UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO
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IMPLICACIÓN DE ANGIOTENSINA II EN LA PROGRESIÓN A ENFERMEDAD RENAL CRÓNICA (ERC) COMO CONSECUENCIA DE LA LESIÓN RENAL AGUDA.

TESIS
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RESUMEN

Antecedentes: La lesión renal aguda (LRA) es una complicación frecuente en los pacientes que se someten a cirugía cardíaca mayor ó en los que presentan hemorragia, deshidratación, shock séptico, diabetes mellitus, o que reciben medicamentos nefrotóxicos o medios de contraste. A pesar de los avances en las estrategias preventivas, la LRA continúa con alta morbilidad y mortalidad. A pesar de la recuperación clínica de un episodio LRA, existe un riesgo inminente de que estos pacientes progresen a enfermedad renal crónica (ERC) y los mecanismos involucrados son poco conocidos.

Objetivo: Establecer si el bloqueo de los receptores AT1 de angiotensina II antes del insulto isquémico puede ser efectivo en prevenir o reducir la severidad de la LRA y con ello la transición de la LRA a ERC, así como estudiar los posibles mecanismos que median la renoprotección.

Metodología: Primero se estudiaron 34 ratas Wistar macho con un peso entre 250-300 g y se dividieron en cuatro grupos: 1) ratas con operación simulada, n = 8, (S); 2) las ratas con operación simulada y que recibieron de losartán (50 mg/kg/día por sonda orogástrica) tres días antes de la cirugía, n = 8, (Los); 3) ratas que fueron sometidos a isquemia renal bilateral durante 45 min, n = 9, (UTxI), y 4) el grupo que recibió losartán 3 días antes de la isquemia renal bilateral, n = 9, (Los-Pre). Estos animales se observaron durante nueve meses. En otra serie de experimentos, se incluyeron 72 ratas que se dividieron en los grupos S (n=21) UTxI (n=26) y Los-pre (n=26) y se estudiaron en cuatro períodos diferentes de: 1, 3, 5 o 15 días después de la cirugía simulada o de isquemia.

Resultados: Nueve meses después de la LRA, las ratas Wistar desarrollaron ERC que se caracterizó por disfunción renal, proteinuria, hipertrofia renal,

glomeruloesclerosis, atrofia tubular y fibrosis tubulointersticial. La LRA se asoció con un aumento en el estrés oxidante, la inflamación renal, la actina de músculo liso α- (αSMA) y la activación del factor de crecimiento transformante β (TGF). La sobre-expresión de TGF-β se observó principalmente en las células epiteliales. Interesantemente y a pesar de que la administración de losartán previa a la lesión isquémica no impidió o redujo la severidad de la LRA, fue eficaz de prevenir la progresión a ERC. Tres días después de que ocurrió la LRA, la disfunción renal, el daño epitelial tubular y la elevación de biomarcadores urinarios de daño renal persistieron. Aunque, el grupo que recibió losartán profilácticamente tuvo una lesión renal similar, la hipoperfusión renal fue completamente restaurada tan pronto como al tercer día. Además, hubo una activación temprana de factor inducible por hipoxia α (HIF1α) y un aumento del factor de crecimiento endotelial vascular (VEGF).

Conclusiones: Nuestros resultados muestran el efecto nocivo de la activación de los receptores AT1 durante un insulto isquémico y su impacto sobre la función y la estructura renal a largo plazo. Los mecanismos por los que, el antagonismo de los receptores de AT1 de angiotensina II impidieron la transición de la LRA a ERC fueron en parte mediados por la rápida recuperación del flujo sanguíneo renal, la menor inflamación, la mayor translocación nuclear de HIF1α y el aumento del VEGF.

INTRODUCCIÓN

Anatomía y Fisiología renal

El organismo humano produce continuamente productos finales de los procesos metabólicos, generalmente estos productos no tienen otras funciones y son peligrosos a altas concentraciones. Algunos ejemplos de esto son: la urea (producto final del metabolismo de las proteínas), el ácido úrico (de los ácidos nucleicos), la creatinina (de la creatina del músculo), etc. Esta función primordial de desecho la realiza el riñón, órgano par, ubicado en la parte posterior del abdomen, retroperitonealmente.

Ambos riñones comprenden menos del 0.5% del peso corporal total, pero reciben alrededor del 20% del gasto cardíaco, a través de la arteria renal, rama directa de la arteria aorta. Este importante aporte de oxígeno es necesario para que los riñones desarrollen su principales funciones: excreción de productos tóxicos, regulación de la presión arterial, mantener el pH sanguíneo, la producción de la hormona eritropoyetina, la absorción de nutrientes, la concentración de la orina (preservando ó no el agua, según las necesidades del organismo) y la regulación de la homeostasis de iones importantes como el sodio, el cloro, el potasio, el calcio, el fósforo y el magnesio[1] [2,3]. Todo esto es posible, en gran medida por el proceso de filtración a nivel glomerular y la secreción y reabsorción de iones y sustancias a nivel tubular. Para entender cómo el riñón realiza todas estas funciones, debemos comprender también su anatomía.

La unidad funcional básica del riñón es la nefrona. Cada riñón contiene 800,000 a 1,200,000 nefronas. Cada nefrona está constituida por la región glomerular y la región tubular. El glomérulo es un conjunto de vasos sanguíneos a partir de los cuales se origina el filtrado del plasma, que pasa hacia el túbulo, estructura epitelial que se subdivide en diferentes segmentos, los cuales contribuirán mediante absorción y secreción de diferentes substancias para dar lugar a la formación de la orina.

Al realizar un corte longitudinal al riñón se pueden identificar dos secciones: la corteza (región externa granular) y la médula (región interna más oscura). La granularidad que se observa en la corteza es debido a la presencia de los glomérulos y de túbulos contorneados. La médula carece de glomérulos y consiste de un arreglo paralelo de túbulos y pequeños vasos sanguíneos [2].

Figura 1.

A **B**

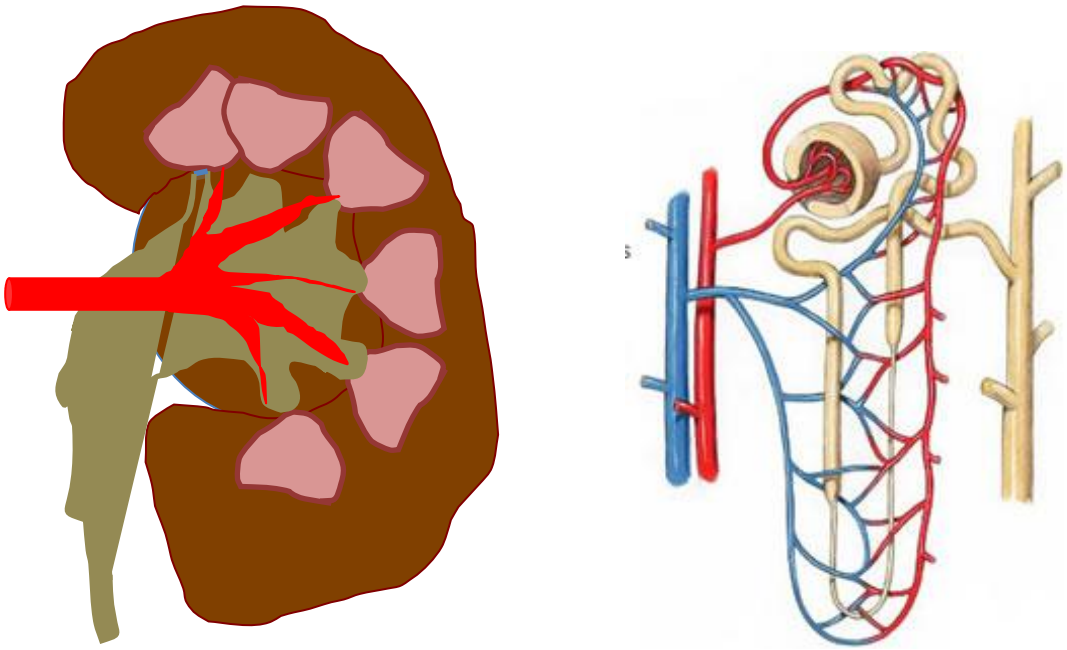
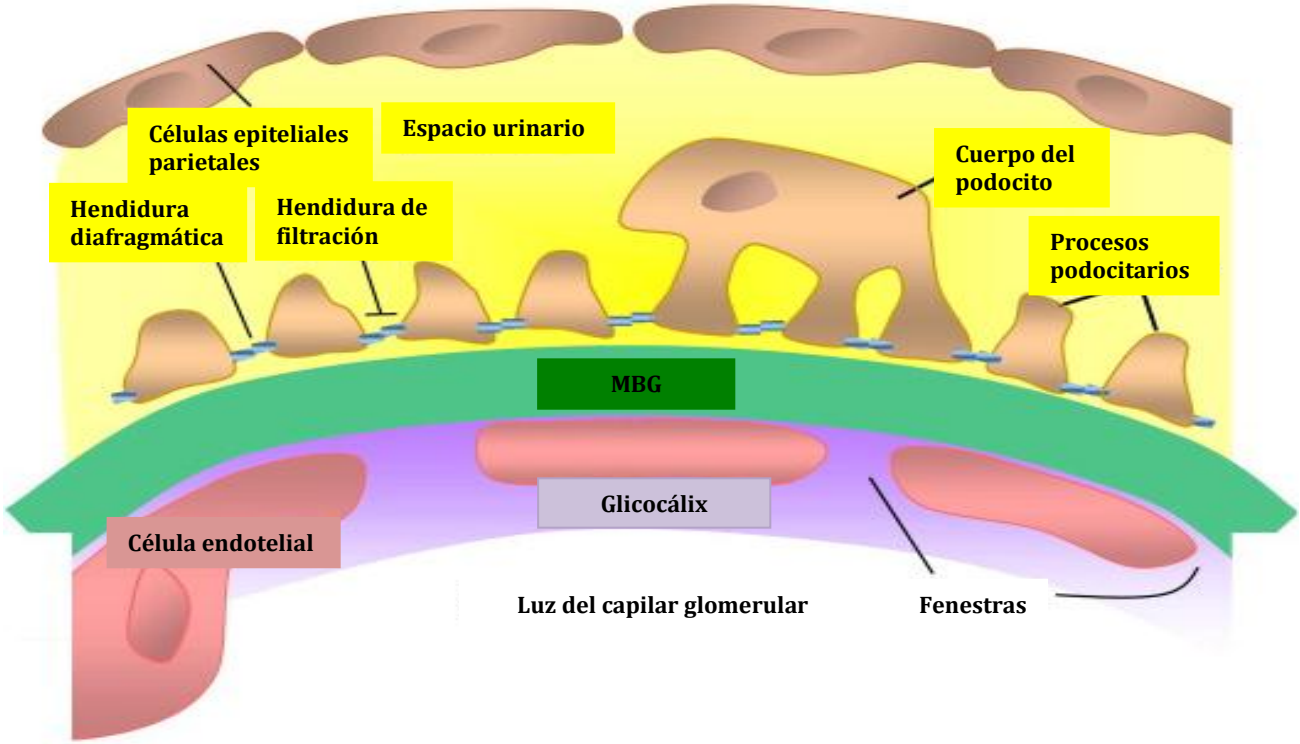


Figura 1. Relación estrecha entre la anatomía y fisiología renal. A) Anatomía del riñón, se identifica la corteza y la médula renal. **B)** Esquema de la anatomía e irrigación de la nefrona (glomérulo y sistema tubular). Imagen tomada de: http://umm.edu/programs/transplant/services/kidney/http://www.goldiesroom.org/Note%20Packets/13%20Human%20Other/02%20Human%20Other%20Systems--lesson%202.htm

Figura 2.



Membrana basal glomerular. Se describen los elementos que conforman a la membrana basal glomerular: endotelio, membrana basal y epitelio visceral (podocitos). MBG: membrana basal glomerular. Imagen tomada de: http://www.ajkd.org/article/S0272-6386(11)00908-5/fulltext

Dentro del glomérulo, el epitelio tubular y el endotelio llegan a un punto ciego llamado cápsula de Bowman o cápsula glomerular. **Figura 2**. Esta cápsula rodea al glomérulo y contiene al espacio de Bowman que se continúa con la luz tubular, sitio a donde pasa el filtrado glomerular. Este filtrado lleva a cabo un recorrido por las diferentes secciones del epitelio tubular: el túbulo proximal, el asa descendente y el ascendente delgada de Henle, el asa ascendente gruesa de Henle, el túbulo contorneado distal y el túbulo conector, después del cual se da origen al túbulo colector inicial, túbulo colector cortical y medular.

El túbulo proximal es un segmento muy rico en mitocondrias y una superficie apical ciliada, lo que, le permite una mayor actividad metabólica, absorbe el 60% del agua que pasa por la luz tubular, así como, la glucosa, los aminoácidos y los iones

importantes para la homeostasis electrolítica. Lo anterior es posible gracias a la presencia de la bomba de Na/K ATPasa localizada en la membrana baso lateral de la célula epitelial, la cual, permite el paso de sodio en y al mismo tiempo, la excreción de potasio en contra de su gradiente osmótico, eventos que dan origen a un gradiente tanto osmótico como electroquímico, ambos permisivos para el paso de agua junto con aminoácidos y la glucosa hacia los capilares peri tubulares, que son formados a partir de la arteriola eferente, quienes van a ser el principal aporte de oxígeno para las células del epitelio tubular. El resto de segmentos tubulares contribuye al procesamiento del filtrado glomerular para obtener como producto final la orina.

Los segmentos tubulares contribuye al procesamiento del filtrado glomerular para obtener como producto final a la orina. Un sistema sin duda importante para esto y para la regulación del volumen intracelular, así como, para mantener el flujo sanguíneo renal en situaciones de hipoperfusión, es el sistema renina angiotensina aldosterona (SRAA).

Sistema Renina Angiotensina Aldosterona, SRAA

El SRAA se activa cuando existe una caída en el flujo sanguíneo renal, lo que produce una reducción en la llegada de cloruro de sodio a la mácula densa, sistema especializado de células tubulares ubicado en la parte terminal de el asa ascendente de Henle que transmiten la señal a las células vecinas endoteliales de la arteriola aferente, lo que a su vez, induce que se libere la renina, enzima que realizará un corte proteolítico al angiotensinógeno (proteína producida en el hígado) dando origen a la angiotensina I, que puede ahora ser procesada por la enzima convertidora de angiotensina, para dar origen a la angiotensina II, sustancia que promoverá entre otras cosas, la vasoconstricción de la arteriola eferente, dando como resultado el aumento de la presión

hidrostática glomerular y así una recuperación del filtrado glomerular.

Existen diversas funciones que ejerce la activación del SRAA en los diferentes tejidos, como el cerebro, el tejido adiposo, el sistema gastrointestinal y el cardio vascular. Sin embargo, es en el riñón donde la Angiotensina II ejerce sus principales funciones como regular: el contenido de líquido corporal, la presión sanguínea), la hemodinámica intra renal y la filtración glomerular. La Angiotensina II estimula la secreción de la hormona antidiurética en la glándula pituitaria con el incremento en la reabsorción de agua en el conducto colector y también incrementa la secreción de aldosterona, una hormona esteroidea sintetizada principalmente en la corteza adrenal y un mediador rio abajo de la Angiotensina II que induce la reabsorción de sodio y la excreción de iones hidrógeno y potasio por el riñón [3].

Casi todas funciones de la angiotensina II, como la vasoconstricción, liberación de aldosterona, estimulación del transporte tubular de sodio, efectos pro inflamatorios, así como acciones profibrogénicas y estimulantes del crecimiento, son mediadas por el receptor AT1. Estudios clínicos y experimentales sobre los efectos de los BRA y estudios en ratones knockout para el receptor AT1 han evidenciado su participación en la progresión de la enfermedad renal crónica [4].

Una gran parte de las acciones de la angiotensina II son llevadas a cabo por la activación de sus receptores AT1 y AT2. Aunque también se han clonado otros dos receptores, el receptor Mas para Ang-(1-7) y el receptor AT4 para Ang IV [5]. Específicamente, el AT1 es un receptor heptahelicoidal acoplado a proteína G. Cuando un agonista se une a este receptor, se liberan múltiples respuestas celulares, predominantemente vía el acoplamiento a las proteínas $G_{q/11}$, y también a las proteínas $G_{12/13}$, y $G_{i/o}$ en los roedores. La señalización por $G_{q/11,}$ que es mediada por inositol fosfato/$Ca2^{+}$, es el mecanismo primario de transducción iniciado por angiotensina II en

sus principales tejidos blancos, incluyendo glándulas adrenales, células renales, cardíacas y de músculo liso [6]. Todo esto ha llamado la atención no solo por las implicaciones fisiológicas, sino también, por las implicaciones fisiopatológicas estudiadas a lo largo de los últimos años.

La función de los receptores AT2 es menos clara, pero se ha propuesto que tienen acciones antagónicas a los receptores AT1, promoviendo un feedback negativo. En línea con esta noción, la activación del receptor AT2 ha mostrado disminuir la presión sanguínea, inhibir el crecimiento celular, inducir la diferenciación celular y mediar la apoptosis. Estas acciones parecen estar mediadas por la activación dependiente de proteína G y la activación independiente de fosfatasas de tirosina y parece involucrar una fosforilación reducida y además reforzar el estatus de activación de los elementos de señalización clave de las vías de sobrevida y crecimiento, como: Akt/PKB y ERK1/2. No obstante, el impacto de los receptores AT2 sobre los procesos de crecimiento celular e inflamación es controversial todavía [4,6].

Actualmente se considera que el SRAA asume los mecanismos de acción parácrino, autócrino e intracrinos en la señalización hormonal. Muchos tejidos y células, incluyendo los riñones, tiene todos los componentes necesarios del SRAA para formar angiotensina II *in situ* [7].

Barrera de filtración glomerular

La barrera de filtración glomerular está constituida por 3 capas: los procesos podocitarios, quienes cubren la cara externa a los capilares glomerulares, la membrana basal glomerular y el endotelio de los capilares glomerulares, cuya superficie está cubierta por glicocálix. Las células endoteliales de los capilares glomerulares están rodeados por la membrana basal glomerular y una capa de los procesos podocitarios.

Los podocitos son células epiteliales modificadas y representan la capa visceral de la cápsula de Bowman. Inician en el polo vascular y se continúan con la capa parietal de la capsula de Bowman, es decir, son continuos con la capa parietal de la cápsula de Bowman.

La membrana basal tiene una importante contribución para las características de la permeabilidad de la barrera de filtración. Tanto el diafragma como la membrana basal están compuestas por una red de proteínas, de tal forma que la membrana basal contribuye a la selectividad de la barrera de filtración, la integridad del diafragma es esencial para prevenir una fuga excesiva de proteínas del plasma (albúmina).

Las células endoteliales de los capilares glomerulares están rodeadas por la membrana basal glomerular y una capa de los procesos podocitarios. Las células endoteliales contienen fenestraciones grandes de 70 nm, espacios que no proveen restricción para el movimiento de agua y solutos pequeños, incluyendo proteínas u otras moléculas más grandes, fuera de la luz de los capilares. El glicocálix consiste de glicosaminoglicanos cargados negativamente, lo cual ayuda a prevenir la fuga de macromoléculas cargadas negativamente. Así, las células endoteliales ayudan a limitar la filtración de los elementos celulares, por ejemplo los eritrocitos, Figura 4, [2,3].

Una vez que se logra pasar la barrera de filtración glomerular, el plasma filtrado llegará a los túbulos renales, cuya principal función es recuperar la mayoría de los fluidos y solutos filtrados en el glomérulo. Si el fluido no fuera recuperado, el riñón excretaría el volumen total de plasma sanguíneo en menos de media hora. La recuperación de la mayor parte del filtrado glomerular ocurre en el túbulo proximal, el cual reabsorbe el cloruro de sodio (NaCl), el bicarbonato de sodio (NaHCO3), los nutrientes filtrados como: la glucosa, los aminoácidos, los iones divalentes ($Ca^{2}+$, HPO_4^{2-}, y SO_4^{2-}), y el agua.

El asa de Henle es conformada por tres secciones: el asa delgada descendente

de Henle [tDLH], el asa ascendente delgada de Henle [tALH] y el asa gruesa ascendente de Henle [TAL]) y su función principal es la de participar en la concentración de la orina. El asa de Henle lleva a cabo esta función al permitir la salida de NaCl hacia el intersticio de la medula, sin la salida de una proporción igual de agua, produciendo así, un intersticio hipertónico. Más abajo, hacia la médula, los conductos colectores aumentan esta hipertonicidad, ya sea por permitir o no el flujo de agua por osmosis hacia el intersticio hipertónico. En los humanos, solo el 15% de las nefronas son yuxtamedulares, es decir, poseen asas descendentes más largas que llegan a la punta de la papila, a diferencia de las corticales que son más cortas. Sin embargo, esta subpoblación de nefronas es extremadamente importante para crear gradientes osmóticos dentro de la papila que permiten el movimiento de agua fuera del lumen de toda la población de conductos colectores medulares. Como consecuencia de este movimiento de agua, la osmolaridad urinaria en los conductos colectores puede exceder de la que hay en el plasma. Las células del TAL secretan la glicoproteína Tamm-Horsfall (THP). Los sujetos normales excretan 30 a 50 mg/día de esta proteína hacia la orina, contribuyendo, junto con la albúmina (<20 mg/día), a la mayor parte de la proteína presente normalmente en la orina. El THP contribuye además a la matriz de los colindaros celulares, definidos como detritus cilíndricos en la orina que toman la forma de la luz tubular en el cual está formado [2].

El túbulo distal y el sistema de conductos colectores llevan a cabo el control fino de la excreción de agua y del NaCl. Aunque solo pequeñas fracciones del filtrado glomerular llegan a estos sitios distales de las nefronas, es en estos segmentos donde actúan varias hormonas como: la aldosterona y la arginina vasopresina conocida también como la hormona antidiurética.

Bajo condiciones normales, la tasa de filtración glomerular total de los dos

riñones es de 125 ml/min o 180 L/día. Tal cantidad es requerida para que todo el líquido extracelular pueda ser expuesto frecuentemente, más de 10 veces/día, a la revisión del epitelio tubular. Si no fuera tal cantidad, solo pequeñas fracciones del volumen sanguíneo serian aclaradas o depuradas de ciertos solutos por unidad de tiempo.

Una forma de medir la tasa de filtración glomerular (TFG) es a través de la depuración de creatinina. La creatinina es un producto generado de la degradación de la la creatina muscular que es un ácido orgánico nitrogenado que se encuentra en los músculos y que es derivado de los aminoácidos. Dado que es una sustancia que prácticamente se filtra toda, se ha usado como una herramienta para determinar la TFG de una persona. Sin embargo, es importante tomar en cuenta que, una parte es secretada por el epitelio tubular y que sus concentraciones en sangre van a variar de acuerdo a la composición y actividad muscular, la edad o el sexo del paciente.

Lesión renal aguda (LRA)

Definición, etiología y epidemiología de Lesión Renal Aguda

La lesión renal aguda es un síndrome que se presenta durante la caía abrupta y transitoria del flujo sanguíneo renal, lo cual provoca alteración en la función del riñón y con frecuencia se acompaña de oliguria [8]. Es una condición seria causada por múltiples y diversas etiologías y está asociada con un incremento temprano y a largo plazo en la morbilidad y mortalidad, así como, con el desarrollo subsecuente de enfermedad renal crónica (ERC) [9], de la transición de ERC a enfermedad renal terminal (ERCT), eventos cardiovasculares y muerte prematura [10,11].

La KDIGO (Kidney Disease Improving Global Outcome) define a la LRA cuando existe un incremento en la creatinina sérica mayor a 0.3 mg/dl en 48 horas; ó cuando el incremento en la creatinina sérica es mayor que 1.5 veces vs. la medición basal, en un

tiempo de 7 días; ó cuando el flujo urinario es menor que 0.5 ml/kg/h en 6 horas [12,13].

La incidencia de la LRA en la población general se encuentra entre 2,147 a 4,085 por millón de habitantes por año (pmp) [14,15]. Estudios recientes realizados en hospitales de países con alto ingreso económico [16-18], reportaron que la incidencia de la LRA se encuentra entre el 3.2 al 9.6% de las admisiones, con una mortalidad global intrahospitalaria alrededor del 20%, mientras que, en las unidades de cuidados intensivos (UCI), la incidencia es de hasta el 60%. La LRA que requiere terapia de remplazo renal ocurre en el 5 al 6% de los pacientes en la UCI, con una elevada tasa de mortalidad, hasta de un 60% [19]. Se estima que cerca de dos millones de personas mueren a causa de la LRA cada año [17 19,20]. Una revisión sistemática de 312 estudios de cohorte, que incluyó un total de 49 millones de pacientes (la mayoría de los cuales eran países con alto ingreso económico), demostró que la LRA ocurría en 1 de cada 5 adultos y en 1 de cada tres niños hospitalizados [21].

La presencia de la LRA en pacientes hospitalizados, incrementa directa e indirectamente los costos al sistema de salud. La LRA está asociada con mayores cuidados, exámenes, estancias no planeadas u hospitalizaciones prolongadas, así como, con un incremento en el riesgo de re hospitalización temprana. En un estudio realizado en Estados Unidos que incluyó 52 pacientes que desarrollaron LRA se demostró un incremento de 6.5 veces en el riesgo relativo ajustado para morir, una estancia hospitalaria más prolongada (>3.5 días) y un costo adicional en la hospitalización de $9,000 dólares en comparación con los pacientes hospitalizados que no desarrollaron LRA. Los costos para la atención de la LRA rebasan las de condiciones más prevalentes como hospitalizaciones por falla cardíaca ($2,200), neumonía ($2,100) y sangrado gastrointestinal ($2,100)

[9,22,23].

La principal causa de la LRA es el fenómeno de isquemia reperfusión (I/R) , evento que se puede presentar tanto en los riñones nativos, como, en los trasplantados[24 25]. La LRA también es frecuente en los pacientes que son sometidos a cirugía cardiaca mayor (30%), que presentan shock séptico, hemorragia, deshidratación; diabetes mellitus o que son sometidos a infusión de medios de contraste [26 27 19,25],.

En países de bajo y mediano ingreso económico, la LRA tiene dos tipos de presentaciones. En áreas urbanas, la principal causa es la isquemia renal debida principalmente a sepsis y frecuentemente es asociada al uso de fármacos nefrotóxicos [28]. En las zonas rurales, la LRA generalmente es una condición que se adquiere en la comunidad, afecta a personas jóvenes e individuos sanos. Las causas especificas de la LRA incluyen enfermedades diarreicas con deshidratación, enfermedades infecciosas (malaria, dengue, fiebre amarilla, leptospirosis, tétanos y virus de la inmunodeficiencia humana), aborto séptico y medicinas naturales [29-31].

La mayoría de los factores que inician la LRA están asociados con pobreza, mala higiene personal, agua contaminada (enfermedades diarreicas), ausencia de educación y una adecuada urbanización.

En México poco a poco se empieza a generar información al respecto a la incidencia de la LRA. En el 2004 Piñon J y colaboradores reportaron una mortalidad hospitalaria del 52%, aunque no se especifica si la LRA fue la causa directa de la muerte o se presentó dentro de una falla orgánica múltiple. [32].

Según el informe de egresos hospitalarios del Sistema Nacional de Salud del 2002, la LRA es una de las principales causas de atención hospitalaria, ocupa el cuarto lugar en los hombres (55 033 casos) y el décimo en las mujeres (50 924 casos), lo que representa una tasa de 115.0 y 101.5 por 100 000 habitantes en hombres y mujeres

respectivamente

Por lo tanto, la LRA constituye un problema de salud pública por su frecuencia creciente, su asociación con graves complicaciones y altos costos, así como, elevada mortalidad a corto y a largo plazo.

Fisiopatología de la LRA

Como se mencionó anteriormente, la LRA es consecuencia de la caída en el flujo sanguíneo renal, lo cual, condiciona el desarrollo de hipoxia, que da lugar a la imposibilidad para que funcione la bomba de Na/K ATPasa, que a su vez, sufre una relocalización en la célula epitelial, desde la membrana basolateral a la porción apical de la misma. Lo anterior condiciona una pérdida del gradiente electroquímico, que imposibilita la absorción de las moléculas necesarias para el organismo.

Anteriormente, había opiniones divergentes con respecto a si eran las células epiteliales o las endoteliales las que sufrían alteraciones durante un evento de LRA. Actualmente se conoce que actúan como un binomio y que ambos tipos celulares son afectados durante un evento isquémico [33-35]. Las células endoteliales sufren varias alteraciones como lo son: la pérdida de sus uniones intercelulares, alteraciones en el citoesqueleto y en el glucocálix; lo que provoca un incremento en la permeabilidad de la microcirculación y edema intersticial. Esto a su vez conlleva a una alteración en el aporte de oxígeno, lo que intensifica el daño renal después de la caída del flujo sanguíneo. Simultáneamente se presenta un incremento en la expresión de moléculas de adhesión que permiten que las células inflamatorias se adhieran al endotelio con la consiguiente infiltración leucocitaria en el intersticio renal [8]. Estas alteraciones aunadas a la caída del flujo sanguíneo, ocasiona una disminución en la perfusión de los capilares peri tubulares, donde el segmento S3 del túbulo proximal es el más

afectado, debido al gran número de mitocondrias que posee [3]. Así mismo, la disminución de la producción del ATP en éstas células epiteliales produce un desacople en la cadena respiratoria con la subsecuente formación de radicales libres, como el peróxido de hidrógeno, la NADPH (Nicotinamida adenina dinucleótido fosfato) etc., lo que, contribuye al inicio de la muerte del epitelio tubular por apoptosis y/o necrosis, lo cual, va muy relacionado a la duración y gravedad del insulto isquémico[36] [37].

En el epitelio tubular se desencadena también un ambiente inflamatorio, propiciado en un inicio por el daño endotelial, en el cual, se aumenta la expresión de moléculas de adhesión, apertura de las uniones intercelulares, permitiendo el paso de células del sistema inmune hacia el espacio intersticial, como polimorfonucleares en un inicio y posteriormente linfocitos T y macrófagos. Todo esto conduce a la presencia de interleucinas pro inflamatorias, como la interleucina-6 (IL-6), la interleucina-18 (IL-18), la proteína quimio atrayente de monocitos (MCP-1) y el factor de necrosis tumoral alfa (TNF-α). Así como, una respuesta anti-inflamatoria, mediada por el factor de crecimiento transformante beta (TGF-β por sus siglas en inglés), las células T-cooperadoras y la producción de la interleucina-10 (IL-10), entre otras [35,38,39].

A nivel funcional esto se traduce en una caída de la tasa de filtrado glomerular, con elevación por ende de la creatinina sérica y la presencia de oliguria, acompañado o no con la presencia de proteínas en la orina. Este proceso se acompaña de la elevación de biomarcadores de daño tubular como son: la molécula de daño renal (Kim-1, por sus siglas en inglés), la proteína de choque térmico de 72 KDa (Hsp72, por sus siglas en inglés), la lipocalina de los neutrófilos asociada a la gelatinasa (NGAL, por sus siglas en inglés) y la interleucina 18 (IL-18), entre otros [40 41-44] o incluso requerir terapia de reemplazo renal, dada la falla de este órgano [42].

A nivel histológico se aprecia la perdida del borde en cepillo de las células epiteliales, la formación de cilindros en la luz tubular, consecuencia del conglomerado de la proteína Tamm-horsfall con el sodio y el agua que no pudieron ser reabsorbidos de la luz tubular, estos cilindros provocan una mayor congestión a nivel de la luz tubular. Además, existe presencia de infiltración de polimorfonucleares, principalmente neutrófilos en las etapas agudas de la LRA, con la llegada posterior de macrófagos y linfocitos T CD8+ [38,45].

Todas estas alteraciones son agravadas durante la reperfusión renal, la cual condiciona un mayor ambiente pro oxidante y pro inflamatorio [46,47]. Esto condiciona un desequilibrio entre las sustancias vasodilatadoras como, el oxido nítrico y las sustancias vasoconstrictoras como, la endotelina y de forma importante la activación del sistema renina angiotensina aldosterona (SRAA) [48-50].. El desequilibrio causado promueve en general una mayor vasoconstricción, la cual propicia aún más la disminución en el aporte de de oxígeno y de nutrientes al endotelio, ocasionando que el número de vasos en la médula externa disminuya e incluso desaparezcan después de la isquemia/reperfusión, fenómeno conocido como rarefacción capilar [33] [51,52]. El fenómeno de rarefacción es el causante de que se pierdan nefronas, lo cual, condiciona que en las nefronas remanentes se lleve a cabo una hiperfiltración compensatoria, condicionada por una elevación de la presión hidrostática intraglomerular, la cual contribuye al daño de la membrana basal glomerular y por ende a la pérdida continua de nefronas a lo largo del tiempo [53,54].

Por la participación tan relevante del endotelio demostrada a través de diversos estudios en años recientes, se ha investigado de forma importante el papel de el factor de crecimiento del endotelio vascular (VEGF) [55], dado su papel importante en la neovascularización y de esta forma evitar el ciclo continuo de hipoxia; inflamación;

fibrosis, consecuencia de un ambiente hipóxico continúo, que ahora sabemos, conduce al deterioro progresivo del epitelio tubular y a la generación de fibrosis túbulo intersticial a largo plazo [54,56].

Si el insulto isquémico es detenido a tiempo y manejado de forma oportuna, se podría revertir los procesos anteriormente mencionados y mejorar la regeneración del epitelio tubular a partir de las células epiteliales sobrevivientes y de la misma forma una recuperación en la función renal.

Tal y como se aprecia, durante la LRA, una serie de vías de señalización son activadas para reparar as estructuras afectadas. No obstante, aún se desconoce con exactitud los mecanismos por los que se da una proliferación celular des regulada , hipertrofia y una producción no controlada de matriz extracelular a largo plazo [33].

Transición de la Lesión renal aguda a enfermedad renal crónica

Anteriormente se especulaba que las personas que se recuperaban de un episodio de LRA, no tenían ninguna consecuencia posterior en la función y estructura renal, sin embargo, evidencia reciente basada en observaciones epidemiológicas en pacientes que sufrieron LRA indican que esto no es así. De hecho, se ha demostrado que la LRA es un factor de riesgo para desarrollar enfermedad renal crónica (ERC), refiriendo algunos autores un incremento en el riesgo de 8 hasta 28 veces si los pacientes necesitaron terapia de reemplazo renal [53], además de que puede promover la transición de ERC a ERC terminal (ERCT) [50,57-59 60,61]. También, se ha descrito que la probabilidad de desarrollar ERC o ERCT es proporcional a la severidad y a la duración de la LRA [62]. De igual forma, el riesgo de desarrollar ERC se ve aumentado cuando los pacientes tienen además una enfermedad renal pre existente [14,63]. En un estudio reciente se reportó que el 54% de los pacientes que presentaron un episodio

de LRA y que no requirieron terapia de reemplazo renal, pero que tampoco recuperaron por completo la función renal, tuvieron un gran impacto en la epidemiología global de la ERC y la ERCT [53]. Estas observaciones también se han visto en la población pediátrica, la cual presenta una elevada incidencia de LRA [64].

Aunado a esto, los estudios epidemiológicos muestran que la incidencia y la prevalencia de la ERC y de la ERCT ha aumentado considerablemente en las últimas tres décadas, al igual que los gastos generados en el cuidado de estos pacientes [65,66]. En Estados Unidos, el programa de ERCT ocupa el 6.7 % del total de los gastos médicos [57](28). Tomado todo en consideración, resulta indispensable el estudio de los mecanismos por los cuales un episodio de LRA puede desencadenar y conducir a la afectación de la función y la estructura renal en forma progresiva, así mismo, es de vital importancia encontrar maniobras farmacológicas que eviten la transición de la LRA a ERC.

Mecanismos involucrados en la transición de la Lesión renal aguda a Enfermedad renal crónica

Se han empezado a dilucidar algunos de los mecanismos que conducen a la transición de la LRA a ERC y que a continuación menciono.

Arresto celular

Cada fase del ciclo celular tiene funciones específicas que ayudan a una adecuada proliferación celular. Cuando las células se encuentran quiescentes, esta fase se conoce como fase 0. Pero, cuando empieza un proceso de reparación, las células deben de entrar a diferentes fases en un orden y tiempo determinado. Si las células se mantienen en una fase por mucho tiempo o salen demasiado pronto, el proceso de reparación y recuperación puede llegar a ser mal adaptativo. Cuando se inicia el arresto

celular, las células evitan el estrés que ocurre durante la división celular y por ende el daño, lo cual vuelve a este fenómeno un tanto protector. Pero, si las células no re inician el ciclo celular y permanecen en arresto celular en G1 o G2, es muy probable que se desarrolle un fenotipo fibrótico [67]. Como se ha expuesto previamente, en la fisiopatología de la LRA, el epitelio tubular tiene una participación muy importante en la regulación de su regeneración una vez que el episodio isquémico ha concluido, sin embargo, si el daño isquémico fue severo, es posible que el epitelio tubular se convierta en el iniciador y el perpetuador del daño a largo plazo con la consecuente progresión a ERC.

De hecho dentro de los mecanismos propuestos para el desarrollo de ERC [37,67-69] que ocurren posterior a un insulto isquémico, se encuentra el fenómeno de arresto celular del epitelio tubular, específicamente en la etapa G2/M del ciclo celular. Esto resulta en la activación de los mecanismos de reparación del DNA, lo cual da paso a la síntesis y secreción de factores profibróticos, como es el caso de TGFβ. El grupo del Dr. Bonventre [68], demostró que a mayor gravedad del insulto isquémico, mayor es la cantidad de células que se encuentran en este arresto celular. Estas células conocidas como senescentes tienen efectos importantes además de llevar a cabo la secreción de TGF-β, pues también indican una respuesta inflamatoria importante denominada como fenotipo secretorio asociado a la senescencia. Y también producen proteínas que favorecen el remodelamiento de la matriz extracelular, causando defectos en la diferenciación y crecimiento celular. La resultante secreción de TGF-β se asocia con un aumento en la expresión de JNK, que justo ocurre durante la fase de arresto del ciclo celular G2/M. Todo esto poco a poco ha llevado al estudio más a fondo de proteínas como p21 y p53 durante un evento isquémico, dado que son importantes reguladores del ciclo celular.

Transdiferenciación de fibroblastos a miofibroblastos y de epitelio a mesénquima

La característica principal de la fibrosis, es la acumulación extensa de matriz extracelular en el intersticio renal. El hallazgo histológico más temprano es la acumulación en el intersticio de fibroblastos con un fenotipo de miofibroblastos. Éstas, son células contráctiles que expresan la actina de músculo liso alfa (α-SMA por sus siglas en inglés) además de poseer fibras de estrés [70]. Uno de los mecanismos propuestos para esta acumulación, es la transdiferenciación epitelio mesénquima (TEM) (Strutz & Neilson 2003; Strutz 2009a,b).

