, CXCL1, CXCL8 (IL8), CCL2 (monocyte chemoattractant protein, MCP-1), reactive oxygen species (ROS), and LTB_4. Notably, some of the former cytokines act as chemotactic factors that promote neutrophil migration [366]. Cigarette smoke stimulates granulocyte production through granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and TGF-β1 released from airway epithelial cells and lung macrophages. TGF-β1 participates in the activation of myofibroblasts and airway smooth muscle cells, causing proliferation and fibrosis [358, 367]. Furthermore, granulocyte production leads to persistent neutrophilic inflammation present in most COPD patients [367]. Additionally, epithelial cells and alveolar macrophages release chemokines such as CXCL9 (induced by IFN-γ), CXCL10 (IFN-inducible protein 10), and CXCL11 (IFN-inducible T-cell alpha chemoattractant) [358]. The upregulation of CXCL10 and its receptor (CXCR3) contributes to the accumulation of CD4+ and CD8+ T cells in patients with this obstructive disease [368]. CD8+ T cells are cytotoxic entities that release perforins, granzyme B, and TNF-α, contributing to alveolar cell apoptosis in emphysema [369]. Matrix metalloproteinases (MMPs), e.g., MMP-9 and MMP-12, similarly contribute to proteolytic attack on the alveolar wall matrix [364, 370]. Furthermore, airway eosinophilia may occur when COPD exacerbations are mediated by viral infections [371]. IL-33 produced by epithelial cells has been suggested to be the key regulators in Th2- and ILC2-mediated eosinophilia in this illness [372]. Moreover, oral corticosteroid therapy has been shown to be effective in reducing eosinophilic inflammation in patients with COPD [373].

15.5.2 Androgens' Effects on Inflammation in Asthma and COPD

Airway smooth muscle (ASM) is one of the major structural elements of the airways. This smooth muscle layer controls airway caliber and tone [95, 295, 374]. Hyperresponsiveness to physical or chemical stimuli is a characteristic feature of asthma. AHR is described as an exaggerated airway narrowing [375] that is usually reversed with the use of bronchodilators [376]. Given the gender differences in the incidence and the outcomes of this illness, sex steroids' (e.g., androgen) non-genomic and genomic effects on the airways have been widely studied. Primordial evidence of non-genomic actions of androgens on ASM was found in 2006 by Kouloumenta et al. They observed that TES is capable of relaxing rabbit tracheal preparations pre-contracted with cholinergic agonists [377]. Further studies showed that TES and their metabolites 5α-DHT and 5β-DHT induce the relaxation of bovine and guinea pig ASM by blocking L-type voltage-dependent Ca^{2+} channels (L-VDCCs) [378]. In this context, published data from Dr. Montaño's research group confirmed that TES, 5α-DHT, 5β-DHT [379], and DHEA [294] relax the KCl and carbachol pre-contracted guinea pig tracheal tissues. Additional studies from the same research group pointed out that TES not only blocks L-VDCCs but interferes with store-operated Ca^{2+} channels (SOCCs) and inositol 1,4,5-trisphosphate (IP_3) receptor (ITPR), and induces the production of PGE_2 [296, 297]. Most recent published data shows that the chronic exposure to a physiological TES concentration increases the expression of β_2-adrenoceptor (β_2-AR) and upregulates delayed rectifier voltage-dependent K^+ channels (K_V) and high conductance

Ca^{2+}-activated K^+ channels (BK_{Ca}), enhancing the relaxing responses to salbutamol in guinea pig ASM [380].

Androgen effects on immune cells and their inflammatory mediators in healthy subjects and asthmatic patients have also gained relevance. For instance, a study on human peripheral blood mononuclear cells (PBMCs) showed that the number of cells secreting IFN-γ is correlated with the serum levels of DHEA-3-sulfate (DHEA-S, a metabolite of DHEA mainly produced in the suprarenal cortex that possesses weak androgen activity) in premenopausal women and men [381]. Moreover, it has been observed that patients with an asthmatic condition have reduced serum DHEA and DHEA-S concentrations compared with healthy subjects [382–384]. Also, DHEA significantly diminishes both Th1 and Th2 responses in cultured PMBCs from patients with asthma [385], and DHEA-S attenuates chemotaxis and migration of peripheral human neutrophils and inhibits chemokinesis of human ASM [386]. These observations point out that these androgens interfere with cell migration and inflammation in airways, maybe leading to the decrease of asthma symptoms. In this regard, the administration of nebulized DHEA-S to patients with poorly controlled moderate to severe asthma diminished the symptoms and improved the control of this disease [387]. Furthermore, asthmatic women with low serum levels of DHEA-S who received a supplementation with DHEA showed an upgraded lung function in asthma outcomes [388]. In allergic asthma, ILC2s seem to play a pivotal role in initiating and mounting the typical Th2 inflammatory response [343], which induces eosinophilic inflammation, AHR, and remodeling of the airways [42, 389]. In fact, ILC2s are known for producing higher quantities of IL-5 and IL-13 compared with Th2 cells [390–392]. Several reports have shown increased numbers and activation status of ILC2s in blood and sputum samples from adult and pediatric asthma patients [390, 393–397]. Likewise, genetic polymorphisms related to asthma susceptibility have also been shown localized to gene regulatory elements in ILC2s [398]. Animal models have served to prove that androgens exert a downregulation on ILC2s and Th2 inflammatory patterns [106, 253, 299, 399]. Two of these studies illustrate that DHEA reduces house dust mite-induced allergic inflammation by decreasing blood eosinophilia, IL-4, IL-5, and IFN-γ levels [399] and suppresses eosinophil infiltration and AHR through the modulation of Th2 cytokines in ovalbumin (OVA)-sensitized mice [400]. Male mice have low numbers of eosinophils and lymphocytes in bronchoalveolar fluid and lower IL-4 mRNA expression levels in splenic cells than castrated males and females [401]. Moreover, male mice show reduced numbers of ILC2 progenitors (ILC2Ps) and less severe IL-33-driven lung allergic inflammation. Furthermore, IL-5 and IL-13 levels, after IL-33-induced activation of ILC2s, are diminished in male mice compared to females [392]. Likewise, ILC2s from male mice display higher expression levels of killer cell lectin-like receptor subfamily G member 1 (KLRG1) and IL-33 receptor (ST2) [299]. Importantly, 5α-DHT through AR signaling limits the differentiation of ILC2Ps into mature ILC2s in the bone marrow [106, 299] (Fig. 15.4). These insights suggest marked differences between ILC2 development from male and female mice. Additionally, TES decreases house dust mite-induced airway eosinophilic and neutrophilic inflammation, IgE production, and AHR in castrated mice [253]. It is well known that mast cells infiltrate the bronchial epithelium and release bronchoconstrictor mediators in allergic asthma. In this regard, the systemic administration of 5α-DHT inhibits mast cell activation and degranulation. Also, this androgen reduces airway hyperplasia and mucus production in a murine model of OVA-sensitized females [402]. Mast cell degranulation leads to the release of leukotrienes, highly potent lipidic mediators also involved in the allergic response, specifically inducing ILC2 activation and lung inflammation [403]. Macrophages, eosinophils, and basophils are capable of releasing these lipidic molecules as well [404, 405]. Two classes of LTs have been described to modulate different targets. While LTB_4 acts as a potent leukocyte chemoattractant and enhances the macrophages' bacterial killing capability, the cysteinyl leukotrienes (Cys-LTs,

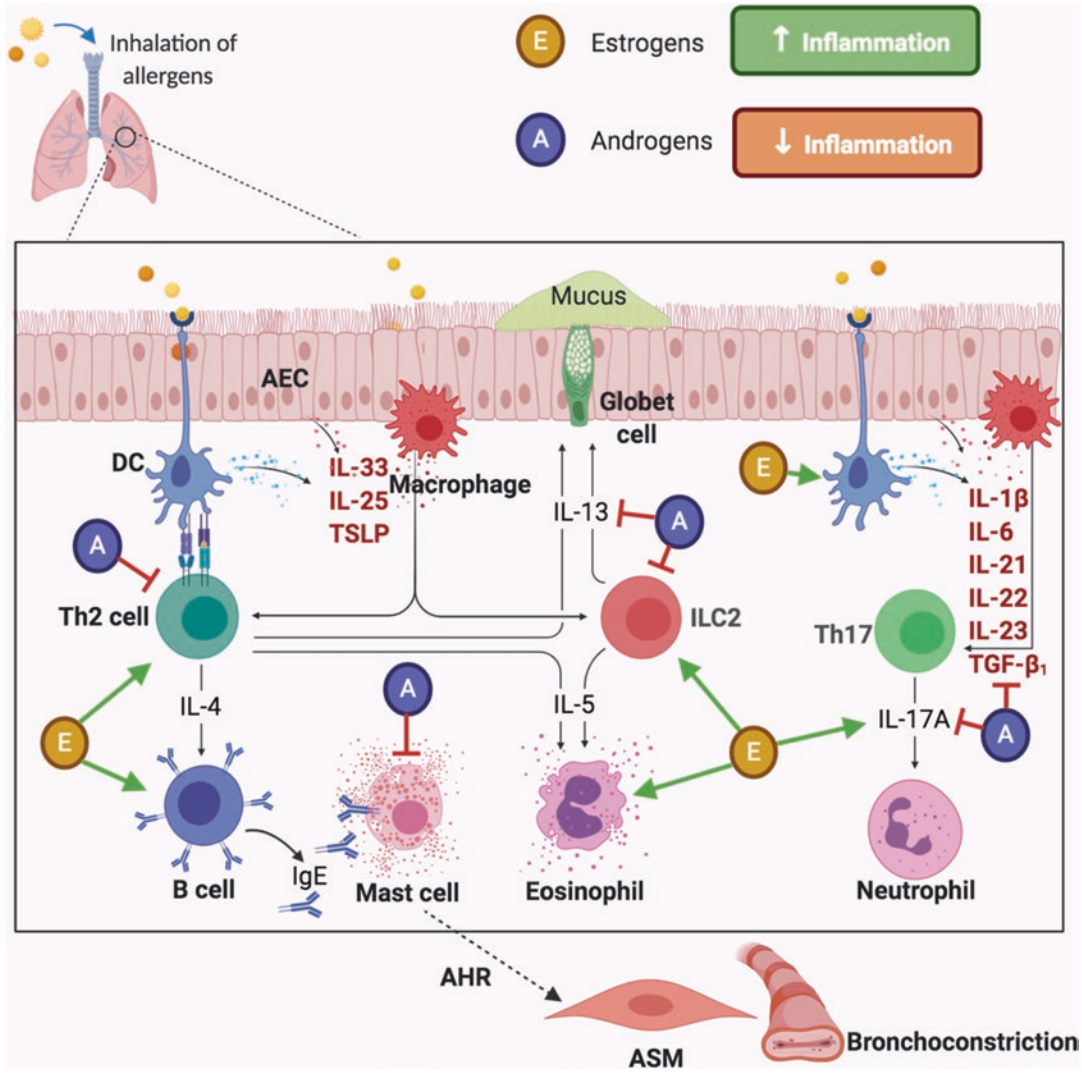

Fig. 15.4 Androgen and estrogen effects on the inflammation in asthma. Asthmatic disease involves an inflammatory-driven response of the airway by the exposure to allergens (pollen, house dust mite, cockroach, fungal antigens, etc.). Dendritic cells (DCs) present allergenic peptides to uncommitted T lymphocytes and stimulate the production of allergen-specific T cell. DCs, airway epithelial cells (AECs), and macrophages secrete epithelial alarmins IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which recruit Th2 cells and group 2 innate lymphoid cells (ILC2s) that secrete IL-4, IL-5, and other cytokines. Eosinophil migration is induced by IL-5, whereas IL-4 and IL-13 mainly favor immunoglobulin E (IgE) production by B cells with the consequent activation of mast cells. The infiltration of mast cells in the airways has been associated with airway hyperresponsiveness (AHR) mediated by the release of bronchoconstrictor substances such as histamine, leukotrienes, and prostaglandin D_2. These mediators activate their own receptors in the airway smooth muscle (ASM) provoking bronchoconstriction. Moreover, exposure to allergens also initiates an immune response through IL-1β, IL-6, IL-21, IL-22, IL-23, and TGF-β1 produced by DCs, AECs, and macrophages favoring naive Th differentiation to Th17. Th17 cells synthesize IL-17A, a key inductor of neutrophilic inflammation by evoking granulopoiesis and neutrophil chemotaxis. The effects of estrogens (E) or androgens (A) on the illustrated immune cells can differ depending on the hormone concentration and the timing and duration of the stimulus. Androgens diminish Th2 and ILC2 cell population, consequently limiting eosinophilic inflammation and IgE production. They also decrease IL-17A synthesis by Th17, lowering neutrophilic inflammation. Estrogens increase Th2 and ILC2 cell differentiation and induce IL-17A production from Th17 cells. Furthermore, estrogens augment total serum IgE, IL-5 production, and eosinophilia. In conclusion, estrogens may display detrimental effects on airway function by enhancing inflammation, and androgens exert opposite effects

LTC_4, LTD_4, and LTE_4) evoke bronchoconstriction and increase vascular permeability [406, 407]. For LT biosynthesis, arachidonic acid (AA) is excised from phospholipids in the plasma membrane by the action of phospholipase A_2 (PLA_2). Once AA is released, the nuclear membrane-bound 5-LOX-activating protein (FLAP) delivers it to 5-lipoxygenase (5-LOX) that converts AA into the different types of LTs [406, 408, 409]. In this regard, 5α-DHT and TES diminish the biosynthesis of LTs by interfering with 5-LOX localization via activation of type 2 extracellular signal-regulated kinase 2 (ERK 2) [407, 410] and by blocking the assembly of 5-LOX/FLAP [411]. The regulation of ERK2 and 5-LO trafficking exerted by androgens may explain the gender differences observed in the anti-leukotriene therapy, where young girls show better outcomes than boys [412]. Moreover, increase in $[Ca^{2+}]_i$ in ASM cells (ASMCs) is a primordial mechanism that triggers the exacerbated bronchoconstriction seen in asthma. In this context, a study in primary human ASMCs made by Kalidhindi et al. indicates that basal expression of AR is greater in males compared to females but increases with asthma or with an inflammatory condition in both genders. More interestingly, ASMCs from asthmatic females display a greater AR expression than males; however, androgen receptor may take minor functionality in females. In addition, TNF-α and IL-13 enhance histamine-induced increase in $[Ca^{2+}]_i$ in ASMCs; nevertheless, TES and 5α-DHT decrease the enhancement through AR signaling. AR effects on $[Ca^{2+}]_i$ increments are explained by the downregulation of stromal interaction molecule 1 (STIM1) and Orai1, key machineries in store-operated Ca^{2+} entry (SOCCE), and the increasing of SARAF (formerly known as TMEM66, a negative regulator of SOCCE) [413].

Not all patients with asthma course with type 2 inflammation but display an IL-17A-mediated neutrophil inflammatory pattern that is resistant to corticosteroid treatment. IL-17A is secreted by CD4+ Th17 cells and is associated with more severe asthma phenotypes [42, 105]. Moreover, macrophages and DCs express receptors for IL-17A and favor the synthesis of IL-6 and TNF-α [414]. Interestingly, the stimulation of the AR by TES decreases IL-17A protein expression and IL-23 receptor (IL-23R) mRNA expression from Th17 cells, reducing neutrophilic airway inflammation [253]. Also, IL-17A induces glucocorticoid receptor β (GR-β) expression and favors corticosteroid therapy resistance in patients with severe asthma [415]. α and β subtypes have been described as the two known glucocorticoid receptors. GR-β acts as an inhibitor of GR-α, and this latter isoform decreases the expression of inflammatory mediator genes [416, 417]. These findings point out that TES (by downregulating IL-17A) may decrease the expression of GR-β, promoting the anti-inflammatory effect of corticosteroids.

Persistent asthma (as seen in a IL-17A-mediated inflammatory response) is characterized by airway remodeling that involves epithelial-mesenchymal transition (EMT), cell mucus hypertrophy, subepithelial fibrosis, deposition of extracellular matrix proteins, and smooth muscle hypertrophy and hyperplasia [418, 419]. These cell modifications lead to pronounced airflow obstruction and epithelial damage that predispose to AHR. TGF-β1 induces EMT, and its increased expression levels correlate with severe asthma phenotypes [420, 421]. In this context, Xu et al. demonstrated that DHEA (via a genomic effect) inhibits the bronchial TGF-β1-induced EMT and preserves the epithelial morphology [422].

The influence of androgens on COPD patients and the inflammatory response in animal models have not been broadly investigated as occurring in asthma. However, since circulating TES levels have been positively correlated with cardiorespiratory function and muscle growth and strength [423, 424], low levels of this androgen may be associated with worse outcomes in patients with COPD [425]. In this context, numerous studies show that men with COPD have medical relevantly lower levels of TES and DHEA-S compared with healthy men [425–431]. Moreover, a recent research suggests that testosterone replacement therapy (TRT) decreases the rate of COPD patient hospitalizations and slows the progression of the disease [432]. COPD patients' usually

display neutrophil-mediated inflammation, mainly located in the lung parenchyma, influenced by the action of IL-17A [433]. Macrophage activity also plays a crucial role in the lung of smokers and is even higher in COPD patients. Macrophages expressing IL-17A receptors have been observed in mice with myocarditis, and the genetic suppression of these receptors interferes with macrophage recruitment [434]. Similar to neutrophils, macrophages contribute to lung damage by synthetizing and releasing pro-inflammatory mediators, e.g., cytokines, chemokines, and ROS [363]. Moreover, cigarette smoke stimulates the activation and recruitment of macrophages, causing emphysema, involving the action of MMP-9, MMP-12, and CCL2 [197, 363, 435, 436]. In COPD, the role of the two distinct described phenotypes of macrophages (M1 and M2) is unclear, showing no sign of predominance for a determined subtype [197, 363]. The classical activation of macrophages (M1) is associated with Th1 immune response producing pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12 [197, 202]. However, Th2 cytokines (IL-4 and IL-13) can alternatively activate macrophages as occurring in allergic asthma [437]. M2 macrophages commonly produce anti-inflammatory cytokines, including TGF-β, IL-10, CCL18, and CCL22 [197, 437]. Interestingly, evidence suggests that alveolar macrophages may be involved in the pathogenesis of COPD in a non-inflammatory manner; i.e., smoking induces a polarization pattern toward the down-regulation of M1-related inflammatory genes (CXCL9, CXCL10, CXCL11, and CCL5) and the induction of genes associated with the M2 polarization mechanisms (MMP-2, MMP-7, and the adenosine A3 receptor) [438]. Nevertheless, M2 alveolar macrophages contain inflammatory mediators that may cause an increase in cell recruitment, mucus secretion, and airway remodeling if they are immoderately discharged, as occurs in humans and mice asthmatic lungs [439, 440]. In this context and contrary to the anti-inflammatory androgen effects, it was revealed that, although 5-αDHT reduces lung inflammation, the same androgen enhances IL-4-stimulated M2 macrophage polarization in OVA-induced allergic mice. Also, the genetic ablation of AR diminishes eosinophil recruitment and lung inflammation due to the compromised M2 polarization [440]. Therefore, further research is required in order to elucidate the role of androgens on alveolar M2 macrophages and in the inflammatory outcomes of diseases such as asthma and COPD.

The evidence indicates that male sex steroids have beneficial anti-inflammatory properties in asthma patients by attenuating innate lymphoid cells Type 2, Th2 cells, IL-17A-mediated response, and the leukotriene biosynthesis pathway, through different mechanisms (Fig. 15.4). Also, androgens might reduce the neutrophilic inflammation in COPD, but additional studies are required. Moreover, even though the use of androgens in order to increase muscle mass and improve cardiorespiratory functions is promising, the effect of these hormones in men with COPD seems to be modest, and more research is indispensable to resolve whether TRT could be an option in COPD treatment. Regarding the use of androgens as an anti-inflammatory treatment in asthma and COPD, it should be considered the metabolic pathway of DHEA and TES, leading to the production of estrogens. Furthermore, 5α-DHT, a reduced metabolite of TES with important androgenic actions, has been associated with prostate cancer [441]. In this context, 5β-DHT, the other reduced metabolite of TES with minor androgenic activity and without estrogenic effects, might be taken into account as a potential therapeutic choice [76], although clinical studies are imperative to support this notion.

15.5.3 Estrogens' Effects on Inflammation in Asthma and COPD

It has been established that the incidence of asthma is more common in young boys and adult women, and that the severity of the symptoms may increase during pregnancy [26, 31, 105]. The transition from childhood to adulthood is distinguished by a higher probability of persistence of wheezing in females [300, 442].

Therefore, the influence of female hormones (mainly estradiol) on the airway biology has been widely explored [95, 141, 142, 145, 443–446]. Interestingly, a contradicting role of estrogen suggests either the induction of AHR and inflammation [214, 447–449] or an improvement of asthma symptoms by downregulating inflammation and favoring ASM relaxation [214, 450–453]. In this regard, several bronchodilator mechanisms have been shown to be affected through the rapid actions of female sex steroids. In 1983, a group of researchers reported that E2 in supraphysiological concentrations enhances the bronchodilator response to adrenaline and noradrenaline in pig bronchus and the increment in the potency of catecholamine-induced bronchodilation may be mediated by an inhibition of catecholamine metabolism or uptake [454]. Later on, it was exhibited that E2 causes relaxation of isolated trachea muscle strips (pre-contracted with acetylcholine or KCl), independently of the adrenergic system, but possibly involving prostaglandin synthesis and cyclic guanosine monophosphate (cGMP) modulation [455]. It is well known that prostaglandins modulate cyclic adenosine monophosphate (cAMP) and cGMP formation. Cyclic nucleotides stimulate protein kinases that influence the inhibition of Ca^{2+} influx channels, which favors bronchodilation. In this context, E2-induced increase in cAMP has been observed in porcine coronary arteries [456]. Also, physiological concentrations of E2 potentiate the relaxation evoked by isoproterenol via cAMP production in human and guinea pig ASM. The cAMP pathway triggered by E2 in a non-genomic way promotes the blockade of Ca^{2+} influx (mainly through L-VDCCs) and the further decrease in $[Ca^{2+}]_i$ [453]. In addition, as demonstrated by Dimitropoulou et al. [457], the increase in cyclic nucleotides leads to the phosphorylation and the opening of K^+ channels. They observed that a physiological E2 concentration inhibits AHR in asthmatic mice via an ER signaling pathway that implicates the activation of protein kinase G (PKG) and the opening of BK_{Ca} channels. Nevertheless, a recent study demonstrates that 17β-estradiol has a little effect on the formation of cAMP in primary cultured human ASMCs. Interestingly, the same study shows that progesterone is capable of promoting the formation of cAMP after 3 minutes of stimulation [458]. In the last years, some studies have further demonstrated that estrogens diminish Ca^{2+} levels in ASMCs from different species via rapid (non-genomic) effects through ERs or by directly blocking membranal Ca^{2+} channels. Supraphysiological concentrations of E2 lower ASM basal tone through the obstruction of L-VDCCs in guinea pig ASM [295]. Moreover, the acute exposure of physiological concentrations of E2 decreases histamine-evoked Ca^{2+} influx via the inhibition of L-VDCCs and SOCCE in ASMCs from women [167]. It has been shown that asthmatic airways are less responsive to nitric oxide (NO) donors; however, E2 through ERβ reverses this phenomenon favoring ASM relaxation [451]. Also, E2 (in a physiological range) rapidly increases NO production in human bronchial epithelium from women and produces relaxation of bronchial rings pre-contracted with acetylcholine (Ach) [459]. In addition, a study has demonstrated that P4 and 5β-pregnanolone prevent histamine- or carbachol-induced contraction in guinea pig ASM [460].