La TEM, es un proceso, mediante el cual, las células epiteliales pasan por un proceso de cambios morfológicos importantes, donde pierden sus características epiteliales como, la polaridad y la expresión de marcadores de unión, e inducen la expresión de marcadores de fibroblastos como la proteína específica de fibroblasto (FSP-1 por sus siglas en inglés), vimentina y proteína actina de músculo liso alfa (α-SMA pos sus siglas en inglés). Una vez que las células comienzan a expresar fibras de estrés, migran a lo largo de la membrana basal para convertirse en miofibroblastos en el intersticio, expresando marcadores como lo es α-SMA, proteína que sólo se expresa en la superficie de los miofibroblastos [68,70-73]. En la génesis de este fenómeno, se encuentra el TGF-β, como citosina inductora del cambio fibroblastos a miofibroblasto, e incluso también en trabajos recientes se ha demostrado que las células endoteliales pueden también contribuir a este proceso de acumulación de fibroblastos en el intersticio [71].

La TEM ha sido estudiada durante la LRA y la progresión a ERC. Diversos trabajos han demostrado, que el ambiente post isquémico es un nicho de citosinas y moléculas, que promueven la TEM . Aunque, existen estudios que argumentan que este proceso no existe y que es la migración de células pluripotenciales desde la médula

ósea, las que sufren este proceso para dar origen a la población de miofibroblastos[74,75].

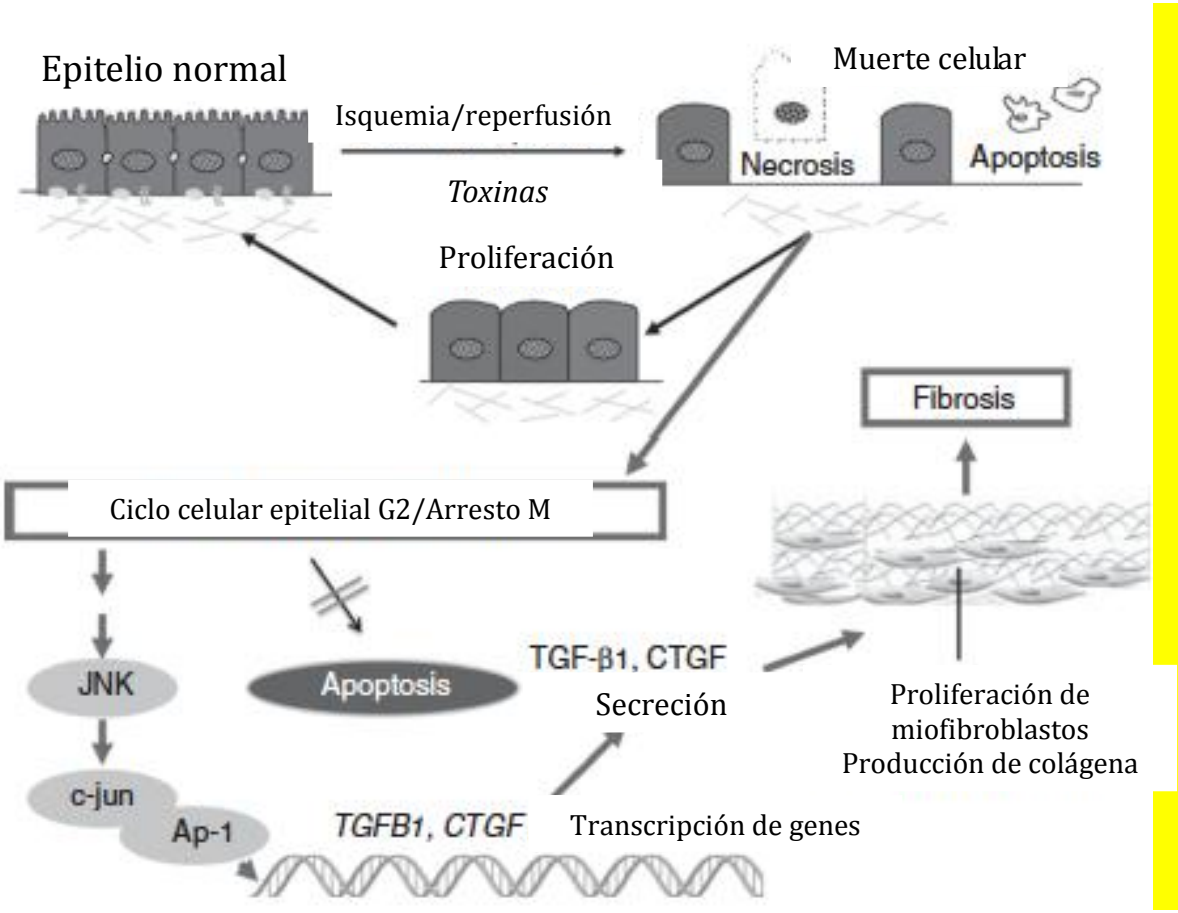


Figura 5. Ciclos de hipoxia-inflamación-daño en el ciclo de LRA a ERC. Se menciona que estos ciclos se repiten a lo largo de la progresión a ERC secundaria a un evento isquémico.

Rarefacción capilar e inflamación

Existe evidencia sobre la importancia que tiene la función endotelial alterada durante el daño renal isquémico, en la tardía recuperación del flujo sanguíneo renal. El

daño endotelial implica la pérdida de adhesión intercelular, así como, la alteración en la función de la barrera capilar. Esta última es importante porque puede participar en la baja perfusión renal al comprimir los capilares peri tubulares y exacerbar el atrapamiento de los eritrocitos. Además, la alteración en la adhesión celular, condiciona alteraciones en la coagulación y favorece aun más el proceso inflamatorio [76].

Uno de los mecanismos que esta tomando relevancia como propiciador de la transición de la LRA a a ERC, es la discrepancia entre la disponibilidad de oxígeno y la demanda del mismo, generando hipoxia crónica. Éste evento, propicia una serie de cambios estructurales y funcionales que pueden dar lugar a fibrosis. La hipoxia tubular es generada por la disminución en el flujo sanguíneo peri tubular, el estrechamiento de la luz de los vasos, la constricción vascular debido a la expresión modificada de factores vasoactivos y moléculas de señalización (endotelina, óxido nítrico, angiotensina II) y de forma relevante la rarefacción de los capilares peri tubulares [77]. Éste último fenómeno, se caracteriza por vasoconstricción permanente de los capilares peri tubulares, con una desaparición de los mismos, ocasionando el desarrollo de nefronas sin irrigación, condicionando la muerte de las mismas, con el desarrollo a la par de mayor inflamación y daño renal [78].

De hecho se han señalado tres posibles mecanismos que contribuyen al desarrollo de fibrosis y que están relacionados con esta pérdida de capilares: 1) exacerbación de hipoxia (un gran promotor de la fibrosis), 2) alteración en el control hemodinámico, principalmente en la médula, zona per se con baja tensión de oxígeno y 3) la proliferación de nuevos fibroblastos secundario a la transición epitelio mesénquima m[78,79].

Las bases celulares de la rarefacción de los capilares peritubulares aún no se conocen por completo. Algunos estudios señalan la importancia del proceso de

apoptosis. Sin embargo, independientemente del mecanismo causal de la rarefacción, tal parece que posterior a la LRA, este fenómeno suele ser permanente, lo cual sugiere que la capacidad de regeneración de los capilares peritubulares es muy limitada, aunado a la presencia de inflamación y de citosinas como TGF-β, con capacidades para una regulación negativa de la proliferación celular, pueden propiciar aún más este fenómeno [8,80].

Mecanismos para evitar el desarrollo de este fenómeno, han sido propuestos, tal es el caso de la participación del factor inducible por hipoxia 1 alfa (HIF-1α por sus siglas en inglés). HIF es un factor de transcripción que posee una subunidad sensible al oxígeno y una subunidad β expresada constitutivamente. HIF 1α y 1β son mediadores clave del proceso de adaptación celular a la hipoxia, entre otras cosas porque facilitan la sobrevida de las células al estimular la eritropoyesis. Además, regulan procesos biológicos importantes para la reparación celular, del tejido, pero también pueden participar en la fibrogenésis y la TEM [54].

El heterodímero de HIF activa la transcripción de genes en respuesta a la hipoxia al unirse a secuencias especificas del DNA, conocidas como elementos de respuesta a la hipoxia (HREs por sus siglas en inglés) y por reclutar co activadores transcripcionales como CBP/p300. HIF es continuamente sintetizado debido a que es degradado bajo condiciones de normoxia [81,82].

Aunque durante el proceso isquémico hay factores que regulan a la baja la regeneración vascular, también existen factores que la propician, como es el caso del factor de crecimiento del endotelio vascular (VEGF por sus siglas en ingles). Se ha mostrado que cuando se inhibe a HIF, la fase de recuperación post isquemia renal se prolonga y la disminución de este factor de transcripción, disminuye la recuperación de los capilares peritubulares [55,83,84].

Aunado a estas alteraciones vasculares, existe una disminución en la adhesión de las células endoteliales, lo que permite el paso de los leucocitos hacia el intersticio renal, en un inicio son principalmente neutrófilos y posteriormente leucocitos y macrófagos. Estas células endoteliales contribuyen a la congestión de los capilares peritubulares, dado que producen moléculas que afectan el tono vascular como las especies reactivas de oxígeno, o al liberar citosinas que contribuyen a dañar el parénquima renal [35,47,85,86].

Existe un desequilibrio entre el proceso inflamatorio y el anti inflamatorio, en el cual predominan las citosinas como el TNF-α, la proteína quimioatrayente de monocitos (MCP-1), IL-18, IL-6 y una menor cantidad de citosinas anti-inflamatorias como, la IL-10. Los macrófagos en un inicio ayudan a la recuperación renal, a través del fenotipo M2, sin embargo a largo plazo, en la progresión a ERC, parecen predominar el fenotipo tipo M1, de características pro inflamatorias, más que, de reparación.

Modificaciones epigenéticas

Actualmente, no es de sorprendernos el encontrar que la regulación epigenética puede participar en la transición LRA a ERC debido a las implicaciones que tiene en la adaptación celular en circunstancias extremas, como el estrés oxidante, hipoxia y daño mitocondrial [87].

Modificaciones en la estructura de cromatina

Durante un episodio de LRA, las células epiteliales tubulares están sujetas a un ambiente hipóxico, lo cual ocasiona cambios no solo en el metabolismo celular, sino también en la estructura de la cromatina y en la unión de diferentes factores de transcripción [88]**.** Se sabe también que existe un incremento en la expresión de citosinas pro inflamatorias como el TNF- α y la MCP-1 después de un episodio de LRA, el cual

persiste hasta los 7 días. Este efecto parece ser el resultado de la regulación epigenética, dado que existe un incremento en el complejo de remodelación multiproteínico de la cromatina que incluye el factor SWItch/Sucrose Non-Fermentable (SWI/SNF). Este complejo depende de una actividad ATP-asa de tipo helicasa y regula la estructura de la cromatina. Las ATPasas de este complejo son la maquinaria que permite los cambios dinámicos en la estructura de la cromatina, activando o no la expresión genética. Específicamente, el complejo humano SWI/SNF es también capaz de desdoblar los nucleosomas a lo largo del DNA, promoviendo los sitios de inicio de la transcripción y haciéndolos más accesibles para genes específicos. Este complejo, contiene el gen 1 relacionado con Brahma (BRG1, por sus siglas en inglés), que tiene una sub unidad de remodelamiento de cromatina ATPasa catalítica. En el ratón, BRG1 es un regulador de los complejos de remodelamiento de de nucleosoma en el gen TNF-α [89]**.** Hallazgos recientes han mostrado que existe también un incremento en la MCP-1, independiente de las causas de LRA.

Modelo animal para estudiar la transición de LRA a ERC

En nuestro laboratorio hemos desarrollado un modelo de LRA que conlleva al desarrollo de ERC. Después de recuperarse del episodio de LRA, los animales mostraron proteinuria progresiva, disfunción renal y alteraciones histológicas significativas, nueve meses después. El bloqueo de los receptores de mineralocorticoides con espironolactona antes de la agresión isquémica impidió el desarrollo de la LRA y por lo tanto, la progresión a ERC. Además, la administración de espironolactona de 1 a 3 horas después de la isquemia también impidió la transición de LRA a ERC, lo que implica la activación del sistema renina angiotensina aldosterona (SRAA) en la progresión a ERC después de un episodio de LRA. También observamos que la función renal se recuperó por completo 10

días después del episodio de LRA, pero los signos de inflamación en el riñón persistieron, lo que se asoció con la progresión a ERC a lo largo de los meses siguientes [90]. Debido al efecto de la angiotensina II, sobre la vasculatura renal y la inducción de inflamación, en el presente estudio se analizó en qué medida el bloqueo del receptor de la angiotensina II con losartán antes el insulto isquémico podría ser eficaz en reducir a severidad de la LRA y su impacto sobre la transición a ERC, después de que el episodio de LRA se resolvió.

Hipotésis

El bloqueo de los receptores de Angiotensina II antes de inducir una lesión renal aguda prevendrá la progresión a enfermedad renal crónica.

Objetivo general

Establecer si el bloqueo de los receptores AT1 de angiotensina II con losartán antes de inducir la lesión renal aguda puede disminuir la severidad del evento isquémico y/o prevenir la transición a ERC, así como, dilucidar los mecanismos responsables del efecto renoprotector de losartán.

Objetivos específicos

Determinar si la administración profiláctica de losartán reduce o evita la lesión renal aguda inducida por isquemia.

Estudiar si la administración de losartán antes de inducir la lesión renal aguda puede reducir o prevenir la transición a ERC.

Evaluar los posibles mecanismos de la renoprotección conferida por el losartán en la reducción ó prevención de la transición de la lesión renal aguda a a ERC.

Estudiar la influencia del antagonismo de los receptores AT1 de angiotensina sobre de la hemodinámica renal, la hipoxia y la inflamación antes de que el episodio de lesión renal aguda haya sido resuelto.

MATERIAL Y METODOS

Todos los experimentos y procedimientos que involucraron animales se llevaron de acuerdo a la Norma Oficial Mexicana NOM-062-ZOO y fueron aprobados por el Comité de investigación de en Animales de nuestras Instituciones.

Fase crónica

Se estudiaron 34 ratas Wistar macho con un peso entre 250-300 g y se dividieron en cuatro grupos: 1) ratas con operación simulada ó cirugía sham, n = 8, (S); 2) las ratas con operación simulada y que recibieron losartán (50 mg/kg/día por en el agua de beber tres días antes de la cirugía, n = 8, (Los); 3) ratas que fueron sometidos a isquemia renal bilateral de 45 min, n = 9, (UTxI), y 4) el grupo que recibió losartán 3 días antes de la isquemia renal bilateral también de 45 min, n = 9, (Los-Pre). Estos animales se observaron durante nueve meses.

Fase aguda

En otra serie de experimentos, se incluyeron 73 ratas que se dividieron en los grupos S (n=21) UTxI (n=26) y Los-pre (n=26) y se estudiaron en cuatro períodos de seguimiento diferentes: 1, 3, 5 o 15 días después de la cirugía simulada o de isquemia. Todos los animales fueron mantenidos en ciclos de luz/obscuridad 12:12 h y tuvieron libre acceso a agua y a comida.

Modelo de Isquemia/Reperfusión: Se utilizaron ratas Wistar macho de un peso entre 250 a 300 g. Las ratas se anestesiaron con pentobarbital sódico (30 mg/kg) y se colocaron en una cama termoregulada. Posteriormente se procedió a realizar una incisión sobre la línea media abdominal hasta llegar al peritoneo. La isquemia renal

bilateral se indujo al interrumpir el flujo sanguíneo en ambos riñones mediante la colocación de un clip en cada arteria renal durante 45 min, posteriormente, los clips se retiraron y se observó que ocurriera una adecuada reperfusión. Los animales fueron suturados de la pared abdominal y se dejaron evolucionar según el periodo establecido. Las ratas sometidas a cirugía falsa (Sham), se manipularon de la misma forma que las que fueron sometidas a I/R, pero sin la colocación de los clips.

Parámetros funcionales: Cada 30 días, las ratas se colocaron en jaulas metabólicas para recolectar la orina de 24 h y cada 90 días se obtuvo previa anestesia con éter, una muestra de sangre de la arteria ocular para determinar la creatinina sérica (CrS). La concentración de creatinina en suero y orina se determinó por medio del ensayo QuantiChrom creatinine kit (DICT-500); junto con la determinación de la creatinina en la orina se calculó la depuración de creatinina (CrCl).

La excreción de proteínas en la orina se determinó en la recolección de 24 horas realizada mensualmente por medio del método de turbidimetría con ácido tricloroacético.

Después de 270 días de realizada la isquemia de 45 minutos o de los tiempos estipulados previamente, las ratas se anestesiaron con pentobarbital sódico (30 mg/kg) y fueron colocadas nuevamente en una mesa termoregulada. Se realizó el registro de la presión arterial media con un transductor de presión cada dos minutos durante 10 minutos (Model p23 db, Gould. Puerto Rico) y se capturó con un polígrafo (Grass Instruments, Quincy, MA). Posteriormente se realizó una incisión longitudinal en la línea media del abdomen, se disecó y expuso la arteria renal izquierda y se colocó una sonda de ultrasonido (1RB, Transonic, Ithaca, NY) alrededor de ésta arteria para realizar el registró del flujo sanguíneo renal cada dos minutos durante 10 minutos.

Estudios histopatológicos. Al finalizar el experimento, el riñón derecho se extrajo, fue separado en corteza y médula renal y se guardó a menos 80 grados centígrados para los posteriores estudios moleculares. El riñón izquierdo se perfundió a través de un catéter en la arteria femoral derecha con buffer de fosfatos, manteniendo la presión arterial media de cada animal, posteriormente se perfundió con formalina al 10% hasta que se complementó la fijación. Los riñones se sumergieron en parafina, se seccionaron a 4μ y se tiñeron con ácido periódico de Schiff (PAS) y rojo de sirio. Se evaluó el tamaño glomerular mediante la medición del diámetro glomerular; para ello se capturaron 10 campos de corteza renal de las ratas utilizando una cámara digital incorporada en un microscopio marca Nikon y se midieron al menos 500 glomérulos en las microfotografías digitalizadas por cada grupo. Se cuantificó el diámetro glomerular y los diámetros fueron agrupados en rangos con el fin de detectar la aparición de hipertrofia glomerular. Así mismo, se determinó el porcentaje de glomérulos con glomeruloesclerosis. En las secciones teñidas con Rojo de Sirio se capturaron 5 campos subcorticales de los riñones de diferentes grupos para evaluar el porcentaje de fibrosis túbulo intersticial mediante morfometría. La fibrosis túbulo intersticial consiste en expansión de la matriz extracelular, deposición de colágena junto con distorsión o colapso de los túbulos, por lo cual se delimitó el área afectada. El porcentaje de fibrosis se calculó al dividir el área fibrótica entre el área total excluyendo el área glomerular y el área de la luz tubular. Para evaluar la proliferación celular se realizó inmunohistoquímica para el antígeno nuclear de células de proliferación (PCNA) y la proteína ki67 (presente en el núcleo de células que replicando su DNA), se realizó el conteo de células positivas para PCNA y/o ki67 tanto a nivel tubular como a nivel glomerular. Todas estas determinaciones se realizaron mediante un análisis doble ciego.

Integridad de los podocitos y detección subcelular de nefrina por microscopía inmunoelectrónica: Para la detección subcelular de nefrina y la integridad de podocitos por microscopía inmunoelectrónica, se utilizó un fragmento de 1x1 mm de tres ratas por grupo estudiadas a los 9 meses, se fijó en paraformaldehído. Se colocaron secciones delgadas (70-90 nm) en rejillas de níquel. Las rejillas se incubaron durante la noche a 4 °C con un anticuerpo específico para nefrina (Abcam ab58968) diluido dos veces en PBS con 1% de albúmina de suero bovino, y 0,5% de Tween 20. Después de enjuagar con PBS, las rejillas se incubaron durante 1-h a temperatura ambiente con cabra anti-IgG de conejo (Sigma Chemical Co., St. Louis, MO) conjugado con partículas de oro 10 nm diluido 1/20 en PBS. Las rejillas se tiñeron con sales de uranio (Microscopía Electrónica de Ciencias, Fort Washington, PA) y se examinaron con un M-10 Zeiss microscopio electrónico (Karl Zeiss, Jena Alemania). La integridad de los podocitos así como los podocitos positivos para nefrina fueron evaluados en forma ciega en por lo menos 100 podocitos y las imágenes representativas fueron fotografiadas.

Microarreglos de tejido renal e inmunohistoquímica para TGFβ y HIF1 α Se construyeron dos microarreglos de tejido con 20 tejidos renales cada uno, embebidos en parafina, pertenecientes a los cuatro grupos estudiados, utilizando un equipo de microarreglos ATA-100 Chemicon semi automático (Advanced Tissue matriz de Chemicon). De cada tejido se realizaron rebanadas por triplicado y se deparafinizaron. La recuperación del antígeno se realizó usando citrato de sodio (0,01 M, pH 6,0). La actividad peroxidasa endógena se inhibió con metanol y peróxido de hidrógeno al 3%. Las muestras fueron bloqueadas por inmersión en solución de bloqueo universal y 1% de albúmina de suero bovino durante 60 min. Las rodajas de tejido se incubaron

durante la noche a temperatura ambiente con anti-TGF-β o anti-HIF1α (Santa Cruz, Santa Cruz, CA. EE.UU., Novus Productos Biológicos, Littleton, CO, EE.UU.). Después de lavar los portaobjetos, se incubaron con el enlace universal de anticuerpo biotinilado (Dako) y luego con estreptavidina conjugada con peroxidasa de rábano (HPR); el color se generó mediante la adición de DAB (diaminobenzidina). La reacción se detuvo, y las muestras se contra tiñeron con hematoxilina. Los tejidos se deshidrataron y las preparaciones se cubrieron con resina y se secaron a temperatura ambiente. Las secciones teñidas fueron digitalizadas con una ampliación de x40 que utiliza un ScanScope CS Aperio (Aperio, Vista, CA). Las imágenes fueron revisadas utilizando un ImageScope (Aperio) y fueron enviados a análisis de imágenes automatizado utilizando Spectrum Software (Aperio). Dentro de la intensidad de tejido, se desarrolló un algoritmo para cuantificar la expresión total de TGF-β ó HIF1α. La salida del algoritmo devuelve un número de mediciones cuantitativas, a saber, la intensidad, la concentración y el porcentaje de tinción positiva presente. Escalas cuantitativas de la intensidad y el porcentaje se clasificaron y se determinaron los valores de corte. La intensidad de la tinción se clasificó como 0 (sin manchas), 2+ (moderada) y 3+ (fuerte). La expresión total final se calculó a partir de una combinación de intensidad y porcentaje de puntuación.

Estudios Moleculares:

Niveles de RNAm de TGF-β, MCP-1, TNFα, IL-10, e IL-6

Extracción de RNA: El RNA se extrajo de los tejidos almacenados a -80ºC mediante homogenización con fenol y tiocianato de guanidina y ultracentrifugación con cloroformo. Para determinar la calidad del RNA se midió su concentración por espectrofotometría de UV (280 nm/260 nm).

RT-PCR (Transcripción Reversa): La transcripción reversa (RT) se llevó a cabo con 10 μg de RNA total del tejido. Primero se llevó el RNA a 65 ºC por 10 min. La reacción se realizó utilizando 200 U de transcriptasa reversa del virus de la leucemia en el mono (Moloney murine leukemia virus reverse transcriptase, MMLV, Stratagene), 100 pmol de hexámeros al azar (random primers, Life Technologies), 0.5 mM de cada dNTP (una mezcla de dCTP, dATP, dGTP, dTTP, Sigma), y 1X de buffer de TR (75 mM KCl; 50 mM Tris-HCl; 3 mM MgCl2; 10 mM DTT, pH 8.3), se incubó a 37ºC por 60 min y se llevó a un volumen final de 20 μl. Una vez transcurrido el tiempo de reacción, las muestras se llevaron a 95ºC por 5 min para inactivar la transcriptasa reversa.

PCR en tiempo real: Se utilizaron sondas TaqMan específicas para amplificar fragmentos de DNAc de Applied Biosystems marcadas con FAM (6-carboxyfluoresceina) o VIC, para el análisis de marcadores de fibrosis y/o inflamación: IL-6, TNF-α, MCP-1, TGF-β y 18S RNAr como amplificación control. FAM y VIC son colorantes fluorescentes utilizados para detectar la amplificación de productos. De esta forma la cantidad de FAM o VIC, liberada por la degradación de la sonda TaqMan por exonucleasa en la reacción de PCR, es medida en función del cada ciclo de amplificación por reacción PCR mediante el uso de un termociclador en tiempo real ABI 7000 Prism (Applied Biosystems). La expresión de cada gen se cuantificó en forma relativa usando el método comparativo de Ct.

Ensayo para determinación de peróxido de hidrógeno urinario

La cantidad de peróxido de hidrógeno (H_2O_2) en la orina se determinó con un kit Amplex Red Hydrogen Peroxide/Peroxidase (Invitrogen, Eugene, OR) de acuerdo a las instrucciones del fabricante. El ensayo utiliza una curva estándar de H_2O_2 (1–10 IM). La cantidad de 50 μl de cada rata fue colocada en una micro-placa; posteriormente se agregaron 50 μl del reactivo Amplex red/HRP, y las muestras se incubaron durante 30

min a temperatura ambiente, protegidas de la luz. En presencia de peroxidasa, el reactivo Amplex red reacciona con H_2O_2 lo cual produce resorufina, aun producto de oxidación rojo-fluorescente. Por lo tanto, la placa se leyó a una longitud de onda de 560 nm. La concentración de H_2O_2 en las muestras se expresó como nanomolas por 24 h.

Análisis por Western Blot

Se aislaron las proteínas de la corteza renal de 4 animales por grupo de estudio al homogenizarse con buffer de lisis. Las proteínas desnaturalizadas fueron separadas con sodium dodecyl sulfate (SDS)–polyacrylamide gel y transferidas a membranas de polyvinylidine fluoridem (Millipore). Las membranas fueron bloqueadas y posteriormente incubadas overnight a 4ºC con anti-α-SMA de ratón (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Col 1AI (1:1000, Santa Cruz), y anti-VEGF (1:1000, Santa Cruz). Después las membranas fueron incubadas con un anticuerpo secundario anti-conejo ó anti-ratón (Santa Cruz Biotechnology) acoplados a IgG HRP (Alpha Diagnostics, San Antonio, TX) según correspondía. La proteína de control que se utilizó fue la actina (1:5000) y el anticuerpo secundario anti-IgG-HRP de cabra (1:5000; Santa Cruz Biotechnology). Las proteínas fueron detectadas con un estuche comercial que acentúa la quimioluminiscencia (enhanced chemiluminescence Millipore) y autoradiografía, siguiendo las indicaciones del fabricante. Todo el análisis de Western blot se realizó dentro del rango lineal de la proteína cargada y el uso del anticuerpo. Las bandas fueron escaneadas para realizar el análisis densitométrico.

Niveles urinarios de Kim-1

Los niveles urinarios de Kim-1 se analizaron utilizando un estuche comercial de ELISA (USCN Life Science Inc.). Todos los procedimientos se realizaron de acuerdo a las instrucciones del fabricante.

Niveles urinarios de Hsp72

Los niveles de Hsp72 en orina se detectaron por Western blot, cada orina se diluyó 1:100 en solución salina 0,9%, y 10 μl de cada dilución se cargó y se revolvió por electroforesis y la electrotransferencia, como se describió anteriormente. Las membranas se incubaron con anticuerpo de ratón anti-Hsp72 (Life Sciences ENZO, 1: 5000 dilución) durante 2-h. A partir de entonces; las membranas se incubaron con un anticuerpo secundario, de cabra conjugado con HRP anti-IgG de ratón (1: 500, Santa Cruz Biotechnology). Se calculó la densitometría de las bandas.

Análisis estadístico:

Los resultados se presentan como el promedio ± SE. Las diferencias entre grupos se evaluaron mediante ANOVA utilizando la corrección de Bonferroni para comparaciones múltiples. Todas las comparaciones pasaron la prueba de normalidad. Las diferencias en los rangos de los diámetros glomerulares entre los grupos se evaluaron mediante análisis de contingencia, y las diferencias se evaluaron mediante la prueba de Xi cuadrada con la corrección de Yates. La correlación entre los datos fue evaluada por el test de Pearson. La significancia estadística se definió cuando el valor de p fue <0.05.

RESULTADOS

Efecto de la administración de losartán 24-h después del insulto isquémico

En la Figura 1A aparece el promedio de las cifras de presión arterial media (PAM) que presentaron los animales 24-h posteriores a la LRA en los distintos grupos estudiados. Como se puede apreciar ninguno de los animales desarrolló hipertensión, esto nos permite contar con un modelo, en donde podemos disecar el papel específico del proceso isquémico en el desarrollo de la ERC, sin una variable confusora, como lo es la presencia de hipertensión sistémica. La LRA renal inducida por 45 min de isquemia bilateral se caracterizó por una reducción significativa del flujo sanguíneo renal (RBF) (Figura 1B) y de la depuración de creatinina (Figura 1C), el aumento de aldosterona en suero (Figura 1D), la elevación de la proteinuria (Figura 1E) y la presencia de lesión tubular severa (Figura 1F-1H). La LRA también se evidenció por la elevación significativa de los biomarcadores de daño tubular urinarios como, Hsp72 y Kim-1 (Figura 1I y 1J, respectivamente). Este tipo de daño fue asociado con la reducción de óxido nítrico (Figura 1K) y la elevación de estrés oxidativo (Figura 1 l), como previamente lo reportamos (21; 22). Todas estas alteraciones no fueron modificadas por el pre tratamiento con losartán, incluyendo la elevación de la aldosterona en suero. Por lo tanto, el desarrollo de la LRA después de un insulto isquémico no fue prevenido o reducido por la administración de losartán.

Efecto del bloqueo antagonismo de los receptores AT1 de angiotensina II en la transición de la LRA a ERC

En otra serie de experimentos, los animales fueron seguidos y estudiados a los 9 meses después de inducir la lesión isquémica con y sin pre tratamiento con losartán y se compararon con sus respectivos grupos control. Como muestra la Figura 2A, en los

primeros 90 días después de la cirugía simulada o la isquemia ninguno de los grupos presentó proteinuria. Sin embargo, a partir de este momento se observó un aumento en el grupo isquémico sin tratamiento (UTxI), en comparación con el resto de los grupos. Es decir, el aumento progresivo de la proteinuria no se observó en los animales expuestos a la isquemia, que previamente fueron tratados con losartán (Los-Pre), a pesar de presentar un grado de LRA similar (Figura 1). Ninguna de las ratas desarrollaron hipertensión (Figura 2B), como se informó anteriormente (20).

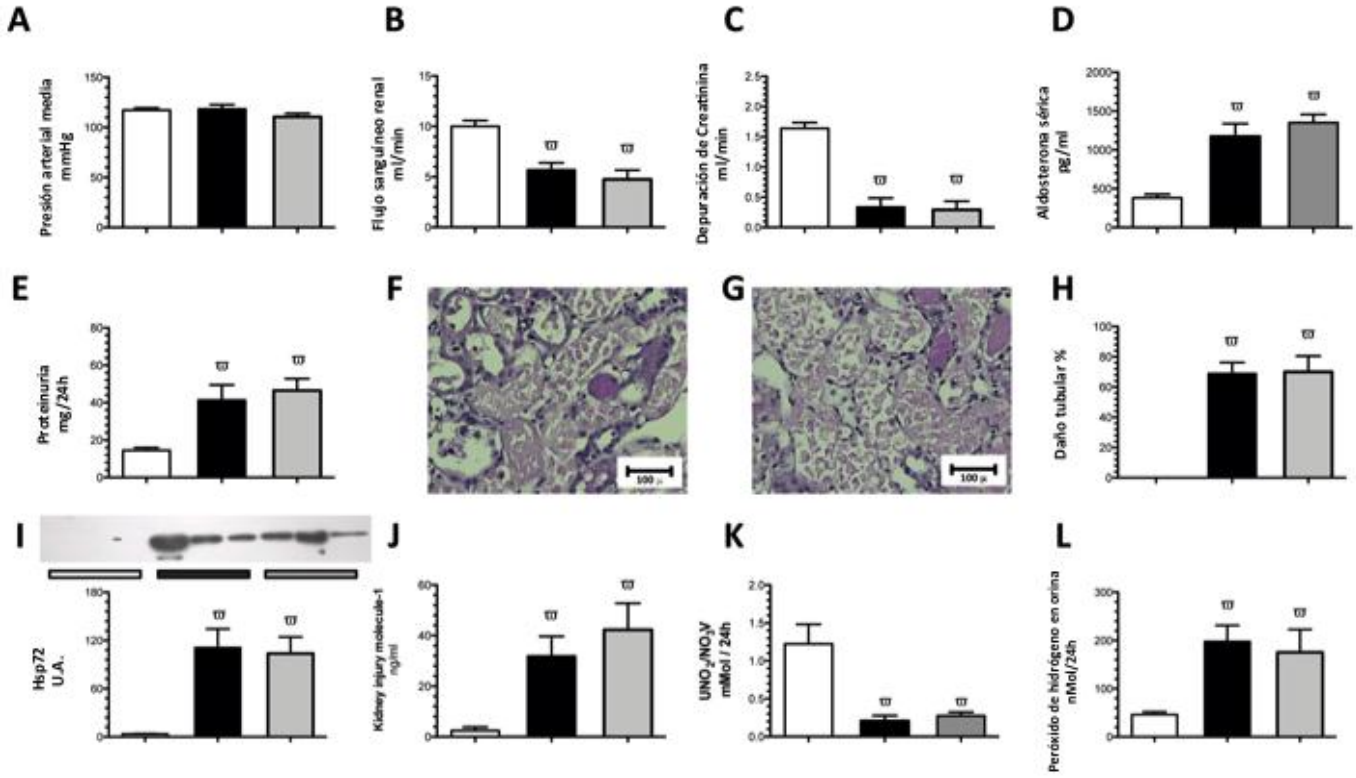


Figura 1. ***La administración profiláctica de losartán no impidió la lesión renal inducida por I/R.*** A) Promedio de la presión arterial media, B) flujo sanguíneo renal (RBF), C) creatinina sérica, D) depuración de creatinina E) proteinuria, F y G) imágenes representativas cortes renales teñidos con PAS de los grupos UTxI y Los-Pre, respectivamente, H) porcentaje lesión tubular en al menos 5 ratas por grupo. I) niveles urinarios Hsp72 por WB (n=6 ratas por grupo), J) excreción urinaria de Kim-1 de (n=8), K) excreción urinaria de NO_2/NO_3 (n=5) y L) excreción urinaria de H_2O_2 (n=6 por grupo) en ratas sham (barras blancas), UTxI (barras negras), Los-Pre (barras grises). Todos los parámetros se analizaron 24-h después de la isquemia y los datos se muestran como promedio ± SE. π p <0.05 vs. grupo Sham.

Por lo tanto, todas las alteraciones funcionales y estructurales resultaron del insulto isquémico. Al final del período experimental, el grupo UTxI presentó una reducción significativa en la depuración de creatinina (Figura 2C), que fue acompañado por una ligera reducción en el RBF. Estos cambios funcionales renales no se observaron en el grupo isquémico que recibió losartán (Los-pre).

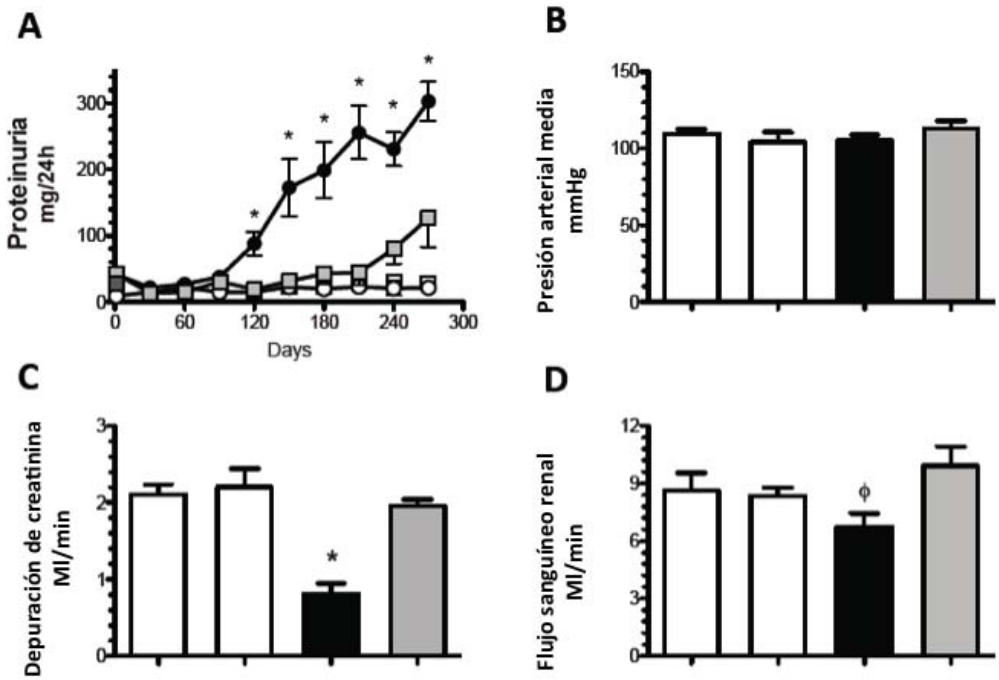


Figura 2. ***La administración profiláctica de losartán previene la progresión a enfermedad renal crónica después de un episodio LRA***. A) Proteinuria determinada cada 30 días: círculos abiertos representan ratas con operación simulada, (n=6); cuadrados abiertos representan ratas que recibieron losartán (50 mg/kg por día), tres días antes de la cirugía simulada (n= al menos 6); círculos negros representan las ratas que fueron sometidos a isquemia renal bilateral, (n=9); cuadrados grises representan las ratas que recibieron losartán tres días antes de la isquemia renal bilateral, (n=8), B) la presión arterial media (n=al menos 6 por grupo), C) depuración de creatinina (n= al menos 7 por grupo) y D) el flujo sanguíneo renal (n = al menos 6 por grupo). Todos los parámetros se determinaron después de nueve meses en sham (barras blancas), Los (barras blancas), UTxI (barras negras) y Los-Pre (barras grises). * P <0.05 frente a todos los grupos, Φp <0.05 vs. grupo Los-Pre.

El análisis histopatológico reveló que el grupo UTxI desarrolló daño estructural severo, tal como: hipertrofia glomerular, atrofia tubular y la formación de

cilindros (Figuras 3C), en comparación con los grupos control (Figuras 3A-3B). Estos cambios estuvieron ausentes en el grupo Los-Pre (Figura 3D). La lesión glomerular en el grupo UTxI fue confirmada por el porcentaje glomeruloesclerosis (18%), mientras que el grupo Los-Pre se protegió de esta lesión (Figura 3E). Se encontró una fuerte correlación entre la glomeruloesclerosis y la proteinuria (Figura 3F, p = 0.0001).

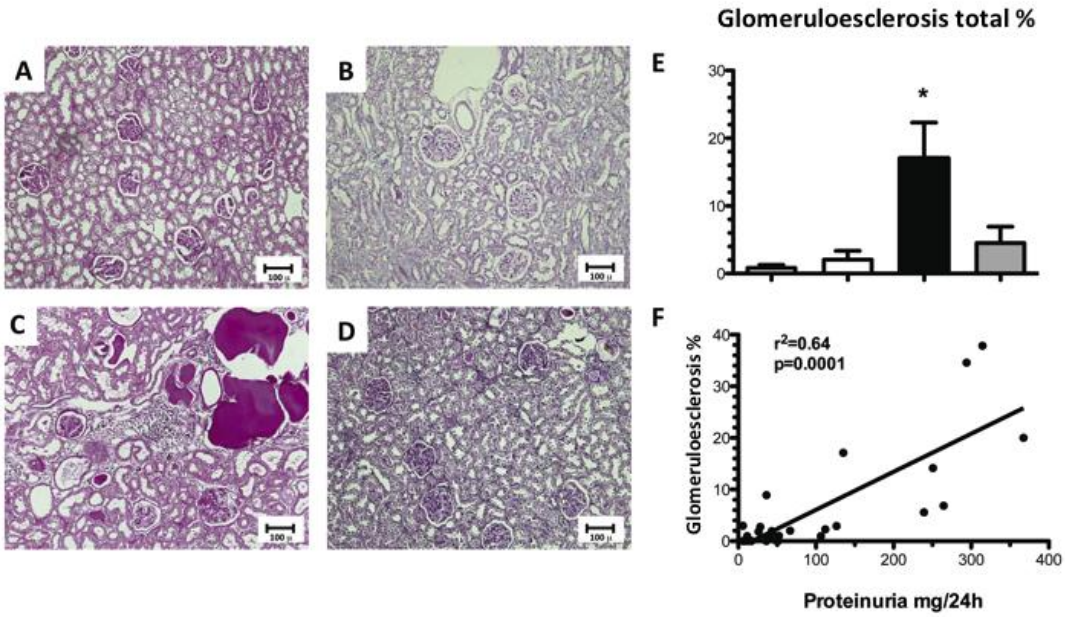


Figura 3. ***Un episodio LRA conlleva al desarrollo de alteraciones estructurales. Prevención por losartán***. Imágenes representativas de secciones de riñón teñidas con ácido periódico de Schiff (PAS) A) Sham B) Los, C) UTxI, y D) Los-Pre (amplificación x100). E) porcentaje de glomeruloesclerosis en sham (n=7, barra blanca), Los (n=8 segunda barra blanca), UTxI (n=7, barra negro) y Los-Pre (n=8 barra gris), F) correlación de Pearson entre el % glomeruloesclerosis y la proteinuria *p <0.05 frente a todos los grupos.

Además, el grupo UTxI desarrolló hipertrofia renal significativa, evidenciada por el aumento del peso del riñón, siendo un 74% más pesado que el del grupo control (Figura 4A). Así mismo, este grupo presentó un mayor porcentaje de

glomérulos con diámetros mayores de 151 micras, en comparación con los dos grupos controles o el grupo Los-Pre (Figura 4).

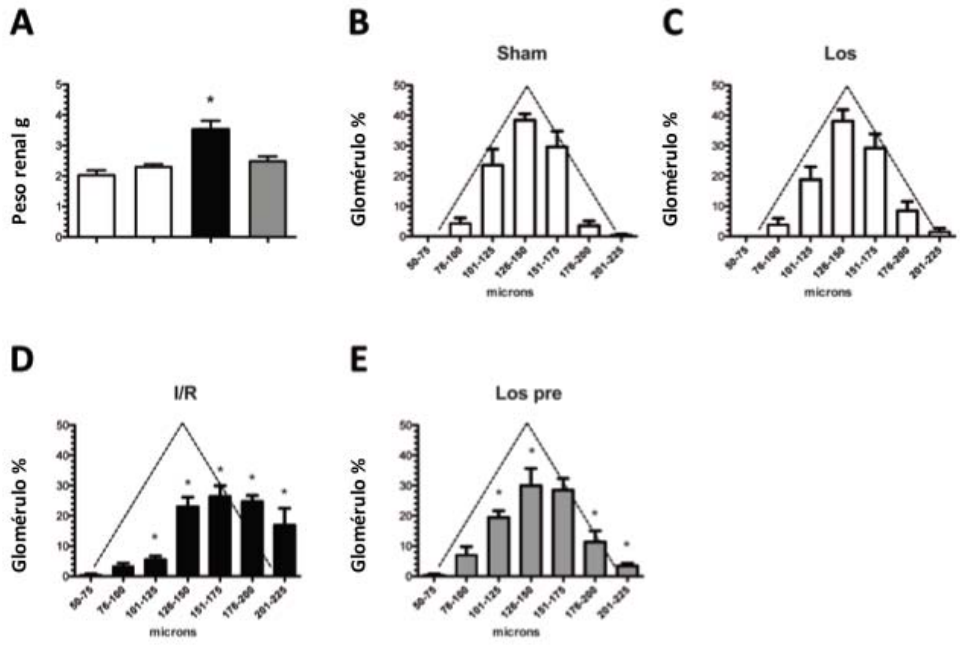


Figura 4. ***La hipertrofia renal y la glomerular inducida por un episodio de LRA fue impedida por la administración de losartán***. A) peso del riñón en Sham (n=8, barras blancas), Los (n=8 segunda barra blanca, UTxI (n 6 barras negras) y Los-Pre (n=7 barra gris). Distribución del diámetro glomerular evaluado en al menos 6 ratas por grupo B) Sham, C) Los D) UTxI y E) Los-Pre. *p<0.05 frente al grupo Sham.

A nivel ultraestructural, el grupo UTxI exhibió extensa fusión de los podocitos (Figura 5B), un efecto que no se observó en el grupo de Los-Pre (Figura 5C). La detección subcelular de la nefrina por microscopía inmunoelectrónica reveló que fusión de podocitos se asoció con una reducción significativa en el contenido de partículas nefrina en los podocitos (Figura 5D). Aunque el grupo Los-Pre exhibió una reducción en la nefrina, las diferencia no fue estadísticamente significativa.

En el grupo UTxI se observó una área extensa afectada por fibrosis túbulo intersticial (Figura 6E y 6F) en comparación con los grupos control (Figura 6A-6D).

Mientras que, el grupo Los-Pre exhibió poca tinción para el rojo de Sirio (Figura 6G y 6H). Estas observaciones fueron confirmadas por el

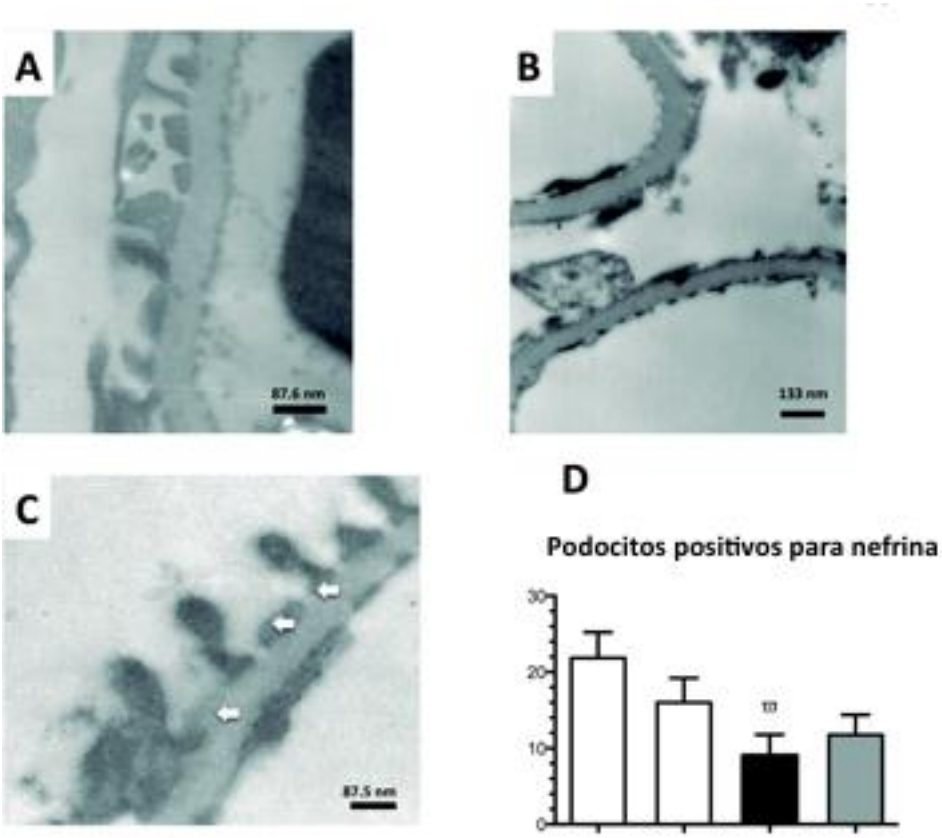


Figura 5. ***Detección subcelular de nefrina y de la ultraestructura podocitos.*** Microfotografías de transmisión electrónica representativas: A) Sham, B) UTxI, y C) Los-Pre Las flechas blancas indican la nefrina detectada por inmunotinción de partículas de oro. D) Representa el número de podocitos positivo para nefrina en al menos 3 ratas por grupo. Aumento original: 20.000. π p <0,05 vs grupo sham.

análisis morfométrico presentado en la Figura 6I. Además, la fibrosis túbulo intersticial correlacionó con la proteinuria (Figura 6J, p = 0.0001). También se observó dilatación tubular significativa el grupo de UTxI, siendo el ancho tubular un 16.7% mayor que el grupo operado de forma simulada o el grupo Los-Pre (54.3 ± 1.4 vs. 47.4 ± 1.1, o 49.2 ± 1.6, respectivamente p <0.05). Además, el grupo UTxI presentó mayor expresión de la actina de músculo liso (α-SMA), que los grupos de

control (Figura 6K). En contraste, el grupo isquémico que reciben tratamiento con losartán no mostró este aumento de α-SMA. El daño estructural renal también fue confirmado por el aumento de cuatro veces en la excreción urinaria de la molécula de daño renal 1 (UKim-1) como se muestra en la Figura 6L. La renoprotección conferida por la administración profiláctica losartán también se demostró mediante la normalización de atrofia tubular y UKim-1.

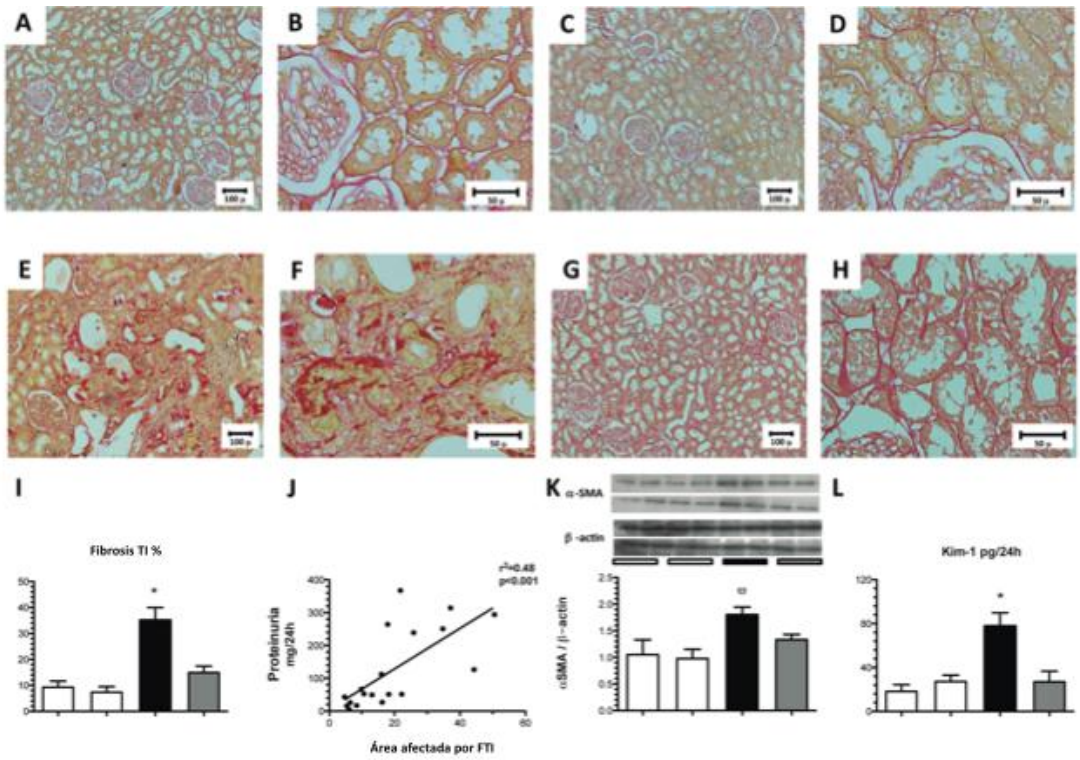


Figura 6. ***La ERC inducida por un episodio LRA se asoció con lesión túbulo-intersticial efecto que no se observó con el pre-tratamiento con losartán***. Microfotografías de luz representativos de cortes histológicos de riñón teñidos con rojo sirio de A y B) sham, C y D) Los, E y F) UTxI, Gy H) Los-Pre (magnificación x100 ó x400, respectivamente). I) Porcentaje de superficie afectada por fibrosis túbulo-intersticial (n= al menos 6 ratas por grupo), J) correlación de Pearson entre la proteinuria y la fibrosis túbulointersticial, K) imágenes del Western blot de α SMA y β-actina, así como, el análisis densitométrico (n=4 por grupo). L) excreción urinaria de Kim-1 (S y Los n=4, UTxI y Los-Pre n=6 por grupo) para: sham (barra blanca) Los (segunda barra blanca), UTxI (barra negra) y Los-Pre (barra gris). * P <0.05 frente a todos los grupos y πp <0.05 vs. grupo sham.

La Figura 7A muestra que el grupo UTxI presentó un aumento significativo de los niveles de RNAm de TGF-β, efecto que fue revertido por el tratamiento con losartán. Este hallazgo fue corroborado por la inmunohistoquímica de microarreglos

para TGF-β, (Figuras 7D-7G). El grupo UTxI presentó mayor inmunotinción TGF-β, principalmente en el epitelio tubular (Figura 7F), en comparación con los grupos control (Figura 7D y 7e). Esta mayor inmunotinción no se observó en el grupo Los-Pre (Figura 7G). En consecuencia, el análisis de imagen digital a partir de estas microfotografías reveló una aumento significativo de TGF-β (Figura 7B) en el grupo UTxI, que no se observó en el grupo Los-Pre. Como resultado de la activación de TGF-β, los niveles de proteína colágeno I aumentaron significativamente en el grupo UTxI; este efecto no se observó en el grupo Los-Pre (Figura 7C). La influencia de TGF-β, sobre la fibrosis renal fue evidenciada por la correlación significativa entre la expresión total de TGF-β y la fibrosis túbulo intersticial (p = 0.001, Figura 7I).

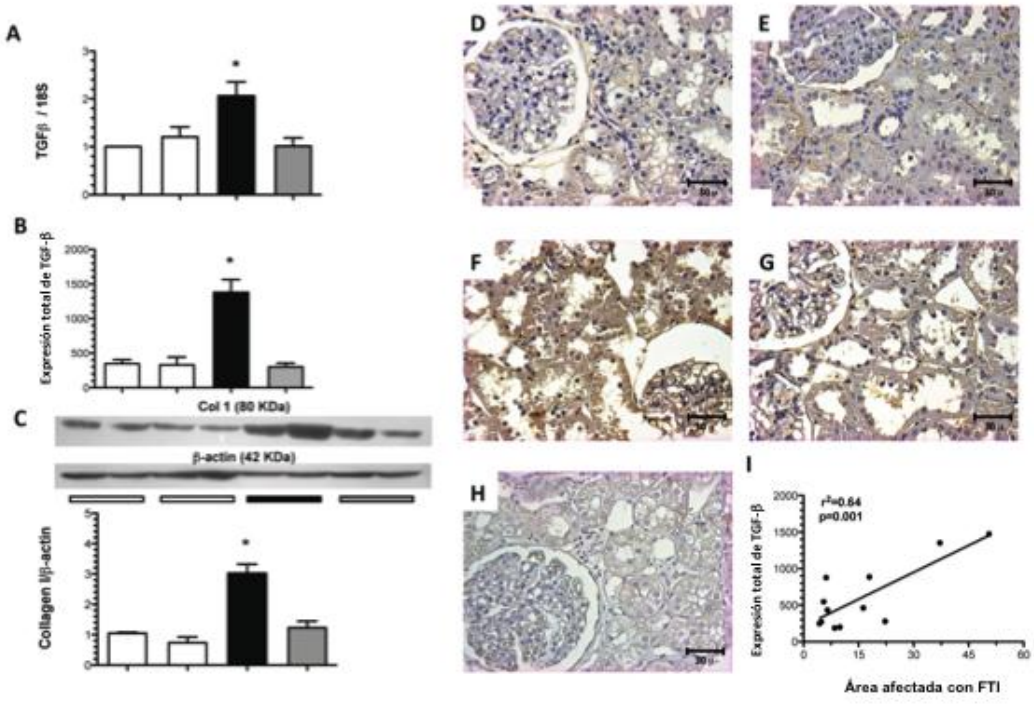


Figura 7. ***Contribución de TGF-β en la transición de LRA a ERC***. A) niveles de RNAm de TGF-β, Sham n=7, Los n=8, UTxI n=6, y el grupo Los-Pre n=7, B) Expresión total de TGF□β evaluada mediante microarreglos de tejidos e inmunohistoquímica en al menos 4 ratas por grupo; los niveles de proteína se cuantificaron como expresión total de la densidad (TDE) de TGF-β, tres secciones diferentes por rata, C) Imágenes representativas del Western blot Col1AI y β-actina, respectivamente junto con el análisis densitométrico (n=4 por grupo). Imágenes representativas de los microarreglos

para TGF-β en D) Sham, E) Los, F) UTxI, G) Los Pre (magnificación x400) y H) control de isotipo. I) correlación de Pearson entre la expresión total de TGF-β y la fibrosis tubulointersticial. Sham (Barra blanca) Sham, Los (segunda barra blanca), UTxI (barra negra) y Los-Pre (barra gris). * p<0.05 frente a todos los grupos.

La proliferación epitelial tubular se evaluó mediante inmunotinción de PCNA y Ki67. La proliferación fue muy baja en los grupos control, como se muestra por microfotografías representativas y contando las células epiteliales tubulares positivas (Figuras 8A-8D). En cambio, se observó un aumento significativo en la proliferación en el grupo UTxI (Figuras 8E y 8G, respectivamente). En el grupo de Los-Pre, la proliferación observada fue similar al grupo control (Figuras 8F y 8H, respectivamente).

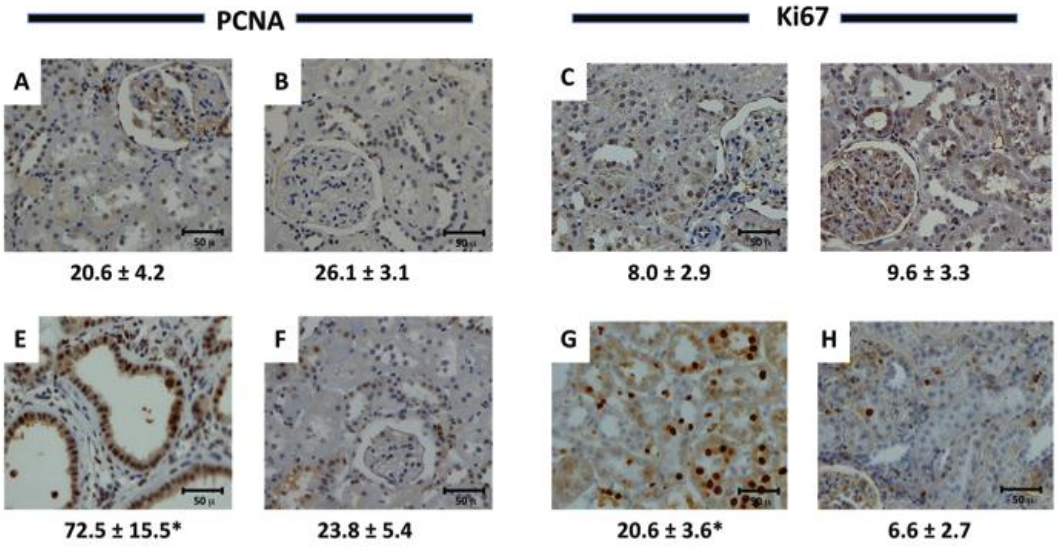


Figura 8. ***La Atrofia tubular observada en el grupo con ERC se asoció con la proliferación de células epiteliales y prevenida por losartán.*** La proliferación tubular fue evaluada por inmunohistoquímica para PCNA y Ki67 como se muestra en las microfotografías representativas de diapositivas renales (Magnificación x400). A y C) representan el grupo sham; B y D) Los E y G) UTxI; F y H) Los-pre. El Promedio de la células epiteliales positivas para PCNA y Ki67 ± desviación estándar muestran bajo la imagen correspondiente. * p<0.05 frente a todos los grupos.

Todas las alteraciones funcionales y estructurales observados en el grupo UTxI se asociaron con un mayor estrés oxidante, que fue evaluada por la excreción

urinaria de H_2O_2 (Figura 9A), a pesar del aumento de los niveles de RNAm de G6PD intra renal (Figura 9B). Interesantemente, el aumento del estrés oxidante no se observó en el grupo tratado con losartán. Otro evento que participan en la progresión a ERC es la activación de la inflamación. En consecuencia, los niveles de RNAm de la proteína quimioatrayente de monocitos 1 (MCP-1) y de la interleucina-6 fueron aumentados en el grupo UTxI (Figura 9C-9D). Este patrón no se observó en el grupo de Los-Pre, que mostró un estado de menor inflamación.

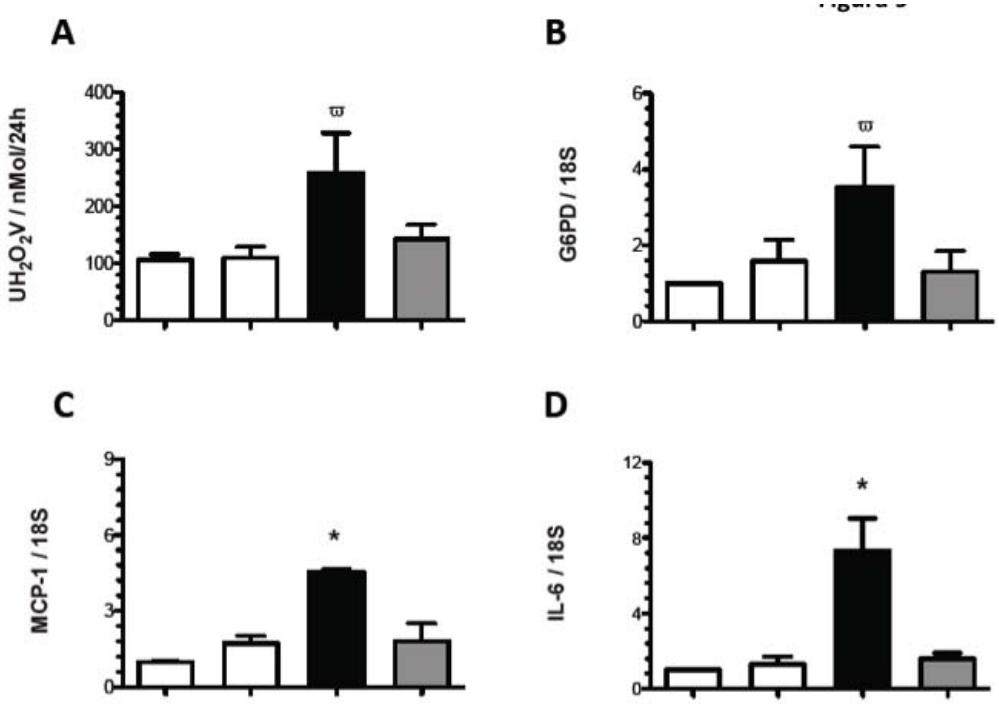


Figura 9. ***El estrés oxidante y la inflamación en el grupo de ERC fueron inhibidas con la administración de losartán antes lesión isquémica.*** A) peróxido de hidrógeno en al menos 6 ratas por grupo. B) G6PD, C) MCP-1, y D) niveles de RNAm de IL-6. Sham y Los grupos (barras blancas), UTxI (barras negras), y Los pre (barras grises). π p <0.05 vs sham, *p <0,05 frente a todos los grupos.

Con el fin de determinar los mecanismos por los que, el losartán impidió la transición de LRA a ERC, a pesar de que el grado de LRA fue el mismo, estudiamos a un conjunto de ratas en los primeros días post isquemia.

Efecto temprano del antagonismo de los receptores AT1 de angiotensina II después de un proceso isquémico

En la Figura 10 se muestran los resultados fisiológicos y bioquímicos a los 3 días post isquemia. Se encontró que en el grupo UTxI, la hipoperfusión renal y disfunción persistieron (Figuras 10B y 10C) sin proteinuria (Figura 10D). A nivel estructural, la lesión tubular fue evidente (Figura 10E) y se correlacionó con la elevación de la excreción urinaria de Hsp72 y de Kim-1 (Figura 10G y 10H). Todas estas alteraciones se observaron de manera similar en el grupo Los-pre, excepto con la pronta recuperación del flujo sanguíneo renal.

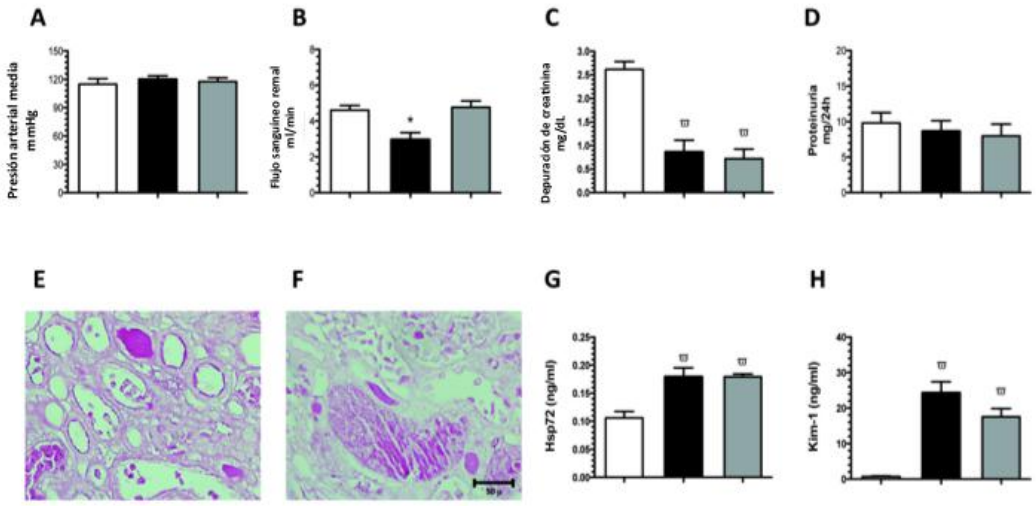


Figura 10. La disfunción renal y las lesiones estructurales persisten después de 3 días de la isquemia, losartán sólo previno la hipoperfusión renal. A) la presión arterial media (MAP), B) de flujo sanguíneo renal (RBF), C) depuración de creatinina, D) proteinuria, E) tinción de PAS representativa de un riñón del grupo UTxI y F) del grupo Los-pre, G) niveles urinarios de Hsp72 y H) de Kim-1. Sham (barras blancas, n = al menos 5); UTxI (barras negras, n= al menos 6); y Los-Pre (barras grises, n= al menos 5 ratas por grupo). *p <0,05 frente a todos los grupos, πp <0,05 vs. Sham.

La Figura 11 muestra los resultados fisiológicos, bioquímicos y moleculares a los 5 días post isquemia. Se encontró que en el grupo UTxI, la disfunción renal

continuó (Figuras 11B y 11C) sin proteinuria (Figura 11D), pero la excreción urinaria de Kim-1 persistió elevada (Figura 11E). Todas estas anomalías no se observaron en el grupo Los-Pre. Aunque la elevación H_2O_2 urinaria en el grupo UTxI no alcanzó diferencia estadística (Figura 11F), IL-6 y TNF-α fueron significativamente aumentados (Figuras 11H-11J). Interesantemente, la recuperación más rápida de la disfunción renal en el grupo Los-Pre se asoció con la normalización de la excreción urinaria de H_2O_2 y de la citosinas pro inflamatorias.

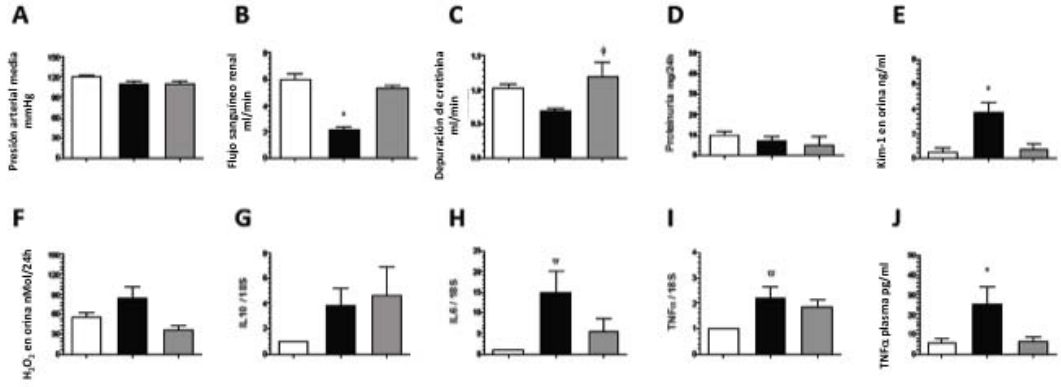


Figura 11. ***La disfunción renal y la inflamación persisten después de 5 días de la isquemia, pero no en ratas pre-tratadas con losartán.*** A) presión arterial media (MAP), B) flujo sanguíneo renal (RBF), C) depuración de creatinina, D) proteinuria, E) excreción urinaria de Kim-1, F) excreción urinaria de H_2O_2, G) niveles de RNAm de IL-10, H) niveles de RNAm de IL-6, I) niveles de RNAm de TNF-α, y J) niveles de TNF-α en plasma. Sham (barras blancas, n=5); UTxI (barras negras n=5) y Los-Pre (barras grises, n=4). *p <0.05 frente a todos los grupos, πp <0.05 vs. Sham y Φp <0.05 vs Los-Pre.

HIF-1α es un factor de transcripción que promueve la transcripción de genes necesarios para la supervivencia de la célula cuando hay una caída en el suministro de oxígeno. Encontramos que los niveles de RNAm de HIF1-α mRNA fueron similares entre los grupos y no se modificaron después de 1, 5 (Figura 12) o 15 días después de la isquemia (Figura 13G). En contraste, los niveles de proteína HIF1-α totales y nucleares, medidos por microarreglos de tejido en la corteza renal aumentaron significativamente después de 15 días en el grupo Los-Pre (Figura 13E-

13F y 13I-13K), un efecto que no se observó en UTxI grupo (Figura 13C-13D y 13I-13K). La mayor parte de la expresión fue localizada en el epitelio tubular. Con el fin de evaluar la actividad transcripcional nuclear de HIF-1, se determinaron los niveles de proteína de VEGF después de 15 días post isquemia. El análisis de Western-blot reveló dos bandas correspondientes a la conformación de monómero y dímero de VEGF (Figura 13H). El análisis densitométrico de tanto dímero y monómero, muestran una ligera reducción en el grupo UTx, pero las diferencias no fueron significativas por ANOVA. De acuerdo a la mayor cantidad de HIF-1 nuclear observada en el grupo Los-Pre, la cantidad de monómero y dímero de VEGF fue significativamente mayor.

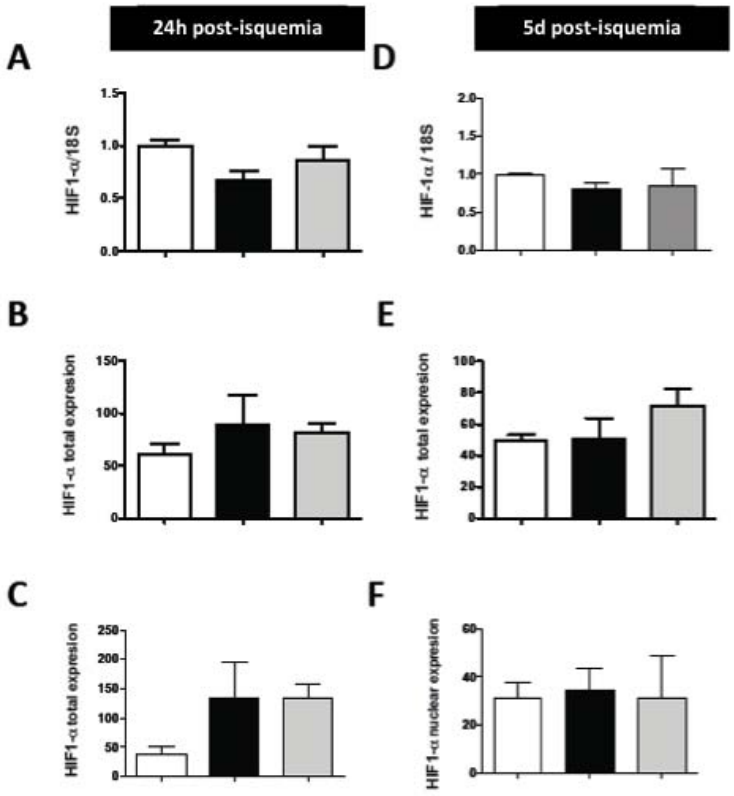


Figura 12. ***Niveles de RNAm y de proteína de HIF-1α en la corteza renal después de 1 y 5 días de la isquemia.*** A y D) los niveles de RNAm de HIF1-α, 1 y 5 días post-isquemia, respectivamente. B y E) microarreglos de tejidos e inmunohistoquímica de HIF1-α 1 y 5 después de la isquemia, respectivamente. C y F) expresión total nuclear HIF1-α en la corteza renal 1 y 5 días post-isquemia, respectivamente. Sham- es representado por barras blancas; n = 5, UTxI por barras negras; n = 5, y Los Pre-por barras grises.

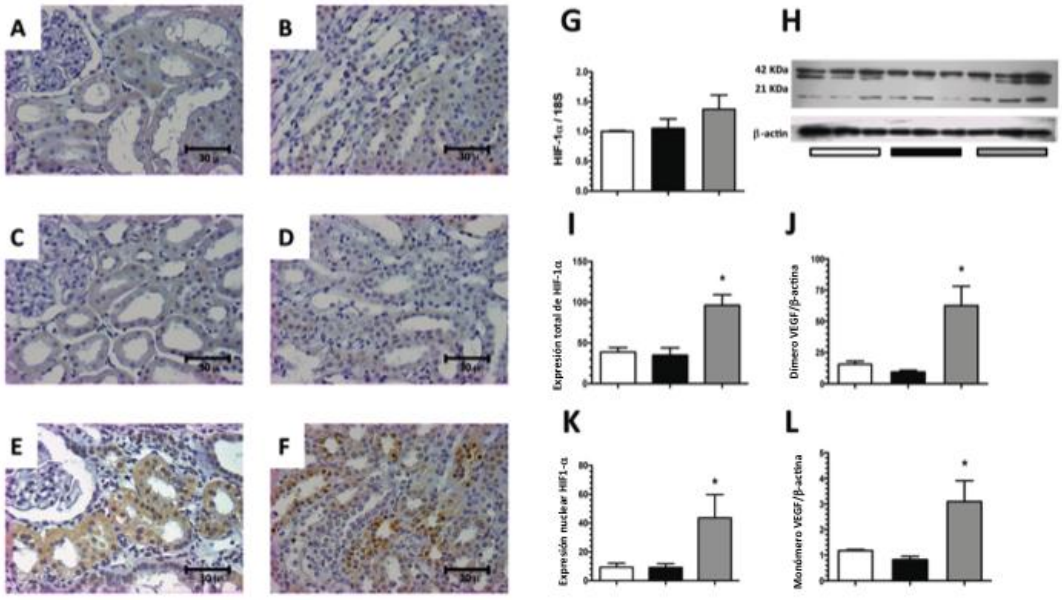


Figura 13. ***HIF-1α y VEGF en la corteza renal después de un evento isquémico.*** HIF1-α / proteína se evaluaron después de 15 días de isquemia por microarreglos de de tejido e inmunohistoquímica en al menos 4 ratas por grupo A, C y E) en la corteza renal y B, D, y F) en la médula renal. En A y B son microfotografías representativas del grupo sham, C y D) del grupo UTX, E y F) grupo Los-pre. G) niveles de RNAm de HIF1-α, I) expresión total de HIF1-α, K) niveles de HIF1-α nuclear. H) Western blot de VEGF y β-actina, respectivamente, n=4. J y L) análisis densitométrico de VEGF/β-actina para la conformación dímerica y monómerica de VEGF, respectivamente. Sham (barras blancas) UTxI (barras negras); y Los-Pre (barras grises). *p<0.05 frente a todos los grupos.

DISCUSION

En este estudio, hemos investigado los mecanismos que conducen a la transición de la LRA a la ERC y también hemos proporcionado evidencia de la importancia de una intervención temprana, como lo es la administración profiláctica de losartán, para detener o frenar la progresión a ERC. De manera interesante, nuestros resultados muestran que a pesar de que el pre-tratamiento con losartán no protegió a las ratas contra la LRA, si fue eficaz para prevenir la transición a ERC. El grupo isquémico sin tratamiento desarrolló ERC que se caracterizó por hipertrofia renal, disfunción renal, hipertrofia glomerular, glomeruloesclerosis, atrofia tubular y fibrosis túbulo intersticial. A nivel ultra estructural, se detectó fusión de podocitos y menor nefrina. Estas alteraciones funcionales y estructurales se asociaron con un aumento de los niveles de proteína αSMA, el estrés oxidativo, la inflamación y la activación de TGFβ.

Anteriormente, demostramos que el bloqueo de angiotensina II con una dosis baja de losartán (8 mg/Kg) no impidió la lesión renal aguda inducida por isquemia unilateral y sólo una dosis más alta (80 mg/Kg) tuvo un efecto menor [91]. En este estudio, encontramos que la administración profiláctica de losartán no impidió, ni redujo el grado de severidad de la LRA inducida por isquemia renal bilateral. Sin embargo, después de nueve meses, el grupo que recibió losartán (Los-Pre) conservó una función renal adecuada, así como una buena preservación de la arquitectura glomerular y tubular.

Con el fin de definir los mecanismos por los cuales, la administración profiláctica de losartán impidió transición de LRA a ERC, se estudiaron a los animales tres o cinco días después de la isquemia y observamos que tres días pos isquemia, la hipoperfusión y la hipofiltración renal persistieron, así como, el daño del

epitelio tubular. La lesión tubular también se evidenció por el aumento en la excreción urinaria de dos biomarcadores diferentes de daño tubular. A pesar de que, el grupo Los-Pre presentó un grado de lesión renal similar al grupo no tratado, la caída del flujo sanguíneo renal fue restaurada mas rápidamente. Además, 5 días después de inducir la isquemia renal bilateral, el grupo UTxI permaneció con evidencia de hipoperfusión, que fue acompañado por inflamación renal (elevación de IL6 y TNFα). Estos hallazgos sugieren que el grupo UTxI mantiene la hipoxia renal durante un período más largo. Esta hemodinámica renal alterada fue impedida por losartán. La restauración temprana del flujo sanguíneo renal inducida por el antagonismo de los receptores AT1 podría explicarse por la activación de los receptores AT2, que median vasorrelajación [92], sin embargo, se requieren más estudios para probar esta afirmación.