Chronic effects of estrogens, or the lack of them, on Ca^{2+} handling and AHR have also been investigated. For instance, the genetic ablation of ERα induces AHR among other lung function anomalies and interferes with airway smooth muscle and nerve physiology, probably involving the dysregulation of M2 muscarinic receptors [461]. Matsubara et al. reported that endogenous estrogens downregulate AHR in OVA-induced asthmatic female mice. Correspondingly, 17β-estradiol suppresses AHR in male mice challenged for 10 days with OVA [462]. Dimitropoulou et al. observed that estrogen replacement therapy prevents the development of AHR and inflammation (important markers in asthma) in ovariectomized asthmatic mice. Also, they found a reduction in TGF-β1 levels from bronchoalveolar lavage fluid (BALF) of estrogen-treated mice [463]. Recently, Bhallamudi et al. [464] found in non-asthmatic and asthmatic human ASMCs that long-term exposure (24 h) to propylpyrazoletriol (an ERα agonist) enhances the Ca^{2+} response to

histamine. Differently, ERβ stimulation with the agonist WAY-200070 (WAY) evokes a decrease in histamine-induced $[Ca^{2+}]_i$ increase. Besides, TNF-α and IL-13 improve $Ca^{2+}]_i$ responses evoked by histamine, Ach, and bradykinin; and ERβ activation abolishes this phenomenon. Interestingly, E2, a non-selective ER agonist, does not show significant changes in $[Ca^{2+}]_i$ when ASM cells are stimulated only by histamine. However, E2 induces a decrease in $[Ca^{2+}]_i$ increase elicited by histamine bradykinin and Ach when ASMCs are pre-treated with TNF-α or IL-13. ERβ effects on agonist-induced $[Ca^{2+}]_i$ increases seem to be mediated by an augment on sarco(endo)plasmic reticulum Ca^{2+} ATPase 2 (SERCA2) function and by the inhibition of L-VDCC [464].

Estrogen actions on the inflammatory response and their cellular mechanisms in asthma have been explored as well. Most studies point out that estrogens, mainly through ERα signaling, increase allergic airway inflammation and allergen-induced AHR [141, 142, 145, 461]. Th2 inflammation in asthmatic patients influence B-cell activation that favors increased levels of IgE [465]. Women have been shown to have higher serum IgE levels during puberty, which are associated with more severe asthma symptoms provoked by histamine, IL-4, and IL-13 released from mast cells [442, 465, 466]. Likewise, OVA-induced female asthmatic mice with increased IgE levels (compared with those observed in males) show minor sensitivity to budesonide treatment against IL-5 production and the development of AHR [447]. The exposure to environmental tobacco smoke (ETS) increases Th2 cytokine response after allergic (OVA) sensitization. Female mice exposed to ETS display more IgE-positive cells in the lungs and augmented levels of IL-4, IL-5, IL-10, and IL-13 than males [449]. Additionally, acute exposure of physiological concentrations of E2 elicits the increase in $[Ca^{2+}]_i$ via ERα activation and the subsequent IgE-induced degranulation and LTC_4 production in a rat basophilic leukemia cell line (RBL-2H3M) and in a human mast cell line (HMC-1) [226]. These insights indicate that females are more susceptible to develop a more damaging inflammatory response after the exposure to tobacco smoke as occurring in COPD. Furthermore, it has been proposed that female sex steroids are responsible for inducing eosinophilia in allergic asthma. A study performed by Riffo-Vázquez et al. shows that gonadectomy decreases total serum IgE, IL-5 production, and pulmonary eosinophilia in female mice sensitized to OVA [214]. In addition, it has been demonstrated in a murine model of allergic asthma that progesterone promotes airway eosinophilia and provokes AHR [215].

Dendritic cells and macrophages are also modulated by estrogen actions. These cells present allergenic peptides to uncommitted T lymphocytes and favor type 2-mediated airway inflammation [51]. In this context, it has been observed that E2 triggers the expression of DC-derived cytokines, such as IL-6, IL-8, and MCP-1 [467], and enhances the production of human DC population, promoting the proliferation and differentiation of Th cells into Th2 cells [468]. Asthmatic patients present significantly increased numbers of M2 macrophages in BALFs compared to normal subjects [469, 470]. OVA-sensitized female mice also have more M2 alveolar macrophages compared to males [471, 472]. In addition, alveolar macrophages from female mice exhibit greater expression of M2 genes, IL-4 receptor (IL-4R)-α, and ERα after OVA challenge. Furthermore, IL-4 induces M2 gene expression in female mice macrophages, and exogenous E2 potentiates this polarization [473].

Female sex hormones have also been associated with the IL-17-mediated inflammatory pattern displayed in some patients with asthma. This phenotype is distinguished by manifesting neutrophilic inflammation as occurring in COPD [42, 304]. In this context, Newcomb et al. found that women with severe asthma have increased numbers of Th2 cells and greater production of IL-17A compared with asthmatic men [474]. Given these insights, the authors claimed that female sex steroids are responsible for increasing Th17 cell differentiation and IL-17A production from these cells. However, they did not observe a correlation between E2 and P4 plasma levels (at the time of obtaining blood samples) with IL-17A

protein expression in Th17 cells from women and further suggested that the increase in the production of IL-17A in women compared to men is due to the exposure of T cells to female sex hormones during the development of Th17 cells in the body. To further determine the mechanism by which sex steroids modulate the production of IL-17A, they performed an experiment where it was observed that the administration of E2 and P4 augments the expression of IL-17A and IL-23 receptor (IL-23R) in Th17 cells from ovariectomized mice [474]. In this regard, it has been established that IL-17A requires IL-23R signaling to maintain and stabilize the Th17 cell phenotype [475, 476]. Moreover, the same authors found an increased expression of IL-23R in Th17 cells from women compared to men [474]. Another study shows that E2 chronic stimulation enhances the expression of IL-6 and IL-17 in peripheral blood mononuclear cells from asthmatic patients, both females and males [477]. Interestingly, it has been demonstrated that the estrogen deficiency occurring in postmenopausal women is associated with increased serum levels of IL-17A [478]. Anti-inflammatory effects of estrogen in asthma have been reported as well. E2 inhibits cell recruitment into the lungs during the allergic inflammatory process, preventing the cell adhesion through the downregulation of E-selectin. Finally, estradiol treatment of ovariectomized OVA-induced allergic rats diminishes the release of LTB_4, IL-10, and TNF-α [267].

More severe asthma symptoms reported in women appear to be associated with hormonal changes occurring during the different life stages, i.e., menstruation, pregnancy, and menopause [38, 276]. During pregnancy, asthma can change its manifestations, and women with severe disease may present worsening of symptoms that do not improve with the use of OC [283]. It has been described that in the third trimester of pregnancy, one third of women with asthma show an improvement, one third show no change, and another third get worse [479, 480]. In this regard, highest estrogen levels during the third trimester of pregnancy may explain the different manifestations of the disease [265]. Cytokines with an anti-inflammatory role such as IL-10 and IL-4 have been shown augmented after the stimulation with E2 (in concentrations occurring during pregnancy) in CD4 T cells from humans and mice [252, 481, 482]. Furthermore, higher physiological concentrations of estrogen reduce the T-cell production of TNF-α [483]. In the menopausal period, asthmatic patients tend to experience more pronounced respiratory complications [484]. It has been proposed that E2 serum levels may function as an appropriate biomarker for asthma severity in postmenopausal women [285]. Height reduction of the thoracic spine due to osteoporosis related to estrogen deficit in menopausal women might be implicated in the decrease of lung function [485]. Menopause hormone therapy based on estrogen administration is used to alleviate climacteric symptoms, including osteoporosis; vasomotor disorders; skin, urogenital, and weight changes; etc. [486]. Nonetheless, risks as developing thromboembolic venous diseases via alterations in blood coagulation must be taken into account when high doses of MHT are used [486–488]. Aminoestrol, butolame, and pentolame, types of 17β-aminoestrogens, have been suggested as an alternative of MHT, considering their weak estrogenic and antithrombotic activity [489–491]. In this regard, Flores-Soto et al. observed that butolame and pentolame but not aminoestrol, cause hyperresponsiveness to carbachol, histamine, and KCl in guinea pig ASM through the activation of L-VDCCs [492]. This finding points out that aminoestrol is a good alternative for MHT, but further studies are required to critically asses the role of aminoestrogens in postmenopausal asthma exacerbations, particularly in the inflammatory response.

Several recent works have shown a differential expression and activation of both ERα and ERβ in non-asthmatic and asthmatic ASMCs that may explain the distinct outcomes regarding airway inflammation, remodeling, and responsiveness in females [141, 142, 145, 161, 443]. Aravamudan et al. quantified the expression of ERα and ERβ in ASMCs from asthmatic and non-asthmatic subjects. They observed that ERβ expression is greater in asthmatic ASMCs and in ASMCs exposed to TNF-α or IL-13 as well. The expression of the ER isoforms is regulated by inflam-

matory signaling pathways such as p42/pp44 mitogen-activated protein kinase (p42/44MAPK), phosphatidylinositol 3-kinase (PI3K), and NF-κB, proteins implicated in airway remodeling and asthma development [161]. Furthermore, the dual effects of 17β-estradiol on smooth muscle have been reported. Some works demonstrate that this estrogen promotes a mitogenic effect in female and male rabbit ASM [493] and induces rat vascular smooth muscle proliferation via ERs and MAPK signaling cascade [494], while other studies show that E2 inhibits the proliferation and migration of vascular smooth muscle cells [495, 496]. Recent studies by Ambhore et al. suggest that ERβ plays a protective role against airway remodeling and hyperresponsiveness. One of these studies confirmed that ERβ but not ERα activation inhibits platelet-derived growth factor (PDGF)-induced proliferation in ASMCs from asthmatic and non-asthmatic males and females by interfering with cell cycle mechanisms and suppressing proliferative proteins [443]. Also, the administration of a selective ERβ agonist (WAY) decreases airway remodeling and AHR in a murine model of allergen-induced asthma; specifically, ERβ activation abolishes the increase in vimentin, fibronectin, and collagen I caused by allergic sensitization [141]. Fibronectin and collagen are molecules highly involved in extracellular matrix (ECM) deposition that leads to airway remodeling and hyperresponsiveness [497–499]. In this regard, another study shows that ERβ activation diminishes TNF-α-induced increased protein expression of ECM proteins such as collagen I, collagen III, and fibronectin in human ASMCs from asthmatic and non-asthmatic subjects. Also, ERβ signaling reduces the activity of MMP-2 and the expression of MMP-2 and MMP-9 in both asthmatic and non-asthmatic ASMCs [142]. These proteolytic enzymes are key regulators in the ECM degradation and the progression of asthmatic airway remodeling [500–502]. Interestingly, the inhibitory effect of ERβ on MMP expression and activity seems to be mediated by the NF-kB signaling pathway [142]. The most recent study about the protective role was performed by Kalidhindi et al., using an allergen-induced asthma model in ERα and ERβ knockout (KO) mice [145]. Initially, they observed that female mice exposed to the allergen show a more pronounced decay in lung function (in terms of airway resistance and compliance) compared to male mice. Correspondingly, KO animals display a deteriorate lung function compared to ERα KO and WT KO animals. In addition, the genetic ablation of ERβ results in a more prominent airway remodeling and responsiveness and increased expression of fibronectin, vimentin, and alpha smooth muscle actin (α-SMA), consistent with the observations made by Ambhore et al. [141, 142]. These explorations help to clarify the role of estrogen receptors both α and β, pointing out a protective role for the latter one, and contribute to explain the gender differences observed in asthma prevalence after puberty, when women present more severe symptoms.

Otherwise, the influence of estrogens in COPD has been poorly explored. Data suggest that women are more likely to have COPD and present a faster decline of lung function than men. Also, epidemiological studies have shown that hormone replacement therapy (HRT) containing estrogens exacerbates COPD [503]. Unlike the reported for asthma, the expression of the ERs seems not to play an essential role in the development of COPD, since the three major subtypes, ERα, ERβ, and GPR30, are expressed in the same grade in lung tissues from COPD patients and normal subjects, including both men and women. However, the intracellular pathways that lead to the production of female sex steroids are upregulated. Aromatase and 17β-HSD1, two of the main enzymes responsible for the generation of estrogens, are increased in alveolar macrophages of COPD patients compared with controls [504]. Moreover, once smoke cigarette is inhaled, chemicals are metabolized in two separate phases. Cytochrome P450 enzymes accomplish the phase I. These enzymes are a large family of proteins with the critical function of metabolizing cigarette smoke and other environmental irritants, turning them into intermediate compounds. Phase II enzymes conjugate and secrete the metabolites produced in the former clearance steps. A downregulation in either

expression or function of phase II enzymes might suppose the accumulation of metabolites produced by CYP in the lung, causing oxidant injuries to the tissue [36, 505]. Interestingly, it has been suggested that E2 upregulates CYP enzymes. For instance, female mice show a more pronounced sensitivity to naphthalene (an important component of cigarette smoke) toxicity and a more prominent pattern of airway epithelial injury than male mice [506]. Also, female mice have more significant expression of CYP enzymes and augmented accumulation of naphthalene metabolites [507]. Furthermore, the stimulation of the ERα by E2 increases the basal and the smoke-induced expression of CYP1A1 and CYP1B1 (two members of CYP family) in human bronchial epithelial cells [508]. Moreover, a CYP1A1 differential metabolic activity and distinct outcomes of the produced metabolites have been claimed. It has been demonstrated that CYP1A1 shows high metabolic activity for E2 2-hydroxylation, followed by 15α-, 6α-, 4-, and 7α-hydroxylation [509]. 4- and 16α-hydroxylated estrogens may contribute to cancer development [510, 511], while 2-hydroxylated estrogens are thought to have anti-cancerogenic activity and a higher rate of excretion the in premenopausal female smokers [512–514]. The augment in the expression of CYP is associated with increased levels of estradiol and oxidants, mediated by the enhancement in the metabolism of cigarette smoke [36, 514]. These insights point out that COPD female patients may have increased production of E2 and a higher production of cigarette smoke metabolites leading to more severe lung damage.

These basic and clinical reports indicate contradictory outcomes regarding anti-inflammatory and pro-inflammatory properties of estrogen. Although several studies show that E2 is capable of relaxing ASM and reducing AHR, this sex steroid may lead to disadvantageous results in asthma patients by enhancing allergic and IL-17A-mediated inflammation (Fig. 15.5). Also, dual hormonal effects exerted by E2 and the differential expression and activation of estrogen receptors may limit the use of female hormones in asthma treatment. Moreover, the findings suggest that women under menopause hormone therapy and experiencing PMA might consider the risk of worsening the lung function. Furthermore, estrogen may promote neutrophilic inflammation in subjects with COPD, but further studies are necessary in order to corroborate this appreciation. Additionally, the use of aromatase inhibitors in a pulmonary illness such as lung cancer has been validated in preclinical assays [515, 516]. In this regard, the possibility of 17β-HSD1 and aromatase serve as potential therapeutic targets in COPD patients should be explored.

15.5.3.1 Pulmonary Fibrosis

Pulmonary fibrosis (PF) is a chronic interstitial lung disease characterized by progressive remodeling of the lung parenchyma with extracellular matrix deposition that leads to an abnormal tissue repair (lung scarring) [517, 518]. This disease affects approximately three million persons worldwide and is more common in men than in women [519–521]. The etiology of this disease has been related to infections [522–524], environmental and occupational pollutants [525, 526], cigarette smoke [527, 528], obstructive sleep apnea, and others [529, 530]. However, it can also manifest without any associated pathology, known as idiopathic pulmonary fibrosis (IPF). The PF symptoms, such as dyspnea and dry cough, progressively worsen, resulting in a median survival time of 3 to 5 years after diagnosis [518, 531]. Spirometry reveals a restrictive respiratory pattern, attributed to the accumulation of parenchymal scar tissue and the subsequent alteration of normal lung architecture [532, 533].

Fibrogenesis in the lung involves the fibroblast hyperproliferation at alveolar injuries and the excessive deposition of extracellular matrix (ECM) components, along with signaling pathways that degrade the ECM [534–536]. The alveolar endothelial injury caused by infections, tobacco smoke, gastroesophageal reflux contents, and environmental pollutants favors an impaired epithelial regrowth above an irregular matrix. During PF development, epithelial-mesenchymal transition (EMT) occurs. In this process, profibrotic cytokines, including TGF-β1, confer to

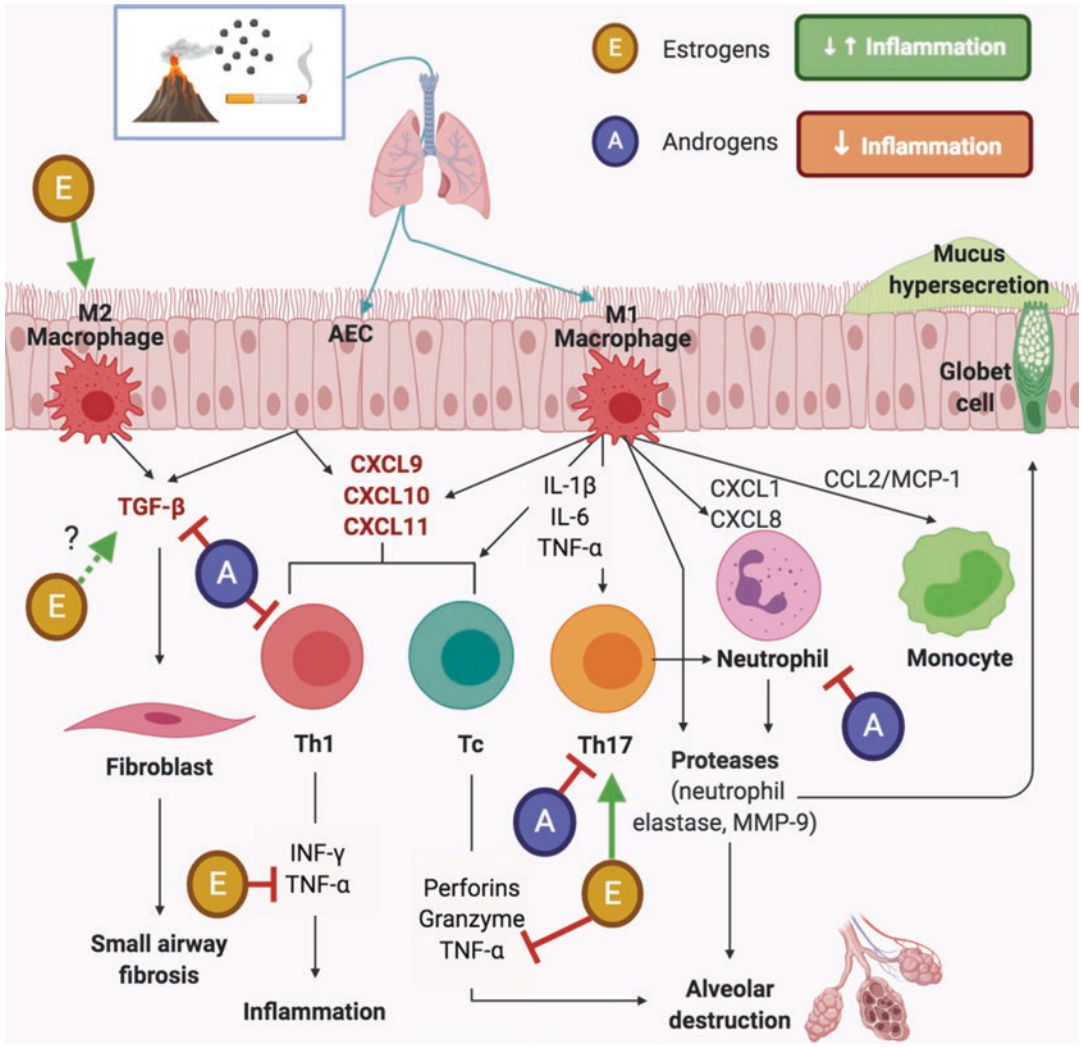

Fig. 15.5 Androgen and estrogen actions on inflammation in chronic obstructive pulmonary disease (COPD). Inhaled cigarette smoke and environmental and occupational exposures activate airway epithelial cells (AECs) and macrophages to release several chemotactic factors that attract T helper 1 (Th1) cells and Th1 CD8+ T cells (Tc) to the lungs, including CXC-chemokine ligand 9 (CXCL9), CXCL10, and CXCL11. Th1 cells release cytokines that induce inflammation, and Tc release perforins, granzyme B, and tumor necrosis factor α (TNF-α), contributing to alveolar cell apoptosis. Cytokines (IL-1β, IL-6, TNF-α) released from M1 macrophages (pro-inflammatory cells) activate Th17 cells, which modulate the neutrophilic inflammation. The attraction of neutrophils and monocytes is mediated by CXCL1, CXCL8, and CC-chemokine ligand 2 (CCL2), respectively. Inflammatory cells secrete matrix metalloproteinase 9 (MMP-9), which causes elastin degradation and emphysema. Neutrophil elastase evokes mucus hypersecretion. AECs and M2 macrophages (anti-inflammatory cells) release transforming growth factor β1 (TGF-β1), which stimulates fibroblast proliferation, provoking fibrosis of the small airways. The effects of estrogens (E) or androgens (A) on these immune cells can change depending on the hormone concentration and the timing and duration of the stimulus. Androgens diminish Th1 responses and inhibit TGF-β1-induced epithelial-to-mesenchymal transition (EMT) and preserve the epithelial morphology. In addition, androgens decrease IL-17A synthesis by Th17 cells, lowering neutrophilic inflammation. On the other hand, estrogens potentiate the M2 polarization of macrophages, favoring an anti-inflammatory condition, and might enhance the secretion of TGF-β1. Moreover, high physiological concentrations of estrogen reduce the T-cell production of TNF-α. Estrogens may present dual effects on airway inflammation in COPD, and androgens may reduce this response

epithelial cells, properties of mesenchymal cells to produce collagen [537–539]. This cytokine also promotes the accumulation of fibronectin in the ECM and the differentiation of fibroblasts into myofibroblasts [540, 541].

Moreover, T cells modulate lung fibrosis development. Th1 cytokines attenuate fibrosis, whereas Th2 cytokines promote fibrogenesis [542, 543]. In this regard, several studies have shown increased IL-13 expression in patients with IPF and in animal models [544–547]. IL-13 is mainly produced by Th2 lymphocytes, epithelial cells, ILC2s, and M2 macrophages [548–550]. This interleukin stimulates the proliferation of fibroblasts and induces pro-fibrotic cytokines, e.g., TGF-β1 and PDGF [551, 552]. In animals, exposure to bleomycin is the standard model of injury-associated PF since it promotes lung damage associated with apoptosis of alveolar epithelium, loss of the epithelial function, and an increased inflammatory response [553–555]. Interestingly, bleomycin also stimulates IL-13 production and myofibroblast differentiation [556]. Additionally, IL-17 has been linked to profibrotic effects through interactions with TGF-β signaling [555, 557, 558]. It has been proved that, by blocking IL-17 production, the progression of PF is delayed in different murine models of lung fibrosis [557, 559, 560].

15.5.3.2 Androgens' Effect on Inflammation in Pulmonary Fibrosis

Gender is an important factor in determining the risk and prognosis for PF. The prevalence of PF is greater in men, who display faster progression and less survival rates compared to women [561, 562]. Moreover, the incidence of this disease increases with age, appearing between the fifth and seventh decades of life [563, 564]. Studies suggest that androgens may contribute to increase lung injury and fibrosis [48, 49]. However, the sex differences in PF have been studied mostly in animal models. In this context, Voltz et al. found in a murine model of bleomycin-induced PF that males show a decreased lung function and an increased fibrosis compared with females. Furthermore, mice castration restores lung function, and 5α-DHT replacement therapy aggravates it [49]. Another research group using the same model observed that aged male mice exhibit augmented collagen deposition and neutrophilic alveolitis, and an elevated mortality, compared to female mice or young mice [565]. Also, young and old mice exposed to bleomycin show exacerbatedlevels of TGF-β, and aged male mice present increments in neutrophil chemoattractants such as IL-17A, chemokine (C-X-C motif) ligand 1 (CXCL1), and CXCL2 [565, 566]. These observations suggest that androgens promote the fibroproliferative response associated with fibrocyte recruitment and favor the susceptibility to present lung fibrosis. In another study, since decreased levels of DHEA have been related to immunosenescence [567], the levels of DHEA and DHEA-S in BALF and plasma from patients with IPF were analyzed. Interestingly, DHEA/DHEA-S ratio is significantly decreased in plasma from males with IPF. Furthermore, the same study shows that DHEA (100μM) reduces human lung fibroblast proliferation and differentiation into myofibroblast induced by TGF-β1 [568]. Recently, Cephus et al. suggested that androgens negatively regulate ILC2 and ILC2-derived IL-13 production. They demonstrated that chronic administration of 5α-DHT to mice reduce IL-13 production from lung ILC2s. Moreover, IL-13+ ILC2s are also significantly decreased in sham-operated male mice compared to gonadectomized male mice and sham-operated female mice [298]. In conclusion, studies show discrepancies, indicating that androgens may play a promoting or protective role in the development of PF, and more information is needed related to androgen effects on the inflammatory response in lung fibrosis.

15.5.3.3 Estrogen Effects on Inflammation in Pulmonary Fibrosis

The development PF has also been thought to be influenced by estrogen actions. Distinct reports indicate a protective role for estrogen; however, animal models have shown mixed results. In this context, Gharaee-Kermani et al. observed that female Fisher rats with bleomycin-induced PF

have higher mortality rates and more severe fibrosis compared to male rats. Furthermore, the authors found that ovariectomized animals presented less fibrosis and estradiol replacement therapy (ERP, 1 and 10 nM) increases procollagen 1, IL-4, and TGF-β1 mRNA expression in fibroblast from bleomycin-treated rats [48]. In contrast, a protective role of estrogen on lung fibrosis has also been proposed. A study conducted in ovariectomized female relaxin gene KO (Rln1−/−) mice revealed an increment in collagen concentration and deposition in the lung. Relaxin is a hormone capable of diminishing fibrosis in several organs [569]. Other research group demonstrated in Sprague-Dawley (SD) rats that ovariectomy exacerbates bleomycin-induced PF and pulmonary hypertension, and 2-methoxyestradiol attenuates this condition [570]. The discrepancy of results in which estrogen may promote or inhibit lung fibrosis can be explained by the difference among the species of rodents used in the former studies since ovariectomized Fisher rats develop a less severe inflammatory response to pneumotoxins and have twice higher plasma levels of E2 than SD rats. Moreover, elevated levels of E2 potentiate the activity of NF-κB, which, along with E2, contribute to a pro-inflammatory state [570–572].

Monocytes and their derivatives participate in the immune response developed in lung fibrosis. Studies confirmed that estrogen might inhibit the expression of monocyte chemoattractants [573–575]. Alveolar macrophages are a vast source of pro-fibrotic molecules such as TGF-β1, PDGF, and MMPs [566, 576]. PDFG is a potent fibrogenic molecule that promotes PF through fibroblast activation [577]. In the lungs of IPF patients, PDGF expression is increased in epithelial cells and macrophages [578, 579]. It has been reported that this growth factor induces Ca^{2+} waves through IP_3 receptors and modulates gene expression of ECM proteins in human pulmonary fibroblasts [580]. In this context, Ambhore et al. postulated that the activation of the ERβ by a selective ERβ agonist suppresses PDGF-elicited proliferation of human ASMCs [443]. Furthermore, estrogen regulation on the main pro-fibrotic cytokine has also been reported. In a rat model of congenital diaphragmatic hernia (CDH) induced by nitrofen, the administration of E2 (0.2 mg/kg) significantly reduces the TGF-β1 expression in lung tissue [581]. Correspondingly, tamoxifen (an anti-estrogen drug used in breast cancer treatment) seems to promote TGF-β1 expression, leading to lung fibrosis [582]. Recently, Smith et al. claimed that E2 might modulate TGF-β1-induced mesenchymal transition in bronchial epithelial cells; however, TGF-β1-induced EMT was not significantly affected by E2 [583]. According to the authors, this fact may be due to the decrease in mRNA expression of ERs (ERα, ERβ, and GPR30) and the reduction in protein expression of ERα elicited by TGF-β1. The authors also found that E2 downregulates the expression of chloride intracellular channel protein 3 (CLIC3) and retinol binding protein 7 (RBP7), proteins associated with pathogenic mechanisms of PF. Recently, Elliot et al. demonstrated that ERα is augmented in lung human tissues and myofibroblasts from IPF patients and in bleomycin-treated mice. Moreover, a decrease in the expression of ERα in human myofibroblasts lessens fibrosis-associated pathways [584]. The evidence so far indicates that E2 mostly downregulates the inflammatory response in PF, conferring a protective status in women. However, the mixed results observed in lung tissues in vitro and in animal models require further exploration.

15.5.3.4 Lung Cancer

Lung cancer (LC) is characterized by morphological cellular transformations and alterations of key pathways in cellular homeostasis. According to the World Health Organization, LC is one of the most frequent causes of cancer-related death in men and women worldwide. Interestingly, about 80–90% of these cases are produced by tobacco smoking; however, only ~15% of smokers develop lung cancer, suggesting a genetic susceptibility [585–587]. The estimated number of new cases and deaths in men continues to exceed those values in women [588]. However, trends suggest that the number of deaths from LC in women will exceed those in men in the future [589, 590]. The augmented incidence in women

is related to an increase in smoking habits. However, non-smokers diagnosed with LC are more likely to be female, pointing out an additional hormonal component [591–593]. Non-smoker women with this pathology diagnosed at early ages have better survival rates than men [594–596].

Lung cancer is categorized in small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC, the most predominant type). NSCLS subtypes are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [597]. In LC, tumor microenvironment, made up of tumor cells, fibroblasts, vascular and lymphatic endothelial cells, growth factors, and others, favors a pro-inflammatory state. Fibroblast exposure to cigarette chemicals or another pro-carcinogenic factor leads to an increased inflammatory response by secreting PGE_2 and IL-8 and promoting the activity of ERK1/2 [598, 599]. Macrophages and neutrophils play a critical role in the inflammatory condition of LC. In this regard, two distinct types of macrophages are involved in the tumoral condition: resident macrophages which play a cytotoxic role against tumor development and tumor-associated macrophages (TAMs) with a pro-tumoral function [600]. TAMs are recruited at the tumor site by monocyte chemoattractant protein-1 (MCP-1/CCL2) [601, 602]. M1 macrophages (activated via IFN-γ) display pro-immunogenic characteristics, and M2 macrophages promote tumor growth, angiogenesis, invasion, and metastasis [603–606]. In addition, neutrophil infiltration into the tumor microenvironment, as described in the adenocarcinoma LC subtype, is associated with lower survival. In this context, neutrophils promote the EMT and potentiate the migration activity of tumor cells, and notably, neutrophil infiltration is associated with hemoptysis (coughing up of blood) [607]. Moreover, these immune cells release pro-inflammatory cytokines, proteases, and ROS, which damage DNA and activate oncogenes [608, 609]. Contrariwise, CD8+ and CD4+ T cells appear to have a protective role in LC, as they improve the survival rate by reducing the progression of the disease [610–612].

Several cytokines that regulate the tumor microenvironment have been studied in LC as well. For instance, TNF-α favors the survival of tumor cells by inducing genes encoding NF-κB-dependent anti-apoptotic molecules and inflammatory cytokines, including IL-1β, TNF-α, TGF-β1, IL-6, and IL-8 [613–615]. TGF-β is overexpressed in LC and displays pleiotropic effects such as cell growth, proliferation, differentiation, and apoptosis [616–619]. IL-10 has been shown to exert dual effects on the tumor microenvironment. In this context, it has been demonstrated that IL-10 possesses immunosuppressive functions by promoting T-cell apoptosis and anti-angiogenic properties [620–622]. Also, higher levels of IL-10 have been shown to be associated with metastasis [623, 624].

15.5.3.5 Androgens' Effects on Inflammation in Lung Cancer

The androgen pathway and its relevance to lung cancer cells have been studied. The AR (a member of the nuclear receptor superfamily) is found in normal and in lung cancer cells [625–627]. The expression of nuclear receptors has been suggested as a prognostic biomarker for survival and relapse of LC patients [628, 629]. In 2012, Jeong et al. proved that the exposure of SCLC and NSCLC cell lines to 5α-DHT (at physiological concentrations) increases the mRNA expression of the AR and stimulates cellular growth [630]. TES is also capable of stimulating the growth of SCLC cell lines expressing the AR [627]. An epidemiological study shows that high concentrations of total serum TES are associated with the presence of LC [631]. Furthermore, the use of 5α reductase inhibitors in patients with LC is associated with long better survival [632]. These insights suggest that the suppression of the androgen pathway may have a direct effect on lung cancer.

The correlation between androgens and inflammation on lung carcinogenesis has been scantly investigated. Regarding the survival of patients, T cells have been related to favorable prognosis in LC [610, 612]. In this context, it has been observed that androgen deprivation posi-

tively regulates the infiltration of T cells in the lung tissue [633]. Furthermore, Wu et al. found that androgen deprivation by castration increases the radiation-induced inflammatory response in mice [634]. They demonstrated that mRNA levels of TNF-α, IL-6, IL-1, and TGF-β are increased after castration. According to the authors, androgens could downregulate the actions of NF-κB and, consequently, inhibit the activation of genes encoding inflammatory cytokines. These data point out that TES might restrict the inflammatory response in LC by limiting the reactivity of T cells and interfering with the NF-κB signaling.

The influence of androgens on macrophages is well known; nonetheless, the relationship between androgens and macrophages in lung cancer has not been explored. In this context, Padgett et al. found that physiological concentrations of DHEA abolish the secretion of TNF-α, IL-1, and IL-6 in murine macrophages [635]. Another study proved that TES at physiological and supraphysiological concentrations suppresses the expression and release of TNF-α from human macrophages [636]. These results point out that androgens possess anti-inflammatory properties that modulate macrophage activity. In LC, IL-10, mainly produced by TAMs, has protumoral qualities and correlates with nonresponse to anti-tumoral therapy [600, 624, 637]. In 2012, Wang et al. proposed that this interleukin promotes tumor malignancy by stimulating T-cell apoptosis and tumor cell survival in the lung [620]. Interestingly, IL-10 transgenic mice injected with Lewis lung (3LL) carcinoma cells develop larger tumors than control mice, pointing out that this interleukin may prevent an adequate response against tumor cells [638]. Moreover, patients with NSCLS show increased IL-10 serum levels (compared to healthy subjects), and this increase is associated with reduced survival [639]. In this regard, the androgen effects on IL-10 have been studied in tissues different from lung cancer cells. It was demonstrated that TES at physiological concentrations acts as an inducer of IL-10 synthesis in mice monocyte macrophages [205]. Furthermore, TES replacement therapy in men with symptomatic androgen deficiency resulted in increased serum concentrations of IL-10 and decreased levels of TNF-α and IL-1 [204]. These studies suggest that probably androgens favor the pro-tumoral IL-10 effects, but specific studies in lung cancer cells are needed to confirm this assumption.

Another target of androgen regulation is the transmembrane prostate androgen-induced protein (TMEPAI). The synthesis of TMEPAI is elicited mainly by androgens, but also by TGF-β1 [640–642]. This oncogenic protein is expressed in lung cancer cell lines. Moreover, TMEPAI plays a vital role in TGF-β1-induced EMT by modulating ROS signaling and causing changes in epithelial cells, including migration, invasion, and proliferation of the tumor [643]. Furthermore, a research group found in lung cancer cells that the ablation of TMEPAI prevents cell proliferation, migration, and invasion. Also, they observed that the expression of TMEPAI in nude mice facilitates tumorigenesis. Finally, they proposed that the activation of TGF-β pathway induces TMEPAI expression and, subsequently, TMEPAI downregulates TGF-β signaling by promoting lysosomal degradation of the TGF-β receptor [644].

In conclusion, the evidence suggests that androgens might positively or negatively regulate lung cancer development. On the one hand, androgens may stimulate the growth of lung cancer cells and might play a role in the EMT by inducing the synthesis of TMEPAI. On the other hand, androgens may interfere with the signaling of NF-κB, leading to the downregulation of inflammatory cytokines.

15.5.3.6 Estrogens' Effects on Inflammation in Lung Cancer

Histological subtypes of lung cancer differ between men and women, being adenocarcinoma and bronchioloalveolar carcinoma, the most common subtypes in women [645, 646]. Estrogens have an essential role in lung carcinogenesis and can be locally synthesized by lung cancer cells [647, 648]. Moreover, ERs are found in lung cancer cells, suggesting that local production of estrogens is a response to a process of car-

cinogenesis [649, 650]. Increasing evidence shows that estrogens are involved in lung cancer proliferation and progression and most human lung tumors express the ERβ subtype and the aromatase enzyme [651–653]. In patients with NSCLC, elevated aromatase and ERβ expression are associated with poorer survival [653, 654]. Furthermore, Stabile et al., using a murine model of lung cancer induced by tobacco carcinogen (NKK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), demonstrated that aromatase is expressed in TAMs, whereas ERβ is found in both macrophages and lung tumor cells. Interestingly, they also observed that the combination of anastrozole (aromatase inhibitor) and fulvestrant (ER antagonist) inhibits tobacco carcinogen-induced lung tumorigenesis [655]. Moreover, the increased ERα expression has been linked to macrophage infiltration into the tumor microenvironment [656]. The infiltration of macrophages is favored by CCL2 and its receptor (CCR2), i.e., the stimulation of ERα by E2, may activate the CCR2 signaling, leading to macrophage infiltration, MMP9 production, tumor progression, growth, and metastasis [656, 657].

During the inflammatory response, IL-6 acts as a regulator of neutrophil trafficking [658]. Some authors have focused on IL-6 and its role as a biomarker of ongoing inflammation. It has been reported that patients with LC have increased serum levels of this interleukin [659, 660]. Another research group observed that ovariectomized mice have decreased total neutrophils and this condition was recovered by E2 replacement therapy [661]. Human NSCLC cell lines and more important human NSCLC samples show tumor expression of IL-6 and its receptor. This interleukin can stimulate and enhance pathways in tumorigenesis, including signal transducer and activator of transcription 3 (STAT3), which regulates cell cycle progression, apoptosis, and tumor angiogenesis [662, 663]. In 2018, Huang et al. confirmed that E2 induces the activation of ERβ and promotes IL-6 expression via the MAPK/ERK and the PI3K/AKT signaling pathways in lung cancer cells. Moreover, they proposed that ERβ/IL6 signaling pathway could be a target for therapeutic intervention [664]. Furthermore, the estrogen-related receptor alpha (ERRα), a protein with a similar structure to ERα, has been implicated in LC. This receptor is expressed in LC cells and can regulate cell proliferation and migration [665, 666]. In 2018, Zhang et al. demonstrated that ERRα was significantly elevated in NSCLC cell lines compared with a normal bronchial epithelial cell line. They also reported that the overexpression and activation of ERRα increase the expression of IL-6 and the inhibition of NF-κB eliminates the ERRα effect in IL-6 synthesis [667]. The role of estrogen action on the inflammatory response in lung cancer is much better understood than the role of androgens. Moreover, it has been proposed that aromatase inhibitors may serve as potential drugs in lung cancer therapy [515, 516]. In this regard, further research on estrogen-targeted therapies that could improve patient survival and reduce tumor invasion is needed.

15.5.3.7 Coronavirus Disease 2019

The coronavirus disease 2019 (COVID-19) is caused by a virus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). According to the World Health Organization, by September 17, 2020, SARS-CoV-2 had infected more than 30,055,710 people worldwide and killed more than 943,433. This disease is characterized by respiratory symptoms and is transmitted from human to human through respiratory secretions and saliva [668, 669]. Patients with COVID-19 exhibit fever, dry cough, difficulty in breathing, myalgia, headache, diarrhea, and nausea [670–672]. Also, clinical data indicate decreased oxygen saturation, blood gas deviation, and abnormalities observed by chest X-rays, lymphopenia, and an increase of C-reactive protein [673].

Severe COVID-19 cases progress to acute respiratory distress syndrome (ARDS), around 8–9 days after symptom onset, and may lead to respiratory failure [674–676]. Essentially, SARS-CoV-2 binds to host cells such as airway epithelial cells, alveolar cells, vascular endothelial cells, and macrophages in the lung by the angiotensin-converting enzyme 2 (ACE2) host

target receptor [677–680]. After the SARS-CoV-2 infection, a reduction in the ACE2 function that is associated with acute lung injury occurs [681–683]. Furthermore, ACE2 may be cleaved by transmembrane serine protease-2 (TMPRSS2), leading to an enhancement in the entry of the virus [684, 685]. On the other hand, there is an association between the gender of COVID-19 patients and fatality rates. In this context, data from the WHO show that a lower percentage of women (1.7%) infected with the virus will die in comparison with men (2.8%). Other investigations reported that less female patients with the severe form of the disease require intensive care or die compared to male patients [60, 686]. In this context, it has been insinuated that there may be alleles that confer resistance to this disease, since ACE2 gene is located on the X chromosome [687]. Interestingly, it also has been demonstrated that E2 downregulated the expression of ACE2 in differentiated normal human bronchial epithelial (NHBE) cells [688], which might explain the lower fatality rate in females.

The severity of COVID-19 is due to the host response featured by an uncontrolled inflammation associated with high levels of circulating cytokines, lymphopenia, and mononuclear cell infiltration in the lungs [676, 689]. When SARS-CoV-2 infects epithelial cells expressing the surface receptors ACE2 and TMPRSS2 in the airway, it activates a local immune response that in most cases resolves the infection [690]. Alveolar endothelial cells and macrophages detect the released PAMPs such as viral RNA and trigger the generation of pro-inflammatory cytokines and chemokines including IL-6, IFN-γ-induced protein 10 (IP-10/CXCL10), macrophage inflammatory protein 1α (MIP1α), and MIP1β in order to recruit immune cells [674, 676, 691]. The infiltration of monocytes, macrophages, and T cells to the site of infection promotes further inflammation [689, 692, 693] and may explain the lymphopenia seen in patients with this disease [670, 694]. In addition, it has been suggested that high exhaustion and a decreased functional diversity of T cells in peripheral blood may be an indicator of developing severe acute respiratory syndrome in patients with COVID-19 [695].

Moreover, higher levels of cytokines and chemokines have been related to the severity of the disease and eventually death [674, 694, 696]. In a study, a research group measured plasma levels of diverse cytokines in patients with severe COVID-19, founding increased levels of IL-2, IL-7, IL-10, G-CSF, IP-10, MCP-1, MIP1, and TNF [674]. Interestingly, Zhou et al. showed that IL-6 levels were elevated in non-survivors compared with survivors [697]. This cytokine storm, along with the cell infiltration, provokes lung damage by the excessive secretion of proteases and ROS [689, 692]. In addition, neutralizing antibodies produced by B cells can block viral infection, a situation that may occur around 1 week following symptoms onset [698, 699]. However, it has been suggested that some patients may not develop long-lasting antibodies to this virus and is unknown whether they are susceptible to reinfection. In this context, Elizaldi et al. conducted a study focused in CD4 T follicular helper (T_{fh}) cells (entities with high importance in the generation of long-lasting and specific humoral protection against viral infections) and reported that following infection with SARS-CoV-2, adult rhesus macaques exhibited transient accumulation of activated proliferating T_{fh} cells toward a Th1 response. They also proposed that a vaccine promoting Th1-type T_{fh} responses that target the S protein of the virus may lead to protective immunity [700]. Since the disease continues to spread worldwide, several immunosuppressive therapies have been used. Clinical trials have been focused on targeting pro-inflammatory mediators such as IL-6 and GM-CSF (clinical trials: ChiCTR2000029765).

15.5.3.8 Androgens' Effects on Inflammation in Coronavirus Disease 2019

More male patients with COVID-19 have higher mortality and develop the severe form of the disease than women [60, 701]. The difference in the number of cases reported by gender augments progressively in favor of male patients [702]. Studies have proposed that androgens modulate

the immune system response and may predispose men to different clinical course and prognosis of COVID-19. Since men with severe COVID-19 are ≥60 years old, decreased TES levels during aging may be involved in a pro-inflammatory condition [703–705]. Furthermore, it is well established that TES concentration in plasma is reduced by comorbidities like obesity, diabetes, and COPD, which are prevalent in COVID-19 patients [706–711]. In this regard, it has been shown that hypogonadism is highly prevalent (22–69%) in male patients with COPD [712]. Meanwhile, testosterone treatment improved the exercise capacity, muscle strength, and oxygen consumption in men [713].