Bajo condiciones de hipoxia, el factor de transcripción HIF-1α juega un papel crucial en la regulación de la expresión de más de 100 genes diana implicados en la proliferación celular, la angiogénesis, el metabolismo de la glucosa, la apoptosis, entre otros (véase la revisión [93]. La expresión de HIF1α es regulada por la degradación proteosomal mediada por la enzima que contiene dominios prolil hidroxilasa (PhD). De hecho, los inhibidores de PhD, no solo inducen la activación HIF, sino que también son capaces de reducir el daño renal inducido por I/R [94], nefrectomía subtotal [95], trasplante renal alogénico [59], o nefritis [60]. Estos datos sugieren que HIF ejerce un papel protector en condiciones de hipoperfusión renal. En este estudio, encontramos que los niveles de HIF-1α mRNA no fueron diferentes entre los grupos después de 1, 5 o 15 días después de la isquemia, pero la expresión total y la nuclear HIF-1α, evaluada mediante microarreglos de tejidos, fue significativamente mayor en el grupo Los-pre después de 15 días de la isquemia.

Esta activación puede ser el resultado de la inhibición de los receptores AT1 por el losartán, porque se ha propuesto que la inhibición PHD reduce la expresión de los receptores AT1 y se observa menor fibrosis perivascular en las arterias coronarias [96]. Nuestros datos también sugieren que después de un insulto isquémico hay una activación inefectiva de HIF1α para hacer frente a la hipoperfusión renal, la rarefacción vascular y la inflamación. En contraste, el tratamiento con losartán fue capaz de inducir la translocación nuclear y la activación de HIF-1α, que se detectó mediante la inducción de la transcripción VEGF. Nuestros datos sugieren que el aumento de VEGF en las ratas tratadas con losartán podría disminuir la rarefacción capilar, mejorar la perfusión renal y reducir la hipoxia crónica. Aunque, no podemos explicar cómo el tratamiento profiláctico con el losartán indujo HIF-1α 15 días post isquemia, es posible que el aumento de este factor de transcripción pueda ser el resultado de: una menor degradación HIF1α, o por una mayor dimerización, o bien por un aumento de la translocación nuclear. Nuestros resultados muestran que la activación de HIF-1α después de la isquemia por el losartán se asoció con la prevención de la transición AKI a la ERC, sin embargo, el mecanismo por el cual el tratamiento profiláctico activó HIF-1α permanece para ser explorado en estudios futuros.

Después de 9 meses, la proteinuria en el grupo UTxI correlacionó con la glomeruloesclerosis y la fibrosis túbulointersticial, lo que sugiere que la proteinuria anormal es el resultado de daño gradual tanto de la barrera de filtración glomerular, demostrada por la fusión de podocitos y la reducción en la expresión de la nefrina podocitaria, así como, por la atrofia y dilatación del epitelio tubular. Estas alteraciones son muy similares a las observadas en la recientemente caracterizada

nefropatía Mesoamericana. Por lo tanto, nuestros hallazgos apoyan la hipótesis de que esta nefropatía se relaciona con uno o varios episodios de LRA (34)

La lesión isquémica renal se ha asociado con la aparición de nefronas atubulares [97]. Por lo tanto, es probable que en la primera semana post isquemia, un número significativo de nefronas se pierdan. La reducción de nefronas significa que las nefronas funcionales restantes deben compensar la función perdida a través hiperfiltración e hipertrofia. De hecho, la hipertrofia renal fue evidente en el grupo UTxI y evitada por la administración profiláctica losartán. Un estudio reciente mostró que la hipertrofia renal inducida por la isquemia depende de la extensión de la muerte epitelio tubular mediada por activación de la vía de señalización TNFα [98]. También encontramos que la hipertrofia glomerular y la glomeruloesclerosis se asociaron con la fusión de podocitos y una reducción inmunotinción con partículas de oro de la nefrina. Estas anormalidades ciertamente aceleran el deterioro de las nefronas funcionales. Por lo tanto, es factible que el grupo de Los-Pre tuviera un menor número de nefronas atubulares, lo que se refleja en una mejor función renal y la preservación de la estructura glomerular. En apoyo a esta hipótesis, Pagtalunan ME, et al. [99] demostraron que el losartán administrado después de un proceso isquémico fue capaz de reducir el número de nefronas atubulares.

Después de un episodio de LRA, las células epiteliales tubulares proximales sufren la muerte por necrosis o apoptosis y otras pierden su polaridad y se desprenden de la membrana tubular. En consecuencia, la de diferenciación y proliferación epitelial se activan con el propósito de restaurar el epitelio tubular. La reparación tubular, sin embargo, se ve alterada por procesos tales como el arresto celular [68] y por cambios epigenéticos [87] que en lugar de mejorar la arquitectura renal, desencadenan la fibrogénesis. Nuestro estudio mostró que el grupo UTxI

mostró un epitelio tubular atrófico que se asoció con un aumento de la proliferación, evaluada por Ki67 y PCNA. Resultados similares se han observado en las biopsias renales de pacientes que padecen LRA [100]. Estos resultados indican que la proliferación tubular se perpetúa como un fenómeno de una mala adaptación, efectos que no fueron observados en el grupo Los-Pre.

La fibrosis túbulointersticial progresiva juega un papel clave en el daño renal crónico e implica la participación de inflamación crónica, de diferenciación celular tubular y la activación de miofibroblastos [101]. Además, la acumulación de leucocitos y su activación sostenida pueden extender los períodos de isquemia debido a la congestión vascular e inducir daño tubular y endotelial directamente mediante la liberación de mediadores inflamatorios [102]. En nuestro estudio observamos que el insulto isquémico promovió el desarrollo de fibrosis túbulo intersticial. Esta alteración fue, en parte, mediada por una mayor expresión y activación de TGF-β. Yang et. al [68] propuso que después de un insulto isquémico grave, el epitelio tubular produce grandes cantidades de TGF-β. Nuestros resultados con microarreglos de tejidos confirmaron estos hallazgos; el aumentó de la expresión de TGF-β fue observada principalmente en el epitelio tubular. Esta respuesta fibrótica no se observó en el grupo de Los-Pre. Bechtel et al. [87] mostró que las células epiteliales lesionadas desencadenan transdiferenciación de los fibroblastos a miofibroblastos. En concordancia con esto, observamos que los niveles de αSMA, un marcador de la transdiferenciación de fibroblastos, se incrementó en el grupo de UTxI y esta sobre regulación no fue detectada en el grupo Los-Pre.

Por lo tanto, nuestros resultados sugieren que los receptores AT1 deben ser bloqueadas en el momento de la lesión isquémica renal para evitar los efectos deletéreos de la angiotensina II sobre el endotelio y la reparación ineficaz del

epitelio tubular a largo plazo. En este sentido y debido a que la LRA se produce en el 30% de los pacientes sometidos a cirugía cardíaca, varios estudios clínicos han examinado el efecto de los inhibidores de la enzima convertidora de angiotensina (IECA) o antagonistas de los receptores AT1 de angiotensina II (ARAII) en la incidencia de LRA. Sin embargo, los resultados publicados no han sido concluyentes ya que en algunos se ha reportado que la LRA aumenta con este tratamiento [103], o no produce un cambio en la incidencia [104,105], o incluso se ha reportado que se reduce [106]. Recientemente, Coca SG, et al. [107] estudiaron el efecto del suspender o continuar el tratamiento con IECA/ARAII en pacientes sometidos a cirugía cardiaca en comparación con los pacientes no tratados. El estudio demostró que los niveles biomarcadores urinarios de daño renal como NGAL, IL-18, Kim-1 y L-FAPB fueron similares entre los pacientes y que solo hubo una reducción en la tasa de filtración glomerular (TFG) en el grupo que continuaron con la terapia IECA/ ARAII. De manera interesante, la reducción de TFG no se ha atribuido como un efecto adverso, debido a que estos fármacos mejoran la perfusión capilar peritubular, lo que a su vez podría reducir la isquemia y la necrosis tubular [61,108]. Desafortunadamente, no se ha informado la evaluación a largo plazo de los pacientes que sufren LRA y que reciben terapia con IECA o ARAII.

Anteriormente, hemos demostrado que la administración profiláctica de espironolactona previno completamente la LRA y la transición a la ERC [90]. En este estudio, el tratamiento profiláctico con losartán antes de la isquemia no impidió ni la elevación de la aldosterona, ni la severidad de la LRA dentro de las primeras 24-h, lo que demuestra que la protección del ARAII es independiente de antagonismo de los receptores de mineralocorticoides y sugiere que la aldosterona y la angiotensina

II diferencialmente regulan la hemodinámica renal y la participación de las vías de señalización para la reparación endotelial y del epitelio tubular.

Nuestros resultados no sólo refuerzan la gran importancia que tiene un episodio de LRA como factor de riesgo para el desarrollo de la ERC, sino también muestran el efecto nocivo de la activación de los receptores AT1 durante un insulto isquémico y su impacto en la función renal y la estructura a largo plazo. Este estudio también muestra la potencialidad del antagonismo de los receptores AT1 en la prevención de la transición de la LRA a ERC por un mecanismo relacionado con la pronta recuperación del flujo sanguíneo renal, la prevención de un proceso inflamatorio, mayor translocación HIF-1α nuclear y la inducción temprana de VEGF después de la lesión isquémica. La identificación oportuna de la LRA, junto con una intervención profiláctica eficaz, tendrá un impacto dramático en el retraso de la progresión a ERC.

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Recovery from ischemic acute kidney injury by spironolactone administration

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Abstract

Background. Prophylactic mineralocorticoid receptor (MR) antagonism with spironolactone (Sp) in rats completely prevents renal damage induced by ischemia. Because acute renal ischemia cannot typically be predicted, this study was designed to investigate whether Sp could prevent renal injury after an ischemic/reperfusion insult.

Methods. Six groups of male Wistar rats were studied: rats that received a sham abdominal operation (S); rats that underwent 20 min of ischemia and reperfusion for 24 h (I/R) and four groups of rats treated with Sp (20 mg/kg) 0, 3, 6 or 9 h after ischemia.

Results. As expected, I/R resulted in renal dysfunction characterized by a fall in renal blood flow and glomerular filtration rate and severe tubular injury which was confirmed by a significant increase in tubular damage biomarkers including kidney injury molecule-1, heat shock protein 72 and urinary protein excretion. The renal injury induced by I/R was in part due to Rho-kinase, endothelin and angiotensin II type 1 receptor upregulation. Interestingly, Sp administration at 0 and 3 h after ischemia completely reversed and prevented the damage induced by I/R. The protection induced by Sp given 6 h after ischemia was partial, but no protection was observed by administering Sp 9 h after ischemia.

Conclusion. Our results show that MR antagonism administered, either immediately or 3 h after I/R, effectively prevented ischemic acute renal injury, indicating that spironolactone is a promising agent for preventing acute kidney injury once an ischemic insult has occurred.

Keywords: AKI treatment; endothelin; renal dysfunction; Rho-kinase

Introduction

Acute kidney injury (AKI) is characterized by an abrupt and sustained decline in the glomerular filtration rate (GFR) that leads to a wide spectrum of acute alterations in kidney function and structure. One prominent cause of AKI is renal ischemia that occurs in patients with low blood pressure as result of a complication of several common clinical situations, such as severe cardiac failure or arrhythmia, generalized sepsis or excessive loss of blood or fluid during surgery or as a consequence of trauma. Thus, ischemic and nephrotoxic injuries are the major causes of AKI in native and transplanted kidneys [1]. AKI occurs in ∼5% of hospitalized patients and up to 40–60% of intensive care unit patients [2]. Despite technical improvements in dialysis and clinical care, the prevalence of AKI has risen significantly in the last 15 years due to aging population and the rising pandemics of obesity, diabetes and hypertension [3, 4]. In addition to high morbidity and mortality during the AKI episode, once resolved, AKI may also lead to the development of chronic kidney disease (CKD) or increases the transition rate from pre-existing CKD to end-stage renal disease [5–8]. In addition, even a small change in kidney function due to a minor AKI is associated with a higher long-term mortality rate [9].

In addition to its renal effects as a mineralocorticoid hormone, aldosterone plays a prominent role in the pathophysiology of renal diseases [10–19]. Recent evidence suggests that aldosterone is a potent renal vasoconstrictor [20–22]. Supporting that, aldosterone modulates the tone of the renal vasculature, we have shown that a mineralocorticoid receptor (MR) blockade with spironolactone not only reduces the structural renal damage associated with cyclosporine (CsA) but also prevents renal dysfunction due to afferent and efferent vasoconstriction [23–25]. We have also shown that prophylactic treatment with spironolactone completely prevents renal dysfunction and histological signs of tubular injury from ischemia–reperfusion (I/R) injuries [26]. To determine whether the protective effect of spironolactone during ischemia is due to blocking MR or to an unknown effect of this drug, we demonstrated that adrenalectomy had similar effects to those observed with spironolactone treatment [27]. Altogether, these results support the hypothesis that spironolactone prevents AKI after I/R by blocking the MR and therefore suggest that aldosterone plays a central role in promoting renal damage induced by a renal ischemic insult.

At present, there are no specific treatments for AKI caused by ischemia or nephrotoxic agents for use in the common clinical practice. In hypotension, once ischemia has damaged the kidney, other than restoring renal perfusion pressure and avoiding nephrotoxics, there is nothing that can currently be done to help prevent further damage or repair what has already occurred in the tubular cells. Therefore, prevention is a challenge clinicians face. In this regard, several treatments and experimental strategies have been advanced for the prevention of AKI, with different degrees of effectiveness, depending on the patient's other risk factors and the strategy used. However, most of these treatments are only effective when they are prophylactically administered [28–33]. Unfortunately, the development of renal ischemia cannot be predicted. Because we previously observed that giving spironolactone several hours before an ischemic insult completely prevents the histological and biochemical consequences of I/R, we designed the present study to investigate whether MR antagonism could reverse the renal injury induced by ischemia/reperfusion once ischemia has already been established and to better understand the molecular mechanisms of the renoprotection involved.

Materials and methods

All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and were approved by our Institutional Animal Care and Use Committee. Sixty-two male Wistar rats (270–315 g) were divided into six groups: sham-operated rats (S); animals subjected to bilateral renal ischemia for 20 min and 24 h of reperfusion (I/R) and four groups of rats that underwent bilateral renal ischemia for 20 min and reperfusion for 24 h, but also received one dose of spironolactone at 20 mg/kg by gastric gavage immediately after or at 3, 6 or 9 h after ischemia (Sp0, Sp3, Sp6 and Sp9, respectively). Two hours after ischemia, the animals were placed in metabolic cages at 22°C with a 12:12-h light–dark cycle and were allowed free access to water. All animals were studied after 24 h of reperfusion. At least six animals per group were used for renal functional and biochemical studies, and the rest were used for molecular studies.

Renal ischemia/reperfusion (I/R) injury (surgical procedure)

The rats were anesthetized by intraperitoneal injections of sodium pentobarbital (30 mg/kg) and underwent abdominal incision and the dissection of bilateral renal pedicles. Renal ischemia was induced by using nontraumatic vascular clamps; placed on each pedicle for 20 min. After the clamps were released, the incision was sutured, and the animals were allowed to recover. Sham abdominal operated rats underwent anesthesia and renal pedicle dissection only.

Functional studies

After recovery, the animals were placed in metabolic cages with 12:12-h light–dark cycle and were allowed free access to water. Twenty-four hours after ischemia, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital and placed on a homeothermic table. The trachea, jugular veins, femoral arteries and bladder were catheterized with polyethylene tubing (PE-240, PE-50 and PE-90). During surgery, the rats were maintained under euvolemic conditions by infusing 10 mL/kg body weight of isotonic rat plasma followed by an infusion of 5% low calorie commercial sugar (METCO, Mexico City, Mexico) at 1.6 mL/h as a marker of the GFR. We have previously shown that this compound has sufficient sensitivity to measure GFR under normal and pathophysiological conditions to a similar extent as the gold standard inutest [34]. The mean arterial pressure was continuously monitored with a pressure transducer (model p23 db; Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA). The left renal artery was exposed via a midline abdominal incision, and an ultrasound transit-time flow probe (1RB; Transonic, Ithaca, NY) was placed around the left renal artery and filled with ultrasonic coupling gel (HR Lubricating Jelly; Carter–Wallace, New York, NY) to record the renal blood flow (RBF). Therefore, GFR was determined by using the Davidson method [35].

In addition, the urine protein excretion was measured from 24-h urine collections by using the trichloroacetic acid (TCA)-turbidimetric method [36]. Serum aldosterone was quantitatively determined by enzyme-linked immunosorbent assay (ELISA) following the procedures described by the manufacturer (EIA-4128; DRG International Inc.). Urine and serum creatinine concentrations were measured with QuantiChrom creatinine assay kit (DICT-500), and renal creatinine clearance was calculated.

Urinary Hsp72 protein levels

Urinary heat shock protein 72 (Hsp72) protein levels were analyzed using a commercially available high-sensitivity ELISA (Assay Designs EKS-715, MI). Briefly, samples and standards were added to wells coated with a mouse monoclonal antibody. Hsp72 was captured by the antibody and then detected by adding a rabbit polyclonal detection antibody. Both antibodies are specific for inducible Hsp72 and do not react with other members of the HSP70 family. A horseradish peroxidase (HRP) conjugate bound to the detection antibody and color development was accomplished by the addition of tetramethylbenzidine substrate and stopped with an acidic stop solution. The optical density of samples was read at 450 nm by a plate reader and was compared to a standard curve generated from known concentrations of recombinant Hsp72 that ranged from 0.1 to 12.5 ng/mL.

Histological studies

At the end of the experiment, one kidney was removed and quickly frozen for molecular studies. The other kidney was perfused with phosphate buffer through the femoral catheter, preserving the mean arterial pressure. The kidney was fixed with 4% formaldehyde. After appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 3 μm and stained with routine periodic acid-Schiff and hematoxylin. Ten subcortical and juxtamedullary fields were recorded from each kidney slice using a digital camera mounted on a Nikon microscope (Nikon Instruments Inc., Japan). Digital microphotographs were recorded for each rat slide to assess the number of tubules with cast formation per field. We counted at least 400 tubules on each slide per animal. The damaged tubular area was expressed as the percentage of tubules with cast formation.

Oxidative stress determination

Renal lipoperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed as previously reported [26]. Briefly, after homogenization of the tissue, the reaction was performed in a 15.4 mM solution of *N*-methyl-2-phenylindole in 15% of hydrochloride acid and heated at 45°C for 40 min. The mixtures were centrifuged at 3000 *g* for 15 min. The supernatant absorbance was read at 586 nm. MDA was quantified using an extinction coefficient of 1.1×10 [4] M^{-1}/cm^{-1} and expressed as nanomoles of MDA per milligram of protein. The tissue protein composition was estimated using the Lowry method.

Urinary hydrogen peroxide assay. The amount of hydrogen peroxide (H_2O_2) in urine was determined with an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Eugene, OR) according to the manufacturer's instructions. The assay employed a standard curve of H_2O_2 (1–10 μM). A 50 μL of each urine standard was placed in a microplate; 50 μL of Amplex red reagent/HRP was then added, and the samples were incubated for 30 min at room temperature, protected from the light. In the presence of peroxidase, the Amplex reagent reacts with H_2O_2 to produce resorufin, a red-fluorescent oxidation product. Therefore, the plate was read at 560 nm. The H_2O_2 concentration in the samples was expressed as nanomoles per 24 h.

Molecular studies

RNA isolation and real-time reverse transcription–polymerase chain reaction. The total RNA was isolated from the kidneys using the TRIzol method (Invitrogen, Carlsbad, CA) and checked for integrity using 1% agarose gel electrophoresis. To avoid DNA contamination, total RNA samples were treated with DNAase (DNAase I; Invitrogen). Reverse transcription was conducted with 1.0 μg of total RNA using 200 U of

the Moloney murine leukemia virus reverse transcriptase (Invitrogen). The messenger RNA (mRNA) levels of kidney injury molecule-1 (Kim-1), Rho-kinase, angiotensinogen and angiotensin II type 1 (AT1) receptor as well as prepro-endothelin and ETA and ETB endothelin receptors were quantified by real-time polymerase chain reaction on the ABI Prism 7300 Sequence Detection System (TaqMan; ABI, Foster City, CA). Primers and probes were ordered as kits: Rn00597703_m1 for Kim-1, Mm00485745_m1 for Rho-kinase, Rn00593114_m1 for angiotensinogen, Rn00578456_m1 for AT1 receptor, Rn00560677_s1 for AT2, Rn00561129_m1 for prepro-endothelin, for the ETA receptor and Rn00569139_m1 for the ETB receptor (Assays-on-Demand; ABI). As an endogenous control, we used eukaryotic 18S ribosomal RNA (predesigned assay reagent, external run; ABI). The relative quantification of all gene expression was performed using the comparative 2[-Delta Delta C(t)] method [37].

Western blot analysis

To obtain a pool of cell homogenized kidneys cells, proteins from the cortex of four kidneys in each group were isolated by homogenization with a lysis buffer. The denatured proteins were separated with a sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to polyvinylidine fluoride membranes (Millipore). The membranes were blocked and then incubated overnight at 4°C with goat anti-Rho-kinase antibody (1:2500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-endothelin A receptor antibody (1:5000; Abcam Inc. Cambridge, MA), rabbit anti-endothelin B receptor antibody (1:5000; Abcam Inc.) or goat anti-angiotensin AT1 receptor (1:500; Abcam Inc.). Then, the membranes were incubated with a secondary antibody donkey anti-goat IgG-HRP (Santa Cruz Biotechnology) or rat anti-rabbit IgG HRP (Alpha Diagnostics, San Antonio, TX). To control protein loading and transfer, all membranes were probed with an actin antibody (1:5000) and a secondary antibody, donkey anti-goat IgG-HRP (1:5000; Santa Cruz Biotechnology). For this purpose, the membranes were stripped and re-probed. For stripping, the membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCL, pH 6.7) and incubated at 50°C for 30 min, then the membranes were washed three times with large volumes of wash buffer and were blocked and incubated with the antibodies as described previously. In the case of Rho-kinase, the lower part of the membrane was used for actin blotting. Proteins were detected with an enhanced chemiluminescence kit (Millipore) and autoradiography, following the manufacturer's recommendations. All western blot analyses were performed within the linear range of protein loads and antibody use. The bands were scanned for densitometric analysis.

Tissue levels of endothelin-1

Endothelin-1 levels were analyzed using a commercially available ELISA kit [Endothelin-1 (1-31) Assay kit; Immuno-Biological Laboratories Inc.] according to the manufacturer's instructions. Tissue homogenates and standards were added to the pre-coated wells and incubated overnight at 4°C. Endothelin-1 was captured by the antibody and then detected by adding the labeled antibody and the chromogen. The optical density of the samples was read at 450 nm by a plate reader and was compared to a standard curve generated from known concentrations of endothelin-1 that ranged from 1.56 to 200 pg/mL. The protein concentration in the tissue homogenates was determined by the Lowry method (BioRad). The endothelin-1 concentration was normalized by the amount of protein added to the well.

Statistical analysis

Results are presented as the means ± Standard error. The significance of the differences among groups was tested by analysis of variance (ANOVA) using Bonferroni's correction for multiple comparisons. Statistical significance was defined as having a P-value <0.05.

Results

Figure 1 depicts results of several physiological parameters analyzed in the studied groups. Neither I/R or spironolactone administration modified the mean arterial pressure values among the groups (Figure 1A). As we previously reported [26, 27], renal ischemia induced a significant increase in the serum aldosterone. A similar increase was observed in all experimental groups receiving spironolactone after the renal I/R insult (Figure 1B). After 24 h of ischemia, rats exposed to I/R exhibited renal hypoperfusion, as demonstrated by a significant decrease in RBF, and hypofiltration, as shown by the reduction in the

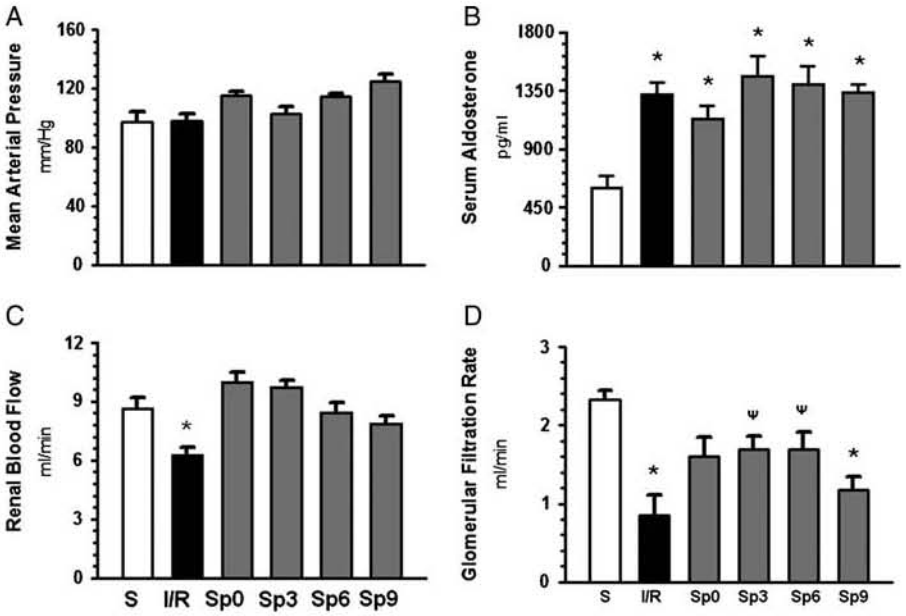

Fig. 1. Functional parameters in rats that underwent I/R and treated with spironolactone after the ischemic insult. (**A**) Mean arterial pressure, (**B**) Serum aldosterone levels, (**C**) RBF and (**D**) GFR in sham-operated rats (white bars), in rats that underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and in rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). Error bars represent the Standard error. *P < 0.05 versus sham-operated rats, ψP < 0.05 versus the I/R group.

GFR (Figure 1C and D, respectively) and creatinine clearance (from 1.65 ± 0.13 to 0.86 ± 0.13 mL/min, $P < 0.05$). RBF reduction was not observed in the groups that received a single dose of spironolactone at 0, 3, 6 or 9 h after renal ischemia. The fall in GFR was partially prevented by spironolactone administration at 0, 3 and 6 h after ischemia as is depicted by GFR mean values (Figure 1D) and creatinine clearances (1.28 ± 0.12, 1.36 ± 0.15 and 1.79 ± 0.44 mL/min, respectively, although only in Sp6 group, the difference was statically different by ANOVA). Even though the renal plasma flow was corrected in the 9-h group, the renal function did not recover.

The upper panel of Figure 2 shows representative images of renal histological slides from the rats that underwent I/R alone (Figure 2A cortex, Figure 2B medulla) and from rats that underwent I/R followed by spironolactone at 0, 3, 6 and 9 h after bilateral renal ischemia (Figure 2C–J, respectively). Light microscopy revealed that renal I/R produced severe tubular damage characterized by lumen dilatation or collapse, loss of the brush border, cellular detachment from tubular basement membranes observed in the renal cortex (Figure 2A, magnification ×400) and extensive cast formation in renal medulla (Figure 2B, magnification ×100). All these lesions were practically absent in rats exposed to spironolactone at 0, 3 and 6 h after ischemic insult (Figure 2C–H, respectively). Consistent with the functional data, at the histological level, no protection was observed when spironolactone was administrated 9 h after ischemia was induced (Figure 2I and J).

Quantitative analysis of the histological images revealed that the percentage of tubules with cast formation in the I/R group was 23.9 ± 5.7%. In contrast, due to spironolactone administration, this percentage was reduced to 2.5 ± 1.3, 6.2 ± 2.2, 11.4 ± 3.7 and 8.7 ± 3.7 in the groups Sp0, Sp3, Sp6 and Sp9, respectively (Figure 2K), but only the values for Sp0 were significantly different from the I/R group, when ANOVA analysis was performed.

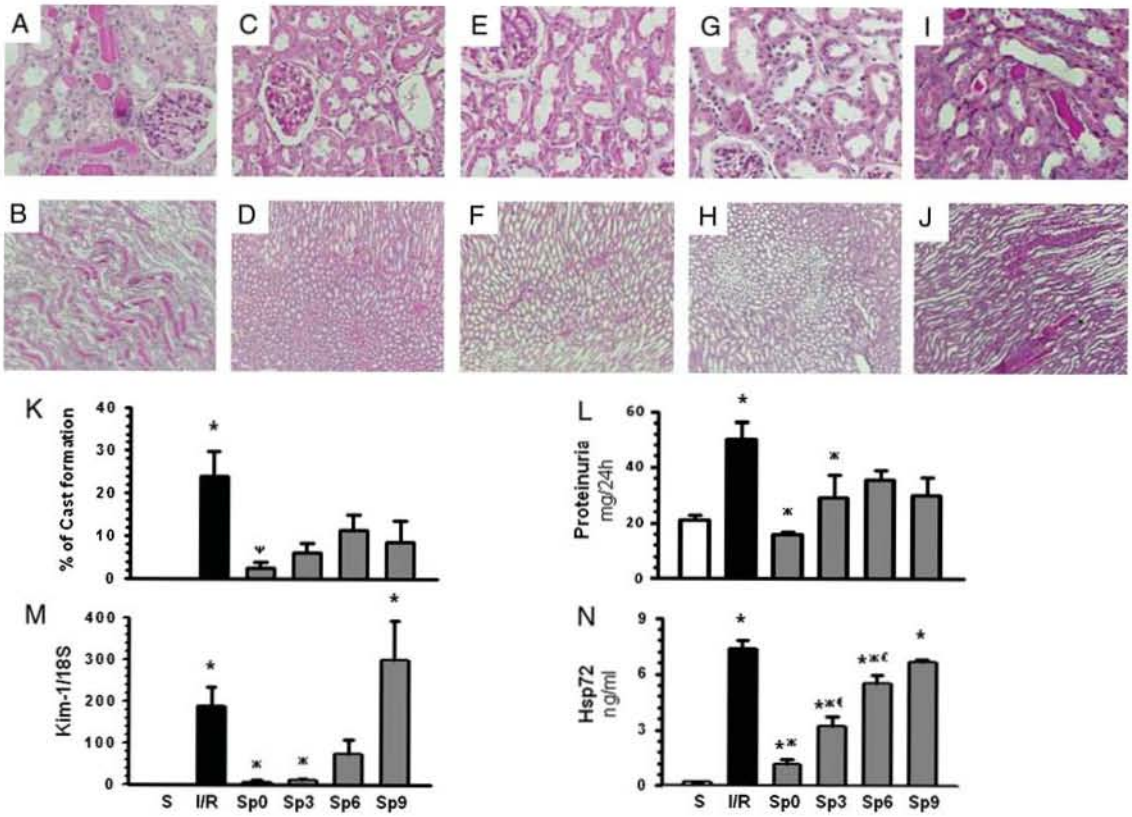

Fig. 2. Effect of spironolactone administration on tubular injury after an ischemic insult induced by I/R. Subcortical and medullary histological sections of kidneys stained with periodic acid-Schiff from studied groups. (**A**) and (**B**) Kidney histology from an untreated I/R rat with detachment of the basement membrane of tubular epithelial cells, tubular dilation, loss of brush border, flattened epithelial cells and the presence of hyaline casts (magnification ×400 and ×100, respectively). (**C**) and (**D**) Representative images of cortical and medullary sections from rats treated with spironolactone immediately after bilateral renal ischemia (magnification ×400 and ×100, respectively). (**E**) and (**F**) Representative images of cortical and medullary sections from rats treated with spironolactone 3 h after bilateral renal ischemia (magnification ×400 and ×100, respectively). (**G**) and (**H**) Representative images of kidney sections from rats treated with spironolactone 6 h after bilateral renal ischemia, (magnification ×400 and ×100, respectively). (**I**) and (**J**) Representative images of kidney sections from rats treated with spironolactone 9 h after bilateral renal ischemia, (magnification ×400 and ×100, respectively). (**K**) Quantification of the tubular percentage of tubules with casts formation, in sham-operated rats (white bars), in rats that underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and in I/R rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively); at least 400 tubules on each histological slide were counted. (**L**) Urinary protein excretion levels. (**M**) Kim-1 mRNA levels and (**N**) Urinary Hsp72 excretion. Error bars represent the Standard error. *$P < 0.05$ versus sham-operated rats, ж$P < 0.05$ versus the I/R group, €$P < 0.05$ versus Sp0 and £$P < 0.05$ versus Sp3.

The biomarkers of tubular injury were consistent with the functional and histological observations. In the I/R group, urinary protein excretion increased by ∼4-fold, when compared with sham-operated rats (Figure 2L). In the rats that underwent I/R and were treated with spironolactone after 0 h, the increase in urinary protein excretion was completely prevented. In the Sp3, Sp6 and Sp9 groups, proteinuria developed, but the levels were significantly lower than those observed in the I/R group; however, only the Sp3 group showed levels that were significantly different. As shown in Figure 2M, Kim-1 mRNA levels increased by ∼150-fold in the I/R group, whereas in the Sp0 and Sp3 groups, Kim-1 mRNA levels were similar to the normal values. Although Kim-1 mRNA levels were increased in the Sp6 group, these values were significantly lower than those of the I/R group. In contrast, in the Sp9 group, Kim-1 upregulation was not prevented. We also assessed the urinary concentration of the Hsp72 because recent observations from our laboratory suggest that this protein is a sensitive and early biomarker of AKI induced by renal ischemia [38]. Accordingly, urinary Hsp72 excretion was significantly increased in the I/R group compared to the sham-operated rats (7.3 ± 1.3 versus 0.1 ± 0.03 ng/mL, respectively, $P < 0.0001$). Spironolactone administrated immediately after bilateral renal ischemia induction prevented Hsp72 upregulation (1.1 ± 0.3 ng/mL), but this upregulation was partially prevented when the mineralocorticoid blocker was administrated 3 or 6 h after the insult (3.2 ± 0.6 and 5.5 ± 1.1 ng/mL, respectively). The decrease in urinary Hsp72 excretion was not observed in the Sp9 group (7.0 ± 0.5 ng/mL).

The renoprotection conferred by early spironolactone administration after renal ischemia was also supported by the measurement of kidney malondialdehyde and urinary H_2O_2 excretion as markers of oxidative stress (Figure 3A and B, respectively). As we previously reported [26, 27], renal injury induced by I/R was associated with an ∼5-fold increase in oxidative stress in both tissue and urine. This increase in oxidative stress was not observed in the groups that received spironolactone at 0 or 3 h after kidney ischemia and this effect was observed in lesser

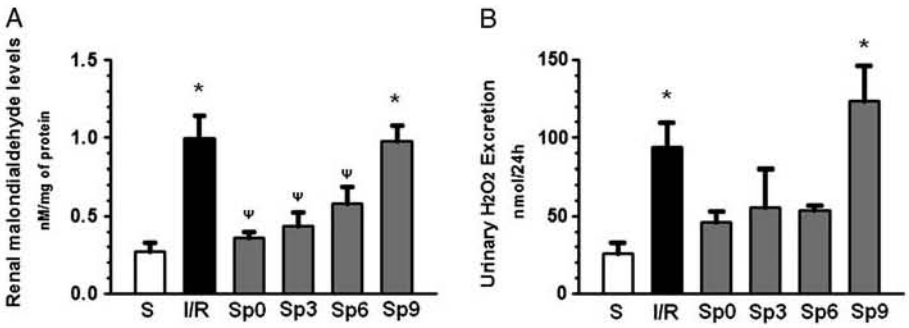

Fig. 3. Effect of spironolactone administration after ischemic insult on oxidative stress induced by I/R. (**A**) Renal malondialdehyde levels in sham-operated rats (white bars), in rats that underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and in I/R rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (**B**) Urinary H_2O_2 excretion in all studied groups. *$P < 0.05$ versus sham-operated rats, ψ$P < 0.05$ versus the I/R group.

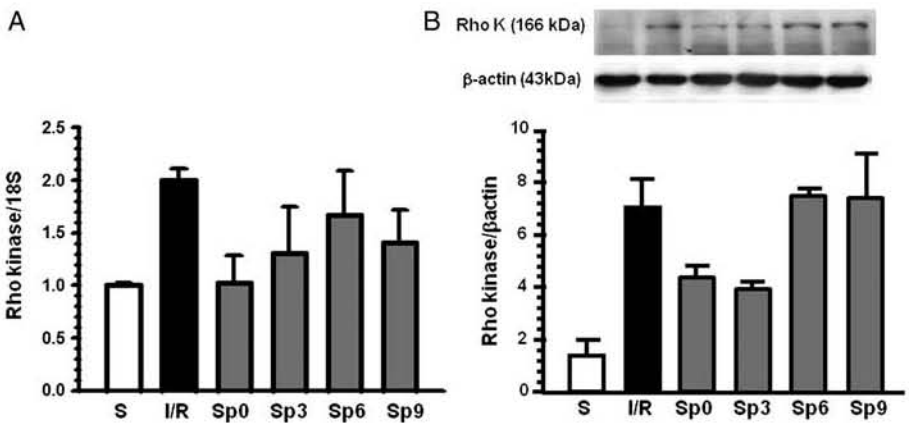

Fig. 4. Ischemia/reperfusion injury is associated with Rho-kinase upregulation and is partially prevented by early spironolactone administration. (**A**) Rho-kinase mRNA levels determined by real-time reverse transcription–polymerase chain reaction in total renal cortex RNA extracted from sham-operated rats (white bars), in rats underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and in I/R rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (**B**) The upper inset is a representative autoradiography image obtained from a western blot analysis of all studied groups. The graph represents the densitometry analysis. Error bars represent the Standard error.

proportion in the rats treated after 6 h. This renoprotective effect was not observed in the Sp9 group.

Recent studies have shown that Rho-kinase is involved in signaling pathways that mediate vasoconstriction [39]. Therefore, its induction by aldosterone might contribute to the renal hypoperfusion and hypoxia observed after an ischemic insult. To determine whether these effects are another potential renoprotective mechanism of spironolactone, the expression levels of Rho-kinase were assessed. Figure 4A shows the mRNA levels of Rho-kinase and Figure 4B the Rho-kinase protein expression. In the kidneys from rats with ischemia and reperfusion, the mRNA and protein levels of Rho-kinase were increased compared to those of the sham-operated rats; however, the differences did not reach significance by ANOVA analysis. Of note, this upregulation was partially prevented in the groups treated with spironolactone immediately and 3 h after renal ischemia, but not in the groups that received spironolactone 6 or 9 h afterward.