It has been established that ACE2 mediates the cell entry of SARS-CoV-2, insinuating a protective role of this receptor against the viral infection [702]. Remarkably, this enzyme is selectively expressed by adult Leydig cells [714], pointing out a possible role of testicular secretion of TES in COVID-19 patients [702]. Also, it has been exhibited that patients with SARS-CoV-2 pneumonia, who were transferred to the intensive care unit (ICU) or died in respiratory ICU (RICU), had lower amounts of total TES and calculated free TES, compared to patients who were transferred to the internal medicine unit or were at a stable condition in RICU [715]. Alveolar endothelial cells and macrophages detect the SARS-CoV-2 and trigger the generation of pro-inflammatory cytokines and chemokines. In this regard, it has been demonstrated a correlation between hypogonadism and augmented pro-inflammatory cytokines and that testosterone treatment reduces IL-1β, IL-6, and TNF-α [704]. Furthermore, a study reported that low serum TES concentrations were significantly associated with elevated levels of TNF-α, MIP1α (CCL3), and MIP1β (CCL4) [716]. In the healthy immune response, these and other cytokines promote the infiltration of monocytes, macrophages, and T lymphocytes to the site of infection, leading to a pro-inflammatory feedback loop [691]. Moreover, it was reported that DHEA (at physiological concentrations) eliminates the release of TNF-α, IL-1, and IL-6 in murine macrophages [635]. Another study proved that TES at physiological and supraphysiological concentrations suppresses the expression and secretion of TNF-α from human macrophages [636]. In relation to T cells, Olsen et al. reported that an increase in peripheral T cells was reversed by androgen replacement [717]. Other research group found that castration of post-pubertal male mice increases T-cell numbers in peripheral lymphoid tissues [718]. In peripheral blood cells, TES treatment reduces the relative number of monocytes and increases CD8+ T-cell number [246]. Therefore, it is possible that androgen actions on CD8+ T cells favor the recognition and elimination of infected cells with SARS-CoV-2.

On the other hand, some critically ill patients have been treated with convalescent plasma, and growing evidence indicates positive results [719–721]. In an observational study, male patients with COVID-19 respond with a lower generation of effective SARS-CoV-2 IgG antibodies compared to women, confirming that a reduced antibody response in men is associated with worse prognosis [722]. This finding suggests a sexual hormone influence on B-cell proliferation. In this regard, androgen/AR actions on B lymphocytes have been studied by Altuwaijri et al. They reported that the lack of AR in B cells in different strains of mice results in increased B cells in the blood and bone marrow [243]. This insight supports the hypothesis that androgen-mediated B-cell maturation is AR dependent.

As previously mentioned, TMPRSS2 is a critical protease for the pathogenesis and spread of SARS-CoV-2 [723, 724]. The gene transcription of TMPRSS2 depends on the activity of the AR. It has been suggested that TES may promote higher expression of this protease in the lung of males, which might improve the ability of SARS-CoV-2 to enter cells [725–727]. The regulation of TMPRSS2 by TES has been suggested to influence on the male predominance displayed in COVID-19 infection [726]. Moreover, the hyperandrogenic condition could explain the severe COVID-19 cases in young males [702]. Also, it has been proposed a role for TMPRSS2 variants and their expression levels in the regulating the severity of COVID-19; however, further experimental research and a hypothesis that fosters

validation on large cohorts of patients with different clinical manifestations are required [727]. Given the fact that TRPMSS2 is found in the lung, the use of inhibitors of this protease (currently employed for cancer prostate) against COVID-19 pneumonia seems a promissory therapeutic tool. Additionally, the assessment of potential drugs that interfere with androgen activity, such as androgen receptor inhibitors, steroidogenesis inhibitors, and 5-alpha reductase inhibitors, has been suggested [702].

Finally, it is well established that androgens play inhibitory roles in the inflammatory response, and the evidence indicates that reduced TES levels associated with age or comorbidities may increase the pro-inflammatory response in men, contributing to the development of a severe form of COVID-19. In this context, the quantification of TES levels may be considered when a COVID-19-positive patient is identified. Moreover, if the values are low, the use of testosterone has been remarkably proposed to reduce the associated pulmonary syndrome, thus preventing the progression to severe COVID-19 disease [702].

15.5.3.9 Estrogens' Effects on Inflammation in Coronavirus Disease 2019

Emerging studies have suggested that women are less susceptible to COVID-19 and exhibit lower mortality than men [670, 671], which may be explained by a potential protective role of estrogens. X chromosome encoding the greatest density of genes related to immune response [728] supposes the immunological advantage of women over males. Interestingly, Channappanavar et al. examined the gender-dependent difference outcomes of the infection by SARS-CoV. They demonstrated that the estrogen depletion by ovariectomy or the use of an ER antagonist increases the morbidity and mortality in SARS-CoV-infected female mice [729]. In addition, they suggested that infected female mice have a sex-specific protection during their reproductive period. These findings strengthen the crucial hypothesis about the protective role of estrogen and the ER signaling against the respiratory virus. Another research group observed that, in an age group of 40–60 years, the transcriptomic profile of female lung tissue has more similarities to that evoked upon SARS-CoV-2 infection compared to male tissue [730]. In this regard, characterizing the most activated intracellular pathways during the viral infection may provide a molecular explanation of the lower incidence of COVID-19 in females.

The estrogen/ER signaling regulates the development of immune cells and the pathways of innate and adaptive immune system [731–733]. It appears that the development and severity of COVID-19 depend on individual propensities for the massive release of pro-inflammatory mediators [674, 676]. Data have pointed out that high doses of E2 may inhibit the production of inflammatory cytokines (IL1, IL6, and TNF-α), whereas stimulation with low doses of E2 enhances the production [734–736]. It was proved that, in the early phase of antiviral immune response against SARS-CoV infection, pro-inflammatory mediators (IL-6, CCL2, and CXCL1) display similar increased levels in both sexes and 72 h post-infection these cytokines are upregulated in the lung of male mice compared with females [729]. To understand this finding, it has been suggested that monocyte-macrophage recruitment is suppressed by estrogens, which favor the downregulation of CCL2/MCP-1 expression and inhibit NF-κB activation in macrophages [737, 738]. Similarly, reduced TNF-α and CCL2 levels are observed in gonadectomized mice treated with estrogen, which protect them from influenza virus infection [739, 740]. Furthermore, in SARS-CoV-infected mice, the predominant sources of these pro-inflammatory mediators are the inflammatory monocyte macrophages (IMMs) [741]. In male mice, these cells are increased in numbers and also produced more mediators compared with female mice [729]. Additionally, increased numbers of IMMs in ovariectomized mice compared with intact female mice suggest that estrogen signaling in females abolishes the accumulation and function of IMMs in the lung [729]. Moreover, it has been reported that the downregulation of IL-6 gene expression by E2

is induced via the interaction of the estrogen receptor with NF-κB [742].

Zheng et al. showed in an observational study that, the humoral response in seriously ill male patients exposes a delayed peak of antibody response with a lower generation of effective IgG compared to women [722]. Females exhibit a predominant Th2 cytokine profile, which could be involved in immune responses characterized principally by the secretion of antibodies [743]. Estrogen significantly enhances the generation of a Th2 response [233, 744], corresponding to the findings that this hormone increases the frequency of antibody secreting B cells in the follicular phase (when the levels of estrogen are high) of the menstrual cycle in rhesus macaques. Furthermore, the stimulation of a CD8 + -enriched cell population induced the expression of IFN-γ and IL-12 [745]. These findings are actually attributable to estrogen influence on B-cell proliferation, activation, and maturation by upregulating the expression of CD22, Src homology region 2 domain-containing phosphatase-1 (SHP-1), and B-cell lymphoma 2 (Bcl-2) [733, 746]. Finally, it has been hypothesized that estrogens could protect women from the most serious complications of COVID-19, especially women before the menopause due to high serum estrogen levels [747].

15.6 Conclusions

Inflammation is a complex biological process that involves multiple immune mechanisms. The literature confirms the influence of sex hormones on the incidence and severity of the inflammatory response in lung diseases. The effects of sex steroids have been observed in pathophysiological conditions of the lung related to diseases such as asthma, COPD, lung fibrosis, lung cancer, and COVID-19. This chapter highlights the importance of sex-specific research taking into account the hormonal status of the patients. Moreover, sex steroid actions depend on very particular circumstances such as the hormone concentration, duration of the stimulus, genomic or non-genomic pathway, and interaction between male and female sex hormones. The evidence indicates that sex hormone actions on the inflammatory response can be beneficial or detrimental. Generally, male sex steroids have beneficial anti-inflammatory properties in asthma, COPD, and other lung diseases. On the other hand, E2 displays anti-inflammatory and pro-inflammatory properties on lung diseases. It is crucial to consider that the effects of sex hormones on the inflammatory responses in lung diseases need to be further explored in order to find novel therapeutic approaches and pursue an individualized medicine in the future.

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Anexo 4. Artículo de revisión.

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Transient receptor potential cation channel subfamily V (TRPV) and its importance in asthma.☆

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ABSTRACT

Transient receptor potential (TRP) ion channels play critical roles in physiological and pathological conditions. Increasing evidence has unveiled the contribution of TRP vanilloid (TRPV) family in the development of asthma. The TRPV family is a group (TRPV1-TRPV6) of polymodal channels capable of sensing thermal, acidic, mechanical stress, and osmotic stimuli. TRPVs can be activated by endogenous ligands including, arachidonic acid derivatives or endocannabinoids. While TRPV1-TRPV4 are non-selective cation channels showing a predominance for Ca^{2+} over Na^{+} influx, TRPV5 and TRPV6 are only Ca^{2+} permeable selective channels. Asthma is a chronic inflammatory bronchopulmonary disorder involving airway hyperresponsiveness (AHR) and airway remodeling. Patients suffering from allergic asthma display an inflammatory pattern driven by cytokines produced in type-2 helper T cells (Th2) and type 2 innate lymphoid cells (ILC2s). Ion channels are essential regulators in airway smooth muscle (ASM) and immune cells physiology. In this review, we summarize the contribution of TRPV1, TRPV2, and TRPV4 to the pathogenesis of asthma. TRPV1 is associated with hypersensitivity to environmental pollutants and chronic cough, inflammation, AHR, and remodeling. TRPV2 is increased in peripheral lymphocytes of asthmatic patients. TRPV4 contributes to ASM cells proliferation, and its blockade leads to a reduced eosinophilia, neutrophilia, as well as an abolished AHR. In conclusion, TRPV2 may represent a novel biomarker for asthma in children; meanwhile, TRPV1 and TRPV4 seem to be essential contributors to the development and exacerbations of asthma. Moreover, these channels may serve as novel therapeutic targets for this ailment.

1. Introduction

Asthma is a chronic pulmonary disease affecting as many as 339 million people worldwide according to The Global Asthma Report (2018). This ailment is classically characterized by immune response driven by type-2 helper T (Th2) cells, causing inflammation, airway hyperresponsiveness (AHR), and remodeling (Fahy, 2015). In allergic asthma, patients show elevated expression levels of Th2 cytokines such as interleukin (IL)-4, IL-13, and IL-5, along with augmented allergen-specific IgE and eosinophilia (Lambrecht and Hammad, 2015; Sokol et al., 2008). In addition to Th2 cells and eosinophils, former cytokines lead to the infiltration of type 2 innate lymphoid cells (ILC2s) and macrophages into the lung (Komlosi et al., 2021). However, subjects with serious disease display IL-17 mediated neutrophilic inflammation. This phenotype is extremely severe and difficult to treat because of its corticosteroid resistance (Lambrecht and Hammad, 2015; Manni et al., 2014). The respiratory symptoms, including wheeze, difficulty breathing, and cough, are associated with an expiratory flow limitation (Bateman et al., 2008).

Exacerbated bronchoconstriction and inflammation in asthmatic subjects have been shown to be partly mediated by the dysregulation of ion channels (Valverde et al., 2011). Several Ca^{2+} channels subtypes are involved in airway smooth muscle (ASM) Ca^{2+} influx and contraction, comprising L-type voltage dependent Ca^{2+} channels (L-VDCCs) (Ding et al., 2019; Montaño et al., 2018; Reyes-García et al., 2018), store operated Ca^{2+} channels (SOCCs) (Montaño et al., 2018; Reyes-García et al., 2018), and transient receptor potential cation channels (TRPs) (Chen and Sanderson, 2017; Lin et al., 2021; Reyes-García et al., 2018). Increasing evidence has demonstrated an essential contribution of the vanilloid TRP (TRPV) family in inflammatory lung diseases (Choi et al., 2018; Rahaman et al., 2014; Xiong et al., 2020). The goal of this review

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Abbreviations

5´6´-EET	5´6´-epoxyeicosatrienoic acid
12-HPETE	12-hydroperoxyei-cosatetraenoic acid
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
AHR	airway hyperresponsiveness
Alp1	alkaline protease 1
ASM	Airway smooth muscle
BALF	bronchoalveolar lavage fluid
CGRP	calcitonin gene-related peptide
CICR	Ca^{2+}-induced Ca^{2+} release
CNS	central nervous system
DAG	diacyl glycerol
DC	dendritic cell
DRG	dorsal root ganglia
DUOX1	dual oxidase 1
EGFR	epidermal growth factor receptor
FMT	fibroblast to myofibroblast transition
GERD	Gastroesophageal Reflux Disease
GPCR	G-protein coupled receptor
HBSMCs	human bronchial smooth muscle cells
HDM	house dust mite
Ig	immunoglobulin
IL	interleukin
ILC2s	of type 2 innate lymphoid cells
IP3	inositol 1,4,5-trisphosphate
$K_{Ca}3.1$	Ca^{2+}-activated K^+ channel
LPS	lipopolysaccharide
LT	leukotriene
L-VDCC	L-type voltage dependent Ca^{2+} channel
NADPH	nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T-cells
NGF	nerve growth factor
NKA	neurokinin A
OVA	ovalbumin
PAR	protease-activated receptor
Phospholipase C	(PLC)
PI3K	phosphoinositide-3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
$PM_{2.5}$	particulate matter $\leq$2.5 μm
QF	Qingfei oral liquid
RARs	rapidly adapting receptors
RSV	respiratory syncytial virus
SNP	single nucleotide polymorphisms
SOCC	store operated calcium channel
SOCE	store operated Ca^{2+} entry
TDI	toluene diisocyanate
TRP	transient receptor potential
TSLP	thymic stromal lymphopoietin
Th2	type-2 helper T cells
TM	transmembrane domain
TMA	trimellitic anhydride
TGFβ1	transforming growth factor β1
TNF-α	tumor necrosis factor α
TRP	transient receptor potential
TRPV	transient receptor potential channel vanilloid family
VR1	vanilloid receptor 1
WT	wild type

is to analyze and discuss the participation of TRPV1, TRPV2, and TRPV4 subtypes in the development and the major features of asthma and to point out these channels as novel therapeutic targets for this ailment.

2. Physiology of TRPV1, TRPV2 and TRPV4

About 30 different TRPs are molecularly identified and grouped into canonical family (TRPC), melastatin family (TRPM), ankyrin family (TRPA), NomPC family (TRPN), mucolipin family (TRPML), polycystin family (TRPP), TRPV family, vanilloid-like family (TRPVL), and sor-omelastatin family (TRPS) (Himmel and Cox, 2020). The TRPV family consists of six members: TRPV1-TRPV6. Structurally, TRPVs contain six transmembrane spans and a pore region between the transmembrane domain (TM)-5 and TM6. TRPVs also possess an N-terminal domain with six ankyrin repeats implicated in protein-protein interactions and a C-terminal TRP domain (Himmel and Cox, 2020). TRPV5, and TRPV6 are Ca^{2+}-selective channels, whereas TRPV1-4 consist of permeable cation non-selective ion channels (NSCCs) (Flores-Aldama et al., 2020; Ramal-Sanchez et al., 2021; Vriens et al., 2008). In general, TRPV1-TRPV4 are involved in sensing mechanic, heat, pH, and chemical (endogenous and exogenous ligands) stimuli, while TRPV5 and TRPV6 are associated with Ca^{2+} handling and homeostasis in the kidney and gut (Lee et al., 2019; Pawlak et al., 2019; Vriens et al., 2008). Among all TRPVs, TRPV1, TRPV2, and TRPV4 have been related to the develop-ment and exacerbations of asthma symptoms (Bonvini et al., 2020; Cai et al., 2013; Choi et al., 2018). This review focuses on the role of these channels in this inflammatory airway disease.

TRPV1 was the first member of the TRPV family identified in mammals in 1997, formerly known as vanilloid receptor 1 (VR1) (Caterina et al., 1997; Ramal-Sanchez et al., 2021). TRPV1 is classically activated by vanilloid compounds, such as capsaicin, the main pungent ingredient in the plants of the genus Capsicum, eliciting a painful thermal stimulus (Caterina et al., 1997). Likewise, endogenous canna-binoids, including anandamide (Li et al., 2021) and N-arach-idonoyl-dopamine (Huang et al., 2002), can activate TRPV1. In addition, the lipoxygenase metabolites generated from arachidonic acid, leuko-triene (LT)-B_4 or 12-hydroperoxyei-cosatetraenoic acid (12-HPETE) stimulate the gating of TRPV1. Furthermore, the opening of this channel subtype is mediated by its phosphorylation through protein kinase C (PKC). (Hwang et al., 2000; Numazaki et al., 2002). The binding site of these agonists in the channel appears to be located on an intracellular domain of the protein (Jung et al., 1999, 2002). Moreover, protons interact with specific amino acid residues on the extracellular surface of the channel, modulating cation conductance (Jordt et al., 2000; Tsvet-kov et al., 2017). In this regard, a low extracellular pH between 6 and 5 promotes the opening of the channel (Bevan and Geppetti, 1994; Tominaga et al., 1998). Also, a specific site in the C-terminal domain of TRPV1 required for phosphatidylinositol-4,5-bisphosphate (PIP_2)-me-diated channel gating inhibition has been identified (Prescott and Julius, 2003). Finally, this vanilloid receptor acts as a thermo-sensor induced by noxious temperatures between 42 and 53 °C and can be activated by ethanol (Caterina et al., 1997; Trevisani et al., 2002).

TRPV1 is predominantly expressed in a subset of primary sensory neurons of the trigeminal, vagal, and dorsal root ganglia (DRG) (Caterina et al., 1997; Mezey et al., 2000). These neurons serve as pol-ymodal receptors because of their ability to detect noxious acidic, chemical, thermal, and mechanical stimuli. TRPV1 is also localized in the hypothalamus, thalamic nuclei, substantia nigra, striatum, and cerebellum (Ho et al., 2012; Mezey et al., 2000). The presence of TRPV1 has also been reported in sensory neurons innervating the bladder and the lung. The opening of TRPV1 leads to the excitation of these nerves causing their depolarization and promoting action potentials that modulate bladder function, cough reflex, and detect airway irritants (Mickle et al., 2015; Takayama et al., 2015). Moreover, TRPV1 has been

identified in ASM (Yocum et al., 2017), airway epithelial cells (Fantauzzi et al., 2020), pulmonary artery (Yang et al., 2006), macrophages and T cells (Zheng et al., 2020). This channel is implicated in the signaling of neurokinin A (NKA), calcitonin gene-related peptide (CGRP), and substance P, contributing to neurogenic inflammation in the lung (Guan et al., 2021).

TRPV2 shares about 50% of molecular identity to TRPV1 and is not activated by vanilloids compounds or protons. However, the gating of this channel requires a noxious heat threshold ≥52 °C (Shibasaki, 2016; Zhang et al., 2016). Cell swelling and changes in osmolarity also lead to TRPV2 activation (Muraki et al., 2003; Zhang et al., 2016). This TRP subtype is expressed in medium-large DRG myelinated fibers. The higher temperatures activating TRPV2 compared to TRPV1 may explain its presence in myelinated neurons (Rau et al., 2007; Zhang et al., 2016). The main function of TRPV2 is to mediate the transduction of noxious heat and pain sensation (Liu and Qin, 2016). Recently, it has been shown that TRPV2 is involved in the β-amyloid-induced cognitive impairment, revealing a potential target for Alzheimer's disease (Thapak et al., 2021). Moreover, this vanilloid channel subtype has been implicated in pulmonary inflammation (Siveen et al., 2019). In the lung, TRPV2 is expressed in alveolar macrophages (Masubuchi et al., 2019), pulmonary arteries (Yang et al., 2006), and polymorphonuclear cells (Siveen et al., 2019).

TRPV4 is another NSCC expressed in several tissues including the heart (Peana et al., 2021), brain (Liu et al., 2020), and lungs (Weber et al., 2020). Particularly, in the lung, this channel has been reported in the ASM (Yu et al., 2017), airway epithelial cells (Weber et al., 2020), and alveolar macrophages (Rayees et al., 2019). As other TRPVs, TRPV4 is a polymodal channel that is activated by warm temperatures (Scarpellini et al., 2019), osmotic and mechanical stimuli (Kawasaki et al., 2021; Shibasaki, 2020), phorbol esters, and the arachidonic acid and their metabolites, such as, 5′6′-epoxyeicosatrienoic acid (5′6′-EET) (Zhao et al., 2014). TRPV4 is also activated at temperatures that approach physiological conditions (~34 °C). As for, this TRP channel is a critical component of regular cellular activity with essential implications in the brain and vascular function (Liu et al., 2021; Shibasaki, 2020). In the lung, TRPV4 regulates macrophages function mediating pulmonary inflammation (Scheraga et al., 2020).

3. Role of TRPV1 on asthma

For a long time, capsaicin has been recognized as a potent stimulant of sensory nerves (Jancso et al., 1967; Lawrence et al., 2021). Although in 1969 the bronchoconstrictive properties of capsaicin were demonstrated (Molnar et al., 1969), in the respiratory tract TRPV1 was initially studied in nerve terminals, such as nociceptive C-fiber nerves (Caterina and Julius, 2001) and axons throughout the airways of guinea pigs and rats (Larson et al., 2003; Watanabe et al., 2005). The activation of this TRPV subtype channel leads to the stimulation of nerve terminals and the release of neurokinin A, substance P, and CGRP (De Swert and Joos, 2006; Guan et al., 2021). Importantly, these mediators favor smooth muscle contraction and mucus hypersecretion (Kay et al., 2007; Schelfhout et al., 2008; Sun and Bhatia, 2014). In addition, TRPV1 can be gated after the stimulation of certain G-protein-coupled receptors (GPCRs) (Gouin et al., 2017; Shin et al., 2002). Bradykinin, a potent activator of bronchopulmonary C-fibers (Kajekar et al., 1999), via the bradykinin B_2 receptor (a GPCR), favors the activation of the phospholipase C (PLC) signaling pathway (Kang and Leeb-Lundberg, 2002; Undem and Sun, 2020). In this context, bradykinin activates TRPV1 by releasing the channel from PIP_2-mediated inhibition, promoting cations influx (Chuang et al., 2001). Furthermore, the PLC activation pathway down-regulates the production of lipoxygenase products of arachidonic acid (Ferreira et al., 2004; Shin et al., 2002), which are structurally similar to capsaicin and can interact effectively with the TRPV1 channel (Hwang et al., 2000).

3.1. Neurogenic inflammation and ethanol-induced asthma

TRPV1 is widely distributed in the lungs, where it is typically co-localized with substance P and CGRP-containing neurons within vagal C-fiber sensory nerves. Interestingly, the loss of the TRPV1-positive axon results in the mitigation of bronchoconstriction induced by capsaicin (Watanabe et al., 2006). TRPV1 physiology has been associated with neurogenic inflammation (Zhang et al., 2008) and irritant-induced chronic cough (Groneberg et al., 2004; McLeod et al., 2006). It has been proposed that neurogenic inflammation contribute to asthma. The evidence shows that the expression of TRPV1 is increased in sensory afferent nerves innervating the airways in an allergic inflammation model (Watanabe et al., 2008). As previously mentioned, ions flowing through TRPV1 may be responsible for the action potential induced by a variety of stimuli in sensory nerves (Wang et al., 2021a). As regards, it has been found that ethanol stimulates sensory nerves via TRPV1 activation, probably causing neurogenic inflammatory in the airways (Trevisani et al., 2002). Interestingly, the same study demonstrated that ethanol (0.3–3%) reduces the threshold temperature for TRPV1 gating by approximately 8 °C, prompting its activation at a physiological temperature (37 °C). Moreover, the effects of anandamide and protons (agonists of TRPV1) are potentiated by the presence of ethanol (Trevisani et al., 2002). These findings may explain that, in susceptible individuals, the ingestion of ethanol triggers asthma attacks (Myou et al., 1996; Saito et al., 2001). In 2004, Trevisani et al. showed that ethanol could induce bronchospasm in guinea pigs by stimulating airway sensory nerves through a TRPV1-dependent activation (Trevisani et al., 2004). This excitatory effect of ethyl alcohol may contribute to the mechanism of ethanol-induced asthma via neurogenic inflammatory responses.

3.2. TRPV1 and bronchoconstriction

Release of sensory neuropeptides by acidification of the airways that contribute to the mechanisms of obstructive pulmonary diseases is also implicated in the stimulation of TRPV1 (Hunt et al., 2000; Ricciardolo et al., 2004). In gastroesophageal reflux disease (GERD), inhalation of acidic media has been associated with asthma (Harding, 2003). In guinea pigs, inhalation of citric acid can stimulate sensory neurons to cause bronchoconstriction, a phenomenon prevented by a TRPV1 antagonist (Satoh et al., 1993). In the same animal model, another study showed that the bronchoconstriction induced by capsaicin or its analogue resinferatoxin was antagonized by the TRPV1 antagonist iodo-resiniferatoxin (Undem and Kollarik, 2002). In addition, capsazepine (a selective TRPV1 antagonist) blocks the tonic plateau phase of ASM contraction induced by 20-HETE (Rousseau et al., 2005), and LTD_4 (Skogvall et al., 2007). As concerns for RESPIR 4–95, an analog of capsazepine, it was demonstrated to be more effective than the long-acting β-agonist formoterol in human airways (Skogvall et al., 2008). Thus, capsazepine analogs may be useful compounds in the pharmacological treatment of airway obstruction as it occurs in asthma. Also, Delescluse et al. found that SB-705498 and PF-04065463TRPV1 (two antagonists of the TRPV1 channel) abrogated the AHR to histamine in guinea pigs sensitized to ovalbumin (Delescluse et al., 2012). The authors postulated that TRPV1 might participate in the AHR mediated by the sensibilization of nerve terminals (Delescluse et al., 2012). Furthermore, it has been shown that the carotid bodies, peripheral autonomic and respiratory oxygen sensors, contribute to bronchoconstriction by inducing allergen-mediated parasympathetic activity (Jendzjowsky et al., 2018). In this regard, Jendzjowsky et al. proposed that IL-4, IL-5, IL-13, and lysophosphatidic acid (LPA) stimulate the petrosal ganglia complex (a carotid body) via the PKCε-TRPV1 signaling pathway. Prominently, the authors demonstrated that the use of the PKCε blocking peptide reduced AHR in OVA-sensitized rats. Thus, pharmacological blockade of PKCε and its phosphorylated target TRPV1 may provide a new therapeutic option for bronchospasm (Jendzjowsky

et al., 2021). Altogether, the evidence suggests that TRPV1 channels found on airway sensory nerves are important in the process of bronchoconstriction and in the development of AHR. Therefore, TRPV1 could serve as a potential target for asthma treatment.

3.3. Chronic cough in asthma

TRPV1 has been shown to play a role in airway disease by eliciting an excess in mucus production (Yu et al., 2012), contraction of ASM (Lin et al., 2009), and stimulation of cough reflex (Groneberg et al., 2004). Cough is also considered as a marker to characterize the severity of asthma (de Marco et al., 2006). The role of TRPV1 in the cough response in capsaicin-induced chronic airway inflammatory disease has been associated in patients with asthma (Doherty et al., 2000; Millqvist, 2000). The cough response is initiated by the stimulation of the afferent nerve fibers containing rapidly adapting receptors (RARs) and non-myelinated sensory C-fibers (Canning, 2002; Canning et al., 2004; Widdicombe, 2002). TRPV1 channels located on sensory nerves in the airways are essential to the cough reflex (Guan et al., 2021). Aerosolized TRPV1 agonists, such as capsaicin, elicit cough in asthmatics (Kunc et al., 2020). Moreover, the cough reflex sensitivity to capsaicin is more significant in patients with cough-variant asthma than in control subjects (Nakajima et al., 2006; Weinfeld et al., 2002). In this sense, it has been reported that the lower airway epithelium of asthmatic children displays elevated basal TRPV1 activity compared to non-asthmatic controls (Harford et al., 2018), as well as an augmented expression in patients with chronic cough (Mitchell et al., 2005). The increased TRPV1 gene expression shows a close relationship with asthma onset in young and adult asthmatic patients (McGarvey et al., 2014; Ren et al., 2015). In 2010, Cantero-Recasens et al. suggested that the TRPV1 genetic variant is relevant to asthma pathophysiology. Carriers of the isoleucine-to-valine mutation at position 585 of the TRPV1 protein (TRPV1-I585V genetic variant) showed a lower risk of childhood asthma or presence of wheezing (Cantero-Recasens et al., 2010). Collectively, the enhancement in function and expression of TRPV1 may be associated with hypersensitivity to chronic cough in asthma.

3.4. TRPV1 and respiratory syncytial virus

Recently, the relationship between TRV1 activity and respiratory syncytial virus (RSV) has been studied in asthmatic patients. Importantly, RSV infection up regulates TRPV1 expression on airway sensory neurons and bronchial epithelial cells (Omar et al., 2017). Harford et al. observed that RSV leads to increased TRPV1 activity in airway epithelial cells from asthmatic children (Harford et al., 2018). Later, the same research group proved that during RSV infection, TRPV1 protein is highly augmented in the plasma membranes of asthmatic human bronchial epithelium compared to non-asthmatic cells (Harford et al., 2021). The authors proposed that the airways of asthmatics possess an augmented activity of TRPV1, contributing to a major Ca^{2+} influx and AHR. Moreover, nerve growth factor (NGF) overexpression during the infection by the RSV promotes the release of additional Ca^{2+} from intracellular stores, exacerbating the ASM response. With this respect, TRPV1 antagonists may serve as a therapeutic target in asthmatic patients with RSV infections (Harford et al., 2018). Remarkably, Jing et al., 2020, demonstrated that a Chinese medicine named Qingfei oral liquid (QF) administrated to asthmatic mice with RSV infection, attenuated mucus hypersecretion, and AHR (Jing et al., 2020). The authors proved that QF inhibited the TRPV1- associated airway inflammatory response by reducing the expression levels of TRPV1 and the glycoprotein Mucin-5AC (MUC5AC), a major component of mucus. QF drug has been used to treat patients with viral pneumonia and asthma for decades, and its compounds include ephedrine/pseudoephedrine, amygdalin, and Mulberroside A (Jing et al., 2020).

3.5. TRPV1 and environmental pollutants

Another approach in which TRPV1 has been associated with asthma is by exacerbating the ailment due to the exposure to environmental pollutants (Liu et al., 2017). There are differences in the prevalence of asthma between individuals with common racial background but distinct environmental exposures, suggesting that air pollutants could be a factor in the etiology of asthma (Commodore et al., 2020; Komlosi et al., 2021). Animal models have served to the study of the role of air contaminants on lung diseases. In this context, particulate matter $\leq$2.5 μm ($PM_{2.5}$) could exacerbate asthma in mice sensitized with OVA through the signaling of TRPV1 channels (Liu et al., 2017). $PM_{2.5}$ is elevated in air pollution, and it might be one of the factors of increased incidence of asthma (Dunea et al., 2016; Gorai et al., 2016). Additionally, trimellitic anhydride (TMA, a typical pollutant) increased the protein expression of TRPV1 and TRPV2, as well as the levels of Th2 cytokines in lung tissue of OVA-induced asthmatic mice (Li et al., 2019b). According to the authors, TRPV channels could be new targets for the clinical treatment of TMA aggravating asthma. Moreover, it has been reported that increased ground-level ozone, a major environmental pollutant, is positively associated with the average number of emergency room visits by asthmatic patients (Tian et al., 2018). In fact, previous studies have shown that continuous exposure to ozone is particularly damaging for asthma patients (Goodman et al., 2018). In this sense, a study showed that TRPV1 expression was markedly elevated in ozone-exacerbated asthmatic mice and that treatment with capsazepine effectively suppressed AHR, airway inflammation, and remodeling (Li et al., 2019a). This study elegantly demonstrated that the upregulation of TRPV1 might represent an essential mechanism in the development of asthma after the exposure to environmental pollutants, and the inhibition of TRPV1 has significant anti-inflammatory effects on ozone-induced asthma exacerbation.

3.6. TRPV1 and airway inflammation

There is increasing evidence that TRPV1 also contributes to the development of airway inflammation during asthma. This protein has been reported to be expressed in immune cells, including mast cells, dendritic cells (DCs), neutrophils, and macrophages (Duo et al., 2020; Li et al., 2019c). Moreover, TRPV1 channel is expressed on the plasma membrane of T cells and regulates the activation of $CD4^+$ cells (Bertin et al., 2014; Majhi et al., 2015; Samivel et al., 2016). T cells play a central role in the development of allergic asthma, and modulation of TRPV1 may mediate the function of these cells (Baker et al., 2016; Majhi et al., 2015). As previously mentioned, most of the asthma features are mediated by Th2 cytokines such as IL-13, IL-4, and IL-5 (Komlosi et al., 2021). However, IL-13 alone might be sufficient to explain almost all characteristics of asthma (Walsh, 2010). Rehman et al. showed that the blockade of TRPV1 abrogated the airway inflammation, AHR, and the epithelial injury elicited by the effects of IL-13 mice (Rehman et al., 2013). Additionally, the blockade of TRPV1 displays anti-inflammatory properties in ozone-induced asthma exacerbation (Li et al., 2019a). In conclusion, inhibiting the activity of this channel may contribute to alleviating the airway epithelial injury and the chronic inflammation seen in asthma.

On the other hand, some studies revealed that the secretion of airway epithelial IL-33 induced by allergens implicates an elaborated Ca^{2+}signaling. Initially, ATP is released as a result of cellular damage, which subsequently stimulates the purinergic receptor (P2Y) type 2 on the epithelial surface, promoting an increase of Ca^{2+}. Later, Ca^{2+} increase activates the dual oxidase 1 (DUOX1), highly expressed in the airway epithelium. DUOX1 triggers the formation of H_2O_2 and the redox-dependent epidermal growth factor receptor (EGFR) activity, eliciting the release of IL-33 (Hristova et al., 2016; Kouzaki et al., 2011). Moreover, it has been proved that the protease-activated receptor (PAR)-2 and TRPV1 channel contribute to allergen-induced ATP release.

Consequently, this damage signal promotes the activation of both DUOX1 and the EGFR, favoring the secretion of IL-33 (Schiffers et al., 2020). In addition, PAR-2 activation facilitates the release of thymic stromal lymphopoietin (TSLP) (Kouzaki et al., 2009), which promotes type 2 responses and leads to the development of allergic asthma (Lai et al., 2020). In 2014, Jia et al. provided evidence showing that the Ca^{2+} influx through TRPV1 triggers nuclear factor of activated T-cells (NFAT) translocation from the cytosol into the nucleus to further enhance TSLP secretion from human bronchial epithelial cells (Jia et al., 2014).

Despite the overwhelming evidence implicating TRPV1 activity in the major features of asthma, a study published in 2011 demonstrated that the absence of this vanilloid receptor enhanced the Th2-biased response after intranasal OVA or house dust mite (HDM) sensitization (Mori et al., 2011). The authors observed that the knockout of TRPV1 channel subtype in mice augmented the serum IgE, and the IL-4 and eosinophils in the bronchoalveolar lavage fluid (BALF). Although these findings suggest a protective role of TRPV1 against asthma development, they certainly denote contradictory outcomes (comparing with all the evidence previously mentioned) and should be taken with caution.

3.7. TRPV1 and Ca^{2+} signaling

Dysregulation of intracellular Ca^{2+} handling and signaling in ASM cells can be implicated in the clinical heterogeneity of asthma. TRPV1 forms a cation channel with selectivity for Ca^{2+}, that depends on particular factors, such as the concentration and the structure of the agonist (Bautista and Julius, 2008). Ca^{2+} influx through this channel may play an important role in asthma. Several chemical and physical stimuli evoke specific responses in the ASM establishing an optimal airway caliber and the regulation of the airflow (Lam et al., 2019). Acetylcholine, histamine, and leukotrienes are endogenous agonists which act via their own GPCRs. These agonists elicit the activity of the PLC enzyme, producing diacylglycerol (DAG) and IP_3. The last messenger promotes the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Montaño et al., 2018). Massive Ca^{2+} increase in the cytosol elicits the release of more Ca^{2+}; this event is known as Ca^{2+}-induced Ca^{2+} release (CIRC) (Reyes-García et al., 2018; ZhuGe et al., 1998). SR depletion induces Ca^{2+} influx via a process known as store operated Ca^{2+} entry (SOCE), which contributes to the sustained contraction (Chen and Sanderson, 2017; Wang et al., 2021b). This contraction requires an elevated intracellular calcium concentration ($[Ca^{2+}]_i$) produced in part by continuous Ca^{2+} waves, called Ca^{2+} oscillations (Perez and Sanderson, 2005). In this regard, it has been reported that capsaicin inhibits increments of Ca^{2+} mediated by PLC activity through blocking thapsigargin-sensitive SOCE (Choi and Kim, 1999). Moreover, it has been demonstrated that TRPV1 is expressed in the SR membrane in human ASM cells and that capsazepine inhibits Ca^{2+} oscillations and sustained contraction (Yocum et al., 2017). The authors suggested that TRPV1 channel cooperates with ryanodine receptors or another channel subtype to promote Ca^{2+} oscillations (Yocum et al., 2017). Nevertheless, further research is needed to unveil the role of TRPV1 in ASM contraction during the asthmatic crisis.

3.8. TRPV1 and airway remodeling

Airway remodeling is one of the main features of asthma, in which ASM cells proliferation plays an essential function (Kaminska et al., 2009). In this regard, Zhao et al. observed that the TRPV1 agonist, capsaicin, enhanced the proliferation of asthmatic ASM cells and decreased apoptosis (Zhao et al., 2013). In addition, $[Ca^{2+}]_i$ in ASM cells of asthmatic mice was significantly increased, and after treatment with capsaicin, $[Ca^{2+}]_i$ was further enhanced. Moreover, McGarvey et al. found a greater expression of TRPV1 channels in bronchial epithelial cells of patients with refractory asthma (McGarvey et al., 2014). They proposed this vanilloid receptor as a novel pharmacological target for exacerbations in asthma. Additionally, inflammatory mediators such as tumor necrosis factor α (TNF-α), lipopolysaccharide (LPS), and IL-1α induce TRPVI expression on bronchial fibroblasts (Sadofsky et al., 2012). This channel is not usually expressed on these cell types. However, fibroblast proliferation is crucial to the development of persistent airway obstruction in chronic asthma, and TRPV1 seems to play an essential role. Remarkably, the silencing of TRPV1 or its pharmacological inhibition with capsazepine diminishes the AHR to methacholine and the airway inflammation in asthmatic mice. Th2 profile composed by IL-4, IL-12, and IL-5 is similarly reduced, as well as IL-33, a master regulator of this inflammatory response. Genetic or pharmacological blockade of TRPV1 lessens airway remodeling, characterized by goblet cell hyperplasia, and collagen deposition (Choi et al., 2018). Collectively, TRPV1 represents a remarkable molecular target for alleviating asthma features, including AHR, airway inflammation, and remodeling.

4. Role of TRPV2 on asthma

The polymodal TRPV2 Ca^{2+} permeable channel is mainly activated by noxious temperatures, although other stimuli promote its gating, including mechanical stress and phosphatidylinositol 3-kinase-activating ligands (Kojima and Nagasawa, 2014). Unlike the numerous studies about the role of TRPV1 on asthma, the evidence of TRPV2 and the development of this ailment is still scant. In the last years, bronchial thermoplasty has been proposed to be included in asthma therapy for severe cases. This therapy consists of delivered heat, i.e., radiofrequency energy into the airways to reduce the contractile capacity of ASM and therefore avoid hyperresponsiveness (Miller et al., 2005). In this context, because of its ability to sense heat stimuli, TRPV2 was thought to mediate the loss of ASM function (Dyrda et al., 2011); yet further research is required. Moreover, the expression of TRPV2 has been demonstrated in human peripheral blood cells (Saunders et al., 2007). A possible role of this channel in inflammatory conditions may be suggested. Cai et al. found an increased expression pattern of TRPV2 in peripheral lymphocytes of boys and girls between 2 and 12 years suffering from asthma (Cai et al., 2013). Moreover, not all the patients in this study showed a high correlation between total serum IgE (a well-known biomarker of asthma) levels with the asthma diagnosis. Interestingly, those patients with normal IgE levels exhibited up-regulation of the TRPV2 gene as well. Thus, TRPV2 might serve as an additional novel biomarker for asthma in children with typical IgE levels (Cai et al., 2013). Recently, in a model of OVA-induced asthmatic mice, the administration of trimetallic anhydride (TMA), a widespread pollutant, augmented the expression of TRPV2 in the lung tissue. The exacerbating effects of chemical contaminants in the air might be related to the regulation of this channel subtype. This insight opens the possibility to investigate the TRPV2 protein as a new pharmacological target in cases of asthma exacerbated by air pollutants (Li et al., 2019b). Recently, Zhang et al. demonstrated that San-ao Decoction (SAD), a traditional Chinese prescription used to treat asthma and cough, reduced the inflammatory cytokine levels of IL-4 and IL-10 in BALF, and diminished TRPV2 expression in the lungs of OVA-induced asthmatic mice (Zhang et al., 2020). Most recently, Xu et al. demonstrated that the knockdown or the inhibition or TRPV2 decreased the release of cytokines induced by SARS-CoV-2 infection, pointing out this channel as a target for lung inflammatory conditions (Xu et al., 2021). Taken together, the evidence points out TRPV2 channels as a novelty biomarker for asthma diagnosis and a potential target for the clinical treatment of TMA aggravating asthma.

5. Role of TRPV4 on asthma

The transient receptor potential vanilloid 4 (TRPV4) is a non-selective cation channel implicated in thermal and osmotic sensitivity (Guler et al., 2002; Liedtke et al., 2000; Strotmann et al., 2000; Vriens et al., 2004). The activation of TRPV4 can be mediated by hypotonic solutions (Jia et al., 2004), phorbol derivatives (Watanabe et al., 2002),

heat (Guler et al., 2002), mechanical stress (Shibasaki, 2020), and several endogenous substances such as arachidonic acid and anandamide (Watanabe et al., 2003). In the lung, TRPV4 is expressed in airway epithelial cells and ASM cells (Jia et al., 2004; Lorenzo et al., 2008). In 2004, Jia et al. described that hypotonic stimulation of human ASM elicited contraction through a mechanism dependent on membrane Ca^{2+} channels and that TRPV4 might function as an osmolarity sensor in the airways (Jia et al., 2004). Moreover, TRPV4 was found to be expressed in lung fibroblasts contributing to pulmonary fibrogenesis, suggesting its manipulation as a therapeutic approach for fibrotic diseases (Rahaman et al., 2014).

Emerging evidence has pointed out an important association of TRPV4 activity with the pathogenesis of asthma. A few years ago, it was reported that EET, an endogenous TRPV4 activator and GSK1016790A (a TRPV4 agonist) induced the proliferation of ASM cells (Zhao et al., 2014). The remarkably detailed mechanism was revealed in the same work. In ASMCs, TRPV4 complexes with the Ca^{2+}/calmodulin–dependent protein phosphatase calcineurin. Ca^{2+} influx through TRPV4 stimulates calcineurin which dephosphorylates the NFAT, allowing its translocation into the nucleus and favoring the proliferation of the ASM (Zhao et al., 2014). Later on, a physical and functional coupling between the intermediate-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}3.1$) and TRPV4 in asthmatic human bronchial smooth muscle cells (HBSMCs) was proposed (Yu et al., 2017). In this regard, $K_{Ca}3.1$ channels have been implicated in the proliferation of several cells including, breast cancer tumors (Mohr et al., 2019), bone marrow-derived mesenchymal stem cells (Jia et al., 2020), and arterial and airway smooth muscle cells (Lasch et al., 2020; Yu et al., 2013). Besides ASM proliferation, it has been reported that TRPV4 contributes to Ca^{2+} handling and contraction in this tissue. For instance, it has been shown that TRPV4 forms a complex with the Na^+/Ca^{2+} exchanger and with the IP_3 receptor regulating the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the muscle tension in mice (Zhang et al., 2019). Also, McAlexander et al. observed that Ca^{2+} influx through TRPV4 promoted the synthesis of leukotrienes and further ASM contraction (McAlexander et al., 2014). In addition, TRPV4 is involved in the contraction response elicited by the Bradykinin receptor 2 signaling pathway (Jentsch Matias de Oliveira et al., 2020). In recent studies performed by our research group (unpublished novelty data), we observed that the stimulation of guinea pig ASM cells with the TRPV4 agonist, GSK1016790A, evoked sustained $[Ca^{2+}]_i$ increments depending on the agonist concentration tested (Fig. 1A–B). Moreover, using an L-VDCC blocker (D-600) we confirmed that GSK1016790A-induced Ca^{2+} increase was due to the opening of the TRPV4 and partly mediated by the gating of L-VDCC (Fig. 1C–D). Since TRPV4 is an NSCC, these results strongly suggest a possible role of this vanilloid receptor subtype in membrane depolarization with the subsequent opening of the L-VDCC. These mechanisms i.e., Ca^{2+} and Na^+ influx through TRPV4, membrane depolarization, and L-VDCC activation, might be implicated in ASM contraction, although further research is required. Collectively, the evidence points out TRPV4 channel as a critical protein involved in ASM contraction and proliferation.