Endothelin is a potent vasoconstrictor in renal vasculature; thus, we also assessed the mRNA levels of prepro-endothelin, the tissue endothelin levels by ELISA and the levels of ETA and ETB receptors by western blot analysis. In accordance with our previous observations [27], prepro-endothelin mRNA levels increased by ~9-fold in the I/R group, as is shown in Figure 5A. The increase in prepro-endothelin transcripts was partially prevented by spironolactone administration, even after 9 h. An increase in endothelin after renal ischemia was also observed by using ELISA and was prevented by the administration of spironolactone immediately after ischemia, the differences between I/R rats and those treated at 3, 6 or 9 h were not significant by ANOVA (Figure 5B). This is probably due to the similarity between the I/R group and most of the groups treated with spironolactone; however, it is evident that endothelin was increased by ischemia, indeed *t*-test was significant when this group was compared with sham-operated values, and that this increase was prevented by spironolactone administered at time 0. Renal ischemia had no effect upon the expression of the ETA receptor (Figure 5C). In contrast, the level of the vasodilatory ETB receptor was significantly reduced in the I/R group (Figure 5D). This effect was partially prevented by spironolactone at 0, 3 and 6 h, but not at 9 h.

Angiotensinogen and the AT1 receptor mRNA levels were measured in the total RNA extracted from the kidneys in each study group. No changes in angiotensinogen mRNA levels were observed (Figure 6A), but the I/R

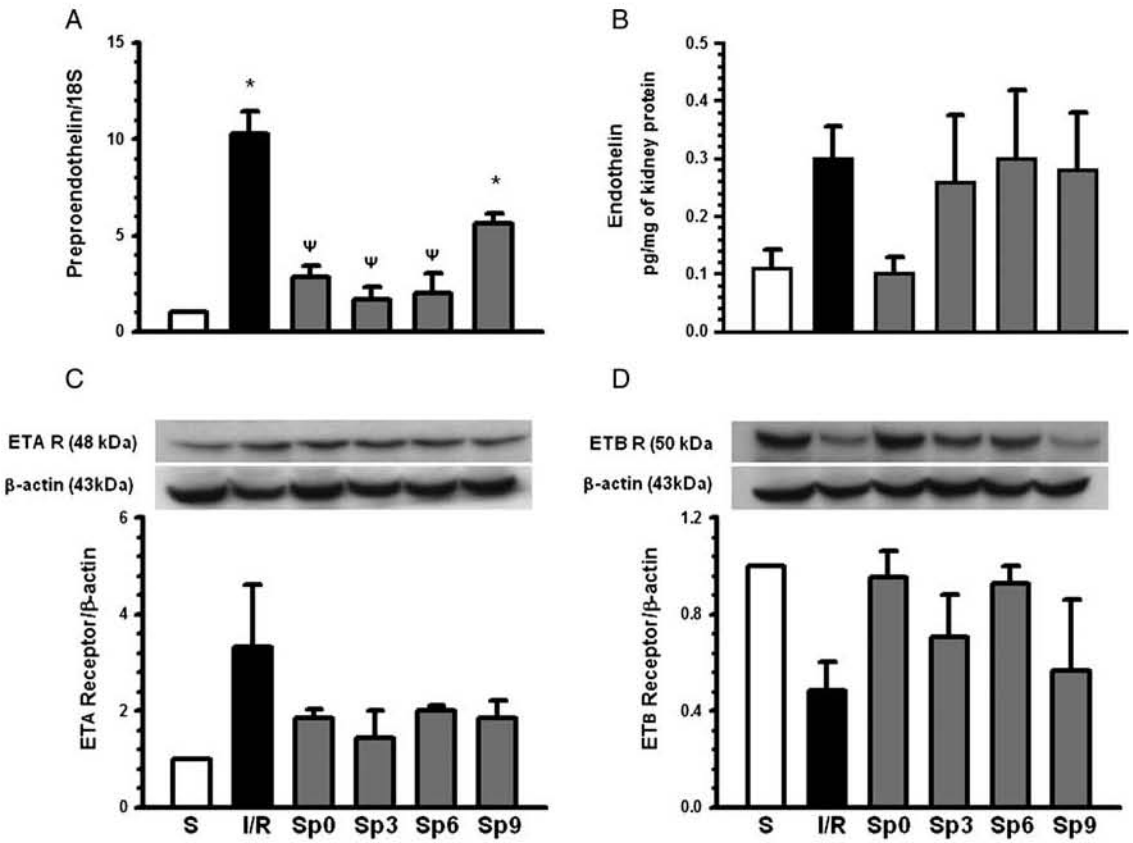

Fig. 5. Ischemia/reperfusion injury is associated with endothelin upregulation and is partially prevented by spironolactone administration. (**A**) Prepro-endothelin mRNA levels determined by real-time reverse transcription–polymerase chain reaction in total renal cortex RNA extracted from sham-operated rats (white bars), in rats underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (**B**) Renal cortex endothelin levels assessed by ELISA in all studied groups. (**C**) and (**D**) ETA and ETB protein levels by western blot analysis; the upper insets are representative images of this chemilumiescent analysis and the lower graphs are the corresponding densitometric analysis. *$P < 0.05$ versus sham-operated rats, ψ$P < 0.05$ versus the I/R group.

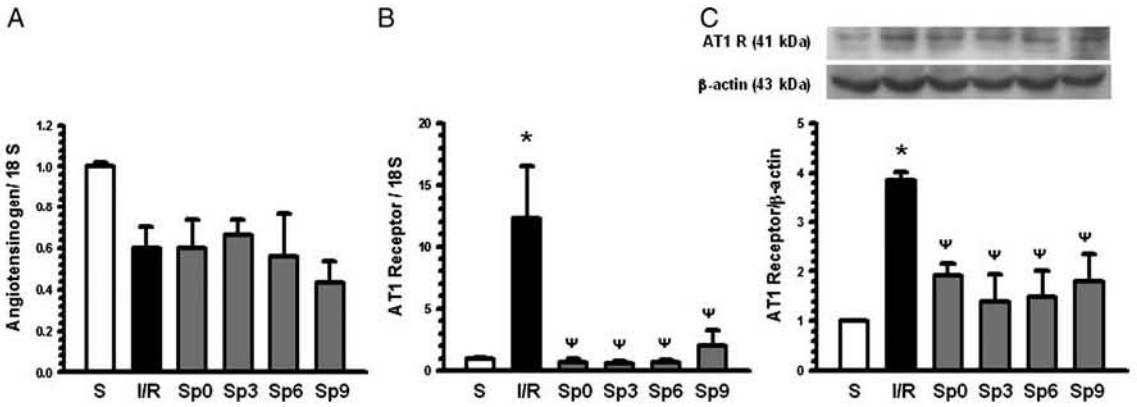

Fig. 6. Ischemia/reperfusion injury is associated with AT1 receptor mRNA levels upregulation and prevented by spironolactone administration. (**A**) The mRNA levels of angiotensinogen assessed by real-time reverse transcription–polymerase chain reaction in total renal cortical RNA extracted from sham-operated rats (white bars), from rats underwent 20 min of bilateral renal ischemia and 24 h of reperfusion (black bars) and from I/R rats that received spironolactone immediately, 3, 6 or 9 h after renal ischemia (gray bars, Sp0, Sp3, Sp6 and Sp9, respectively). (**B**) The mRNA levels of the AT1 receptor. (**C**) AT1 receptor protein levels by western blot analysis, the upper insets are representative images of this chemiluminescent analysis and the lower graph is the corresponding densitometric analysis. *$P < 0.05$ versus sham-operated rats, ψ$P < 0.05$ versus I/R group.

group exhibited more than a 10-fold upregulation of AT1 mRNA, and this effect was completely mitigated in all of the groups treated with spironolactone after the ischemic insult, as depicted in Figure 6B. In accordance with these findings, western blot analysis depicted in Figure 6C, showed that AT1 receptor protein level was significantly increased in the I/R group by 3-fold. In contrast, AT1 receptor upregulation was significantly reduced in all the groups treated with spironolactone.

Discussion

In this study, we show that blocking the MR with spironolactone immediately after or even 3 h after renal bilateral ischemia completely prevented renal dysfunction, tubular injury and oxidative stress. The expected increase in sensitive biomarkers of renal injury was also prevented. A direct relationship between renal protection and the blockage of MR is also supported by the fact that spironolactone conferred partial renoprotection when administered after 6 h and did not confer protection after 9 h.

AKI is characterized by vascular and tubular abnormalities that take place after ischemia and during the reperfusion process. Intra-renal vasoconstriction that leads to a reduction in GFR, together with vascular congestion in the outer medulla and activation of tubulo-glomerular feedback constitute part of the vascular defects, which are likely caused by the increased release of vasoconstrictor factors (mainly endothelin, adenosine and angiotensin II) and decreased production of vasodilators (such as nitric oxide, prostaglandin, acetylcholine and bradykinin) (for a review [40]). Considering that hypoperfusion is a major stimulus of the renin–angiotensin–aldosterone system and that we previously showed that both prophylactic spironolactone administration and adrenals removal prevented renal dysfunction induced by I/R [26, 27], our data suggest that aldosterone plays a primary role in sustaining renal vasoconstriction in this model of ischemic damage. In our previous study using I/R as a model of AKI, the effects of aldosterone were blocked before the ischemic insult took place, and the resulting prevention of AKI was so remarkable that we reasoned that it is possible that spironolactone given after renal ischemia could be helpful to reduce or prevent functional and structural injury. Considering that renal ischemia often occurs unexpectedly in the clinical setting, we evaluated the ability of spironolactone to protect the kidney after establishing an ischemic insult. We found that the decline in RBF with the subsequent reduction in GFR induced by I/R was completely prevented when spironolactone was administrated immediately or 3 h after the renal ischemic insult had occurred. Administering spironolactone 6 h after the ischemic insult produced a lower degree of protection, which, however, was still significant. In this study, we used a low calorie commercial sugar as a GFR marker since we previously showed that this compound has enough sensitivity to measure GFR in normal and pathophysiological conditions to a similar extent of the gold standard polyfructosan [34, 41]. Moreover, our data were further confirmed when the renal function was assessed by creatinine clearance.

It is well known that the tubular epithelium suffers functional and morphological alterations as a consequence of renal hypoperfusion induced by ischemia. Hence, the I/R group exhibited the classical picture of acute tubular necrosis that was accompanied by increased urinary protein excretion, together with upregulation of sensitive biomarkers such as Kim-1 and Hsp72 [38, 42–44]. The morphological alterations and the elevation of sensitive tubular markers of renal injury were also prevented or reduced when spironolactone was administrated immediately and until 6 h after renal ischemia had occurred. Hsp72 was the most consistent biomarker with the time-dependence protection of spironolactone evidenced by the histological injury. These discrepancies among the biomarkers used could be due to a greater sensitivity of Hsp72 to stratify renal injury than Kim-1 or proteinuria, as we previously reported [38] or with different kinetics of induction of each biomarker because it has been

reported that some biomarkers are induced very quickly and returned to normal values after 24 h of renal injury or contrariwise [45]. Taken together, our observations provide evidence about the potential effect that the mineralocoriticoid blockade may have on the improvement of renal function and structure when spironolactone is administrated immediately or even 6 h after an ischemic insult is established. This action spectrum might be more notable in humans, considering the short life of the rats.

As expected [46], the extensive tubular damage observed in the I/R group was associated with a significant increase in oxidative stress, as was evidenced by the increase in renal thiobarbituric acid reactive substances and in the urinary excretion of H_2O_2. This was also prevented by the MR blockade 0, 3 or even 6 h after renal ischemia, suggesting that aldosterone promotes a cellular oxidative milieu. In support of these findings, a specific role for aldosterone in mediating oxidative stress has become apparent [47], specifically; it has been shown that aldosterone induced reactive oxygen species generation by inducing the activation of NADPH oxidase in cultured mesangial cells [48] and by decreasing glucose-6-phosphate dehydrogenase activity [22].

Recent advances in vascular cell biology have demonstrated the substantial involvement of the small GTPase Rho and its downstream effector Rho-kinase in promoting vascular smooth muscle cell contraction by inactivating myosin phosphatase and subsequently increasing myosin light chain phosphorylation (for a review, see [39]). Specifically for AKI, during renal I/R, Rho-kinase mRNA and protein levels increase [27, 49]. Renal injury induced by long-term aldosterone administration has also been associated with increases in myosin phosphate target subunit-1, a marker of Rho-kinase activity [50]. Thus, the Rho/Rho-kinase pathway has recently attracted great attention in various research fields, due to its participation in vascular tone regulation [51] and more recently, due to its role in mediating both extensive foot-process effacement and histologic features of focal segmental glomerulosclerosis [52]. In the present study, we detected that in kidneys isolated from I/R rats that exhibited considerable increases in aldosterone, Rho-kinase mRNA and protein levels were significantly upregulated. This effect was partially prevented when spironolactone was administrated at 0 and 3 h after renal insult. Consistent with this observation, adrenalectomy prevents the Rho-kinase upregulation induced by I/R [27], and eplerenone was able to reduce the Rho-kinase mRNA upregulation observed in salt-induced hypertension in Dahl salt-sensitive (DS) rats [53]. Moreover, the Rho-kinase inhibitor fasudil attenuated the renal injury induced by I/R [54]. All these studies together suggest that aldosterone mediates Rho-kinase upregulation and may mediate, at least in part, the renal vasoconstriction and dysfunction induced by I/R. Even though the GFR was partially recovered in the Sp6 group, it was not associated with the restoration of Rho-kinase levels, suggesting that the possible induction of this kinase by aldosterone occurred very early after ischemia, pointing out that blocking angiotensin and endothelin pathways (Figures 5 and 6) are also involved in the beneficial effect of spironolactone.

As was previously mentioned, the reduction in RBF is primarily mediated by an imbalance in vasoactive substance release. Endothelin is a largely vasoconstrictive peptide that has been implicated in renal pathophysiology [55]. An increase in endothelin levels during I/R-induced renal injury has been observed by us as well as by others [27, 56]. Here, we corroborated that I/R-induced renal injury was related with an upregulation of prepro-endothelin mRNA levels and renal endothelin level. These transcriptional changes were prevented in the animals that underwent ischemia and received spironolactone at 0, 3 and 6 h, suggesting that aldosterone mediates this transcriptional effect. In fact, several studies have demonstrated an increase in both endothelin-1 transcript and protein in response to aldosterone in vascular smooth muscle [57], cardiac [58] and renal [56] tissues as well as in inner medullary collecting duct cells [59]. Interestingly, responsive elements to MRs have been identified in the endothelin promoter [60]. Moreover, endothelin upregulation may also increase Rho-kinase activity [58], prolonging the vicious cycle that contributes to the adverse effects of aldosterone under pathophysiological conditions.

As mentioned above, angiotensin II is a mediator of renal injury induced by I/R. We previously showed that blocking AT1 receptor with losartan partially prevents renal dysfunction in rats that have undergone ischemia [61], suggesting that this peptide partially participates in the renal vasoconstriction observed in this model of renal damage. Although we did not observe changes in the angiotensionogen mRNA levels, the AT1 receptors were upregulated at the mRNA and protein levels in rats that underwent ischemia/repefusion, an effect that was completely prevented by spironolactone.

We recently observed that the prophylactic treatment with MR blocker spironolactone completely prevented I/R-induced AKI in rats [26]. Here, we showed a similar degree of protection when spironolactone was administrated after the ischemic insult had been established. The renoprotection conferred by spironolactone was mediated by slowing down endothelin, AT1 receptor and Rho-kinase renal levels that contributed to preventing renal hypoperfusion and the concomitant generation of free radicals. These data together with our previous findings show that MR antagonism should be further studied as a strategy for preventing AKI following renal ischemia.

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Conflict of interest statement. None declared.

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ARB protects podocytes from HIV-1 nephropathy independently of podocyte AT1

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Abstract

Background. Angiotensin I-converting enzyme inhibitors and angiotensin receptor blockers protect podocytes more effectively than other anti-hypertensive drugs. Transgenic rats overexpressing angiotensin II Type 1 (AT1) receptor selectively in podocytes have been shown to develop glomerulosclerosis. The prevailing hypothesis is that angiotensin II has a capacity of directly acting on the AT1 receptor of podocytes to induce injury. We therefore investigated the mechanism of reno-protective effect of AT1 receptor in a mouse model of HIV-1 nephropathy.

Methods. We generated transgenic mice carrying the HIV-1 gene (control/HIV-1) or both HIV-1 gene and podocyte-selectively nullified AT1 gene (AT1KO/HIV-1). In these mice, we measured urinary protein or albumin excretion and performed histological analysis.

Results. At 8 months of age, AT1KO/HIV-1 ($n = 13$) and control/HIV-1 ($n = 15$) mice were statistically indistinguishable with respect to urinary albumin/creatinine ratio (median 2.5 versus 9.1 mg/mg), glomerulosclerosis (median 0.63 versus 0.45 on 0–4 scale) and downregulation of nephrin (median 6.90 versus 7.02 on 0–8 scale). In contrast to the observed lack of effect of podocyte-specific AT1KO, systemic AT1 inhibition with AT1 blocker (ARB) significantly attenuated proteinuria and glomerulosclerosis in HIV-1 mice.

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Clinical Study

De Novo Donor-Specific HLA Antibody Development and Peripheral $CD4^+CD25^{high}$ Cells in Kidney Transplant Recipients: A Place for Interaction?

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The aim of this study was to determine whether the abundance of regulatory T cells (Tregs) ($CD4^+CD25^{high}$) affects the *de novo* development of anti-HLA donor-specific antibodies (DSAs) in kidney transplant recipients (KTRs). *Methods*. Unsensitized (PRA ≤ 10%, no DSA) adult primary KTRs who received a living (83%) or deceased (17%) KT in our Institution during 2004/2005 were included. DSA testing was performed monthly, and Tregs were quantified by flow cytometry every 3 months, during the 1st year after KT. All patients received triple drug immunosuppressive therapy (CNI + MMF or AZA + PDN); 83% received anti-CD25. *Results*. 53 KTRs were included; 32% developed DSA during the 1st year after KT. Significantly lower 7-year graft survival was observed in those who developed DSA. No difference was observed in Treg numbers up to 9 months after KT, between DSA positive and negative. However, at 12 months after KT, DSA-negative patients had significantly higher numbers of Treg. *Conclusions*. Early development of DSA was not associated to variations in Treg abundance. The differences in Treg numbers observed at the late time point may reflect better immune acceptance of the graft and may be associated to long-term effects. Additional inhibitory mechanisms participating earlier in DSA development after KT deserve to be sought.

1. Introduction

Effective immunosuppressive regimens have greatly improved the early survival of renal allografts. However, the rate of late allograft loss has remained relatively constant [1]. This is probably related to the fact that late allograft dysfunction not only results from immune-mediated damage, but also occurs as the consequence of a complex series of events that include arterial fibrointimal thickening, interstitial fibrosis, and tubular atrophy [2–4].

The presence of donor-specific antibodies (DSAs) directed against human leukocyte antigens (HLAs) has been associated in a growing number of reports to poor prognosis of renal allografts [5–7]. The association between anti-HLA antibodies and poor renal allograft evolution is explained by diverse alloantibody-mediated clinical syndromes, ranging from hyperacute rejection [8], early and late acute alloantibody-mediated rejection [9–11], and chronic humoral rejection [12].

In contrast to the well-known acute and devastating effects of preformed antibodies, *de novo* produced antibodies against an implanted graft do not cause immediate failure [7]. However, *de novo* antibodies may eventually cause chronic graft rejection [13, 14].

The presence of DSA implies that B-cells bearing a B cell receptor able to bind to donor HLA have effectively presented

alloantigens through the indirect pathway and have received T-cell help [15]. However, presentation of alloantigens may result in T-cell activation or in the generation of a tolerogenic response. The factors that determine if a proinflammatory or a regulatory response will prevail in an individual patient are unknown [16]. With this scenario as a background, we were interested in studying whether the *de novo* development of DSA could be influenced by the number of peripheral regulatory T cells (Tregs) during the first year after transplantation in kidney transplant recipients. Therefore, the aims of this study were to document (i) the development of *de novo* DSA during the first year after kidney transplantation, (ii) the abundance of peripheral Tregs during the same period, (iii) the temporal relationship between peripheral Treg numbers and the *de novo* development of DSA, and (iiii) the function and survival of renal allografts in a group of patients who received a kidney transplant during 2004 and 2005 at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán and were followed during at least 5 years.

2. Subjects and Methods

2.1. Patients and Sera Samples. We included in this prospective study all adult patients (>18 yrs) who received a primary kidney transplant from either a living or deceased donor in our institution during 2004 and 2005 and met the following criteria: current negative T-cell and B-cell AHG-CDC crossmatches; PRA ≤ 10%; absence of DSA class I and class II. During the first year after transplantation, monthly blood samples were drawn for DSA testing, and once every 3 months for Tregs quantification. Clinical data, gathered both at baseline and prospectively, included demography, cause of renal failure, type of renal replacement therapy, pretransplant blood transfusions, pregnancies, donor source, shared haplotypes for living related donors, or HLA mismatches for living unrelated and deceased donors, use of induction therapy, immunosuppressive schedule, biopsy-proven acute rejection episodes during the entire followup, graft function at 3, 12, and yearly (≥60 months posttransplant) thereafter until last followup, time and cause of graft loss or death. Institutional Review Board approval was obtained to conduct this trial, and all participant patients signed an informed consent.

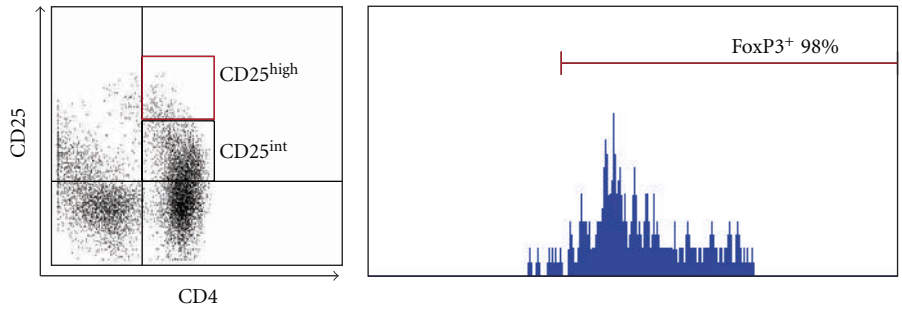
2.2. Immunosuppressive Regimen . Induction therapy with 2 mg/Kg (total dose) of Daclizumab was administered to all kidney transplant recipients (KTRs), except in 2 cases that had a 2-haplotype match. The immunosuppressive treatment included (a) Cyclosporine (target plasma levels 175–200 ng/mL during the first 3 months and ∼150 ng/mL thereafter) or Tacrolimus (target plasma levels 8–12 ng/mL during the first 3 months and ∼5 ng/mL thereafter); (b) an antiproliferative drug, either azathioprine (1.5–2 mg/Kg), or mycophenolate mofetil (2 g/day when combined with cyclosporine; 1.5 g/day with tacrolimus); (c) methylprednisolone 10 mg/Kg on transplant day, followed by daily boluses of 500 mg, 250 mg, and 125 mg, followed by prednisone starting on 100 mg on the 5th posttransplant day and gradually tapering down to 5 mg/day after 3 months.

2.3. Donor-Specific Antibodies Assessment. KTRs and their donors were HLA typed before the transplant using LABTypeSSO (One Lambda) according to the manufacturer's instructions. All pre- and posttransplantation sera were tested for the presence of HLA class I and class II IgG antibodies using LABScreen Mixed according to the manufacturer's instructions (One Lambda, Inc., Canoga Park, CA). All sera positive for HLA antibodies (class I or II) were additionally tested for DSA with single antigen LABScreen beads (One Lambda Inc., Canoga Park, CA). Briefly, 20 μL of serum samples were incubated with HLA class I-coated and HLA class II-coated microspheres, respectively, for 30 minutes in the dark under gentle agitation. The specimens were then washed five times before being incubated with anti-human IgG-conjugated phycoerythrin in the same conditions as in the first incubation. The Labscan 100 flow analyzer (Luminex, Austin, TX) was used for beads and data acquisition. Data were then analyzed with HLA Visual software (One Lambda). The cut-off level was defined as a baseline normalized >500 mean fluorescence intensity units (MFI). The presence of DSA was assigned by comparing the various HLA specificities proposed by the software analysis with the HLA typing of the donor for all the transplanted patients.

2.4. Peripheral Tregs Quantification. Peripheral blood mononuclear cells (PBMCs) were obtained from patients and healthy donors by density-gradient centrifugation (Lymphoprep). PBMCs were stained with anti-CD25-PE (Clone M-A251, BD Pharmingen) and anti-CD4-PerCP (BD Pharmingen). In some experiments, cells were fixed and permeabilized with cytofix/cytoperm (BD Pharmingen) and intracellular staining (anti-FoxP3-FITC) was performed according to the instructions of the manufacturer. Data were collected on a FACScan flow cytometer (BD Biosciences). A $CD25^{high}$ gate that included >95% $FoxP3^{+}$ cells was defined in normal subjects (Figure 1) [17]. This gate, that included ∼2% of $CD4^{+}$ T cells, was used for the quantification of $CD25^{high}$ cells in all patients.

2.5. Acute Rejection Definition and Treatment. Acute graft dysfunction was defined as an unexplained increase ≥ 25% in serum creatinine (SCr). In all cases, graft biopsy was performed and urinary obstruction and/or infection were ruled out. Acute rejection was defined according to Banff '97 and the '03 update working classification of renal allograft pathology [18, 19]. Treatment for acute rejection consisted of 3 days of high dose I.V. methylprednisolone (12 mg/Kg/day). Acute cellular (ACR) steroid-resistance rejections were treated with thymoglobulin. Acute antibody mediated rejection (AMR) episodes were treated with 3 sessions of plasmapheresis (PP), followed each by 100 mg/Kg body weight IVIG, and Rituximab.

2.6. Graft Function at Followup. Data required for glomerular filtration rate estimation (eGFR) according to the Modification of Diet in Renal Disease (MDRD) formula, at 1, 12 months, and last followup after KT was prospectively gathered from medical records.



(a) (b)

Figure 1: Regulatory T cells were defined as $CD4^+CD25^{high}$ T cells. (a) Cells were quantified using the gate indicated in red. A representative dot plot is shown. (b) Virtually all $CD4^+CD25^{high}$ cells express high levels of FoxP3.

Table 1: Patient characteristics.

	All $N = 53$ (%)	DSA negative $N = 36$ (%)	DSA positive $N = 17$ (%)	P
Recipient female	30 (56.6)	20 (55.6)	10 (58.8)	1.000
Donor female	30 (56.6)	21 (58.3)	9 (52.9)	0.942
Recipient age, mean ± SD	32.3 ± 12.4	31.1 ± 12.2	34.8 ± 12.7	0.317
Donor age, mean ± SD	38.5 ± 10.9	37.9 ± 10.3	39.8 ± 12.1	0.558
ESRD etiology				
Diabetes	6 (11.3)	4 (11.1)	2 (11.8)	0.693
Lupus	3 (5.7)	3 (8.3)	0 (0)	0.556
Glomerulonephritis	3 (5.7)	3 (8.3)	0 (0)	0.556
Hypertension	2 (3.7)	2 (5.6)	0 (0)	0.827
Other	3 (5.7)	1 (2.8)	2 (11.8)	0.494
Unknown	36 (67.9)	23 (63.9)	13 (76.4)	0.548
Sensitizing events				
Blood transfusions	35 (66.0)	23 (63.9)	12 (70.6)	0.865
Pregnancies ($N = 30$)	11 (36.7)	5 (25.0)	6 (60.0)	0.108
Donor type				
Living donor	44 (83)	29 (80.6)	15 (88.2)	0.701
Deceased donor	9 (17)	7 (19.4)	2 (11.8)	
HLA mismatches				0.040*
0	6 (11.3)	6 (16.7)	0 (0)	0.186
1	5 (9.5)	4 (11.1)	1 (5.9)	0.917
2	6 (11.3)	5 (13.9)	1 (5.9)	0.693
3	15 (28.3)	9 (25)	6 (35.3)	0.652
4	2 (3.8)	2 (5.6)	0 (0)	0.827
5	13 (24.5)	6 (16.7)	7 (41.1)	0.111
6	6 (11.3)	4 (11.1)	2 (11.8)	0.693
Immunosuppression				
Daclizumab	42 (79.2)	26 (72.2)	16 (94.1)	0.082
CsA+Aza+Pdn	9 (17.0)	6 (16.7)	3 (17.6)	0.762
CsA+MMF+Pdn	3 (5.7)	3 (8.3)	0 (0)	0.556
Tac+Aza+Pdn	18 (34.0)	13 (36.1)	5 (29.4)	0.865
Tac+MMF+Pdn	22 (41.5)	14 (38.9)	8 (47.1)	0.791
Other	1 (1.8)	0 (0)	1 (5.9)	0.698

All the patients had a PRA < 10% at transplantation and absence of donor-specific antibodies (DSAs). *P value by Chi square for trend.

2.7. Statistical Analyses. For continuous variables, differences between two groups were evaluated by independent sample Student's t or Mann-Whitney U tests according to data distribution (normal or abnormal, resp.). We used Chi-square test for categorical variables. The Fisher exact test was used when expected values were under 5. The association between HLA mismatches and DSA status was evaluated with Chi-Square for trend. To compare all measurements of Treg during 1 year, we used Kruskal-Wallis test. Graft survival was analyzed using the Kaplan-Meier method with log rank test for comparison of survival curves. A $P < 0.05$ value was considered statistically significant.

3. Results

3.1. Study Population. Seventy-six first kidney transplants were performed in our center from January 2004 to December 2005. Fourteen out of 76 KTRs had a pretransplant PRA > 10% and therefore were not included. Sixty-two patients were enrolled in the study, but 9 were not considered in the final analysis. Reasons for excluding these patients were as follows: death during the first 12 months ($n = 2$) and followup in another institution ($n = 7$) during the 1st year after transplant. Therefore, the total number of KTRs included in this analysis was 53.

Baseline characteristics of the patients are shown in Table 1. No differences were observed between patients who remained DSA negative and those who developed DSA during the first year after transplant, regarding demography, ESRD cause, antecedent of sensitizing events, donor type, HLA mismatches, or immunosuppressive treatment. According to the inclusion criteria, all patients should have had a pretransplant PRA ≤ 10% and absence of DSA. PRA was 0% for 46 (86.8%) patients; 7 (13.2%) patients had a PRA class I between 2 and 7%, and only 1 of them had a PRA class II of 3%.

3.2. DSA Development during the First 12 Months after Transplantation. Seventeen patients (32%) developed DSA during the first year after KT. Class I DSA, class II DSA, or both were detected in 11, 4, and 2 patients, respectively. As shown in Figure 2(a), development of DSA during the first year after KT was significantly associated to lower graft survival ($P = 0.021$). Graft survival was also lower in patients who developed class I DSA (Figure 2(b)); however, the difference did not reach statistical significance ($P = 0.079$). No difference was associated to development of class II DSA ($P = 0.494$) (data not shown).

3.3. Quantitative Profile of Regulatory T Cells ($CD4^+CD25^{high}$) during the First 12 Months after Transplantation and Its Relationship to DSA Development. Tregs were considered as absolute number (cells/υL) and as percentage of $CD4^+$ T cells. The median (interquartile range) absolute number detected at baseline and during months 3, 6, 9, and 12 were 6.45 (1.7–10.9), 1.49 (0.2–5.0), 5.98 (2.3–11.9), 5.51 (3.9–8.6), 4.81 (2.1–14.6), respectively ($P < 0.001$); these correspond to 1.20% (0.4–1.7), 0.16% (0.02–0.52), 0.97% (0.34–1.44), 0.80% (0.41–1.69), and 0.68% (0.29–1.84), respectively ($P < 0.001$). There was a decrease in Treg number at 3 months. This was probably associated to the effect of anti-CD25 induction therapy. At month 6, the abundance of peripheral blood Tregs tended to increase and remained similar to the baseline number up to the end of the followup (12 months).

Between month 6 and the end of the first year, the numbers of peripheral blood Tregs differed between the patients who developed DSA and those who did not. A progressive decrease in Treg numbers was observed in patients who developed DSA. In contrast, an increase in this population was observed in patients who remained DSA negative (Figures 2(c) and 2(d)). Importantly, the decrease in Treg numbers was specifically associated with development of class I DSA ($P = 0.023$).

Table 2: Acute rejection episodes.

	DSA− $N = 36$	DSA+ $N = 17$
Number (%) of patients with acute rejection episodes	2 (5.6)	6 (35.3)*
Number of acute rejection episodes	3	9
Banff 97 grade and the 03 update working classification		
Acute/active cellular rejection		
Mild acute (IA)	1	1
Mild acute (IB)	0	3
Moderate acute (IIA)	1	1
Moderate acute (IIB)	0	0
Severe acute (III)	0	1
Antibody-mediated rejection		
I	0	0
II	1	2
III	0	1

*$P = 0.010$ (Fisher exact test).

3.4. Acute Rejection Episodes. The number of biopsy-proven acute rejection episodes that occurred during the entire followup was 11. Table 2 describes the patients who developed acute rejection episodes, the number of events confirmed in these patients, and the type of acute rejection documented, according to DSA status. There was a significant difference in the number of patients who experienced acute rejection episodes in the DSA-positive group compared to those in the DSA-negative group ($P = 0.01$).

3.5. Graft Loss and Patient Death. Five grafts were lost during followup. The causes of graft loss in these patients corresponded to biopsy-confirmed chronic antibody-mediated rejection (1), grade III chronic allograft nephropathy (1), grade III acute and chronic antibody mediated rejection plus thrombotic microangiopathy (1), acute cellular rejection 1B superimposed to a chronic vascular rejection (1), and acute and chronic cellular mediated rejection (1).

It is worth mentioning that 4 out of these 5 patients belonged to the group that developed DSA during the first 12

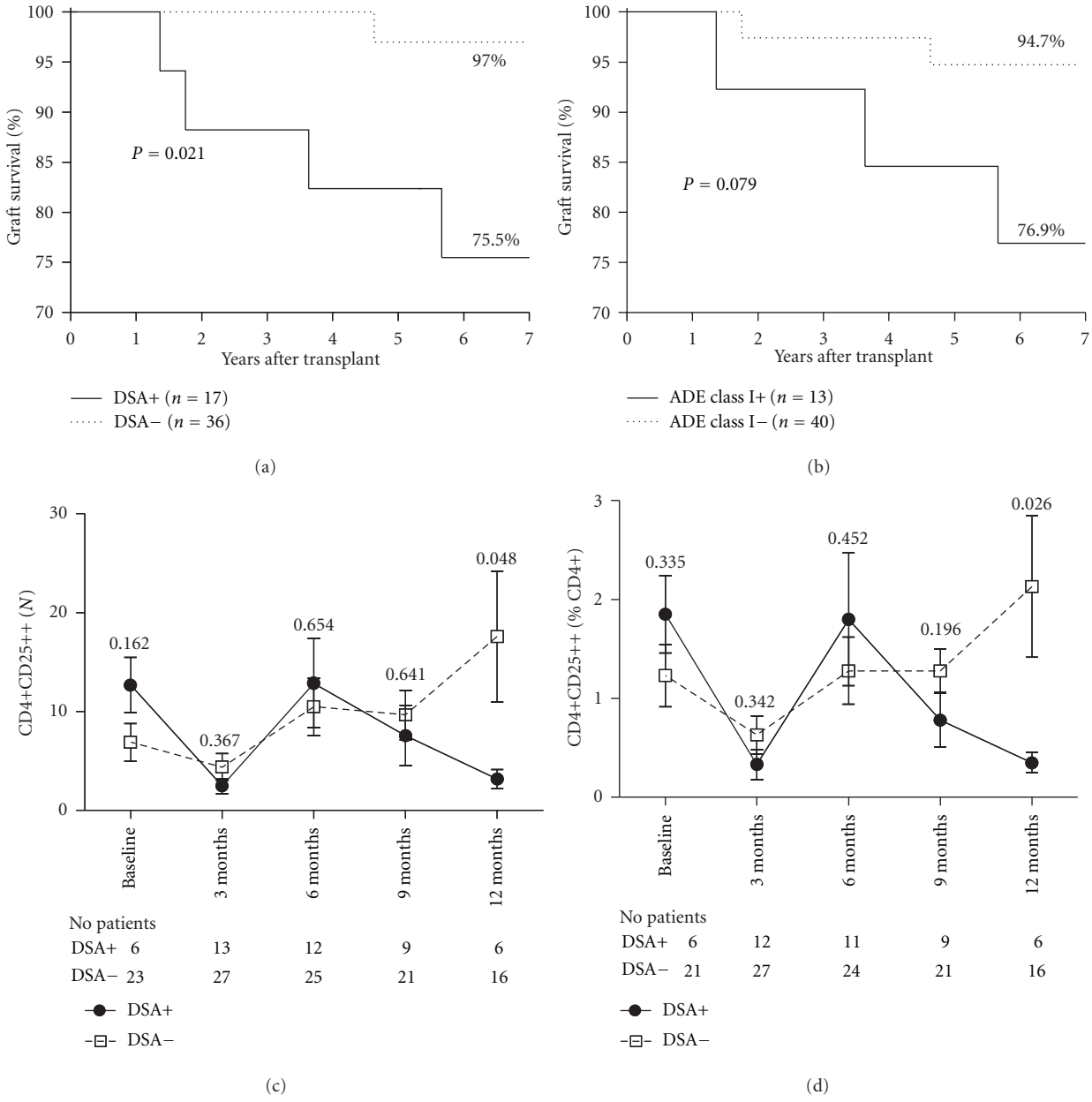


Figure 2: (a) Graft survival according to DSA development during the first year post-KT; DSA+ve versus DSA−ve, $P = 0.021$. (b) Graft survival according to class I DSA development during the first year post-KT, $P = 0.079$. Treg numbers (c) and percentages (d) at different time points during the first year post-KT in patients who developed DSA and those who remained DSA−ve. At 12 months, the difference in Treg numbers and percentages was significantly different between DSA+ve versus DSA−ve patients, $P = 0.048$, and $P = 0.026$, respectively.

months after transplantation (Figure 2(a)). Patient survival was 100%.

3.6. Graft Function. One of the most pursued aspects of the surveillance of these patients was the evolution of renal function for at least 5 years after transplantation. Overall, graft function assessed through SCr and MDRD eGFR shows that regardless DSA status during the first year, there was an increase in SCr as time elapsed. Albeit not significant, deterioration of graft function was more evident in the DSA-positive group according to this parameter (Table 3). When we analyzed delta eGFR (last SCr obtained in 2010—1 month after transplantation), the DSA-negative group showed a mean positive slope of 13.14 mL/min, while a mean slope of −0.41 mL/min was observed in the DSA-positive group. This analysis only included patients with graft function.

4. Discussion

We studied prospectively a cohort of DSA-negative renal transplant patients and quantified the numbers of peripheral blood regulatory T cells (defined as $CD4^{+}CD25^{high}$) at

Table 3: Graft function according to DSA development (1st year after KT).

	All $N = 53$ (%)	DSA negative $N = 36$ (%)	DSA positive $N = 17$ (%)	P
Graft function at 1 month				
SCr (mg/dL)	1.22 ± 0.33	1.27 ± 0.33	1.11 ± 0.30	0.096
eGFR (by MDRD, mL/min)	68.93 ± 27.18	65.37 ± 23.55	76.48 ± 33.15	0.167
Graft function at 12 months				
SCr (mg/dL)	1.23 ± 0.40	1.20 ± 0.32	1.29 ± 0.53	0.481
eGFR (by MDRD, mL/min)	65.69 ± 20.04	66.27 ± 19.53	64.44 ± 21.64	0.759
Deltas (12 months versus 1 month)				
SCr (mg/dL)	.0002 ± 0.39	−0.07 ± 0.36	0.15 ± 0.43	0.053
eGFR (by MDRD, mL/min)	−2.35 ± 21.65	0.62 ± 19.25	−8.66 ± 25.51	0.759
Graft function in 2010				
SCr (mg/dL)	1.95 ± 2.56	1.54 ± 1.91	2.79 ± 3.46	0.181
eGFR (by MDRD, mL/min)	74.41 ± 29.92	79.45 ± 26.96	64.03 ± 33.75	0.081
Deltas (2010 versus 1 month)				
SCr (mg/dL)	0.72 ± 2.59	0.26 ± 1.99	1.67 ± 3.40	0.129
eGFR (by MDRD, mL/min)	8.71 ± 27.59	13.14 ± 25.47	−0.41 ± 30.29	0.097

different time points during the first year. We found that, as expected, Treg numbers dropped following administration of anti-CD25, but their numbers recovered 6 months after transplantation. Moreover, we observed that, in a subset of the patients, Treg numbers remained stable and tended to increase towards the end of the first year. These patients remained DSA negative, and their renal allograft had a better outcome. We also detected a group of patients whose Treg numbers decreased in the second semester after transplantation. DSA development was associated with this phenomenon.

Currently, one of the most challenging aspects in the field of kidney transplantation is the unmet need to translate the fantastic improvements achieved in first-year graft survival—exceeding 90%—into long-term graft survival [1]. Antibody-mediated injury is increasingly recognized as a factor implicated in long-term graft attrition [20, 21]. Important contributions have identified the clinical significance of DSA before the transplant [22] and when their production follows the procedure [14].

One of the purposes of our study was to evaluate the significance of *de novo* DSA developed during the first-year after kidney transplantation. A significantly higher number of patients in the DSA-positive group developed acute rejection episodes compared to the patients that remained DSA negative. Also, long-term graft survival was reduced significantly in the former group of patients. Three out of four biopsies performed in DSA-positive patients who lost the graft revealed acute and/or chronic AMRs combined with findings of T-cell-mediated rejection. In the other patient, a T-cell-mediated rejection dominated the picture. In general, all these rejections corresponded to late AMR that have been associated to *de novo* DSA [23]. Also, it has been suggested that late AMR episodes pose a worse long-term prognoses compared to early AMR episodes. In this study, *de novo* DSA represented a biomarker for graft loss, as has been previously suggested [24]. Most probably DSA development detected during the posttransplant evolution translates a heightened immune response, which, associated to nonadherence or to other factors, provides an opportunity for intervention to prolong graft survival.

The impact that *de novo* DSA conveys to graft survival has been demonstrated previously [14, 25]. Nickerson et al. [26] described 3 presentation patterns related to *de novo* DSA. The histological findings in the grafts of the patients included in our study where acute and/or chronic AMRs are combined with findings of T-cell-mediated rejection suggest that nonadherence to treatment could have been a participant factor [23, 25].

One of the aims of our study was to explore the behavior of the number of Tregs during the first year after transplantation in nonsensitized kidney transplant recipients, and the temporal relationship between Treg numbers and *de novo* DSA development. Naturally occurring $CD4^+$ Tregs constitutively express high levels of the IL-2 receptor alpha chain (CD25), are generated in the thymus, and display a powerful suppressive capacity [27]. Tregs are able to regulate the activity of several types of immune cells including effector T cells and B cells [28].

We were able to detect a drop in the number of $CD4^+CD25^{high}$ T cells at month 3 after transplant. This finding is in agreement to the data published by Segundo et al. [29] and most probably represents the effect of the anti-CD25 monoclonal antibody administered as induction therapy. Previous studies have shown that the majority of Tregs in humans express high levels of CD25 [30], and Bluestone et al. showed that basiliximab caused a transient loss of both $FoxP3^+$ and $FoxP3^-$ $CD25^+$ T cells [31]. In the patients studied in our series, the number of $CD4^+CD25^{high}$ recovered by month 6 and remained stable during the first year after transplant.

No differences in Treg numbers were evident during the first 6 months between the patients who developed or not DSA (Figures 2(c) and 2(d)). However, a progressive increase

in Treg number apparent at the 9th month was observed in the group of patients who remained DSA negative. In sharp contrast, Treg numbers dropped in the group that eventually developed DSA. The difference in $CD4^+CD25^{high}$ T cells between DSA positive/negative groups was significant at month 12. It is interesting to note that more DSA-positive patients received induction therapy with anti-CD25 compared to DSA-negative patients (94% versus 72%). Even though the difference did not reach statistical significance, the trend suggests that administration of anti-CD25 therapy might in some cases cause a prolonged decrease in Treg numbers.

Immunophenotyping was not carried out in the performed biopsies, and protocol biopsies were not performed in patients whose renal function remained stable and were DSA negative. This would have provided us with valuable material to compare the cellular infiltrate in both conditions (rejection versus stable grafts). This represents a weakness of the study. Another weakness of the study is that Tregs were defined as $CD25^{high}$. Since CD25 is a cell activation marker, $CD25^+$ cells may represent activated T cells. Other markers, in particular FoxP3, are more specific for Tregs. Nevertheless, we believe that we did not overestimate the number of Tregs because we gated in $CD25^{high}$ cells that were virtually all $FoxP3^+$ (Figure 1) and because we found that a lower number of Treg was associated with more inflammation. If we were including activated T cells, the bias would have been in the inverse direction.

Summing up, we have presented data that suggests that $CD4^+CD25^{high}$ cells display a protective role against DSA development during the 1st year after KT. Additional inhibitory mechanisms participating earlier in DSA development after KT deserve to be investigated.

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Spironolactone prevents chronic kidney disease caused by ischemic acute kidney injury

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Acute kidney injury (AKI) has been recognized as a risk factor for the development of chronic kidney disease (CKD). Aldosterone has a critical role in promoting renal injury induced by ischemia. Here, we evaluated whether spironolactone administered before or after AKI caused by ischemia protects against CKD. In the first set of experiments, Wistar rats underwent a sham operation without or with prior spironolactone treatment, or underwent 45 minutes of bilateral renal ischemia without or with spironolactone treatment before ischemia and assessed over 270 days. The second set of rats received low (20 mg/kg) or high (80 mg/kg) doses of spironolactone at three different times after the sham operation or bilateral renal ischemia and were assessed after 90 days. Untreated animals developed CKD following ischemia-induced AKI as characterized by a progressive increase in proteinuria, renal dysfunction, podocyte injury, glomerular hypertrophy, and focal sclerosis. This was associated with increased oxidative stress, an upregulation of tumor growth factor (TGF)-β, followed by upregulation of the TGF-β downstream effectors phospho-Smad3, collagen I, fibronectin, and proinflammatory cytokines. Treatment with spironolactone either before or after ischemia prevented subsequent CKD by avoiding the activation of fibrotic and inflammatory pathways. Thus, spironolactone may be a promising treatment for the prevention of AKI-induced CKD.

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Ischemic kidney injury is the primary cause of acute kidney injury (AKI) in hospitalized patients and is associated with high morbidity and mortality rates. In addition, the incidence of AKI has significantly increased in the past 15 years owing to an aging general population and the increased prevalence of obesity, diabetes, and hypertension, which predispose patients to an AKI event.[1–5] AKI occurs in approximately 15% of hospitalized patients and in up to 40–60% of intensive care unit patients.[6,7]

For many years, it was commonly thought that patients surviving episodes of AKI recover their renal function without further consequences. However, recent evidence based on epidemiological observations in patients who suffer from AKI strongly suggests otherwise. AKI has thus been proposed to be a risk factor for developing chronic kidney disease (CKD) and, in particular, for promoting the transition of CKD to end-stage renal disease.[8–11] In support of this hypothesis, the probability of developing CKD or end-stage renal disease over time is proportional to the severity and duration of the AKI event.[8,12,13] Moreover, of great concern is the recent evidence demonstrating that 6.6% of AKI patients who had a complete renal function recovery exhibited a greater risk of death and *de novo* CKD after 2–4 years of follow-up.[12] Until now, however, the progression of AKI to CKD in rats with two intact kidneys, which would allow elucidating the mechanisms causing AKI to progress to CKD, has been rarely explored. Such a model would also be beneficial for identifying pharmacological interventions to prevent injury due to AKI and/or stop the development of CKD and end-stage renal disease.

Several studies involving experimental models have demonstrated that aldosterone has an important role in the physiopathology of renal injury induced by ischemic process, including acute and chronic cyclosporine A nephrotoxicity and ischemia/reperfusion (I/R).[14–21] Accordingly, we have shown that prophylactic spironolactone treatment[16] or adrenal gland removal[17] completely prevents AKI in rats undergoing bilateral renal ischemia. Furthermore, spironolactone administration at different postischemia intervals prevents or reduces functional and structural renal injury,[22] suggesting that aldosterone is a key molecule in mediating ischemic renal

injury and that mineralocorticoid receptor (MR) antagonism, even after ischemia, is a helpful strategy to prevent AKI.

In this study, we characterized a model of CKD induced by a single episode of ischemic AKI and the molecular mechanisms that lead to the development of CKD. Our data reveal that spironolactone administration before or after the ischemic insult is a useful strategy to prevent or reduce the development of CKD.

RESULTS

We have previously shown that, after 24 h of bilateral ischemia, rats develop severe renal dysfunction and tubular injury.[16] Figure 1 shows that renal dysfunction induced by ischemia was completely resolved after 9 days of reperfusion, similar to conditions that often occur in clinical settings. Urinary protein excretion was significantly elevated after 24 h of reperfusion in rats subjected to ischemia and continues to progressively decline until normal values were reached after 6 days of reperfusion (Figure 1a). Proteinuria in spironolactone-pretreated rats was 50% lower than the proteinuria in the untreated group, and normal levels were reached faster. Consequently, the rats undergoing ischemia recovered renal function after 10 days, as demonstrated by normal values of renal blood flow, serum creatinine, and creatinine clearance (Figure 1b–d). Despite the complete improvement in renal function, interleukin (IL)-6 mRNA and phosphorylated Smad3 levels were significantly higher than those in the sham-operated group. Cytokine upregulation was not observed in spironolactone-pretreated rats (Figure 1e–f). These results suggest that although renal function recovered, proinflammatory and profibrotic pathways remained active. These pathways may be able to perpetuate renal injury, which can lead to CKD, a situation that did not occur in spironolactone-pretreated animals.

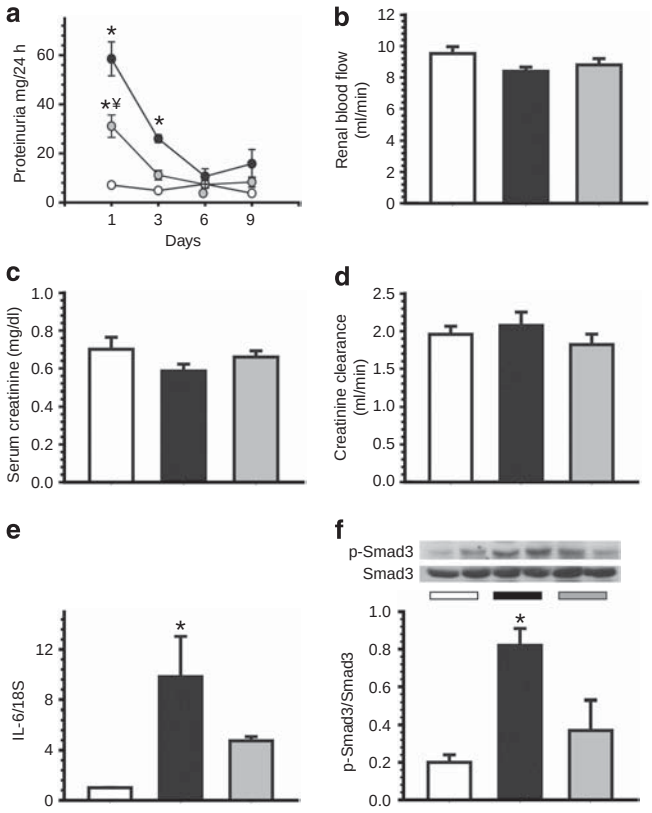

Figure 1 | Renal dysfunction induced by ischemia was completely resolved after 10 days of reperfusion, but proinflammatory and profibrotic cytokines remained enhanced. (**a**) Urinary protein excretion 1, 3, 6, and 9 days after reperfusion, in the sham (o), bilateral renal ischemia (•), and ischemia + Sp pretreatment (•) groups. At 10 days after reperfusion, the rats were killed, and renal function was determined. (**b**) Mean renal blood flow, (**c**) serum creatinine, and (**d**) creatinine clearance. (**e**) Interleukin (IL)-6 mRNA levels were measured in the renal cortex. (**f**) The upper insets show representative autoradiographs from the phosphorylated-Smad3 (p-Smad3) and Smad3 western blot analysis for sham (white bars), bilateral renal ischemia (black bars), and ischemia + Sp pretreatment (gray bars) groups. The lower graphs display the corresponding densitometric analysis. *$P < 0.05$ vs. the sham group and ¥$P < 0.05$ vs. the ischemia group.

Ischemic insult leads to progressive renal dysfunction that can be prevented with spironolactone pretreatment

We assessed whether an AKI episode induced by ischemia leads to CKD. Because we have previously shown that I/R injury is prevented by spironolactone pretreatment or adrenal gland removal,[16,17] we evaluated whether spironolactone pretreatment before ischemia also prevents CKD development. There was considerable mortality in the A-to-C group in the first 10 days after ischemia (57%), and survival was markedly improved by spironolactone pretreatment (15%) (Figure 2a). Proteinuria was assessed every 30 days. The animals that survived the ischemic insult developed a progressive increase in proteinuria, from 20.2 ± 1.5 mg/day at 30 days to 164.8 ± 11.3 mg/day at 270 days. This increase was not observed in the A-to-C + Sp group (9.2 ± 1.0 mg/day at 30 days and 27.1 ± 2.0 mg/day at 270 days) (Figure 2b). Despite the similar body weights of all rats at the beginning of the study, the A-to-C group exhibited a lower average body weight at the end of the study (583 ± 16.3 g) than did the S, Sp, and A-to-C + Sp groups (752 ± 28.3, 729 ± 19.2, and 721 ± 11.8 g, respectively). After 9 months, the A-to-C group developed renal dysfunction that was characterized by a significant reduction in renal blood flow and creatinine clearance (Figure 2c and d). Interestingly, the A-to-C + Sp group failed to develop renal dysfunction. As shown in Figure 2e, at the end of the study, the mean arterial pressure was similar among the groups. Therefore, the renal injury observed in the rats that developed CKD was not due to hypertension. In agreement with these findings, the rats that developed CKD exhibited an increase in the levels of urinary kidney injury molecule-1, an effect that was not observed in the A-to-C + Sp group (Figure 2f).

Ischemic insult leads to severe renal structural injury: prevention by spironolactone pretreatment

Representative light microscopy sections from rat kidneys stained with periodic acid–Schiff are shown in Figure 3a–d.

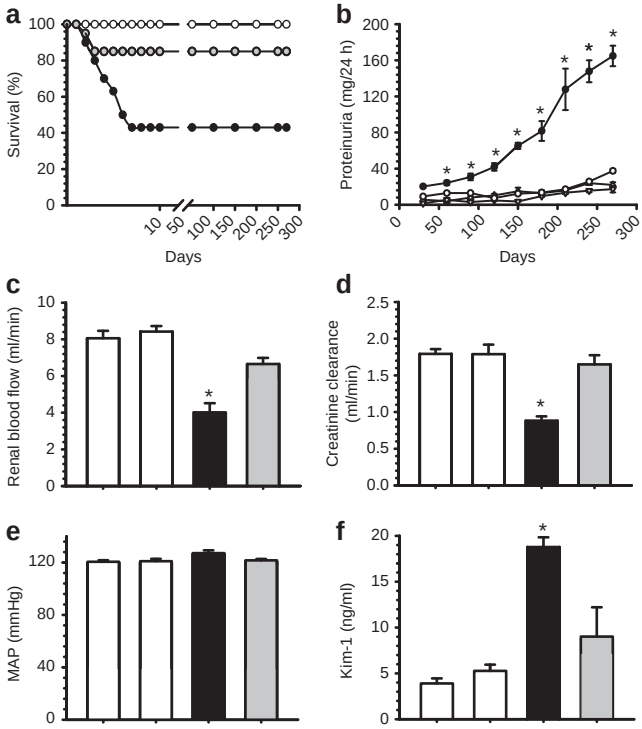

Figure 2 | Acute kidney injury leads to the development of chronic kidney disease (CKD), which can be prevented by spironolactone pretreatment. Four groups were included: sham surgery (S), $n = 9$; rats receiving spironolactone 3 days before sham surgery (20 mg/kg per day), $n = 9$ (Sp); rats undergoing renal bilateral ischemia, $n = 28$ (A-to-C); and rats receiving spironolactone 3 days before bilateral ischemia, $n = 13$ (A-to-C + Sp). (**a**) The survival rate in the A-to-C group (black circles) was 43%, compared with 85% in the A-to-C + Sp group (gray circles) and 100% in the sham and Sp group (overlaid white circles). (**b**) Urinary protein excretion was measured every 30 days during follow-up: sham (▽), Sp (Δ), A-to-C (•), and A-to-C + Sp (o). At the end of the experimental period, (**c**) renal blood flow, (**d**) creatinine clearance, (**e**) mean arterial pressure, and (**f**) urinary kidney injury molecule-1 (Kim-1) levels were determined in the sham (white bars), Sp (white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). *$P < 0.05$ vs. the S and Sp groups.

Rats undergoing ischemia exhibited severe structural alterations characterized by glomerular hypertrophy, glomerulosclerosis, cast formation, severe tubular dilation, and tubulointerstitial fibrosis. By contrast, the A-to-C + Sp group exhibited glomerular and tubular architecture similar to that of control rats. These findings were corroborated by quantification of the number of dilated tubules, percentage of glomerulosclerosis, and glomerular diameter size (Figures 3 and 4). Tubular dilation was present in 42.3 ± 5.0% of the total tubules in the A-to-C group, whereas only 13.1 ± 2.7% of the A-to-C + Sp group displayed dilation ($P < 0.01$). Similarly, the A-to-C group exhibited a significantly higher glomerulosclerosis percentage (10.0 ± 4.4%) than the S and A-to-C + Sp groups (0% and 0.4 ± 0.4%, respectively).

The degree of glomerular hypertrophy was evaluated by measuring glomerulus diameter and by generating a distribution of glomerular size. Figure 4a shows the normal diameter distribution of the control group, wherein most of the glomeruli were in the range of 101–125 μm (38.3%), and only a minor proportion was found in the ranges of 76–100 μm (19.5%) and 126–150 μm (27.5%). The histogram for the control group exhibits a typical bell-shaped Gaussian distribution, as we have shown previously.[21] By contrast, in the A-to-C group, the glomerular diameter distribution was shifted to the right, reflecting glomerular hypertrophy. Accordingly, 43.3% of the glomeruli were >151 μm in diameter. In addition, a lower proportion of glomeruli were found in the diameter ranges of 101–125 μm (20.4%), 76–100 μm (8.3%), and 50–75 μm (0%). All of these differences were statistically significant according to contingency analysis, as shown in Figure 4b. The glomerular size distribution of the A-to-C + Sp group was similar to that of the control group, but not to that of the A-to-C group (Figure 4c), indicating that glomerular hypertrophy was nearly absent. In agreement with these findings, renal hypertrophy in the A-to-C group was also evidenced by a significant elevation in the mean kidney weight (2.5 ± 0.2 g) compared with the S and Sp groups (1.6 ± 0.1 and 1.6 ± 0.1 g, respectively); this increase was not observed in the A-to-C + Sp group (1.5 ± 0.1 g).

Transmission electron microscopy of rat kidneys with CKD revealed ultrastructural alterations that included microvillus degeneration and effacement and detachment of podocyte foot processes (Figure 4e). These alterations were rarely observed in the A-to-C + Sp group (Figure 4f). Furthermore, an extensive tubulointerstitial area was affected by fibrosis in the A-to-C group (Figure 5a and b), but a lower extent of fibrosis was observed in the A-to-C + Sp group (Figure 5c and d). These observations were confirmed by the morphometric analysis represented in Figure 5e. The A-to-C group exhibited fibrosis in 44.8 ± 16.0% of the tubulointerstitial area, compared with 18.7 ± 4.5% in the A-to-C + Sp group; this difference was significant.

Tubular dilation is due in part to cellular proliferation

To determine whether the severe tubular dilation observed in the A-to-C group was associated with tubular cell proliferation, immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) was performed. The A-to-C group exhibited considerable tubular cell proliferation as demostrated by the number of PCNA + cells, an effect that was almost absent in the A-to-C + Sp group (Figure 6a–c). These findings were confirmed by calculating the percentage of nuclei that stained positive for PCNA (Figure 6d). The positive cells were primarily located in the dilated tubules, suggesting that enhanced cell proliferation is promoting tubular dilation in the A-to-C group. This assumption is also supported by a significant correlation between the percentage of dilated tubules and the percentage of PCNA + cells (Figure 6e, $r^2 = 0.87$).

Tubulointerstitial fibrosis is mediated by TGF-β pathway activation

The role of the tumor growth factor (TGF)-β pathway in promoting tubulointerstitial fibrosis was also evaluated.

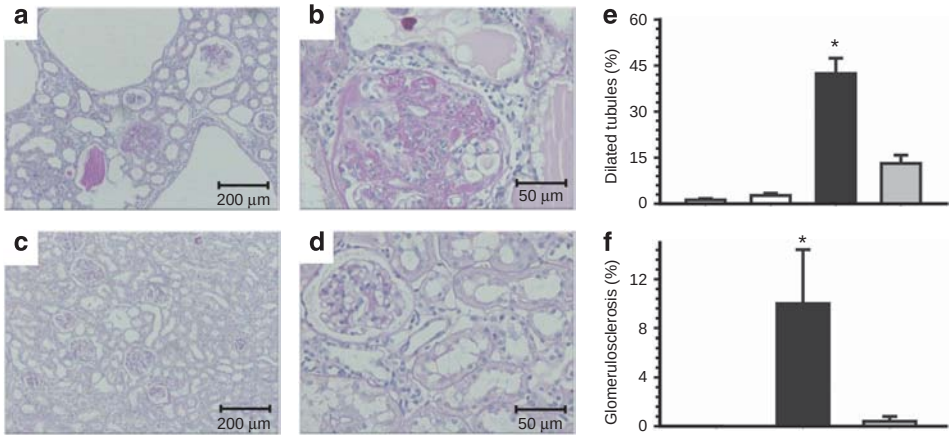

Figure 3 | An acute kidney injury (AKI) episode leads to severe structural damage, which can be prevented by Sp pretreatment. (**a**, **b**) Representative images of periodic acid–Schiff (PAS)-stained sections from the A-to-C group, and (**c**, **d**) sections from the A-to-C + Sp group. (**e**) The percentage of dilated tubules was quantified by counting the number of dilated tubules as a proportion of the total number of tubules. (**f**) Glomerulosclerosis percentage for the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). *$P < 0.05$ vs. the S and Sp groups.

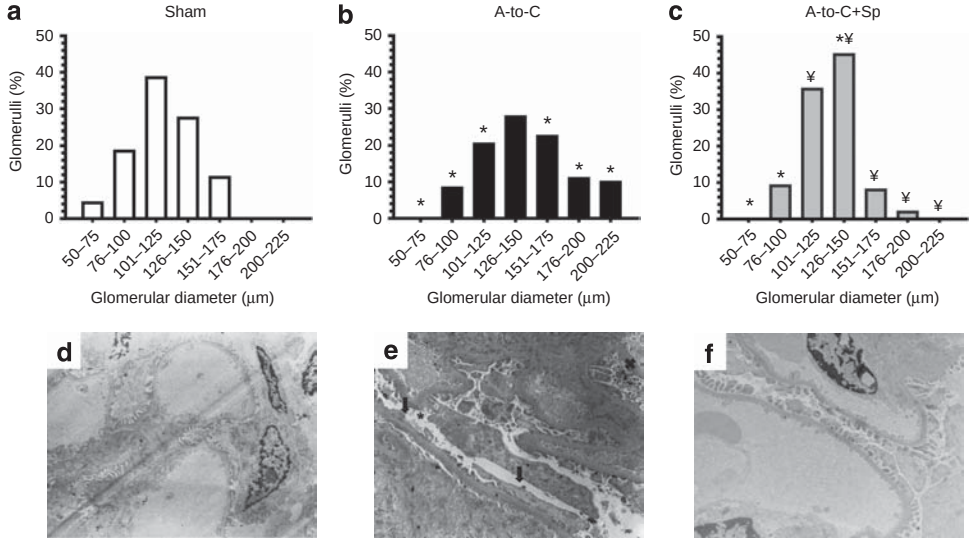

Figure 4 | Glomerular hypertrophy and ultrastructural lesions in rats with chronic kidney disease (CKD). (**a**) The glomerular diameter distribution is represented in white bars for the sham group, (**b**) in black bars for the A-to-C group, and (**c**) in gray bars for the A-to-C + Sp group. Representative transmission electron micrographs are shown in (**d**) the sham group, (**e**) the A-to-C group, and (**f**) the A-to-C + Sp group. Black arrows indicate foot process fusion, and asterisks indicate foot process detachment. Original magnification: ×6,300. *$P < 0.05$ vs. the S group and $^{¥}P < 0.05$ vs. the A-to-C group.

The A-to-C group exhibited a significant twofold elevation in TGF-β mRNA levels. This increase was not observed in the A-to-C + Sp group (Figure 7a). To assess whether this pathway was efficiently activated, the renal levels of downstream effectors of the TGF-β pathway, including phosphorylated Smad3, fibronectin, collagen I, and α-smooth muscle actin (α-SMA), were measured by western blot analysis as shown in Figure 7b–f. The levels of all of these proteins were significantly elevated in the rats that developed CKD. Intriguingly, the activation of the TGF-β pathway was completely prevented in the A-to-C + Sp group. Recent studies have shown that when Smad2 is phosphorylated, it can act as an antifibrotic modulator of the TGF-β pathway.[23] We found that the A-to-C + Sp group, which exhibited less tubulointerstitial fibrosis, also displayed a significant elevation in phospho-Smad2 levels.

Renal injury induced by an ischemic insult is also mediated by increased oxidative stress

Urinary H_2O_2 excretion in the A-to-C group was significantly elevated compared with the S and Sp groups (64.1 ± 7.5 vs. 10.4 ± 1.1 and 5.9 ± 1.0 nmol/min, respectively) (Figure 8a). However, the elevation in urinary H_2O_2 excretion was abrogated in the A-to-C + Sp group (14.8 ± 2.5 nmol/min). In addition, catalase activity was significantly reduced in the A-to-C group (Figure 8b), and this effect was almost entirely prevented in the

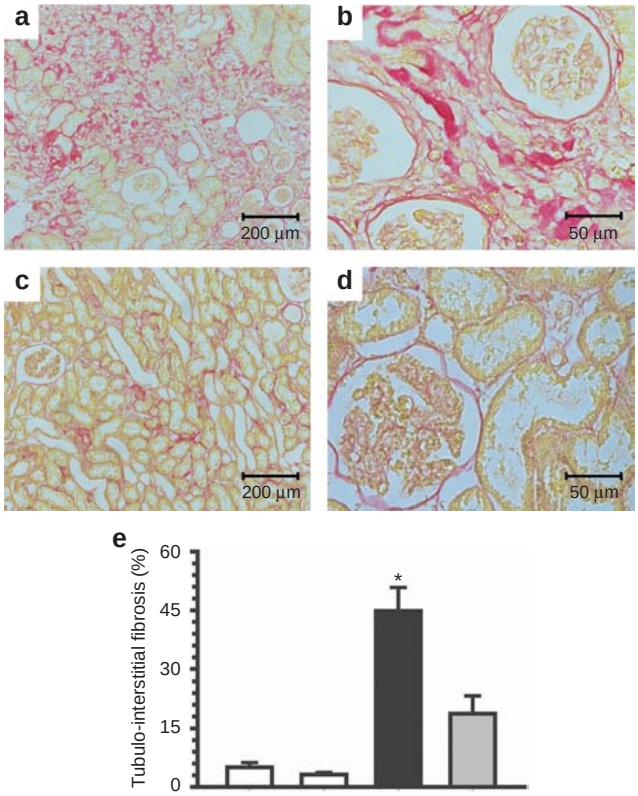

Figure 5 | Tubulointerstitial fibrosis in rats with chronic kidney disease (CKD). (**a**, **b**) Representative light micrographs after Sirius red staining showing the presence of fibrosis (in red) from the A-to-C group, and (**c**, **d**) micrographs from the A-to-C + Sp group. (**e**) The percentage of tubulointerstitial fibrosis in each of the four groups at the end of the 270-day experiment was quantified by morphometric analysis for sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). $^{*}P < 0.05$ vs. the S and Sp groups.

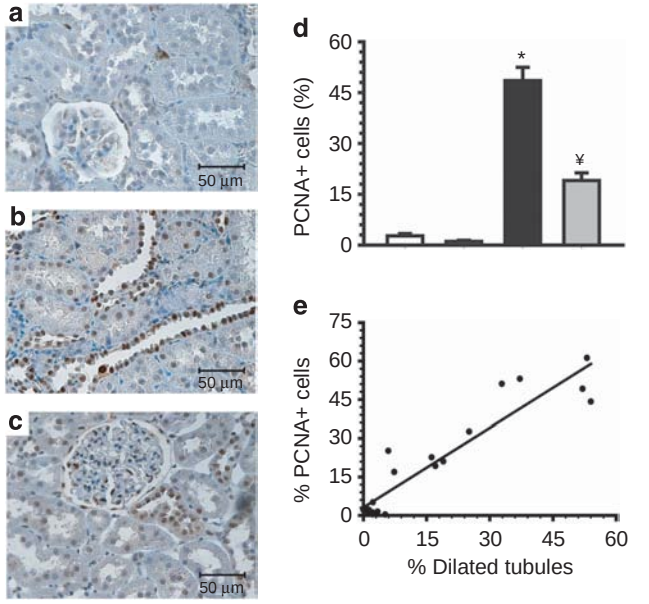

Figure 6 | Tubular cell proliferation as assessed by proliferating cell nuclear antigen (PCNA) immunohistochemistry. To evaluate whether epithelial cells were proliferating and provoking tubule dilation, immunohistochemistry for PCNA was performed in kidney sections from the four groups. (**a**) Representative images from the sham, (**b**) A-to-C, and (**c**) A-to-C + Sp groups. (**d**) The percentage of PCNA + cells in the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). (**e**) The correlation between the percentage of PCNA + cells and the percentage of dilated tubules ($r^2 = 0.87$). $^{*}P < 0.05$ vs. the S and Sp groups, and $^{¥}P < 0.05$ vs. the A-to-C group.

A-to-C + Sp group. No differences in glutathione peroxidase activity were observed among the groups (Figure 8c).

Inflammatory cytokine upregulation is involved in ischemia-induced CKD

Tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), and IL-6 mRNA and protein levels were assessed by real-time reverse transcription–polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. As shown in Figure 9, the mRNA levels of these cytokines were upregulated in the A-to-C group by 1.6-fold for TNF-α and more than 13-fold for MCP-1 and IL-6, compared with the S group. By contrast, the A-to-C + Sp group exhibited mRNA levels similar to the control group. These observations were corroborated at the protein level; a similar pattern was observed in the enzyme-linked immunosorbent assay results.

CKD induced by AKI was also reduced by spironolactone administration after the ischemic insult

Spironolactone administered at 0 or 1.5 h after the ischemic insult prevented the development of proteinuria compared with the untreated A-to-C group either at low or high doses (Figure 10a and d). A low dose of spironolactone administered 3 h after ischemia was unable to prevent, but did significantly reduce, the progressive elevation of proteinuria (Figure 10a). These findings were not associated with significant changes in renal blood flow (Figure 10b and e) or creatinine clearance (Figure 10c and f), probably because 3 months is an early stage of the CKD, often exhibiting proteinuria, without renal dysfunction, as was observed after a longer period of observation after similar ischemia (Figure 2). Despite the lack of significant differences among these groups, spironolactone-treated animals exhibited better renal function than the ischemic group.

At 3 months after inducing bilateral renal ischemia, the untreated A-to-C group, compared with the sham-operated group, exhibited morphological alterations, such as tubular dilation, cast formation, glomerular hypertrophy, and extensive tubulointerstitial fibrosis (Supplementary Figure S1 online). All of these changes were reduced in the animals treated with a low dose of spironolactone (Supplementary Figure S2 online) and were prevented in animals treated with a high dose of spironolactone (Supplementary Figure S3 online). In fact, glomerular hypertrophy (measured as the distribution of glomerular diameters) was prevented in the groups that received a low dose of spironolactone at 0 h

(Figure 11c) or high dose at either 0 or 1.5 h after renal bilateral ischemia (Figure 11f and g, respectively). Although kidney changes were not completely prevented, these renoprotective effects of spironolactone were also observed with a low dose at 1.5 and 3 h after ischemia (Figure 11d and e, respectively). Morphometric analyses demonstrated that, 3 months after inducing ischemia, the untreated rats exhibited fibrotic damage in 33.3% of their tubulointerstitium compared with damage in 3.7% of the tubulointerstitium in the sham-operated rats (Supplementary Figure S1 online). In contrast, a low dose of spironolactone administered either at 0 and 1.5 h post ischemia reduced the tubulointerstitial fibrosis to 14.8% and 15.1%, respectively, and the 3-h administration reduced the fibrosis to a lesser extent, 20.9% (Supplementary Figure S2 online). A high dose of spironolactone at 0 or 1.5 h post ischemia was more effective in reducing the area affected by tubulointerstitial fibrosis to 11.8% and 7.8%, respectively (Supplementary Figure S3 online).

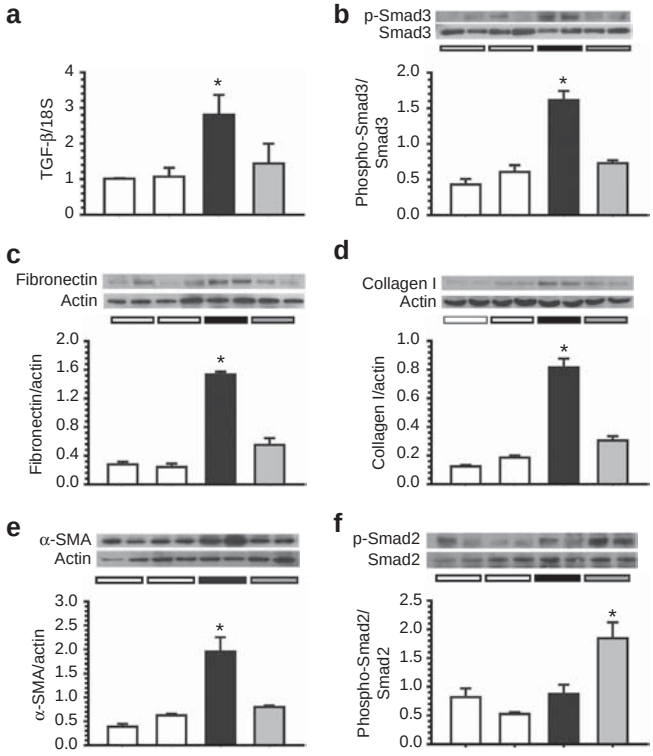

Figure 7 | Association of renal fibrosis with the tumor growth factor (TGF)-β pathway activation. (**a**) TGF-β mRNA levels were quantified by real-time reverse transcription–polymerase chain reaction (RT-PCR). Densitometric analysis of the western blots was performed for (**b**) phospho-Smad3 (p-Smad3), (**c**) fibronectin, (**d**) collagen I, (**e**) α-smooth muscle actin (α-SMA), and (**f**) phospho-Smad2 in the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). In each panel, the upper insets depict representative blots of the corresponding proteins. *$P < 0.05$ vs. the S and Sp groups.

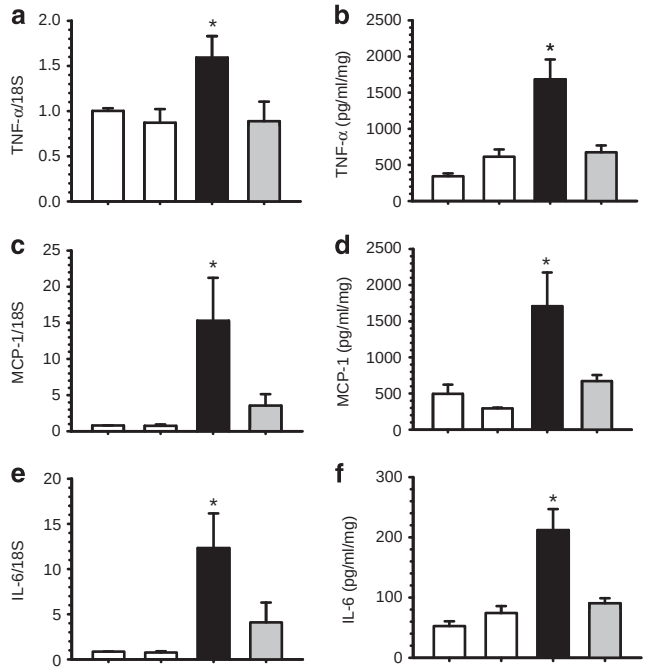

Figure 9 | The contribution of the inflammatory response to chronic kidney disease (CKD) development. (**a**) TNF-α, (**c**) MCP-1, and (**e**) IL-6 mRNA levels measured in total kidney RNA extracted from the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). (**b**) TNF-α, (**d**) MCP-1, and (**f**) IL-6 protein levels quantified by enzyme-linked immunosorbent assay (ELISA) in tissue kidney homogenates. *$P < 0.05$ vs. the S and Sp groups.

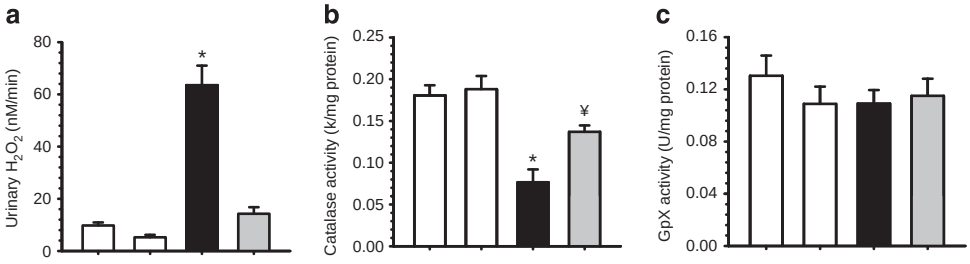

Figure 8 | The contribution of oxidative stress to chronic kidney disease (CKD) development and prevention by spironolactone pretreatment. (**a**) Urinary hydrogen peroxidase (H_2O_2) excretion after 270 days of follow-up. (**b**) Catalase activity and (**c**) glutathione peroxidase (Gpx) activity in the sham (white bars), Sp (second set of white bars), A-to-C (black bars), and A-to-C + Sp groups (gray bars). *$P < 0.05$ vs. the S and Sp groups; ¥$P < 0.05$ vs. the A-to-C group.