Some patients with asthma may experiment exercise-induced bronchoconstriction. One of the most accepted explanations for the occurrence of this phenomenon is the osmotic theory. Increased ventilation in the airways during exercise provokes water loss by dehydration, thus specific mechanisms responsible for bronchoconstriction are elicited (Aggarwal et al., 2018). Continuous inspiration of humid air may induce ASM contraction in asthmatic patients as well (Hayes et al., 2012). In this context, TRPV4 has been proposed as one of the key mechanisms involved in this kind of ASM response, since this cationic channel is sensitive to hypoosmotic stimulus. In fact, single nucleotide polymorphisms (SNPs) in the TRPV4 gene affect the susceptibility to hypoosmotic-induced bronchospasm in asthmatic subjects (Naumov et al., 2016). Moreover, the combination of high relative humidity and paraformaldehyde enhances airway hyperresponsiveness and Th2 response in a model of allergic asthma (Duan et al., 2020). This phenomenon seems to be mediated by the TRPV4-p38 MAPK signaling pathway. In addition, comorbid conditions such as GERD may enhance asthma crisis (Pinyochotiwong et al., 2021). TRPV4 inhibitors, GSK222069 and GSK2337429A, have been shown to reduce the increased levels of macrophages, neutrophils, and associated cytokines in the BALF of mice after the induction of acid aspiration injury by exposure to hydrochloric acid (Balakrishna et al., 2014). TRPV4 might be considered as a target to alleviate GERD related asthma symptoms; however, this statement requires deep investigation.

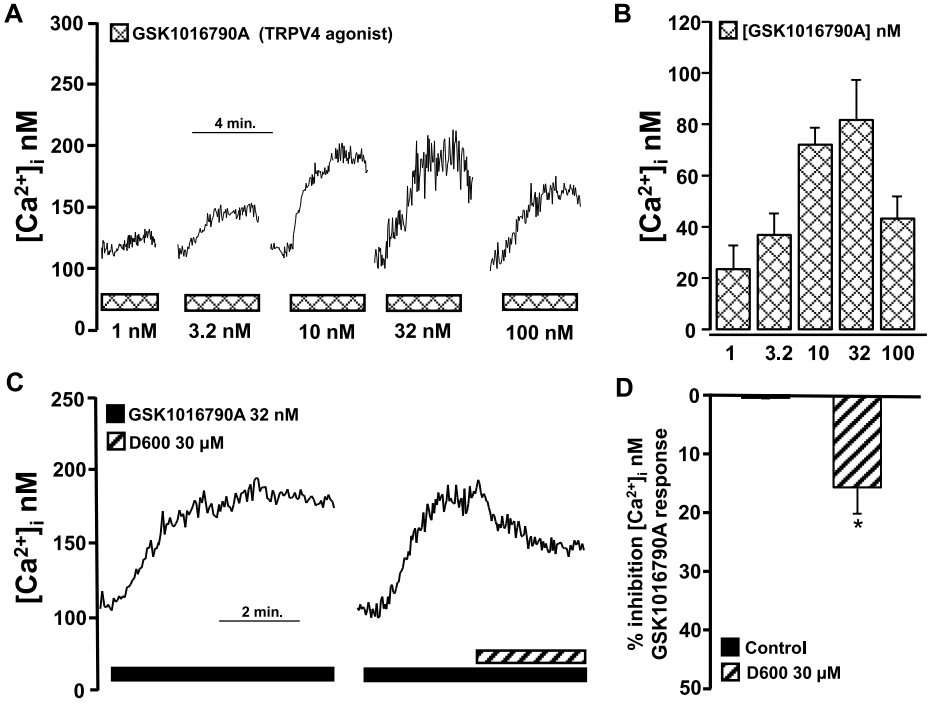

Fig. 1. L-type Ca^{2+} channels (L-VDCCs) contribute to TRPV4-induced $[Ca^{2+}]_i$ increments in guinea pig ASM cells. A) Representative recordings of GSK1016790A (TRPV4 agonist)-evoked $[Ca^{2+}]_i$ increments in single cell at nanomolar (nM) concentrations. Cells stimulated with several GSK1016790A concentrations produced a sustained increment of $[Ca^{2+}]_i$. **B)** Bar graph summarizes the TRPV4 agonist-evoked $[Ca^{2+}]_i$ at different nanomolar (nM) concentrations (1–100 nM, n = 5). **C)** Original recording depicting D-600 effect on $[Ca^{2+}]_i$ increment induced by GSK1016790A (32 nM) stimulation. **D)** Statistical analysis of the D-600 effect on the GSK1016790A-induced increase in $[Ca^{2+}]_i$. *p < 0.05 was found when 30 µM (n = 4) were compared with its respective control (the sustained TRPV4 Ca^{2+} response without D-600). Bars represent mean ± standard error of the mean.

Despite the role of TRPV4 in exacerbating asthma symptoms, a study claims this channel seems not to be implicated in the development of this ailment. Palaniyandi et al. found no differences between TRPV4 KO and wild-type (WT) asthmatic mice. Both groups displayed the same levels of IgE, eosinophilia, and goblet cell hyperplasia (Palaniyandi et al., 2020). Nevertheless, it has been reported that TRPV4 is indispensable and enough for club cells (clara cells) to initiate allergic lung inflammation induced by the aeroallergen alkaline protease 1 (Alp1) of *Aspergillus* sp (Wiesner et al., 2020). Damaged junctions provoked by the allergen are detected as mechanical stress by TRPV4 in club cells, triggering Ca^{2+}-calcineurin pathway and promoting allergic sensitization in mice. Likewise, the apical membrane expression of TRPV4 in club cells is associated with fungal sensitization and asthma in children (Wiesner et al., 2020). Moreover, the expression of TRPV4 in the airway epithelia and in the infiltrating inflammatory cells is increased in a model of toluene diisocyanate (TDI)-induced occupational asthma (Yao et al., 2019). In the same model, the blockade of TRPV4 led to reduced eosinophilia, Th2 response and abolished airway hyperreactivity. Meanwhile, TDI inhalation diminished the membrane levels of E-cadherin and β-catenin. This fact was reverted by a TRPV4 blocker, pointing out an essential contribution of this ion channel to E-cadherin and β-catenin dysfunction (adherens junction dysfunction) in the TDI-induced asthma model (Yao et al., 2019). Furthermore, alveolar macrophages initiate the inflammatory signs in the lung after pathogen infection, which may lead to the formation of edema. In this regard, TRPV4 plays an essential role in the epithelial barrier function protecting the lung against edema (Weber et al., 2020).

TRPV4 is also implicated in non-atopic asthma (IgE independent). In human airway smooth muscle cells, the stimulation of TRPV4 increases $[Ca^{2+}]_i$ and releases ATP, which activates P2X4 receptors on mast cells. These receptors evoke the release of leukotrienes, promoting ASM contraction (Bonvini et al., 2020). Furthermore, TRPV4 mediates lung fibroblast differentiation and airway remodeling in asthma by integrating transforming growth factor (TGF)-β1 and NADPH oxidase (NOX)-4 signaling (Al-Azzam et al., 2020). In asthmatic subjects, lung fibroblast activation in response to continuous injury and repair cycle leads to myofibroblast formation because of fibroblast to myofibroblast transition (FMT) (Michalik et al., 2018). TGFβ1 triggers phosphoinositide-3-kinase (PI3K), eliciting NOX4-dependent generation of reactive oxygen species (ROS). Interestingly, this last process is mediated by TRPV4. This vanilloid receptor subtype is also involved in mediating TGFβ1-induced fibrotic gene expression of collagen 1A1, α-actin, and fibronectin, causing airway remodeling (Al-Azzam et al., 2020). All these findings indicate that TRPV4 participates in the development of asthma and airway remodeling and further suggest that the inhibition of TRPV4 could alleviate exacerbations in asthmatics.

6. TRPV blockers and asthma

Several biomedical studies have pointed out that selective blockers of TRPV1, TRPV2, and TRPV4 subtypes may be useful to alleviate asthma symptoms and reduce its development. As for TRPV1 reports, it has been shown that Capsazepine (a selective TRPV1 antagonist) abrogates AHR and airway inflammation in mice (Choi et al., 2018). Additionally, this drug prevents bronchoconstriction induced by low pH (Satoh et al., 1993) or 20-HETE (Rousseau et al., 2005) in guinea pig ASM. Moreover, it relaxes human small bronchial preparations pre-contracted with LTD_4, PGD_2, or histamine (Skogvall et al., 2007). Moreover, RESPIR 4–95 (an analog of capsazepine) exerts a bronchodilator effect 10-fold greater than that of capsazepine when human bronchi are stimulated with acetylcholine, LTD_4, PGD_2, or histamine, and displays a more prolonged effect compared with formoterol (a long-acting β-agonist) (Skogvall et al., 2008). Furthermore, the TRPV1 selective antagonists SB-705498 and PF-04065463 decrease AHR, probably by interfering with the sensibilization of airway nerve endings in guinea pigs. (Delescluse et al., 2012). Likewise, SB-705498 significantly diminishes the capsaicin-induced cough in patients with chronic coughing (Khalid et al., 2014). On the other hand, the blockade of TRPV2 with SKF-96365 or its genetic ablation decreases the secretion of macrophages' cytokines such as TNF-α, IL-13, and IL-17A (Xu et al., 2021). Finally, the TRPV4 antagonists, GSK222069 and GSK2337429A, attenuate lung inflammation and edema by lowering the number of macrophages and neutrophils in the BALF of mice (Balakrishna et al., 2014). Thus, the evidence so far suggests the possibility of conducting clinical trials to assess the use of selective TRPV1 blockers to induce bronchorelaxation and relieve chronic cough and selective TRPV2 and TRPV4 blockers to counteract the inflammation and edema in asthmatic patients.

7. Conclusions

Variations in osmolarity, temperature, pH, and ASM tension have been associated with asthma exacerbations in patients and animal models. Increasing evidence demonstrates the essential function of TRPV1, TRPV4, and to a lesser extent TRPV2 in the airway physiology, and more important, their contribution to the major features of asthma such as inflammation, AHR, and remodeling. The role of these TRPV subtypes in asthma development and exacerbations is summarized in Fig. 2. TRPV1 signaling increases mucus production, ASM contraction, and cough reflex. Moreover, carriers of TRPV1-I585V genetic variant display a reduced risk of asthma or the incidence of wheezing. In this regard, SNPs in TRPV4 gene influence the development of asthma symptoms. TRPV2 is highly expressed in peripheral lymphocytes of asthmatic boys and girls. Thus, TRPV2 is proposed as a novel biomarker for asthma in children. Remarkably, the inhibition of TRPV2 lessens the production of cytokines after SARS-CoV-2 infection. Additionally, the pharmacological blockade or the gene silencing of TRPV1 and TRPV4 display anti-inflammatory properties in some asthma phenotypes, including allergic and ozone-induced asthma. Likewise, the use of TRPV1 and TRPV4 inhibitors decreases AHR and remodeling. Therefore, TRPV1, TRPV2, and TRPV4 are potential targets to attenuate the major asthma symptoms and their development. Although further clinical research is needed, the evidence highly points out TRPV channels as essential effectors in asthma disease. Moreover, the exacerbations influenced by osmotic and thermal variations seen in this ailment, might represent a new treatment option that should be based on targeting TRPV channels.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Abril Carbajal-García and Jorge Reyes-García contributed equally to this work. JRG: Methodology, Data curation, Writing-Original draft preparation. ACG: Data curation, Writing- Original draft preparation. LMM: Conceptualization, Visualization, Supervision, Writing- Reviewing and Editing.

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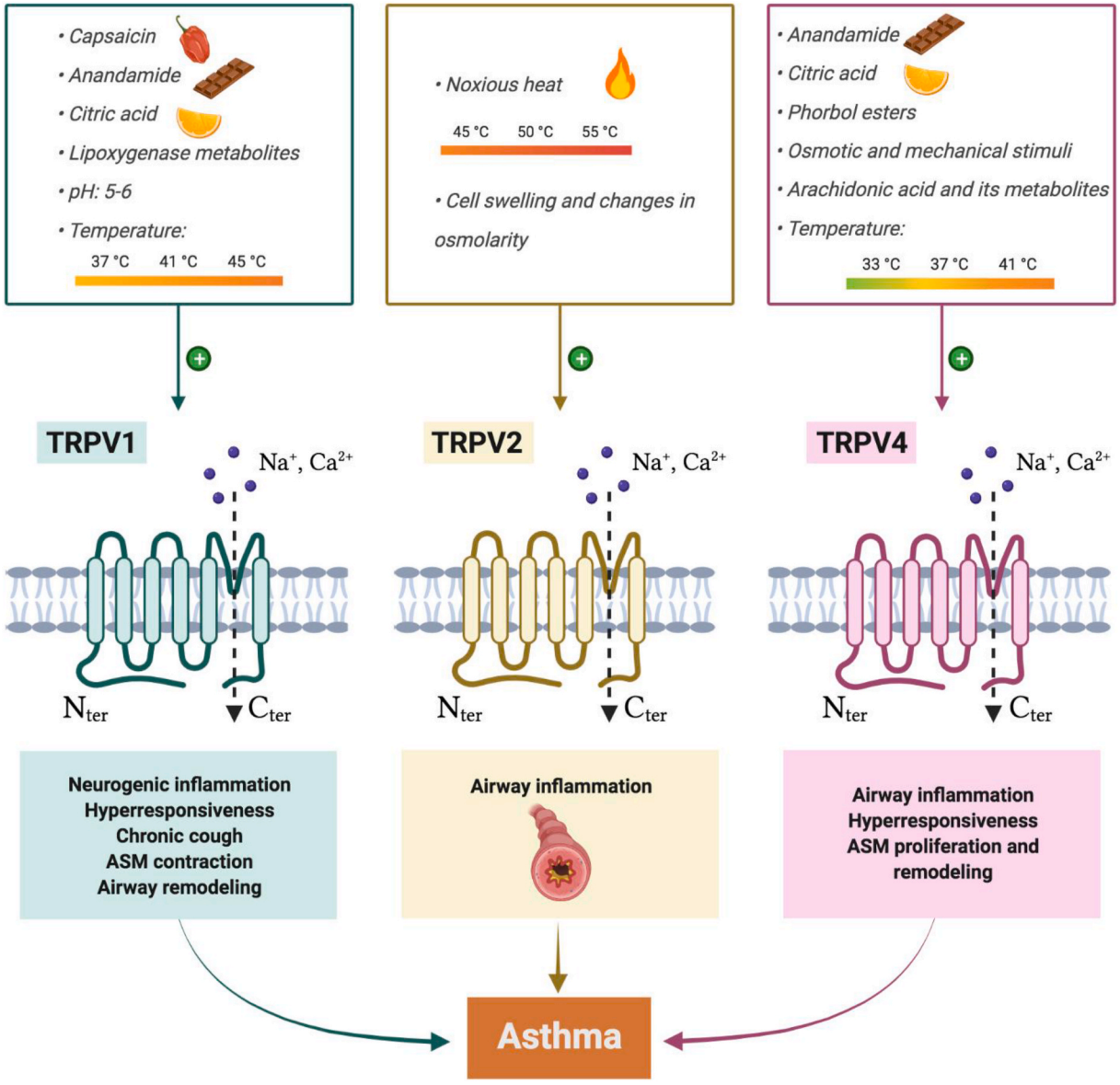

Fig. 2. Role of the transient receptor potential cation channel subfamily V (TRPV) in asthma. Among the six recognized members of TRPV family, TRPV1, TRPV2, and TRPV4 have been related to the development and exacerbations of asthma symptoms. Structurally, TRPVs contain six transmembrane spans and a pore region between the transmembrane domain (TM)-5 and TM6. TRPVs also possess an N-terminal domain (Nter) with ankyrin repeats implicated in protein-protein interactions and a C-terminal TRP domain (Cter). TRPV1, TRPV2, and TRPV4 consist of permeable cation non-selective ion channels: Interestingly, Ca^{2+} influx through these channels may play a critical role in asthma. TRPV1 is classically activated by capsaicin, citric acid, lipoxygenase metabolites generated from arachidonic acid, and endogenous cannabinoids, including anandamide. Moreover, low extracellular pH promotes the opening of the channel as well as temperatures between 37 and 50 °C. TRPV1 signaling increases neurogenic inflammation, hyperresponsiveness, mucus production, ASM contraction, cough, and airway remodeling. The gating of TRPV2 requires a noxious heat, cell swelling, and changes in osmolarity. The main function of TRPV2 is to mediate the transduction of noxious heat and pain sensation. A possible role in asthma of TRPV2 in inflammatory conditions may be suggested and has been proposed as a novel biomarker for asthma in children. TRPV4, another polymodal channel that is activated by warm temperatures approaching physiological conditions, osmotic and mechanical stimuli, phorbol esters, anandamide, and the arachidonic acid and its metabolites. TRPV4 activation induces the proliferation of airway smooth muscle (ASM) cells, allergic inflammation, and airway remodeling. Therefore, TRPV1, TRPV2, and TRPV4 are essential effectors in asthma that can be potential targets to attenuate the symptoms and development of this disease.

Declaration of competing interest

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Declaration of competing interest

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Anexo 5. Artículo de revisión.

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Review

Important Functions and Molecular Mechanisms of Mitochondrial Redox Signaling in Pulmonary Hypertension

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Abstract: Mitochondria are important organelles that act as a primary site to produce reactive oxygen species (ROS). Additionally, mitochondria play a pivotal role in the regulation of Ca^{2+} signaling, fatty acid oxidation, and ketone synthesis. Dysfunction of these signaling molecules leads to the development of pulmonary hypertension (PH), atherosclerosis, and other vascular diseases. Features of PH include vasoconstriction and pulmonary artery (PA) remodeling, which can result from abnormal proliferation, apoptosis, and migration of PA smooth muscle cells (PASMCs). These responses are mediated by increased Rieske iron–sulfur protein (RISP)-dependent mitochondrial ROS production and increased mitochondrial Ca^{2+} levels. Mitochondrial ROS and Ca^{2+} can both synergistically activate nuclear factor κB (NF-κB) to trigger inflammatory responses leading to PH, right ventricular failure, and death. Evidence suggests that increased mitochondrial ROS and Ca^{2+} signaling leads to abnormal synthesis of ketones, which play a critical role in the development of PH. In this review, we discuss some of the recent findings on the important interactive role and molecular mechanisms of mitochondrial ROS and Ca^{2+} in the development and progression of PH. We also address the contributions of NF-κB-dependent inflammatory responses and ketone-mediated oxidative stress due to abnormal regulation of mitochondrial ROS and Ca^{2+} signaling in PH.

Keywords: pulmonary hypertension; mitochondrial ROS; ketones; Ca^{2+} signaling

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1. Introduction

Mitochondria are important organelles that contribute to cellular homeostasis; thus, the dysregulation of mitochondrial function can lead to cellular or tissue injury, and further systemic affections [1]. Mitochondria are best known for being the powerhouses of aerobic cells. Producing adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) [2] is not the only function of these organelles, yet they are involved in the regulation of Ca^{2+} signaling, redox potential, and the control of reactive oxygen species (ROS) production and levels [3].

ROS are important byproducts of O_2 metabolism occurring in the environment of all cells. Major sources of these species are mitochondria and cytoplasmic enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) [4]. For a long time, mitochondrial O_2 derivatives were considered harmful cellular chemical entities; nevertheless, there is increasing evidence showing that ROS are not only toxic oxidants

but serve as signaling molecules implicated in several processes, such as cytosolic Ca^{2+} signaling [5–9], gene transcription [10] and protein synthesis [11]. The impairment of mitochondria function (mitochondrial dysfunction) affects almost any functional tissue in the body, e.g., pancreas, skeletal, cardiac and smooth muscle, nerves, kidney, and lungs among others [12]. Mitochondrial dysfunction due to alterations in ROS production and mitochondrial DNA (mitDNA) damage underlies critical pathophysiological mechanisms in numerous diseases such as diabetes [13,14], fibromyalgia [15], chronic heart failure [16], Alzheimer's disease [17], chronic kidney disease [18], atherosclerosis [19], and pulmonary hypertension (PH) [5,20]. In this regard, the role of ROS has been extensively investigated in vascular biology. The production of ROS is highly increased in PH experimental models and clinical hypertension, and more specifically, the evidence highlights that fluctuations in intracellular ROS concentration ($[ROS]_i$), mostly elicited by mitochondrial dysfunction in pulmonary artery (PA) endothelial cells (PAECs) and PA smooth muscle cells (PASMCs), contribute to the progression of PH [6–9,21–23].

In addition to the generation of ATP and ROS, mitochondria are also involved in amino acid metabolism, release of tricarboxylic acid (TCA) cycle metabolites, fatty acid oxidation (FAO) and ketone bodies synthesis [24,25]. Ketone bodies, including β-hydroxybutyrate (β-HB), acetoacetate and acetone, are primarily produced by β-oxidation of fatty acids (FA) in the mitochondria of hepatocytes, even though enterocytes, astrocytes and kidney epithelial cells may produce them as well [24,26]. Interestingly, it has been shown that β-HB decreases the senescence of vascular cells [27], and that ketones abolish the generation of mitochondrial ROS [28]. This review focuses on the participation of mitochondrial ROS (mitROS) and Ca^{2+} signaling in the inflammatory process leading to PH development, as well as their mutual interactions, and the possible role of ketone bodies on mitochondrial Ca^{2+} and/or ROS signaling.

2. Pulmonary Hypertension

PH is a rare and fatal disease with an estimated prevalence of 5 to 50 cases per million individuals [29–32]. This ailment has been defined by the increase in the mean pulmonary arterial pressure (mPAP) $\geq$25 mm Hg at rest or $\geq$30 mm Hg during/after workout [33]. Most patients suffering from PH are women, covering around 60% to 80% of all cases [30,34,35]. Therefore, female sex has been considered a risk factor for the development of PH. Nevertheless, male sex has been associated with poorer survival rates and this could be explained by the fact that men affected by this illness exhibit a low right ventricular function recovery [36,37]. The latest update in 2013 of World Health Organization's clinical classification system has catalogued PH into five categories/groups depending on the main underlying cause, hemodynamics, clinical features, and therapeutic responsiveness [21,33]. As shown in Table 1, Group I refers to pulmonary arterial hypertension (PAH) and encloses idiopathic PH (IPAH), drug-induced PH, heritable PH, and PH associated with other systemic diseases. PH due to left heart disease corresponds to group II. Groups III and IV include PH due to lung diseases and PA obstructions, respectively. Lastly, group V describes PH with unclear multifactorial mechanisms. Apparently, a number of pathological causes may cause and promote dysfunctions of PA endothelial cells and PASMCs via numerous distinctive signaling mechanisms, thereby leading to the initiation and development of PH, right ventricular failure (RVF) and even death [21,33].