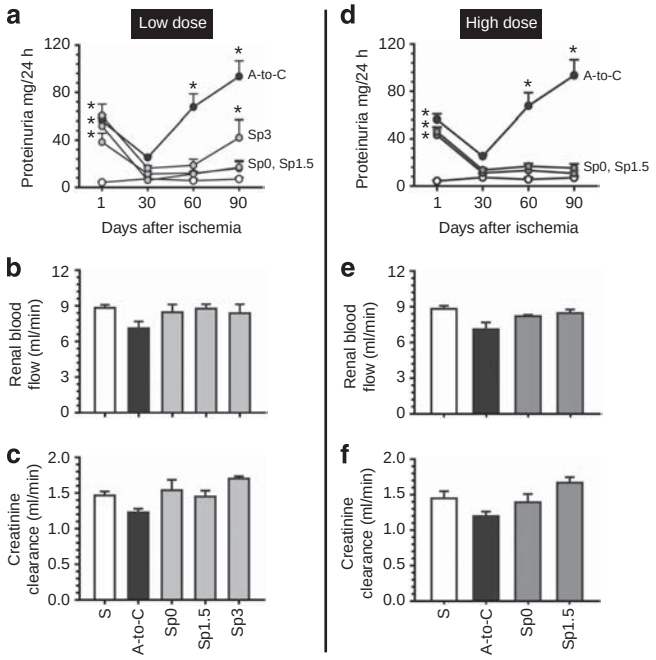

Figure 10 | Spironolactone administration after the ischemic insult prevents the development of proteinuria. (**a**) Urinary protein excretion at 1, 30, 60, and 90 days in sham (white circles), in A-to-C (black circles), and in rats receiving Sp (20 mg/kg) at 0, 1.5, or 3 hours after ischemia (gray circles) or (**d**) treated with a high dose of Sp (80 mg/kg) (dark gray circles) at 0 or 1.5 h after bilateral ischemia. After 90 days of follow-up, (**b**, **e**) renal blood flow and (**c**, **f**) creatinine clearance were determined in the sham group (white bars), A-to-C group (black bars), and the groups receiving a low dose of Sp (gray bars) at 0, 1.5, or 3 h after ischemia or a high dose of Sp (dark gray bars) at 0 or 1.5 h after bilateral ischemia. *$P < 0.05$ vs. sham-operated rats.

Molecular markers of renal fibrosis and inflammation also provided evidence of the protection conferred by spironolactone. The upregulation of TGF-β mRNA levels was prevented or reduced by a low or high dose of spironolactone administered at 0 or 1.5 h after ischemia (Figure 12a and e). However, increased phospho-Smad3 levels remained elevated in rats treated with a low dose, but not with a high dose, of spironolactone (Figure 12b and f). The renoprotective effect of spironolactone was also associated with the prevention of α-SMA and MCP-1 upregulation (Figure 12c–h).

DISCUSSION

In this study, we characterized a rat model of CKD induced by a single episode of AKI. We observed that an episode of AKI was resolved in surviving animals within a period of 10 days. However, although physiological parameters returned to normal values, activation of proinflammatory and profibrotic signals persisted. During the following months, progressive deterioration of renal function and renal structures was observed in rats surviving AKI, leading to the development of CKD. Thus, this model resembles what is currently believed to occur in the clinical setting.[11–13,24–27] Patients who survive an episode of AKI and apparently recover renal function may develop CKD later in life.[8,24] In this study, we observed that spironolactone administration before or after the ischemic insult prevented or significantly diminished the severity of an AKI episode, without signs of proinflammatory or profibrotic activation after 10 days of ischemia. Thus, spironolactone pretreatment resulted in a reduction in rat mortality and in the prevention of CKD. These observations demonstrate the relevance of the prevention of AKI episodes to a reduction in the prevalence of CKD.

CKD development in rats surviving AKI was characterized by a progressive increase in urinary protein excretion, renal dysfunction, glomerular hypertrophy, severe tubular dilation, tubulointerstitial fibrosis, and podocyte injury. Our study supports the hypothesis that an ischemic insult is sufficient to lead to progressive CKD in the rat, despite apparent recovery from the AKI episode. Because it was possible that CKD resulted from irreversible renal artery damage caused by the clamping, a group of rats was studied after 10 days of ischemia, confirming that this was not the case. After 10 days, renal function returned to normal values. Although renal histopathology was not analyzed at this point, we have previously reported that after 3 days of I/R, the tubular epithelium had almost recovered its normal structure.[28,29] However, in this study, we found that profibrotic and inflammatory cytokines remained at high levels despite a return to normal renal function values. In addition, cytokines such as TNF-α and IL-1β, IL-6, IL-12, IL-15, IL-18, and IL-32 are known to be induced as a result of the enhanced leukocyte activation and leukocyte–endothelial adhesion observed after I/R. Moreover, the renal tubular epithelium may generate proinflammatory cytokines such as TNF-α, IL-6, IL-1β, and TGF-β.[30–33] It is important to note that Basile *et al.*[34,35] and Hörbelt *et al.*[36] have demonstrated a persistent reduction in vascular density after I/R that maintains a continuous hypoxic and inflammatory state. Furthermore, Conger *et al.*[37] demonstrated that the postischemic kidney does not properly autoregulate the blood flow. All of these adverse conditions perpetuate continuous cycles of hypoxic damage and inflammation that injure the surrounding tissues and eventually lead to CKD, as has been clearly discussed by Bedfoord *et al.*[38] In agreement with all these findings, we found that, after 9 months, the rats that developed CKD exhibited greater levels of the proinflammatory cytokines compared with the rats that were pretreated with spironolactone. Interestingly, MCP-1 upregulation observed 3 months after ischemia was also prevented when spironolactone was administered after the insult. Our results suggest that the beneficial results of spironolactone in preventing chronic inflammation are a result of its ability to attenuate I/R-induced acute inflammation.

The CKD induced by an AKI episode was characterized by a progressive enhancement in urinary protein excretion and renal dysfunction without changes in mean arterial pressure.

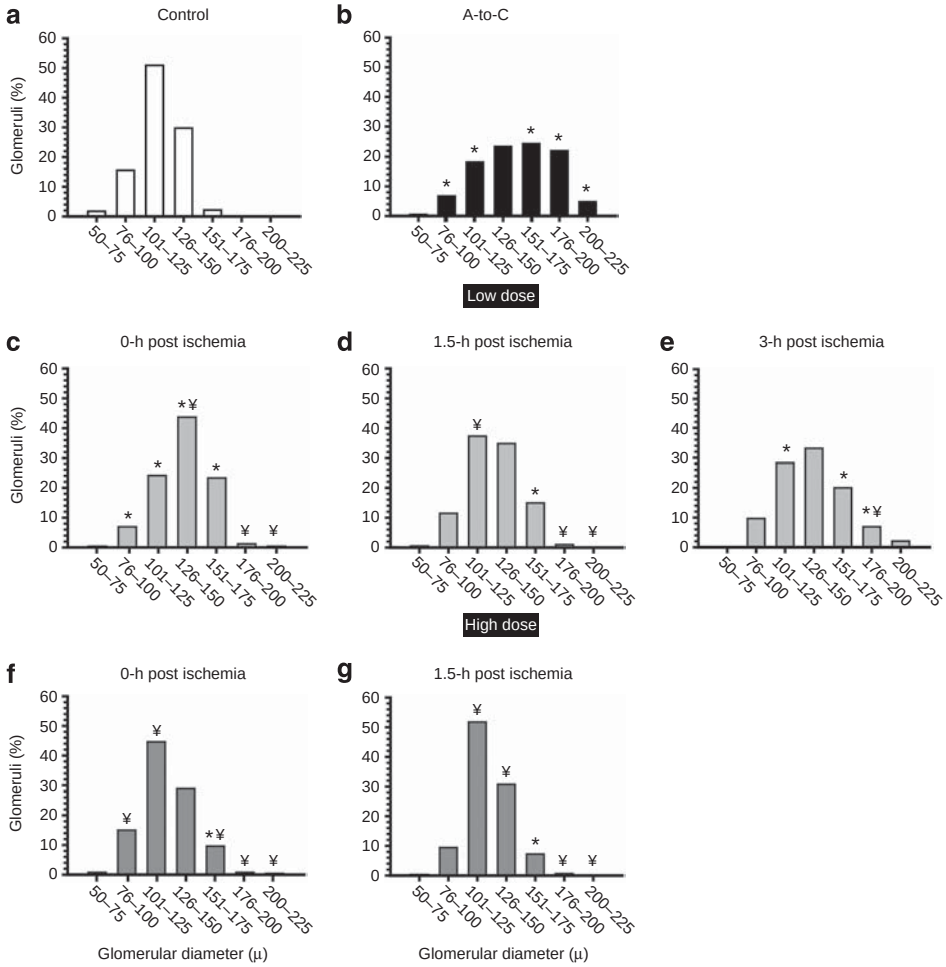

Figure 11 | Glomerular injury is prevented or reduced by spironolactone administration after renal ischemia. Glomerular diameter distribution in sham (white bars), A-to-C (black bars), low spironolactone dose (gray bars), and high spironolactone groups (gray dark bars). *$P<0.05$ vs. the S group and ¥$P<0.05$ vs. the A-to-C group.

These conditions provide us with an experimental model that dissects the detrimental effect of an AKI episode on renal function and structure without changes in blood pressure. Accordingly, the animals that suffered CKD exhibited severe structural injury. Glomerular hypertrophy and glomerulosclerosis were observed 9 months after inducing ischemia. These alterations were also associated with ultrastructural changes. It is plausible that this glomerular injury is a consequence of the endothelial injury and the endothelial-to-mesenchymal transition that occurred after the ischemia.[34,35] Interestingly, in this study, we found that spironolactone administration both before and after renal ischemia prevented the transition of AKI to CKD.

Aldosterone binding to the MR has been suggested to influence endothelial function and vascular tone.[17,19,39,40] Supporting this finding, mice overexpressing MR in the endothelium exhibit altered vascular tone,[40] and aortic wall thickness has been reported in patients with primary hyperaldosteronism.[41] Consistent with this result, aldosterone infusion in mice reduces the expression of glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme in maintaining the balance between nitric oxide and reactive oxygen species production.[42] Therefore, aldosterone has been proposed as a risk factor of vascular injury.[43] The mechanisms involved, however, have not been clearly elucidated. In this regard, we have previously demonstrated that MR antagonism precludes the characteristic hypoperfusion and oxidative stress induced by I/R, suggesting that aldosterone has a pivotal role in mediating renal dysfunction.[16,22] It is possible that the endothelium and the renal plasma flow of spironolactone-treated rats in this study was minimally affected as a consequence of the I/R injury; therefore, the glomerular structure remained unaffected in the long term.

Tubulointerstitial fibrosis is a common feature of CKD progression (for a review, see Rodriguez-Iturbe and Garcia[44]). In fact, severe tubular dilation and an extensive area affected by tubulointerstitial fibrosis were found in the A-to-C group. These results suggest that the tubulointerstitium is more susceptible to the effects of an AKI episode and contributes to the progression and severity of CKD in rats suffering from AKI. Accordingly, the severity of tubular damage reportedly exhibits a more significant correlation

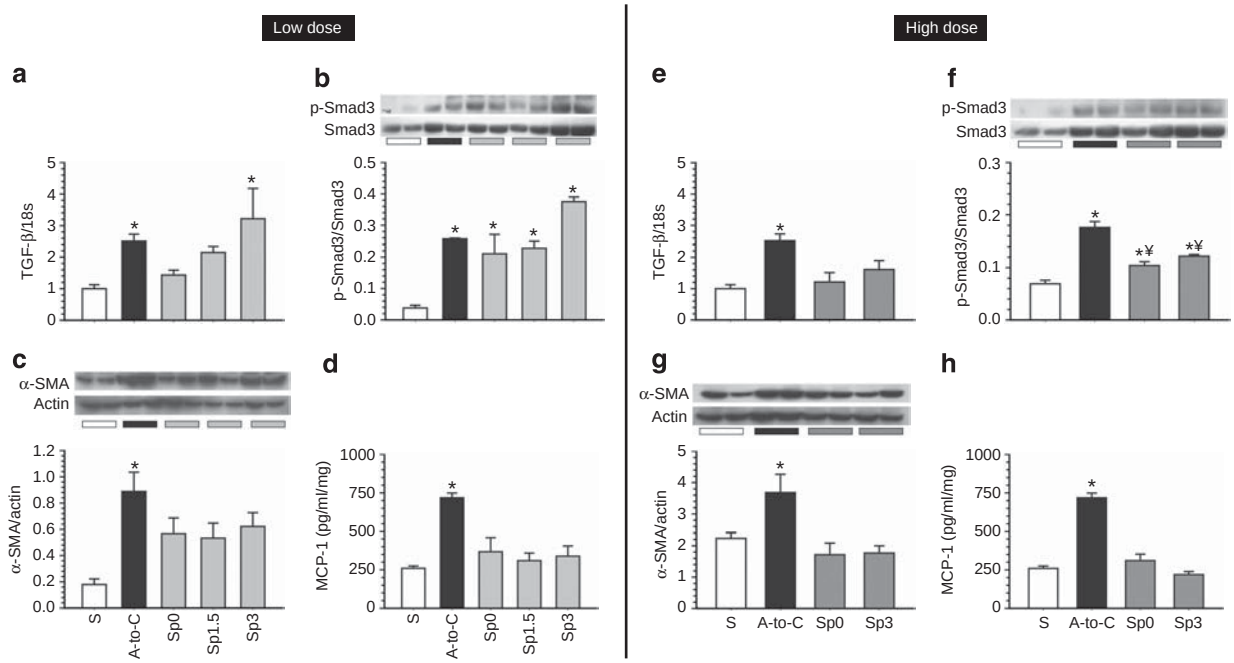

Figure 12 | Tumor growth factor (TGF)-β and inflammation pathways in rats treated with spironolactone after the ischemic insult. (**a**, **e**) TGF-β mRNA levels quantified by real-time reverse transcription–polymerase chain reaction (RT-PCR). Densitometric analysis of the western blots performed for (**b**, **f**) p-Smad3 and (**c**, **g**) α-SMA. (**d**, **h**) MCP-1 protein levels quantified in renal tissue by enzyme-linked immunosorbent assay (ELISA). Sham (white bars), A-to-C (black bars), low Sp dose (gray bars), and high Sp dose (dark gray bars). $^{*}P<0.05$ vs. the S group and $^{¥}P<0.05$ vs. the A-to-C group.

with the reduction in creatinine clearance than with glomerular injury scores.[45,46] These tubular defects were associated with an increase in tubular cell proliferation, as demonstrated by the significant increase in PCNA + staining and by the strong correlation with tubular dilation ($r^2 = 0.87$). In this study, however, the location of PCNA + cells in the epithelium of the dilated tubules strongly suggests that the tubular dilation observed in the A-to-C group was mediated in part by uncontrolled tubular cell proliferation triggered during the regeneration process after the ischemic insult. Although a previous study proposed that the increase in cellular proliferation is a necessary process to regenerate tubular epithelium in a period that typically is completed within 4 weeks after ischemia,[47] recent evidence demonstrated that ischemic, nephrotoxic, and obstructive injuries to the kidney induce a G2/M cell cycle arrest in the proximal tubular epithelial cells, sustaining proliferation indefinitely.[48] In this regard, Wynn[49] has proposed that this cell cycle arrest converts normal epithelial cells to a phenotype that promotes the growth and activation of fibroblasts, turning-on the fibrotic process after an ischemic insult. In addition, TGF-β has been recognized as a key mediator of the genesis of renal fibrosis.[32,50] TGF-β also contributes to the fibrogenesis through inducing epigenetic modifications in fibroblasts in a process that includes the hypermethylation of the gene encoding RASAL1 by the methyltransferase Dnmt1.[51] Accordingly, we found that tubulointerstitial fibrosis developed in the A-to-C group and was associated with increased expression and activation of TGF-β, as evidenced by increases in phospho-Smad3 and its target ECM proteins. Interestingly, activation of the TGF-β signaling pathway was not observed in the animals treated with spironolactone before or after (high dose) ischemia. These data highlight the contribution of TGF-β in mediating renal fibrosis after an ischemic insult. Although Smad2 and Smad3 interact and mediate TGF-β signaling, one recent line of evidence has shown that phospho-Smad2 may act as an antifibrotic effector of the TGF-β pathway.[23] In agreement with those results, the renoprotection conferred by spironolactone was associated with increased levels of phospho-Smad2.

The epithelial-to-mesenchymal transition has been suggested to promote tubulointerstitial fibrosis.[52,53] Under pathological conditions, tubular cells may dedifferentiate into myofibroblasts. TGF-β appears to promote this process by activating the Smad, ILK, and ERK pathways, as observed in tubular cells.[53] To monitor epithelial-to-mesenchymal transition, α-SMA protein levels were measured in the kidney. As expected, the renal fibrosis observed in the A-to-C group was associated with increased α-SMA protein levels. CKD progression has also been linked to an imbalance in free-radical production and antioxidant defense.[44,54] We confirmed that the rats that developed CKD exhibited greater urinary H_2O_2 excretion and a reduction in catalase activity. These changes in α-SMA levels and antioxidant factors were not observed in the A-to-C + Sp group.

In summary, we characterized an experimental model of CKD induced by AKI in the rat. Several mechanisms were responsible for CKD development, including increased

tubular cell proliferation, increased TGF-β pathway activation and oxidative stress, and overexpression of proinflammatory cytokines. By using this model of CKD, we demonstrated that the prevention of AKI with spironolactone completely prevents the progression to CKD. Moreover, we also demonstrate that administering an MR blocker after the ischemic insult also prevented CKD. Our results suggest that the treatment of patients with spironolactone after an AKI episode could be of help in preventing the development of CKD.

MATERIALS AND METHODS

All experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, 1996) and were approved by the Animal Care and Use Committee at our Institution.

Protocol 1

In all, 62 male Wistar (weighing 270–300 g) rats were divided into four groups: (1) rats subjected to sham surgery, $n = 9$ (S); (2) rats treated with spironolactone at 20 mg/kg per day by gastric gavage 3 days before sham surgery, $n = 9$ (Sp); (3) rats undergoing bilateral ischemia for 45 min, $n = 28$ (A-to-C); and (4) rats that received spironolactone 3 days before bilateral ischemia, $n = 13$ (A-to-C + Sp). All animals were observed for 9 months. In addition, four rats from the S, A-to-C, and A-to-C + Sp groups were included and observed for 10 days. All animals were kept in a 12:12 h day–night cycle and with free access to water and food.

Protocol 2

Because we recently demonstrated that postischemia MR blockade is beneficial in the prevention of AKI, with the best renoprotection observed in the first 3 h after ischemia,[22] this set of experiments was designed to evaluate whether postischemia spironolactone administration confers protection against the development of CKD. In all, 33 male Wistar (270–315 g) rats were divided into seven groups: sham-operated rats (S); rats subjected to bilateral renal ischemia for 45 min (A–C); and five groups of rats that underwent bilateral renal ischemia for 45 min, but also received one dose of spironolactone at 20 mg/kg by gastric gavage at 0, 1.5, or 3 h after ischemia (A-to-C, 0 h; A-to-C, 1.5 h; and A-to-C, 3 h, respectively), or that received a higher dose of spironolactone (80 mg/kg) at 0 or 1.5 h after ischemia (A-to-C 80, 0 h and A-to-C 80, 1.5 h). These animals were followed up for 3 months.

All other methods are described in the Supplementary Materials online.

Statistical analysis

The results are presented as the mean ± s.e. The significance of the differences between the groups was assessed by analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. All of the comparisons passed the normality test. The differences in the ranks of glomerular diameters among the groups were evaluated by contingency analysis, and the differences were assessed using the χ^2 test with the Yates correction. Statistical significance was defined as P–value < 0.05.

DISCLOSURE

All the authors declared no competing interests.

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Disclaimer

The results presented in this paper have not been published previously in whole or in part, except as an abstract presented at the Annual Meeting and Scientific Exposition 2011 of the American Society of Nephrology (Philadelphia, PA).

SUPPLEMENTARY MATERIAL

Figure S1. Renal injury observed 90 days after inducing ischemia.
Figure S2. Glomerular and tubular injury is reduced by a low dose of spironolactone administrated after ischemia.
Figure S3. Glomerular and tubular injury is prevented by a high dose of spironolactone administrated after ischemia.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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Short communication

Seroprevalence of *Trypanosoma cruzi* in kidney transplant donors and recipients in Mexico City

R. Rodríguez-Romo, L.E. Morales-Buenrostro, P.A. Reyes, C. Gracida, M. Medeiros, E. Mancilla, C. De Leo, J. Alberú.
Seroprevalence of *Trypanosoma cruzi* in kidney transplant donors and recipients in Mexico City.
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Abstract: Infectious diseases are common causes of morbidity and mortality among kidney transplant recipients. Chagas disease (CD) has been recognized as an emerging infectious complication of transplantation caused by the parasite *Trypanosoma cruzi*. CD is prevalent in Mexico, particularly in the southern coastal region. The impact on Mexican kidney transplant programs has not been previously studied prospectively. From 2009 through 2010, serum samples from 59 kidney transplant donors and 405 renal transplant recipients were screened for antibodies against *T. cruzi*. Serum was initially screened using a locally developed ELISA test; positive results were confirmed by an indirect immunofluorescense test, in accordance with Panamerican Health Organization/World Health Organization guidelines. None of the donors were seropositive for *T. cruzi*, while 8 (1.97%) kidney transplant recipients were confirmed to be seropositive for *T. cruzi*. None of them have developed clinical manifestations of CD, although specific screening of recipients was not performed. A prospective study is planned to define the epidemiology and outcome of CD among kidney transplant donors and recipients in Mexico more thoroughly.

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Key words: *Trypanosoma cruzi* prevalence; kidney transplant recipients; Chagas disease; Mexico

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Infectious diseases are increasingly recognized as important causes of morbidity and mortality among kidney transplant recipients (1). Parasitic infections are an emerging cause of post-renal transplant infections, particularly in endemic regions. One parasitic infection that has generated significant study and development of guidelines for screening and management is Chagas Disease (CD). CD is caused by infection with *Trypanosoma cruzi* and causes a wide range of clinical syndromes in both immunocompetent and immunocompromised patients. Although CD is prevalent in Mexico, particularly in the southern coastal region (2), data remain inadequate to define the epidemiology and clinical impact of the infection in Mexican transplant programs.

In the general population, primary infection is limited and resolves spontaneously but, without specific treatment, the infection can persist and progress to a chronic phase. During the chronic phase, patients can remain asymptomatic (undetermined phase) or may have specific end-organ damage including myocardiopathy

(most frequent complication, 30%) gastrointestinal or visceral involvement (megaesophagus and megacolon, 6%), and peripheral nervous system damage (3%) (3).

Given the prolonged period of time during which the disease may remain asymptomatic, *T. cruzi* screening in transplant donors and recipients has been recommended in high-prevalence populations (4). Infection in the donor may be transmitted to the recipient and result in donor-derived CD transmission. Screening of recipients of organs from Chagas-infected donors is generally recommended and may help identify recipients with asymptomatic parasitemia. Early institution of therapy during asymptomatic parasitemia may improve the outcomes of recipients in this setting.

Alternatively, organ recipients may be asymptomatically infected prior to transplantation and the infection may reactivate in the post-transplant period (5). Such cases may present as asymptomatic parasitemia or manifest acutely with panniculitis, myocarditis, and encephalitis (6). Screening of such recipients for parasitemia may identify patients who would benefit from early treatment. Moreover, pretransplant treatment of seropositive transplant candidates has been proposed, but data supporting this practice are too limited at this time to make a clear recommendation as to which approach is preferable.

Unfortunately, data on the optimal approach for screening of donors and recipients from high-prevalence populations and the optimal post-transplant treatment for recipients with prior *T. cruzi* exposure remain limited. Despite the prevalence of CD, there is very little experience from Latin American transplant centers currently available in the literature (7–14). Based on the limited available experience from Latin America and the poor outcomes of unrecognized cases of donor-derived CD transmission, guidelines for the screening of donors and recipients for pre-existing infection coupled with close post-transplant monitoring and treatment have been developed (4, 6).

Given the existence of regional guidelines, the prevalence of CD in Mexico, and the lack of published experience relating to CD in transplant patients from Mexico, a prospective screening study was designed. We hoped to define the epidemiology and clinical sequelae of CD among kidney transplant donors and recipients in Mexico City.

Patients and methods

Patients

Samples from 3 cohorts of patients were included as follows:

Pretransplant donor samples (Group 1) included predonation serum samples from 29 deceased and 30 living organ donors cryopreserved at −70°C.

Pretransplant living-donor kidney transplant recipient samples (Group 2) included pretransplant serum samples from 30 recipients cryopreserved at −70°C.

Post-transplant kidney recipient samples (Group 3) included samples obtained from 375 kidney transplant recipients ≥12 months post transplant from January 2009 to December 2010. After provision of informed consent, a blood sample (5 mL) was obtained from each patient. The samples were centrifuged at 1300 × g for 5 min, and 100 μL of serum was collected and maintained at −70°C.

Clinical data

The following information about kidney transplant recipients was obtained from the clinical records: age, gender, date of kidney transplant, duration of follow-up since kidney transplant, and time from transplant to sample collection, number of kidney transplants, donor source (living or deceased), haplotype match, immunosuppression scheme, and current kidney function as evaluated by serum creatinine and glomerular filtration rate, estimated by the MDRD formula for adults or the Schwartz formula for children (estimate glomerular filtration rate) (15–17). The following demographic information was also gathered: place of birth, place of residence for the first 5 years of life, overcrowding, number of people living together, living conditions, transfusions, and current place of residence. In those patients whose serology for *T. cruzi* was confirmed as positive, current and/or previous symptoms or current clinical information suggestive of CD were investigated.

Laboratory procedures

All the samples were transported to the Laboratory of Molecular and Proteomic Immunology of the Department of Molecular Biology of the Instituto Nacional de Cardiología Ignacio Chávez (INCICh), where tests for antibodies against *T. cruzi* were carried out using a non-commercial enzyme-linked immunosorbent assay (ELISA); all tests were conducted with standardized methods, within the same institution (18). Samples that were found to be reactive in this assay were analyzed with an indirect immunofluorescense test (confirmatory test), also developed at INCICh. Based on international standards, samples that showed

reactivity in both tests were considered to be seropositive (6, 19–21).

Ethical considerations

The study was designed and coordinated by the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ) and was approved by the Bioethical Institutional Committee on Investigation in Human Beings of INCMNSZ and by the corresponding committees of participating institutions. Serum samples were coded to avoid bias in the analysis of test results.

Statistical analysis

The database was managed using Microsoft Excel and the SPSS statistical program, version 18 (SPSS Inc, Chicago, Illinois, USA) was used for further data analysis. Descriptive statistics were computed for demographic characteristics. Given the limited number of seropositive patients, analysis of risk factors associated with seropositivity was not performed.

Results

Group 1/Predonation cohort

Living donors were more commonly women and most were in their 40s, whereas deceased donors were younger (29.7 ± 18.2 years; see Table 1). Most donors were born in Mexico City or the State of Mexico, and had not moved from this region since birth. None of the living or deceased donors were seropositive for *T. cruzi*.

Group 2/Pretransplant living-donor recipient cohort

Living-donor recipients were predominantly male and most were in their 40s. Most recipients were born in Mexico City or the State of Mexico and had not moved from this region since birth (Table 1). None of the blood samples were positive for *T. cruzi* antibodies, although 1 patient was initially reactive by ELISA but negative by confirmatory indirect immunofluorescense test. This patient was born in Mexico City, did not have known risk factors for CD, and was asymptomatic. None of the live donor kidney transplant recipients developed evidence of clinical CD at a median 27 months of follow-up.

Demographics of Group 1 (donor population) and Groups 2 and 3 (recipient population)

Variables	Group 1 (59)	Groups 2 and 3 (405)
Age years, no. ±SD	32.74 ± 14.8	37 ± 13.5
Female gender (%)	30 (50.8)	183 (45)
Type of donor, no. (%)		
LRD	29 (49)	314 (76)
ERD		26 (6)
DD	30 (51)	65 (17)
HAP, no. (%)		
0		116 (29)
1		257 (63)
2		32 (8)
No. of KT (%)		
1		369 (91)
2		36 (9)
IS, no. (%)		
TAC + MMF + PDN		131 (32)
TAC + AZA + PDN		21 (5)
CsA + MMF + PDN		20 (5)
SIR + MMF + PDN		5 (1)
Other		228 (56)
SCr (median) mg/dL	1.1 (0.7–1.5)	1.6 (0.58–1.63)
eGFR (median ± SD) mL/min	104.8 ± 31.3	60 ± 30
ELISA, no. (%)	1 (1.6)	20 (5)
IIF, no. (%)	1 (1.6)	8 (2)
Overcrowding, no. (%)	3	11 (3)
Transfusions, no. (%)	18 (10.6)	326 (80)
Unknown	30 (17.7)	79 (20)
Place of birth, no. (%)		
Mexico City	16 (27)	163 (40)
State of Mexico	4 (7)	59 (15)
Hidalgo	–	13 (3)
Unknown	35 (59)	45 (11)
Other states	4 (7)	125 (31)
Place of residence, no. (%)		
Mexico City	10 (17)	148 (37)
State of Mexico	16 (27)	74 (18)
Hidalgo	–	10 (2)
Unknown	29 (49)	68 (17)
Other states	4 (7)	105 (26)

No., number; SD, standard deviation; LRD, living-related donor; ERD, emotionally related living donor; DD, deceased donor; HAP, haplotypes shared between donor and recipient; KT, kidney transplant; IS, immunosuppression; TAC, tacrolimus; MMF, mycophenolate mofetil; PDN, prednisone; AZA, azathioprine; CsA, cyclosporine; SIR, sirolimus; SCr, serum creatinine; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence.

Table 1

Group 3/Post-transplant kidney transplant recipient cohort

The average time from kidney transplant to sample collection was 12 months (104 ± 76.5). In this population, 20 patients had a positive ELISA test but only 8/20 had a positive confirmatory test, resulting in a total seroprevalence of 2.13%. None of the seropositive patients have presented or developed signs or symptoms of CD to date (Table 2).

Discussion

This is the first study, to our knowledge, conducted to define the epidemiology and clinical outcomes of CD among kidney transplant patients in Mexico. The overall prevalence of *T. cruzi* seropositivity in the kidney transplant recipient population was 2%, similar to the prevalence reported in studies carried out among blood donors and in the general population in Mexico (22). None of these individuals developed clinical disease post transplant, and none of the donors were found to be seropositive for *T. cruzi*. Nonetheless, this study provides preliminary insight into the epidemiology of CD among Mexican transplant patients.

Based on studies conducted among blood donors, it is clear that patients are at variable risk of latent CD, likely because of the regional endemicity of the disease in Mexico. This regional pattern of endemicity has been taken into consideration in developing policies for screening blood donors and may, therefore, inform similar practices for organ donors (23). The information obtained herein might help support the initiation of studies to screen deceased donors in areas of high endemicity, as the expected yield would be higher. It is particularly important to consider that the time available for screening is much shorter in the deceased-donor population; however, the information obtained from this practice would be relevant for the care of the recipient.

Primary infection with *T. cruzi* can lead to a fatal outcome if the possible transmission through an infected donor is unnoticed and the recipient is not

Patients positive for *Trypanosoma cruzi*

Patient no.	1	2	3	4	5	6	7	8
Age in years	22	21	56	22	25	48	25	41
Gender	M	M	M	M	F	M	M	M
No. of KT	1	1	1	1	1	1	1	1
Type of donor	LRD	LRD	LRD	LRD	DD	LRD	LRD	LRD
Institute of origin	INCMNSZ	INCMNSZ	INCMNSZ	CMNSXXI	CMNSXXI	INCMNSZ	INCMNSZ	INCMNSZ
IS	SIR, MMF, PDN	TAC, AZA, PDN	MMF, PDN	TAC, MMF, PDN	TAC. MMF, PDN	SIR, AZA, PDN	CsA, AZA, PDN	TAC, MMF, PDN
SCr (mg/dL)	1.20	1.49	0.86	On dialysis[1]	0.88	1.34	1.68	1.21
GFR (mL/min)	109.61	85.80	146		48.5	89.06	64.4	101.8
Place of birth	State of Mexico	State of Mexico	Bolivia	Morelos	Mexico City	Mexico City	Morelos	Mexico City
Place of residence	Morelos	State of Mexico	Hidalgo	Mexico City	Mexico City	State of Mexico	Morelos	Queretaro
Overcrowding	No	No	No	No	No	Yes	No	No
Transfusions	Yes	No	No	Yes	No	Yes	Yes	No
Symptoms	No	No	No	No	No	No	No	Pyrosis, regurgitation

[1]Graft loss because of chronic allograft dysfunction occurred 10 years after the transplant; the patient is currently on the waiting list for a second KT.
No., number; M, male; F, female; KT, kidney transplant; LRD, living-related donor; DD, deceased donor; INCMNSZ, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México; CMNSXXI, Centro Médico Nacional Siglo XXI, México; IS, immunosuppression; SIR, sirolimus; MMF, mycophenolate mofetil; PDN, prednisone; TAC, tacrolimus; AZA, azathioprine; CsA, cyclosporine; SCr, serum creatinine; GFR, glomerular filtration rate.

Table 2

monitored (24). However, prior experience in the use of non-cardiac organs from donors who were identified as *T. cruzi* seropositive via screening suggests that favorable outcomes can be attained in recipients when they are closely monitored for parasitemia after the transplant and treated as soon as parasitemia is documented (4). Additionally, such studies could help define the cost-benefit of screening over a range of prevalence of disease in the donor population.

Unfortunately, no systems are in place to collect data on and define the rate of post-transplant complications or patient and graft survival in Mexico, as these data are not collected centrally by health authorities. Developing such a system would allow the study of questions regarding the impact of CD on transplantation in Mexico and would be consistent with the World Health Organization's guiding principles for organ transplantation.

In the United States, migrant populations from Mexico have been identified as having the highest prevalence of *T. cruzi* among blood donors (25). Based on this information, and on the clinical cases reported in kidney transplant recipients who contracted CD via their graft, organ procurement organizations in the US have begun screening high-risk individuals, including immigrants from Mexico and Central and South America, for *T. cruzi* (21, 25). The data provided in this study add valuable insight for countries with a large number of Mexican immigrants.

We should note the study's limitations. First, the study mostly included patients from Mexico City and included only a small number of kidney transplant patients and an even smaller number of living and deceased donors. As such, the prevalence may be slightly underestimated and may not reflect prevalence in all regions of Mexico. Second, it is not possible to determine whether most of the seropositive kidney transplant recipients were infected prior to transplant or acquired infection during or after the procedure. Individuals could have been infected by reduviid insect bites, transfusions, or the graft itself. Third, patients were not monitored prospectively post transplant and, as a result, asymptomatic parasitemia without clinical sequelae may have been missed. Furthermore, serotesting for CD at least 1 year after transplantation may be of no value, as negative serology has been observed in post-transplant follow-up in previously positive recipients (14).

In conclusion, the results obtained in this first analysis of seroprevalence of *T. cruzi* in kidney transplant recipients in Mexico yielded a prevalence of 2%. None of these individuals developed symptomatic CD during the study period. None of the kidney donors in this study were seropositive, although the sample size was small. Nonetheless, these data, along with recent guidelines, suggest that screening of transplant donors and candidates in Mexico should be considered. A large-scale, prospective study should be conducted to determine the seroprevalence of *T. cruzi* in kidney transplant recipients and donors throughout the various states of Mexico. The information provided in this study adds valuable insight for countries with a large number of Mexican immigrants.

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Research Paper

Mild ischemic Injury Leads to Long-Term Alterations in the Kidney: Amelioration by Spironolactone Administration

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Abstract

Administration of the mineralocorticoid receptor antagonist spironolactone prevents the development of chronic kidney disease (CKD) after a severe ischemic injury. However, whether brief periods of ischemia lead to CKD and whether spironolactone administration after ischemia may be a useful therapeutic strategy to prevent the gradual deterioration of structure and function remains unexplored.

Nineteen male Wistar rats were divided into four groups: rats that underwent renal bilateral ischemia for 10, 20, or 45 min were compared with sham operated rats. Additionally, thirteen male Wistar rats that underwent renal bilateral ischemia for 20 min were divided into an untreated ischemic group (I) and two groups receiving spironolactone, 20 mg/kg by gavage, at either 0 (Sp0) or 1.5-h after ischemia (Sp1.5). The rats were followed up and studied after 9 months.

Mild (20 min) and severe (45 min) ischemia induced a progressive increase in proteinuria at varying magnitudes, whereas minor ischemia (10 min) did not modify proteinuria. CKD induced by moderate ischemia was characterized by renal hypertrophy and tubulointerstitial fibrosis. These effects were associated with activation of the transforming growth factor β (TGFβ) signaling pathway and up-regulation of endothelin receptor A (ETA) and alpha smooth muscle actin (αSMA). Spironolactone treatment immediately or 1.5-h after the ischemic insult prevented the onset of these disorders.

Our results show that moderate ischemic insult leads to long-term structural and molecular changes that may compromise renal function in later stages. Additionally, we demonstrate that spironolactone administration after mild ischemia prevents this detrimental effect.

Key words: Aldosterone, fibrosis, acute kidney injury, chronic kidney disease

Introduction

Ischemia/reperfusion injury is the main cause of acute kidney injury (AKI) occurring in approximately 15% of hospitalized patients, and its incidence rises to 40-60% in intensive care unit (ICU) patients (1) (2) (3). Despite recent advances in new therapies and the knowledge of the mechanisms involved in AKI, this syndrome has high morbidity and mortality rates (4).

More worrisome is the recent accumulating evidence indicating that patients who survive an AKI episode have a higher risk of developing chronic

kidney disease (CKD) in the following years (5). Therefore, AKI has been recognized as a risk factor for the development of CKD (6) (7). A recent study showed that 6.6% of AKI patients who had complete recovery of renal function had a greater risk of death and de novo CKD in the following months (8). A recent meta-analysis that included thirteen large studies found that AKI is an independent risk factor for CKD (9). Worldwide, 20% of patients with an AKI episode will develop CKD after 3 years, which represents 300,000 patients in high-income countries, and this value might be higher than 1.8 million in low- and middle-income countries (10).

In support of several epidemiological studies, animal models have shown that after a renal ischemia/reperfusion event, the recovery process may be incomplete, producing progressive renal dysfunction, tubulointerstitial fibrosis and chronic inflammation (for review (11)). We have previously shown that aldosterone plays a key role in the physiopathology of renal injury induced by ischemia. In this regard, we showed that adrenal gland removal or mineralocorticoid receptor (MR) blockade with spironolactone before or even after ischemia prevents the acute (24 h) functional and structural injury induced by I/R (12-14). Interestingly, CKD was prevented when spironolactone was administered upon severe ischemic injury, and the untreated ischemic group developed progressive renal dysfunction, proteinuria, glomerular hypertrophy, glomerulosclerosis, aberrant tubular dilation and tubule-interstitial fibrosis (15). These results suggest that MR blockade is a powerful strategy to prevent CKD induced by a longer period of ischemia in the rat (15). However, this severe ischemic injury model might only be applicable to patients undergoing cardiovascular surgery or renal transplantation.