The pathophysiology of PH involves continuous pulmonary vasoconstriction, endothelial cells (ECs) injury, vascular smooth muscle (VSM) damage and proliferation, intimal fibrosis, remodeling, and inflammation. These alterations, i.e., pulmonary vascular remodeling (PVR), promote an augmented pulmonary vascular resistance (by the occlusion of blood vessels), increasing the right ventricle afterload and leading to right ventricular hypertrophy and failure, and eventually death [38,39]. The enhanced pulmonary vascular reactivity is also associated with malfunctioning of endothelial cells, leading to an imbalance in the production of nitric oxide (NO), prostaglandin (PG)-I_2 (also named prostacyclin), and endothelin-1. More specifically, it has been shown that patients suffering from PAH

display reduced expression of endothelial nitric oxide synthase (eNOS) and NO levels in the lungs [40,41]. In addition, the expression of prostacyclin synthase is diminished in patients with severe PH [42]. Importantly, PGI_2 triggers the synthesis of cyclic adenosine monophosphate (cAMP) and stimulates the peroxisome proliferator activated receptor-γ (PPARγ), leading to an antiproliferative effect in VSM cells (VSMCs) [43]. Eventually, this endothelial perturbance results in a diminished endothelium dependent pulmonary vasculature relaxation [44,45].

Table 1. WHO classification of pulmonary hypertension (PH).

WHO Group	Clinical Classification	Subtypes
I	Pulmonary arterial hypertension (PAH)	Idiopathic; Drug and toxin-Induced; Heritable; Associated with connective tissue diseases, HIV infection, portal hypertension, schistosomiasis; PAH responder to Ca^{2+} channel blockers; Associated with pulmonary venous/capillaries occlusion; Persistent pulmonary hypertension of the newborn.
II	PH due to left heart diseases	Heart failure; Valvular heart disease; Congenital or acquired cardiomyopathies; Failure with preserved/reduced ejection fraction.
III	PH due to lung disease or hypoxia	COPD/hypoxia that includes COPD; Restrictive lung disease; Pulmonary disease with obstructive and restrictive pattern; Interstitial lung disease; Hypoxia without other lung diseases.
IV	PH due to the obstruction of pulmonary artery	Chronic thromboembolic pulmonary hypertension (CTEPH); Other pulmonary artery obstructions.
V	PH due to unclear/multifactorial mechanisms	Hematologic disorders; Metabolic disorders; Others.

Contractile VSM mechanisms are also modified during PH. For instance, the expressions of RhoA and Rho-associated protein kinase (ROCK), important elements of Ca^{2+} sensitization, are elevated in a mouse model of PH [46]. Likewise, Ca^{2+}-activated K^+ ($K_{Ca}{}^{2+}$) channel 3.1 subtype is increased during PH contributing to VSM proliferation and remodeling [47]. On the other hand, the activity of voltage-gated potassium (K_V) channels is lessened [48], leading to persistent PAs contraction. PVR is caused by hypertrophy and hyperplasia of VSMCs, loss of small pre-capillary arteries, neointimal formation, adventitial thickening and plexiform lesions due to disturbances in the apoptosis and proliferation of VSMCs and ECs [49]. Muscularization and wall thickening of peripheral pulmonary arteries set the basis for the increased vascular resistance and persistent contraction seen in PH. Furthermore, the development of this disease has been related to pro-inflammatory or infectious etiologies such as scleroderma, human immunodeficiency virus (HIV), and schistosomiasis which often have systemic vascular complications [50–52]. Researchers have established animal models of PH using monocrotaline injection (MCT), chronic hypoxia, and Sugen 5416 (a vascular endothelial growth factor inhibitor) with chronic hypoxia. These three animal models, which replicate key features of PH in humans, have been assessed in intact animals, PAs, PASMCs and PAECs. The findings have led to significant insights into the development, mechanisms, diagnosis, and treatments of PH [53–56].

During heart failure, a shift from FAO-based metabolism to glycolysis occurs. Under normal circumstances, the ATP produced in cardiomyocytes is primarily generated from FAO; nevertheless, during stress conditions, such as an enhanced ROS environment, FAO may be reduced as glycolysis increases [57]. Metabolism of FA and ketones predominates in patients suffering from PAH with a low glucose control [58].

3. Inflammation in Pulmonary Hypertension

The inflammatory response is implicated in the development of PH, particularly in PAH subtype due to diverse molecular pathologies [59]. Perivascular inflammation precedes pulmonary vascular lesions. Endothelial injury favors the participation of chemokines involved in the recruitment of inflammatory cells and intravascular infiltration. Chemokine (C-X3-C motif) ligand 1 (CX3CL1/Fractalkine) serves as a cell adhesion molecule that acts through its own receptor (CX3CR1) and promotes the recruitment of monocytes, dendritic cells (DCs), mast cells, and subpopulations of T-cells [60,61]. In this regard, distinct chemokines have been found to be increased in PAH patients. For instance, CX3CL1 is upregulated in circulating CD4+ and CD8+ T-lymphocytes [60]. Chemokine (C-C motif) ligand (CCL) 5 (CCL5/RANTES) is also augmented [62], and is responsible for regulating the activation of T-cells and neutrophils [63,64]. Moreover, CCL2/MCP-1 was found to be elevated in patients with PH and activates macrophages to induce the expression and secretion of adhesion molecules and other cytokines [65]. In addition, CX3CR1 deficiency reduces monocyte recruitment and macrophage polarization in hypoxia-induced PH [66]. Circulating inflammatory cells, such as DCs and mast cells, recruited by the above-mentioned chemokines are directed to sites of endothelial injury. In IPAH patients, vascular lesions exhibit immature DCs infiltration [67]. DCs may be implicated in the presentation of antibodies against endothelial cells, fibroblasts, and naive T-cells. Infiltration of T lymphocytes, and particularly CD3+ and CD8+ T cells, has been observed in the lungs of patients with PAH [68,69]. Furthermore, regulatory T cells (Treg) can modulate the endothelial function of the pulmonary artery, inflammation, and SMC proliferation [70]. Studies have shown that patients affected by IPAH present a higher proportion of circulating Treg than healthy subjects [71]. Although mast cells mainly play an essential role in allergic inflammation, these cells are also found to be increased in patients with PH [72]. Mast cells degranulation release interleukin (IL)-4, which stimulates B cells to secrete anti-endothelial cell antibodies contributing to hypoxic PVR and PH [73].

Cytokines released from recruited inflammatory cells mediate communication between endothelial and other vascular cells, e.g., VSMCs. In PH, increased levels of IL-1, IL-6 and tumor necrosis factor (TNF)-α are exhibited [74–76]. It has been suggested that the heightened levels of IL-1 and IL-6 derives primarily from lung microvascular endothelium [77]. In a PH rat model induced with MCT, excessive amounts of IL-1 were found in the lungs [78]. Furthermore, elevated levels of IL-6 have been related with poor survival in patients with PH [79]. All these inflammatory alterations result in vascular remodeling, leading to increased pulmonary vascular pressures and resistance. In this context, mitochondria undergo physiological and structural changes during PH [80], and correspondingly ROS levels are altered [81,82].

Nuclear Factor κB (NF-κB) is a master regulator of inflammation implicated in the development of PH. NF-κB is a family of inducible transcription factors considered as the main controllers of innate immunity [83]. Five structurally related family subunits have been identified: p50, p51, RelA (p65), RelB, and c-Rel. The activation of these family members depends on the degradation of the inhibitor of NF-κB proteins (IκBs), which hold inactive NF-κB dimers in the cytosol upon the stimulation of determined cells.

Two major signaling pathways have been described for NF-κB. The canonical pathway involves the release of the p65/p50 subunits from the IκB complex, promoting the translocation of the heterodimer to the nucleus [84]. The activation of the canonical NF-κB signaling cascade induces the expression of several genes encoding pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6 and other inflammatory mediators, such as anti-apoptotic factors, cell cycle regulators, and adhesion molecules [85–87]. On the other hand, the noncanonical (alternative) NF-κB pathway does not require the degradation of inhibitory IκB complex, but the processing of p100, the protein precursor of p65 [85].

Regarding the role of NF-κB in PH, Sawada et al. in 2007 reported that the stimulation of NF-κB, leading to the activation of the vascular cell adhesion molecule (VCAM)-1, is related to the development of MCT-induced PH in rats. Moreover, the use of the NF-

κB inhibitor, pyrrolidine dithiocarbamate (PDTC), decreases PH symptoms [88]. Later, Huang et al. using the same animal model demonstrated that PDTC restores endothelial cell membrane integrity by rescuing caveolin-1, leading to PH [89]. Likewise, the inhibition of NF-κB with PDTC has proven to be effective in decreasing arterial lumen obliteration in SU5416-induced PH [90]. A novelty technology implemented by Kimura et al., based on a decoy directed against the NF-κB binding site in the promoter region, attenuated inflammation, proliferation, and pulmonary artery remodeling in rats. The implemented nanotechnology may serve as an advanced molecular approach for the treatment of PAH patients [91]. Moreover, Hosokawa et al. demonstrated that IMD-0354, a NF-κB inhibitor, blocks p65 translocation to the nucleus and decreases the proliferation of PASMCs associated with PH [92].

Interestingly, the role of NF-κB in a hypoxia-induced PH model has been investigated as well. It is well known that chronic hypoxia can lead to apoptosis, vascular remodeling and ultimately PH [93]. Hypoxia-inducible factor (HIF)-1α augments its transcriptional activity in response to oxygen decline in the lung [94]. In this context, Luo et al. demonstrated that NF-κB mediates the transcriptional program of HIF-1α promoting vascular remodeling in a PH model [95]. Eventually, it has been shown that the abnormal activity and regulation of NF-κB exacerbate the inflammatory and Ca^{2+} responses in PASMCs from PAH patients [96].

4. Mitochondria in Vascular Remodeling during PH

The mitochondria of the vascular smooth muscle cells (VSMCs) and PAECs, as in any other cells, are responsible for the synthesis of ATP, the key energetic molecule, thus a strict control of metabolism is exerted by these double-membrane-bound organelles [97]. In addition, mitochondria take an important place in the production and regulation of ROS, Ca^{2+} signaling, metabolism of glucose and FA, and apoptosis. These mechanisms are essential players in the development of PVR seen in PH disease [49]. During PH, a disfunction in mitochondria's metabolism occurs, particularly a shift in energy production from OXPHOS to glycolysis and lactic acid fermentation in order to maintain ATP production and cell survival [98]. This phenomenon, known as the Warburg effect, was described in 1956 by Otto Warburg in tumor cells under normal oxygen conditions to support the uncontrolled growth of neoplastic tissue [99,100]. Hyperproliferation, survival and metabolic reprogramming of PASMCs and PAECs set the basis for the pathophysiology of PH [101,102]. Moreover, mitochondria in PASMCs sense the oxygen levels, and patients suffering from PH display abnormalities in this mechanism [103]. Furthermore, PVR involving structural changes in intima, media and adventitia is linked to a marked inflammatory process in pulmonary hypertension [49,104]. However, the precise mechanisms underlying this relationship are still uncertain and mitochondrial dysfunction may serve as an explanation.

Alterations in mitochondrial respiration can lead to variations in mitochondrial membrane potential (MMP) [105]. MMP has shown to be either hyperpolarized [106–108] or depolarized [109] in PH models. Mitochondrial uncoupling proteins (UCPs) participate in the control and regulation of MMP and ROS production. Five UCP homologues have been characterized in mammals (UCP1-UCP5) [110]. In this regard, Pak et al. showed that the genetic ablation of UCP2 promotes the proliferation of PASMCs in mice. Additionally, they found that MMP and ROS production are increased in PASMCs from patients and in animal models of MCT- and hypoxia-induced PH [80]. Recently, it has been shown that heat-shock protein 90 (HSP90), in response to stress, accumulates in the mitochondria of PASMCs from PH patients to protect mitochondrial DNA (mitDNA) and preserve mitochondrial functions leading to cell survival. Moreover, the inhibition of mitochondrial HSP90 (mtHSP90) diminishes mitDNA content, restores mitochondrial bioenergetics and limits the hyperproliferative state of PASMCs [111].

As well as mitochondrial dysfunction, endoplasmic reticulum (ER) stress is implicated in PH pathophysiology, and an interesting interplay between mitochondrial and ER stress drives some aspects of this disease. For instance, the loss of function of bone

morphogenetic protein receptor type II (BMPRII) has been shown to induce ER stress, being a critical genetic factor predisposing to PAH [112]. Restoring BMPRII performance and the abolishment of ER stress have been remarkable suggested as a potential treatment against PH [113,114]. Mitochondrial fragmentation complemented with ER stress have been observed in PASMCs from hypoxic-induced PH rats [115]. Moreover, the same work exhibits that mitochondrial fragmentation promotes ER stress through a ROS dependent mechanism and the abolishment of ER stress improves PASMCs function under hypoxic condition. More interestingly, the mitochondrial division inhibitor (Mdvi-1) decreases mitochondrial fragmentation, ER stress and improves PASMC performance [115]. It is known that ER stress can cause unfolded proteins to accumulate in the ER and then activate the unfolded protein response (UPR). The persistent UPR conduces to the dysfunction of mitochondria accompanied by the disturbance of mitochondria-associated ER membrane (MAM), ultimately leading to cell apoptosis [116,117]. In this context, it has been observed that S-nitroso-L-cysteine (CSNO), a derivative of NO, improves ER stress and regulates the expression of contractile smooth muscle proteins in the lungs of MCT-induced PH rats. CSNO also leads to smooth muscle relaxation via anti-inflammatory pathways, ameliorating PVR [22]. Furthermore, the disruption of MAMs diminishes mitochondrial aberrations in ECs under hypoxic stimulus through an augment in NO release and the inhibition of the proinflammatory profile induced by hypoxia [118]. Lastly, mitochondria and ER morphology and dysfunctions in PASMCs and PAECs arise as novel potential therapeutic targets for the treatment of PH, although further research is needed.

5. Mitochondrial ROS in Pulmonary Vasoconstriction and Endothelial Dysfunction

Variations in [ROS]i in pulmonary vascular cells play a role in the pathogenesis of PH. Oxidative stress and ROS signaling in PASMCs are involved in PA vasoconstriction and remodeling and, therefore in the development and progression of PH [119]. It is well established that mitochondria account for the most production of ROS in PASMCs [9,120]. Mitochondria are considered an important factor in PVR due to their participation in numerous proliferative signaling pathways, such as regulating ROS production, ATP balance, apoptosis, metabolism of glucose and FA, or controlling Ca^{2+} homeostasis. In particular, ROS produced by mitochondrial complexes I, II and III have been suggested to play an important role in the development of PH. For instance, genetic deletion of the core subunit of mitochondrial complex I, NADH dehydrogenase (ubiquinone) iron–sulfur protein 2 (NDUFS2), was reported to decrease mitROS (H_2O_2) production and abolish hypoxia-induced pulmonary vasoconstriction (HPV) in mice and rats [121]. In this context, HPV is known to redirect blood flow from hypoxic to better ventilated areas of the lung. Moreover, HPV and arterial occlusion are important causes of PH as they decrease blood flow and increase vascular resistance. HPV appears to be mediated in part by an increase in intracellular Ca^{2+} and ROS signaling. [122]. In addition, Paddenberg et al. showed that Succinate dehydrogenase (ubiquinone) cytochrome b small subunit, (SDHD), is necessary for optimal functioning of the mitochondrial complex II and for HPV [123]. Furthermore, the mitochondrial complex III, and in particular the Rieske iron–sulfur protein (RISP), has shown to be required for mitochondrial ROS production in PASMCs [7,124,125]. Interestingly, our research group has shown that RISP is essential for the development of chronic hypoxia-induced PH. Knockdown of this sulfur protein in vivo reduces HPV and abolishes the hypoxia-induced increase in right ventricular pressure and the increase in right ventricular weight [5]. The balance between mitochondrial fusion and fission is essential for the physiology of this organelle. The role of dynamin-related protein 1 (DRP1) in mitochondrial fission may contribute to the disruption of ECs function and hyperproliferation of VSMCs involved not only in PH. The aggressive ROS environment triggers DRP1 signaling and mitochondrial fission, eliciting the enhancement of ROS production (ROS-induced ROS generation) [126]. On the other hand, the nuclear factor erythroid 2-related factor 2 (Nrf2) is one of the systems involved in the regulation of

antioxidant genes and mitochondrial fission. In this regard, it has been shown that the activation of Nrf2 prevents PVR by blocking endothelial-to-mesenchymal transition [127].

6. Mitochondrial Ca^{2+}, ROS, and Glutaminolysis

The homeostasis of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is crucial to maintain the vascular tone. At rest, basal $[Ca^{2+}]_i$ is tightly regulated to be around 100 nM. After cellular stimulation with a vasoconstrictor agonist, such as norepinephrine, endothelin, vasopressin, etc., $[Ca^{2+}]_i$ increases reaching values between 500 nM and 1 mM [128].

Dr. Wang's research group and other investigators have demonstrated that ROS facilitates the dissociation of FKBP12.6 from ryanodine receptor 2 (RyR2) to activate the channel [125,129–131]. Moreover, we have demonstrated that Rieske iron–sulfur protein (RISP) knockdown (KD) abolishes the hypoxic ROS formation in isolated PASMCs, whereas RISP overexpression produces the opposite effect. RISP KD also inhibits the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs [124,132]. Most recently, we showed that the dissociation of the FKBP12.6/RyR2 complex (induced by chronic hypoxia) causes sarcoplasmic reticulum (SR) Ca^{2+} leak and increases $[Ca^{2+}]_i$ in PASMCs (Figure 1), thereby leading to subsequent pulmonary artery remodeling and vasoconstriction. These events may occur due to the mitochondrial RISP-dependent ROS generation and the subsequent RyR2 oxidation [5,9]. Furthermore, FKBP12.6 is also specifically bound to big-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in VSMCs. These channels as well as RyRs are essential proteins in mediating vascular smooth muscle tone.

Mitochondrial Ca^{2+} uniporter (MCU) regulates mitochondrial Ca^{2+} ($mitCa^{2+}$) allowing Ca^{2+} uptake. We have reported that Ca^{2+} release mediated by hypoxic or RyR simulation evokes an improved performance in the activity of MCU. The increased MCU leads to the generation of mitROS dependent on ROS, provoking a positive feedback mechanism to potentiate hypoxia-initiated mitROS in PASMCs [9]. This finding indicates an important role of mitROS and MCU in HPV and associated PH. Alterations in ROS production can alter the physiology of ion channels in PASMCs and induce a large increase in $[Ca^{2+}]$i [133]. Mitochondrial ROS production (after hypoxia) block voltage dependent K^+ (K_V) channels [134]. Expression of $K_V1.2$, $K_V1.5$, and $K_V2.1$ channels is reduced in human and animal models of PH [135,136] and in PASMCs following a hypoxic stimulus [137]. Inhibition of these channels causes membrane depolarization and opening of voltage dependent Ca^{2+} channels (VDCCs) with a subsequent large increase in $[Ca^{2+}]$i and vasoconstriction [138].

Glutaminolysis is a mitochondrial process responsible for obtaining cellular energy from the deamination of glutamine to glutamate by glutaminase (GLS1) [139]. Subsequently, glutamate is converted to α-ketoglutarate (α-KG) by glutamate dehydrogenase. This process (anaplerotic reactions) helps to replenish the intermediates of the TCA cycle after they have been consumed and provides energy especially for proliferating cells. The increase in glutaminolysis leads to increased expression of GLS1 and increased uptake of glutamine by the pulmonary vasculature, resulting in increased glutamate production by pulmonary vascular cells and promoting PH. In addition to glutamate accumulation, the N-methyl-d-aspartate receptor (NMDAR) is overexpressed and overactivated in remodeled pulmonary arteries [140]. Moreover, stiffening of the extracellular matrix of vessels directly regulates glutaminolysis via mechanical activation of Yes-associated protein 1 (YAP) and TAZ. Activation of the former transcriptional coactivators triggers upregulation of GLS1 and leads to glutaminolysis, which maintains the hyperproliferative state and migration of pulmonary vascular cells in PH (Figure 2) [141]. As for PH, the relationship between ROS and glutaminolysis has not been studied. However, glutaminolysis has been shown to trigger the formation of ROS and make hyperproliferative cells (cancer cells) sensitive to ROS [142]. Whether glutaminolysis leads to the formation of ROS in pulmonary vascular cells is unknown and represents an interesting area of research.

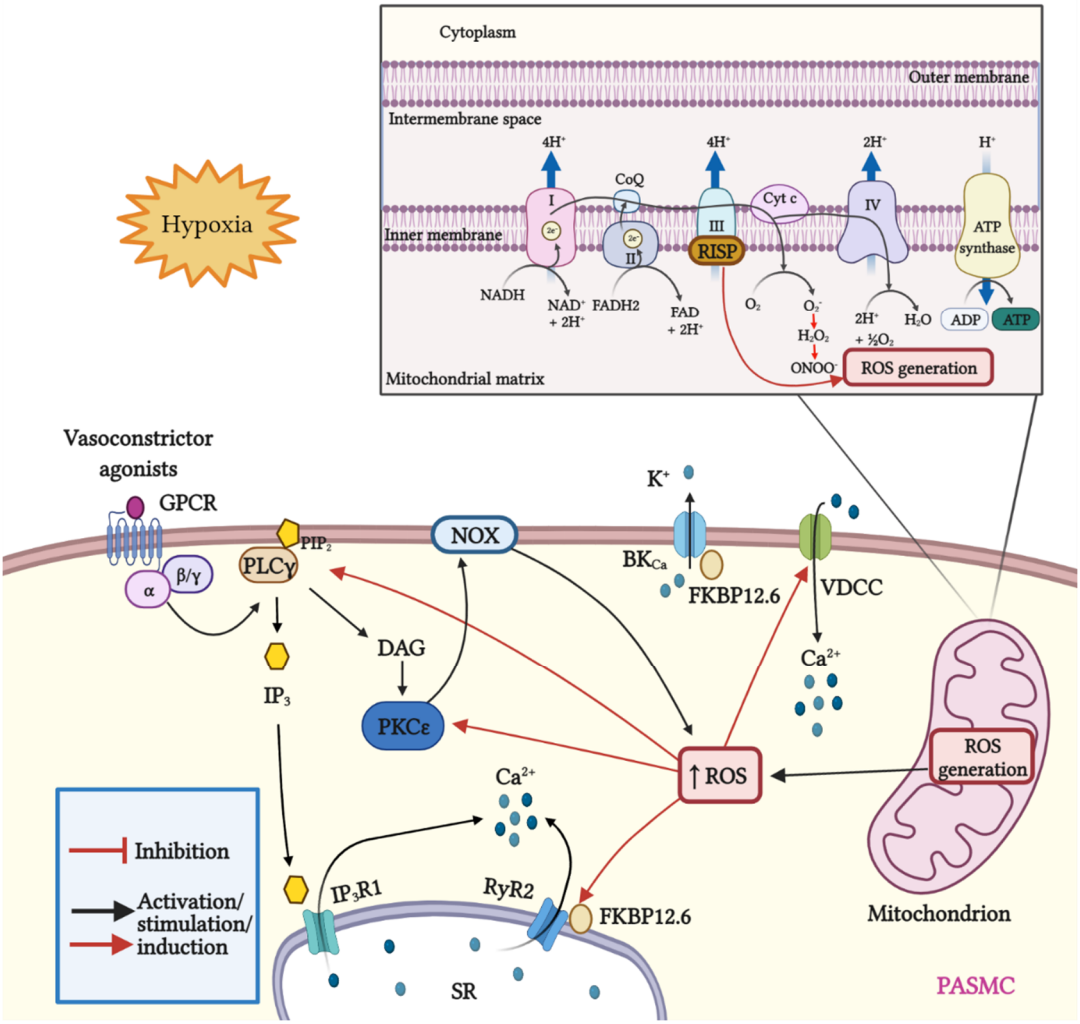

Figure 1. Schematic representation of mitROS generation and signaling; crosstalk between ROS and Ca^{2+} signaling in PASMCs. Mitochondria are the major source of ROS in pulmonary artery smooth muscle cells (PASMCs). During ATP synthesis in the electron transport chain (ETC), coupling between the proton gradient on either side of the inner mitochondrial membrane leads to the production of ROS. Briefly, electrons are transferred from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) to molecular oxygen. In this process, protons are pumped from the mitochondrial matrix into the intermembrane space, and oxygen is reduced to H_2O. Hypoxia increases the production of mitROS, contributing to the increase in $[Ca^{2+}]_i$ and to hypoxia-induced pulmonary vasoconstriction. The Rieske iron–sulfur protein (RISP), a catalytic subunit of the complex III of the mitochondrial ETC serves as a primary molecule in intracellular ROS generation in PASMCs, especially under hypoxic conditions. In addition, mitROS and vasoconstrictor agonists stimulate the PLCγ and PKCε signaling pathways via GPCR activation. PLCγ induces the formation of IP_3 and DAG, causing the opening of IP_3R1 and the release of Ca^{2+} from the sarcoplasmic reticulum (SR). Moreover, mitROS augment the activity of PKCε, which in turn stimulates NOX and promotes the formation of ROS in a process named ROS-induced ROS generation (RIRG). In addition, ROS enable the dissociation of FK506 binding protein 12.6 (FKBP12.6) from ryanodine receptor 2 (RyR2) favoring the opening of this channel and enhancing Ca^{2+} release. Furthermore, FKBP12.6 is physically bound to high conductance K^+ channels (BK_{Ca}) and regulates their open probability. Finally, ROS upregulate voltage-dependent Ca^{2+} channels (VDCCs) which further contribute to the increase in $[Ca^{2+}]_i$, leading to persistent vasoconstriction observed in PH.

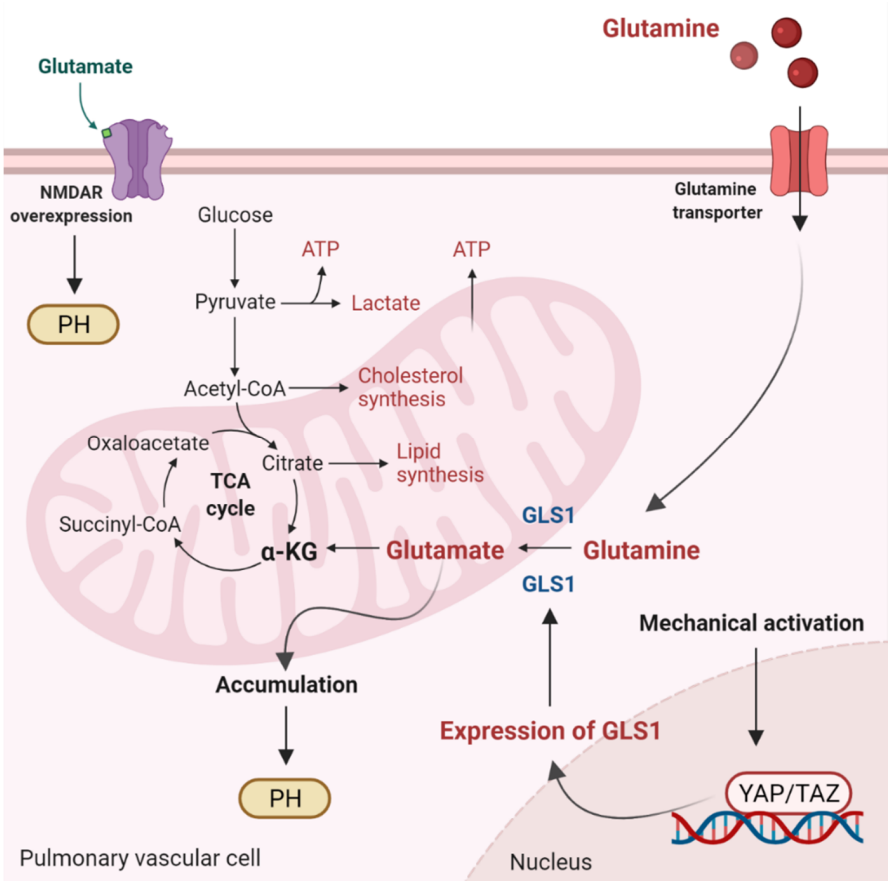

Figure 2. Glutaminolysis and glutamate accumulation contribute to pulmonary hypertension (PH). Glutaminolysis is a mitochondrial process responsible for obtaining cellular energy from the breakdown of glutamine. In this cellular pathway, glutamine is converted into glutamate, aspartate, CO_2, pyruvate, lactate, alanine, and citrate. Initially, glutamine enters the pulmonary vascular cells via a glutamine transporter and is deaminated to glutamate by glutaminase (GLS1). Subsequently, glutamate is converted to α-ketoglutarate (α-KG) by glutamate dehydrogenase. α kg enters the tricarboxylic acid (TCA) cycle, where it is decarboxylated by α kg dehydrogenase to succinyl-CoA and CO_2, providing energy for proliferating cells. Accumulation of glutamate in pulmonary vascular cells promotes PH. In addition, stiffening of the extracellular matrix in remodeled pulmonary cells activates the transcriptional coactivators Yes-associated protein 1 (YAP) and TAZ, leading to upregulation of GLS1 and enhanced glutaminolysis. Furthermore, in remodeled pulmonary arteries, the N-methyl-d-aspartate receptor (NMDAR) is overexpressed and overactivated.

7. Ketones and Mitochondrial Signaling

Ketone bodies, or simply ketones, are highly polar molecules produced by β-oxidation of fatty acids in the mitochondria of the liver cells. However, these molecules may be produced by enterocytes, astrocytes, and kidney ECs to a lesser extent [24,26,143]. Ketones are produced in response to reduced glucose availability, e.g., during periods of prolonged fasting, high-performance exercise, or a pathophysiological state, such as type I diabetes [144,145]. It has been postulated that patients with PAH have reduced oral glucose tolerance and lipid and ketone metabolism predominate over the glucose control [58].

Ketone's metabolism is divided in ketogenesis and ketolysis. Ketogenesis, which mainly occurs in perivenous hepatocytes, produces three molecules: acetone, acetoacetate, and β-HB [146], which represents the most abundant ketone body [147–149]. It is well known that adipocytes store great amounts of energy as fatty acids [150]. When fasting or exercising, glycogen stores are used in the beginning. Once glycogen is depleted, fatty acids from adipocytes are transferred into the liver by the enzyme carnitine palmitoyltransferase

(CPT-1), where they are metabolized in the mitochondria to form ketone bodies [151,152]. These lipid derivatives enter the systemic circulation and reach highly metabolic tissues, e.g., muscles and nervous system, which convert ketones into acetyl coenzyme A (acetyl-CoA) for alternative energy metabolism. The detailed process occurs as follows: two molecules of acetyl-CoA are biotransformed in acetoacetyl-CoA by the action of the acetyl-CoA acetyltransferase (ACAT), a thiolase [153]. Then, 3-hydroxy-3-methylglutaryl Co-A synthase (HMGS2) condenses acetyl-CoA with acetoacetyl-CoA to produce HMG-CoA. Afterward, HMG-CoA is broken into acetoacetate via HMG-CoA lyase (HMGCL). Finally, acetoacetate is further bioconverted to acetone (by decarboxylation) or to β-HB by the action of 3-hydroxybutyrate dehydrogenase (BDH1) [148].

On the other hand, throughout ketolysis, acetoacetate and β-HB are used as an energy source by the mitochondria of several extrahepatic tissues [148,154]. B-HB is transformed to acetoacetate by the BDH1, and this last product is turned into acetoacetyl-CoA by the enzyme beta-ketoacyl-CoA transferase (OXCT1 or SCOT). Acetoacetyl-CoA is separated by thiolase in two molecules of acetyl-CoA [155], that enters the TCA, and subsequently the oxidative phosphorylation, resulting in the generation of ATP [148,156]. Importantly, acetone (the other ketone body) cannot be biotransformed into acetyl-CoA and ends up eliminated through urine or exhaled [157,158].

It is well understood that glucose is an essential contributor for the precise energetic balance in VSM [159]. However, in 1981, Chace et al. demonstrated in PASM from rabbit that the absence of extracellular glucose increases the oxidation rate of β-HB, highlighting the role of ketones as energy substrates for PASMCs. Moreover, when other substrates such as β-HB, palmitate, leucine, glutamine and isoleucine are present in the external medium, glucose accounts for barely 5% of O_2 consumption [160]. The authors also found that ketone bodies may supply for the 8–16% of the O_2 consumption in the VSM.

High glucose levels may elicit the over production of ROS, mainly through NOX4 activity in endothelial cells [161]. In addition, in these cells, hyperglycemia triggers NF-κB signaling pathway leading to the upregulation of proinflammatory cytokines and endothelial adhesion molecules [162]. The induction of endothelial selectin (E-selectin), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and increased mononuclear-endothelial adhesion in addition to ROS generation and NF-κB activity increase vascular permeability and facilitates endothelial barrier dysfunction [162]. Amusingly, red and white wine pomace products (rWPPs and wWPPs) have been shown to reduce the expression of NOX4, thus diminishing ROS production in hyperglycemic endothelial cells [163]. Furthermore, rWPPs and wWPPs improve E-cadherin expression and E-cadherin cell–cell junctions in endothelial cells after INF-γ-induced barrier disruption. Remarkably, these results suggest the potential protective effects of rWPPs and wWPPs in vascular inflammatory ailments where endothelial barrier dysfunction play an essential role [163].

The role of ketones in the development of PH remains elusive. In this regard, the evidence indicates a relationship between the RVF occurring in PH and metabolic disorders (risk factors/comorbidities in human PH), such as high blood sugar [58], insulin resistance [164], dyslipidemia [165], and abdominal obesity [166]. Given these insights and the fact that RVF is the main determining factor in morbidity and mortality in PAH, it has been proposed (and studied) that FA metabolism and its byproducts (ketones) play an important role in RVF and PAH development [167,168]. Hereof, the expression of BDH1 and the oxidation of β-HB are augmented in a model of heart failure, highlight the importance of ketones metabolism in cardiac conditions [169]. Moreover, total blood and myocardial β-HB are increased in patients suffering from heart failure [170].

It is well known that 70–90% of ATP produced in heart comes from the oxidation of FA in mitochondria. The residual percentage is generated by the metabolism of glucose, ketone bodies and amino acids [171]. In this context, Peroxisome proliferator-activated receptor γ (PPARγ) has been shown to regulate glucose and FA metabolism in adipocytes, hepatocytes, skeletal muscle, and pancreatic β cells [172–174]. PPARs belong to the superfamily of nuclear receptors serving as ligand-activated transcription factors. This receptor subfamily

is composed of three members, PPARα, PPARγ and PPARδ, which combine with retinoid X receptors (RXRs) forming heterodimers and binding to specific DNA sites to promote genic transcription [173]. PPARγ is a master regulator of adipogenesis expressed mostly in adipose tissue and liver, as well as PPARα. PPARδ is ubiquitously expressed and all three subtypes are expressed in heart [175]. Emerging evidence highlights that PPARγ acts as a strong, protective regulator in PAH [176], PASMCs [177], and PAECs [178]. As it concerns, Legchenko et al., in a SU5416/hypoxia-induced PAH rat model, demonstrated that the oral administration of pioglitazone (a PPARγ agonist) fully abolishes severe PAH and vascular remodeling, and prevents RVF (Figure 3). Moreover, pioglitazone reverses vessel loss, cardiac hypertrophy, and fibrosis, and normalizes glucose uptake. In addition, the authors showed that, the PPARγ agonist augments mitochondrial fatty acids oxidation and ATP production, counteracting the inefficient metabolism of mitochondria characteristic in PAH (Figure 3). To accomplish its function, pioglitazone upregulates the expression of Cpt1b and Fabp4, proteins implicated in the oxidation of FA and their transport in cardiomyocytes, respectively [179].

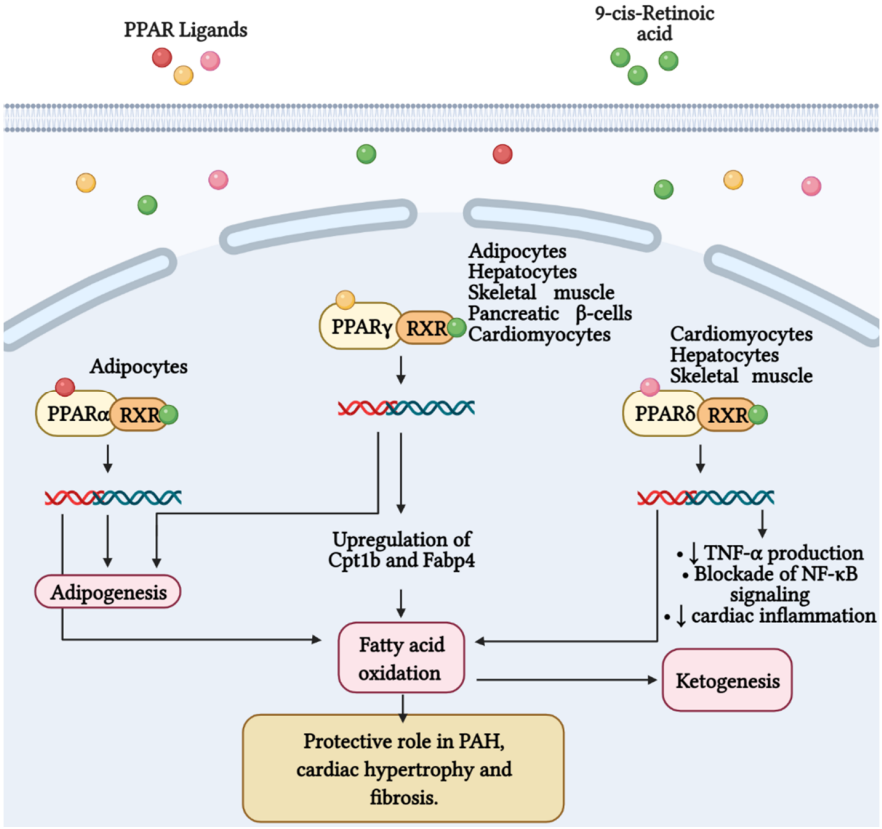

Figure 3. Fatty acid metabolism and its byproducts (ketones) play an important role in the development of right ventricular failure (RVF) and pulmonary arterial hypertension (PAH). Peroxisome proliferator-activated receptors (PPAR) belong to the superfamily of nuclear receptors that serve as ligand-activated transcription factors and consist of three members, PPARα, PPARγ, and PPARδ. These receptors together with retinoid X receptors (RXR) form heterodimers and bind to specific DNA sites to promote genetic transcription. PPARα is a master regulator of adipogenesis expressed mainly in adipose tissue and liver, as is PPARγ. Additionally, PPARγ regulates glucose and fatty acid metabolism in adipocytes, hepatocytes, skeletal muscle, and pancreatic β-cells. PPARγ agonists, such as pioglitazone, increase the expression of Cpt1b and Fabp4, proteins involved in fatty acid oxidation and transport in cardiomyocytes. These effects favor mitochondrial fatty acid oxidation and ATP production, leading to reversal of cardiac hypertrophy, fibrosis, and eliminating severe PAH. Furthermore, PPARδ stimulates fatty acid oxidation, decreases right ventricle hypertrophy and pulmonary congestion. In cardiac inflammation, PPARδ blocks nuclear factor κB (NF-κB) activation and inhibits tumor necrosis factor (TNF)-α synthesis.

Furthermore, the activity of PPARδ in cardiac metabolism has also been studied. It has been observed that the agonism of this PPAR subtype stimulates fatty acid oxidation [180,181]. Moreover, Ding et al. showed that synthetic ligand of PPARδ (GW0742) abolishes the production of TNFα induced by the stimulation with lipopolysaccharide (LPS) in cardiomyocytes. The authors also found that PPARδ signaling blocks the LPS-induced degradation of IκBs and the subsequent activation of NF-κB. These insights suggest the important role of PPARδ in cardiac inflammation [182]. In addition, genetic ablation of PPARδ in mice leads to the downregulation of essential genes implicated in fatty acids oxidation, highlighting a lipotoxic cardiomyopathy [183]. Most recently, it has been demonstrated that the stimulation of selective PPARδ diminish hypertrophy in the RV and pulmonary congestion in a rodent model of congestive heart failure [184].

Emerging evidence indicates that ketone bodies, and in particular β-HB, at low concentrations, potentially contribute to ameliorating endothelial and vascular function in metabolic disease, while elevated concentrations of ketone bodies as observed in diabetic ketoacidosis contribute to the diabetic vasculopathy and diabetic vascular complications. In mammals, β-HB decreases the senescence associated secretory phenotype (SASP) and the senescence of vascular cells. The homeostasis of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is crucial to maintain the vascular tone. At rest, basal $[Ca^{2+}]_I$ is tightly regulated to be around 100 nM. After cellular stimulation with a vasoconstrictor agonist, such as norepinephrine, endothelin, vasopressin, etc., $[Ca^{2+}]_i$ increases, reaching values between 500 nM and 1 mM [128].

8. Conclusions

Mitochondria are involved in essential cellular regulatory and homeostatic process in the cardiovascular system and particularly in VSMCs. Mitochondrial dysfunction has been widely associated with several diseases including PH. These organelles exert a strict control of ROS and ketones production. Uncontrolled ROS generation and its crosstalk with Ca^{2+} signaling have been shown to contribute and aggravate pulmonary hypertension. Thus, anti-ROS therapies targeting implicated proteins such as RISP should be investigated as novel alternatives for the treatment of this ailment. On the other hand, ketone bodies seem to offer a protection against oxidative stress damage in vitro and in vivo; however, they also may exert anti-inflammatory or pro-inflammatory roles in the cardiovascular system, and further research is needed to understand their different roles. Collectively, a better comprehension of the unique roles of RISP-dependent mitochondrial ROS and their specific interactions with mitochondrial Ca^{2+} signaling, NF-κB-mediated inflammatory responses, and ketone-associated oxidative stress can significantly improve our understanding of the molecular pathogenesis of PH and associated RVF. This new knowledge may also help to develop and implement innovative therapies in the treatment of PH and other vascular diseases.

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Abbreviations

AcAC—acetoacetate; ACE—angiotensin-converting enzyme; Ang II—Angiotensin II; AT1—angiotensin II type 1 receptor; ATP—adenosine triphosphate; βHB—beta-hydroxybutyrate; BK_{Ca}—large conductance K^+ channel; BMPRII—bone morphogenetic protein receptor type II; CAT—catalase; $[Ca^{2+}]_i$—intracellular Ca^{2+} concentration; Ca_V—voltage dependent Ca^{2+} channel; CH—chronic hypoxia; CoQ—Coenzyme Q/ubiquinol-cytochrome-c reductase; Cyt—cytochrome; DAG—1,2-diacylglycerol; DNA—deoxyribonucleic acid; EC—endothelial cell; ER—endoplasmic reticulum; ET-1 endothelin-1; ETC—electron transport chain; FAD—flavin adenine dinucleotide; FADH—flavin adenine dinucleotide semiquinone; FADH2—flavin adenine dinucleotide hydroquinone form; FKBP12.6—FK506 binding protein 12.6; FOXO3A—forkhead box O3; GPCR—G protein coupled receptor; HCAR2—hydroxy-carboxylic acid receptor 2; HDAC—class I histone deacetylases; HIF-1—hypoxia-inducible factor 1; HPV—hypoxia-induced pulmonary vasoconstriction; HUVEC—human umbilical vein endothelial cells; ICAM-1—intercellular adhesion molecule 1; IH—intermittent hypoxia; IPAH—idiopathic pulmonary arterial hypertension; IP_3—Inositol 1,4,5-trisphosphate; IP_3R—Inositol 1,4,5-trisphosphate (receptor); LDL—low density lipoprotein; MCP-1—Monocyte chemoattractant protein; SOD—superoxide dismutase; KD—ketogenic diet; MAM—mitochondrial associated membrane; MAPK—mitogen activated protein kinase; MCU—mitochondrial Ca^{2+} uniporter; mitROS—mitochondrial reactive oxygen species; NADH—nicotinamide adenine dinucleotide; NADPH—nicotinamide adenine dinucleotide phosphate; NF-κB—nuclear factor kappa B; NOX—NADPH oxidase; OXPHOS—oxidative phosphorylation; PAH—pulmonary artery hypertension; PAEC—pulmonary artery endothelial cell; PASM—pulmonary artery smooth muscle; PASMC—pulmonary artery smooth muscle cell; PDK1—gene coding for pyruvate dehydrogenase kinase 1; PDTC—pyrrolidine dithiocarbamate; PG—prostaglandin; PHD—prolyl hydroxylase; PKCε—protein kinase C-epsilon; PLC—phospholipase C; PPARγ—peroxisome proliferator activated receptor-gamma; PPARδ—peroxisome proliferator activated receptor-delta; PVR—pulmonary vascular remodeling; RISP—Rieske iron–sulfur protein; ROS—reactive oxygen species; $[ROS]_i$—intracellular ROS concentration; RVF—right ventricular failure; RyR—ryanodine receptor; SR—sarcoplasmic reticulum; TCA—tricarboxylic acid; TNF-α—tumor necrosis factor-alpha; TRP—transient receptor potential channels; UCP—mitochondrial uncoupling protein; VCAM-1—vascular cell adhesion protein-1; VDCC—voltage dependent Ca^{2+} channel; VSM—vascular smooth muscle; VSMC—vascular smooth muscle cell.

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