The probability of developing CKD or end-stage renal disease (ESRD) over time is proportional to the severity and the duration of the AKI event (16). Until now, studies performed in rats have explored the effects of severe ischemic injury (45 to 60 min of renal ischemia) on long-term renal functional and structural deterioration (15;17-24). Because in most of the patients AKI occurs unexpectedly, and renal injury appears as a result of a lower degree of hypoperfusion, we addressed the following issues in this study: whether mild ischemic injury (20 min) is able to induce chronic renal injury, and whether spironolactone administration post-ischemia is effective in preventing the long-term effects of mild ischemia.

Methods

All experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, 1996) and were approved by the Animal Care and Use Committees at our institutions (Comisión de Investigación en Animales del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubiran and Comisión Institucional para el Cuidado y Uso de Animales del Laboratorio del Instituto de Investigaciones Biomédicas).

To test the impact of various durations of ischemia on the progressive increase of proteinuria, nineteen male Wistar rats (270–300 g) were divided into four groups: sham-operated (n=4), I/R of 10 min (n=5), I/R of 20 min (n=5), and I/R 45 min (n=5). To investigate the efficacy of MR antagonism on the long-term effects of mild ischemia, thirteen male Wistar (270–300 g) rats were divided into three groups: rats that were subjected to bilateral renal ischemia for 20 min (I, n=5) and two groups of rats that underwent bilateral renal ischemia for 20 min receiving only one dose of spironolactone (20 mg/kg by gastric gavage) either immediately or 1.5 h after ischemia (Sp0, n=4 and Sp1.5, n=4, respectively). These groups were compared with the sham-operated group used in the first set of experiments.

Ischemia/reperfusion model

Rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad to maintain rat core body temperature at 37 °C. Renal pedicles were isolated and bilateral ischemia was induced by the collocation of a non-traumatic clamps during 10, 20 or 45 minutes. Ischemia was verified visually by change in kidney color. Reperfusion was achieved by release of the clips and confirmed by return of blood to the kidney. The incision was closed in two layers with 3-0 sutures. For sham surgery, anesthesia, laparotomy and renal pedicle dissection, without clamp collocation was performed. After the surgery the rats were allowed to recover and followed up for 270 days.

Functional parameters

Urinary protein excretion was determined every 30 days throughout the follow up in all studied groups using the urine collected over a 24-h period. At the end of the experimental periods (9 months), rats were anesthetized with sodium pentobarbital (30 mg/kg) and placed on a homoeothermic table. The left femoral artery was catheterized with polyethylene tubing (PE-50). The mean arterial pressure (MAP) was monitored with a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA). An ultrasound transit-time flow probe was placed around the left artery and filled with ultrasonic coupling gel (HR Lubricating Jelly,

Carter-Wallace, New York, NY) to record the renal blood flow (RBF). Blood samples were taken at the end of the study. Urine and serum creatinine concentrations were measured with Quantichrom creatinine assay kit (DICT-500), and renal creatinine clearance was calculated by the standard formula C = (U X V)/P, where U is the concentration in urine, V is the urine flow rate, and P is the serum concentration. Urinary protein excretion was measured by the TCA turbidimetric method (25).

Light microscopy analysis

Histopathological analysis was performed in all rats after 9 months. The right kidney was removed and the cortex and medulla were isolated; then, the tissue was frozen in liquid nitrogen and stored at -80° C. The left kidney was perfused through the femoral catheter with a physiological solution. Following blanching of the kidney, the perfusate was replaced by freshly prepared 10% neutral-buffered formalin, and perfusion was continued until fixation was completed. After appropriate dehydration, renal tissue was embedded in paraffin, sectioned at 4 μm and stained with periodic acid-Schiff (PAS) reagent or Sirius red stains. The degree of tubulointerstitial fibrosis was evaluated by morphometry in Sirius red-stained preparations (magnification x400). Accordingly, five to eight subcortical fields per section were randomly selected in kidneys from the groups studied. Tubulointerstitial fibrosis consisted of extra cellular matrix expansion with collagen deposition together with distortion and collapse of the tubules; fibrosis was evidenced by red coloration in Sirius red stained slides. The affected area was delimited, and the percentage of tubulointerstitial fibrosis was calculated by dividing the fibrotic area by the total field area, excluding the glomerular and tubular luminal areas. All of the slides were blindly analyzed.

Western Blot analysis

Total renal proteins were isolated from renal cortex from each rat and homogenized in lysis buffer (50 mM HEPES ph 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, and complete protease inhibitor (Roche). Protein samples containing 50 μg of total protein were resolved by 8.5% SDS-PAGE electrophoresis and electroblotted onto polyvinylidinedifluoride membranes (Millipore). Membranes were then blocked with 5 % blotting-grade non-fat dry milk. After that membranes were incubated in 0.1 % blotting-grade non-fat dry milk with their respective antibodies. Specific antibodies against α-smooth muscle actin (Sigma A2547, 1:5000), Smad3 (sc-101154, 1:500), phosho-Smad3 (Millipore, 1:500), TGF-β (sc-146, 1:500), ET_A (Abcam, 1:5000) and ET_B (Abcam, 1:5000) were used. After incubation with primary antibody, membranes were washed and incubated with their respective secondary antibody. As a loading control, membranes were incubated overnight at 4 °C with goat anti-actin antibody (Santa Cruz Biotechnology, 1:5000 dilution). Actin was detected using donkey anti-goat IgG-HRP (1:5000, Santa Cruz Biotechnology). Proteins were detected with an enhanced chemiluminescence kit (Millipore) and autoradiography, following the manufacturer's recommendations. The bands were scanned for densitometric analysis.

Endothelin ELISA

Endothelin-1 levels were analyzed using a commercially available ELISA kit (Endothelin-1 (1-31) Assay kit; Immuno-Biological Laboratories Inc.) according to the manufacturer's instructions. Tissue homogenates and standards were added to the pre-coated wells and incubated overnight at 4°C. Endothelin-1 was captured by the antibody and then detected by adding the labeled antibody and the chromogen. The optical density of the samples was read at 450 nm by a plate reader and was compared to a standard curve generated from known concentrations of endothelin-1 that ranged from 1.56 to 200 pg/mL. The protein concentration in the tissue homogenates was determined by the Lowry method (BioRad). The endothelin-1 concentration was normalized by the amount of protein added to the well.

Statistical analysis

The results are presented as the mean ± S.E. The significance of the differences between the groups was assessed by analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. All of the comparisons passed the normality test. Statistical significance was defined as having p values <0.05.

Results

Mild and severe ischemic insult leads to progressive increase in proteinuria.

As expected, 45 min of renal ischemia induced a progressive increase in proteinuria from 21.8 ± 3.4 (one month) to 320.4 ± 22.8 mg/24 h (nine months post-ischemia) (Figure 1). A brief period of ischemia (20 min) also induced a progressive increase in urinary protein excretion, although the extent was significantly lesser than in the group with 45 min ischemia, from 12.1 ± 2.2 (one month) to 189.6 ± 36.2 mg/24 h (9 months post-ischemia). In contrast, rats that suffered a minor ischemic insult (10 min) did not develop proteinuria after the nine-month follow-up.

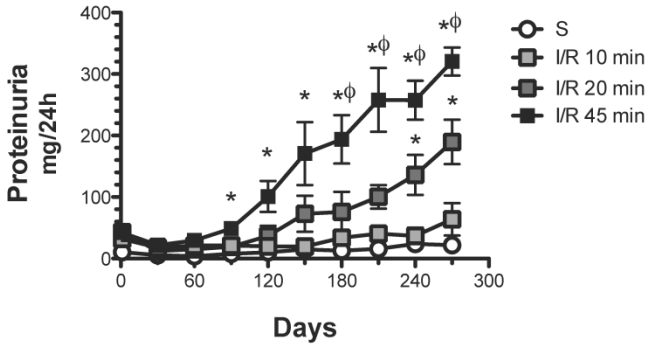

Figure 1. Effect of various durations of ischemia on proteinuria development. Four groups were included: sham (S), and rats that underwent bilateral renal ischemia of 10, 20 or 45 min duration. The urinary protein excretion was determined every 30 days: sham (white circles, n=4), I/R 45 min (black squares, n=5), I/R 20 min (dark gray squares, n=5) and I/R 10 min (gray squares, n=5). *p<0.05 vs. Sham-operated rats and ϕ p<0.05 vs. I/R 45 group.

The progressive increase in proteinuria induced by a brief ischemic period is reduced by spironolactone administration after the ischemic insult.

We next evaluated the effectiveness of post-ischemic treatment with spironolactone to prevent long-term functional, structural and molecular damage. The progressive increase of proteinuria induced by 20-min of ischemia was significantly lessened after nine months in the groups treated with spironolactone immediately (Sp0) or 1.5-h (Sp1.5) after ischemia (63.7 ± 18.5 and 66.1 ± 19.2 mg/day, respectively) (Figure 2A). Despite the presence of proteinuria in the untreated ischemic group, the rats did not exhibit renal dysfunction at the end of the experiment; similar values of renal blood flow and creatinine clearance were observed among the groups (Figures 2B and 2C). None of the rats presented with an increase in mean arterial pressure, indicating that phenotypic changes are directly related to the duration of ischemia and not secondary to systemic hypertension (Figure 2D).

Long-term renal structural changes induced by a brief ischemic period

Representative light microscopy sections from rat kidneys stained with periodic acid–Schiff are shown in Figure 3A-D. IR induced structural changes characterized by glomerular hypertrophy, glomerulosclerosis, and cast formation (Figure 3B). In contrast, the Sp0 and Sp1.5 groups exhibited glomerular and tubular architecture similar to those observed in sham-operated rats (Figure 3C-D). Accordingly, the untreated ischemic group exhibited an increase in the percentage of glomerulosclerosis (14.4%), which was not observed in rats treated with spironolactone (Figure 3E). Renal hypertrophy was evaluated by kidney weight. Despite similar body weights among the groups, the kidney weight and body weight ratio (KW/BW) was 40% higher in the IR group than in the sham operated group (0.0042 ± 0.0004 vs. 0.00291 ± 0.0001, p=0.03), as shown in Figure 3F. This renal hypertrophy was not observed in any of spironolactone-treated groups (0.0030 ± 0.0001 and 0.0032 ± 0.0001 for Sp0 and Sp1.5, respectively).

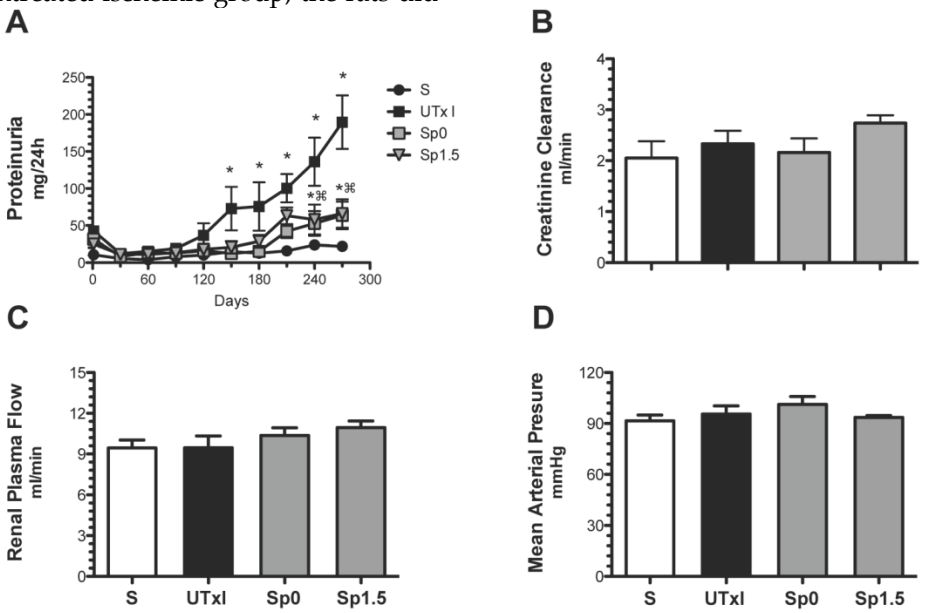

Figure 2. Mild acute kidney injury leads to the development of proteinuria, and the effect was ameliorated by spironolactone administration. Four groups were included: sham (S, n=4), rats that underwent bilateral renal ischemia for 20 min (UTxI, n=5) and rats that received spironolactone (20 mg/kg) at 0 or 1.5 hours after ischemia (Sp0, and Sp1.5, respectively, n=4). A) Urinary protein excretion was determined every 30 days during the follow-up: sham (black circles), A-C (black squares), Sp0 (gray squares) and Sp1.5 (gray triangles). At the end of the 9-month period, B) creatinine clearance, C) renal blood flow and D) mean arterial pressure were determined in the sham (white bars), untreated ischemic group (black bars), and spironolactone-treated groups (gray bars). *p<0.05 vs. sham operated rats and ⌘P<0.05 vs. the UTxI group.

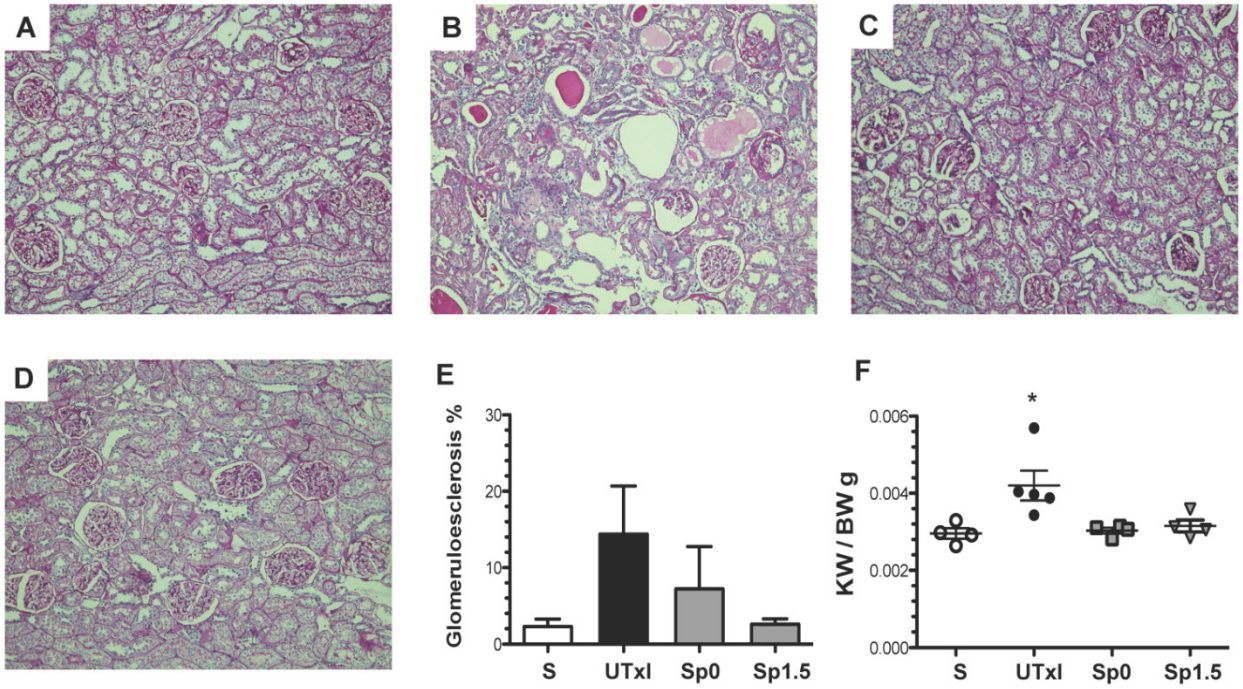

Figure 3. Twenty minutes of bilateral renal I/R led to renal structural injury, which can be prevented by spironolactone administration. Representative images of periodic acid–Schiff (PAS)-stained sections from A) Sham (n=4), B) untreated ischemic group (n=5), C) Sp0 (n=4) and D) Sp1.5 (n=4) groups. The main effects observed were: Tubular dilation, tubular cast formation and glomerular sclerosis. Original magnification: X100. E) Glomerulosclerosis percentage and F) Ratio between kidney weight and body weight (KW/BW). *p<0,05 vs. all the groups.

Figure 4 shows the representative microphotographs from kidney slides stained with Sirius red and the morphometric analysis of the various groups. The untreated ischemic group exhibited a significant area affected by tubulointerstitial fibrosis (Figure 4C-4D). In contrast, the spironolactone-treated groups showed practically no staining for Sirius red (Figure 4E-4H). These observations were confirmed by the morphometric analysis presented in Figure 4B.

Long-term molecular changes induced by a brief ischemic period and prevention by spironolactone.

The role of the TGF-β pathway in promoting the observed fibrosis was assessed. The ischemic untreated group exhibited a significant increase in TGF-β protein levels (Figure 5A). To assess the activation of this pathway, the renal levels of a downstream effector of the TGF-β pathway, phospo-Smad-3, were evaluated by Western blot analysis (Figure 5B). Accordingly, phospho-Smad3 was significantly elevated in the untreated ischemic group. Similar to the effect observed at the TGF-β level, the increased phosphorylation of Smad-3 was not observed in the spironolactone-treated groups. Additionally, the structural injury was associated with a significant up-regulation of renal α-SMA protein levels. This progressive increase was prevented in the spironolactone-treated groups (Figure 5C).

Recently, it was suggested that endothelin-1 activation and enhanced ET_A expression may contribute to the progression of AKI to CKD (18). For this reason, the protein levels of ET-1 and its receptors ET_A and ET_B were measured. Although no difference in the intra-renal content of ET-1 was observed (Figure 6A), we found an up-regulation of ET_A receptors in the untreated ischemic group, and the effect was prevented by spironolactone treatment (Figure 6B). Regarding the ET_B receptor, an up-regulation in all ischemic groups was observed (Figure 6C).

Discussion

The development of CKD in the years following an AKI episode is a major public health issue, and it is associated with poor quality of life in the patients and increased expenses to the health system. The association between AKI and CKD has been consistently recognized in several epidemiological studies. For example, complete recovery of renal function after an episode of AKI in patients with normal baseline kidney function is associated with increased risk of the development of incident stage 3 CKD (5). Moreover, Chawla LS et al. showed that the severity of the AKI episode is strongly associated with the risk of CKD progression (16). However, most of the experimental studies, including a study from our laboratory (15;19;26-29), investigating the possible association between AKI and CKD have focused on the long-term effects of a severe ischemic lesion; therefore, more

information on the effects of a brief period of ischemia on chronic renal injury is required. In this study, we first characterized whether the severity of the ischemic insult was associated with the presence and intensity of chronic renal injury induced by renal bilateral ischemia. Rats that underwent a longer period of ischemia (45 min) developed heavy proteinuria beginning in the third month after ischemia, as we previously reported (15). A mild period of ischemia (20-min) also induced a progressive increase in proteinuria, but this was evident only after 5 months of the initial insult and was less severe compared with the longer-period ischemia group.

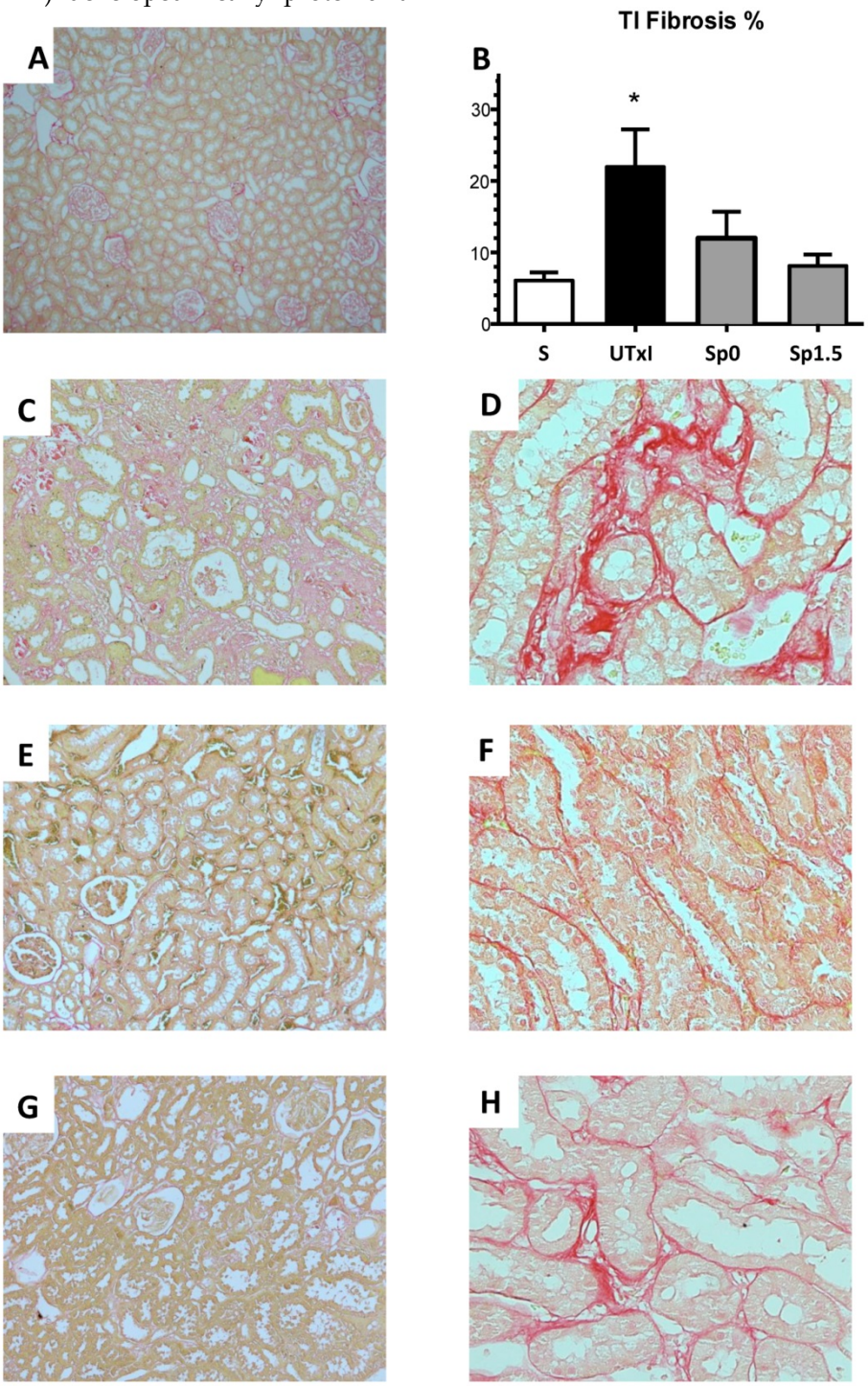

Figure 4. Tubulointerstitial fibrosis development after 9 months of renal ischemia. Representative light micrographs after Sirius red staining showing the presence of fibrosis (in red) from the A) Sham (n=4), C and D) ischemic untreated ischemic group (n=5), E and F) Sp0 (n=4) and G and H) Sp1.5 (n=4). B) The percentage of tubulointerstitial fibrosis in each of the five groups at the end of the 270-day experiment was quantified by morphometric analysis for sham (white bars), untreated ischemic group (black bars), and spironolactone-treated groups (gray bars). Original magnification: X100 (A, C, E, G) and X400 (D, F, H). *p<0,05 vs. all the groups.

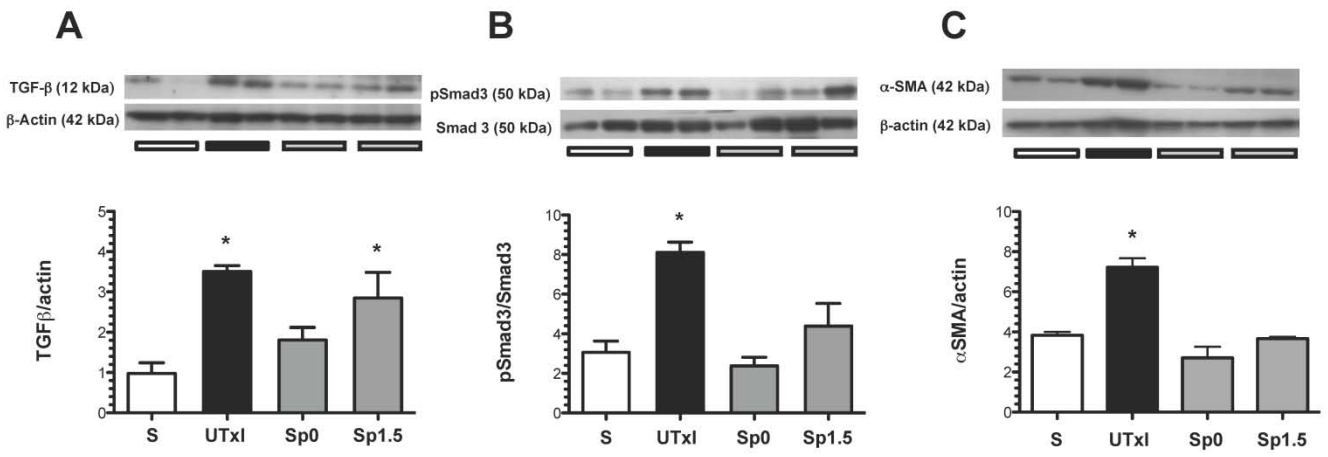

Figure 5. Molecular changes associated with the pro-fibrotic TGF-β pathway activation. A) TGF-β protein levels were quantified by Western blot, B) p-Smad3 and C) α-smooth muscle actin (α-SMA); the densitometric analysis is depicted in the graphs for the sham (white bars, n=4), untreated ischemic group (black bars, n=5), and spironolactone groups (gray bars, n=4). In each panel, the upper insets depict representative blots of the corresponding proteins. *p<0.05 vs. sham operated rats.

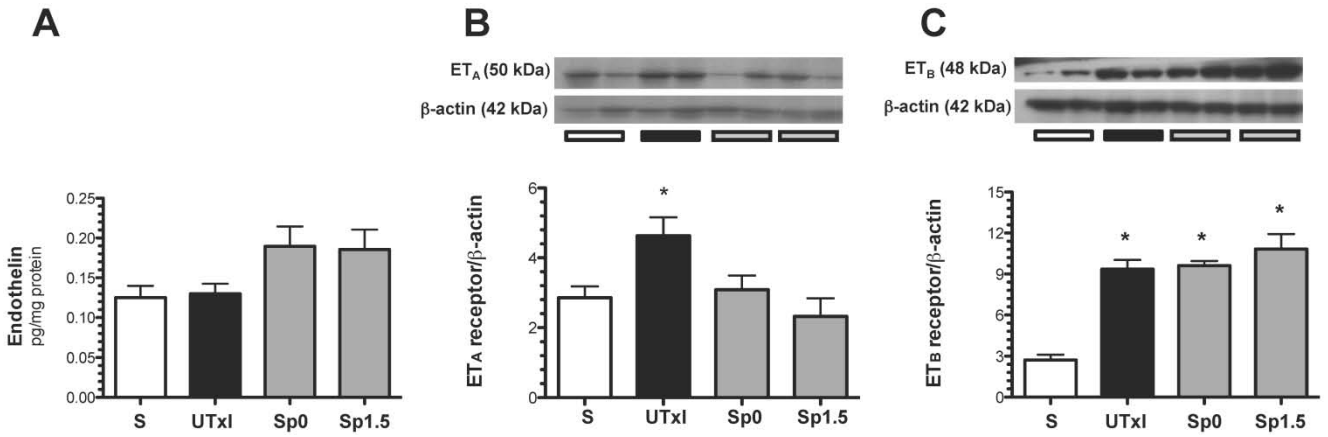

Figure 6. Molecular changes associated with the endothelin pathway. A) Protein levels of endothelin were quantified in renal tissue by an ELISA assay, B) endothelin receptor A (ET_A), and C) endothelin receptor B (ET_B) protein levels. Sham (white bars, n=4), untreated ischemic group (black bars, n=5), and spironolactone-treated groups (gray bars, n=4). * p<0.05 vs. sham operated rats.

In contrast, a brief period of ischemia (10 min) did not lead to proteinuria by the conclusion of the experiment (9 months after ischemia); we cannot exclude the possibility, however, that rats would develop CKD after a longer period of time. This finding is important because many patients in whom AKI may not be accurately diagnosed may be at risk of developing chronic renal injury that may compromise renal function. Indeed, a recent study from Linder A. et al. (30) showed that patients who underwent a mild AKI episode have significantly decreased long-term survival than critically ill patients with no signs of AKI. Although proteinuria developed in rats that underwent 20 min of ischemia, the renal function of the rats was normal nine months after ischemia. These data suggest that the structural injury might be masked by the glomerular compensatory hypertrophy and the nephron functional reserve at this time after mild ischemia. Therefore, although renal dysfunction was not present at this time, these animals exhibited structural and molecular damage, evidenced by glomerular and tubular injury, as well as by effects on pro-fibrotic signaling pathways.

We previously reported that spironolactone administration before or after ischemia may be a useful strategy to prevent the development of CKD induced by a longer period of ischemia. However, this ischemic insult can only be extrapolated to renal transplant or cardiac surgery patients. To investigate a model closer to the common clinical situation, such as patients receiving contrast media or hospitalized patients with lower degree of hypoperfusion, in this study, we investigated spironolactone efficacy in preventing chronic renal injury induced by mild ischemic injury when administered after ischemia. A brief period of ischemia induced structural changes, such as glomerular hypertrophy, glomerulosclerosis, and extensive tubulointerstitial fibrosis. All of these changes were completely prevented by spironolactone administration immediately or up to 1.5-h after

ischemia. Various mechanisms underlying the association between AKI and CKD have been proposed and include chronic hypoxia, abnormalities in the cell cycle of epithelial cells, inflammation, endothelial injury and capillary rarefaction (19;21;29;31). Recently, Kramann et al. (32) showed a reduction in the total number of capillaries and the single capillary area and its perimeter in mice that underwent a severe ischemia, whereas in moderate ischemia, although the number of cortical capillaries did not change, the size of the capillaries was significantly smaller, suggesting that mice with moderate ischemia may also be a target of chronic hypoxia cycles that eventually induce capillary rarefaction after a longer period of time. Indeed, we observed that a mild ischemic insult led to chronic renal injury.

After an ischemic injury, many pathways are activated to promote tubule regeneration; however, some of these pathways remain activated even if the repair is complete, typical of the TGF-β pathway. This mechanism is of particular importance because TGF-β may promote fibroblast trans-differentiation into myofibroblasts and the consequent pro-fibrotic phenotype (33). Indeed, we found that the protein levels of TGF-β, as well as the downstream effector p-Smad3, are increased in rats that underwent a brief period of ischemia. Moreover, an increase in α-SMA, a myofibroblast marker, was also observed. These effects were prevented in the spironolactone-treated rats except for the TGF-β elevation in the Sp1.5 group, however the SMAD-3 phosphorylation was lesser than the untreated ischemic group, suggesting inhibitory SMADs could be inhibiting SMAD-3 phosphorylation.

A recent report suggested that endothelin-1 activation and enhanced ET_A expression may be implicated in the progression from AKI to CKD (18). Therefore, we studied the levels of the endothelin-1, ET_A and ET_B receptors. We could not detect an increase in endothelin-1 levels; however, this discrepancy may be explained by the stage of CKD in which endothelin expression is assessed. In severe CKD, when an extensive area is injured, these cells do not contribute to endothelin production. The differences between rat vs. mice and the effect of bilateral ischemia vs. unilateral ischemia may also account for these results. However, we cannot exclude the idea that endothelin is indeed participating in the progression of CKD, because enhanced expression of the ET_A and ET_B receptors in the rats that underwent mild ischemia was found. Interestingly, the spironolactone treated rats did not present an up-regulation of the ET_A receptor. These data suggest that after ischemia, ET_A remains active and the balance of the receptors may favor the vasoconstrictor effect, which may enhance the chronic hypoxia. In contrast, in spironolactone treated rats, this balance may favor the ET_B receptor, thereby promoting normal perfusion of the kidney and preventing chronic renal injury.

In summary, we provide evidence that in an experimental model of AKI, the duration of ischemia is correlated with the strength and timing of the onset of proteinuria. A mild ischemic lesion was enough to produce progressive proteinuria, renal hypertrophy, glomerular injury, and tubulointerstitial fibrosis. The structural changes were associated with increased TGF-β pathway activation and ET_A receptor up-regulation. These changes were prevented or reduced by spironolactone even if administered after the ischemic insult had occurred. Our data demonstrate the effectiveness of MR antagonism in preventing chronic renal injury induced by a mild ischemic insult in a model of a clinical situation.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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NEPHROLOGY

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Review Article

Epigenetic regulation in the acute kidney injury to chronic kidney disease transition

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DNA condensation, histone acetylation/deacetylation, histones, methylation.

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SUMMARY AT A GLANCE

A fairly comprehensive review focusing on acute kidney injury and epigenetic regulators.

ABSTRACT:

Epigenetic modifications have emerged as a new, important contributor to gene expression regulation in both normal and pathophysiological conditions. Epigenetics have been studied in many diseases and conditions such as acute kidney injury (AKI), a syndrome with a high prevalence that carries a poor prognosis with increased morbidity and mortality. In addition, it has recently been shown that AKI increases the risk for the development of chronic kidney disease (CKD). The specific molecular mechanisms by which AKI increases the risk of CKD and end stage renal disease (ESRD) remain unknown, although there is new evidence supporting a role of epigenetic changes. The most studied epigenetic regulations in AKI are chromatin compaction, DNA methylation, and histone acetylation/deacetylation. These modifications predominantly increase the production of pro-inflammatory and profibrotic cytokines such as: monocyte chemoattractant protein-1 (MCP-1), complement protein 3 (C3), transforming growth factor β (TGF-β) that have been shown for perpetuating inflammation, promoting epithelial-to-mesenchymal transition (EMT) and ultimately causing renal fibrosis. A review of epigenetic mechanisms, the pathophysiology of AKI and recent studies that implicate epigenetic modifications in AKI and in the transition to CKD are discussed below.

EPIGENETICS

In the early 1940s, Conrad Hal Waddington used the term epigenetic, epi (above) and genetics (genetic), for the first time.[1] An epigenetic modification is defined as any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA.[2] The most described epigenetic regulations included chromatin compaction, DNA methylation, histone acetylation/deacetylation, and the noncoding sequences of RNA. Methylation could occur at DNA or at histones. In DNA, a methyl group is added to the cytosine or adenine nucleotides, whereas, in histones, a methyl group is transferred to lysine or arginine of histone proteins. Methylation can occur in different ways: mono-, bi-, or trimethylation in lysine residues and mono- or dimethylation in arginine residues. In contrast, acetylation only occurs in the lysine residues of histones, in which an N-terminal tail protruding is acetylated. The modifications in the amino tail of histones H3 and H4 play a crucial role in gene expression regulation. There are at least eight types of histone post-translational modifications: methylation on lysine or arginine, acetylation and deacetylation of lysines, phosphorylation on serine or threonine residues, ubiquitylation and sumoylation of lysines, and there are more than 60 different residues in which these modifications have been detected. Other mechanisms such as the small, mitochondrial or long non-coding RNA, are implicated. The precise moment when these post-transductional modifications occur depends on cell conditions and they never happen at the same time or in the same histone.

Complex organisms must condense their DNA using proteins that form a complex called chromatin. Chromatin is mainly composed by histones, which condense or relax the DNA in order to form two types: the heterochromatin (compact-chromatin) and the euchromatin (relaxed-chromatin).[3] The functional and basic unit is the nucleosome, which is formed by an octamer of histones. There are different histones: H2A, H2B, H3, H4 and H1. A pair of each of the former four comprises an octamer in

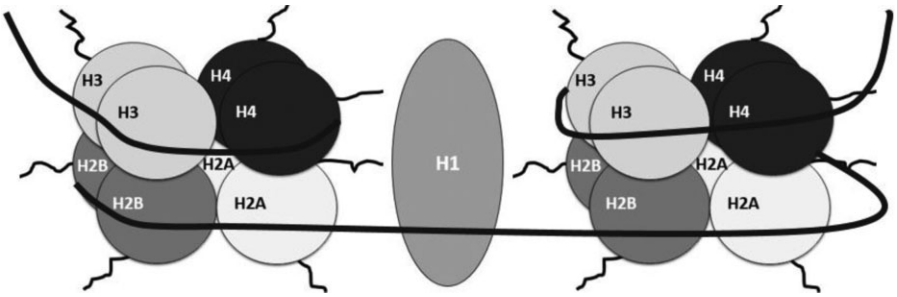

Fig. 1 Histones and DNA pearl necklace arrangement. Chromatin structure is formed by sets of nucleosomes. Each nucleosome contains a pair of histones: H2A, H2B, H3, and H4, which conform the functional octamer. Around the nucleosomes, DNA is wrapped two times. The H1 protein contains DNA from 10 to 80 bp of length that connects one nucleosome with the other one.

which DNA is wrapped two times. A DNA linker chain, connects the nucleosomes. This structural arrangement is condensed 10 times more than the relaxed DNA. The histone H1 functions as a 'linker' protein and assists in the formation of a chromatin fibre, which is formed by a tubular arrangement of nucleosomes and condensed 50 times compared to the relaxed DNA[3,4] (Fig. 1).

The DNA methylation seems to be the most understood method of epigenetic regulation. This occurs in the cytosines of cytosine-guanine (CpG) sites. DNA sections rich in CpG sites are associated with 76% of the promoter regions in the genome. The enzymes responsible for the methylation of DNA are called methyl-transferases (Dnmt). Dnmt1 and Dnmt2 are considered as maintenance methyltransferases, whereas Dnmt3 acts as a 'novo' methyl-transferase. Dnmt1 and Dnmt2 are responsible for attaching methyl groups to DNA hemi-methylated portions during replication, while Dnmt3 acts after replication.[5]

The histones acetylation state is regulated by the activity of histone acetylases (HAT) and deacetylases (HDAC). Normally, the acetylation of H3 and H4 increases the expression of genes involved in the open structure of the chromatin.[6]

Influence of the environment in epigenetic patterns

Manuel Esteller *et al*. demonstrated that monozygotic twins who were exposed to different environmental conditions during their lives had different patterns of methylation and acetylation within their genome affecting the gene expression of each twin. Conversely, when twins grow under the same conditions, patterns of methylation and acetylation are almost identical.[7] Additional evidence is found in studies of bees, where it has been shown that the larvae that are bound to be future queens are separated from the rest and fed a special mixture of honey, commonly called royal jelly, which has a high content of HADC10. This diet changes the acetylation pattern and phenotypic characteristics typical of a queen bee.[8]

RNA as an epigenetic regulator

In the last decades, the noncoding sequences of RNA (ncRNA) have been proven to play a key role in epigenetic regulation. It is now known that they serve a wide range of functions including the facilitation of chromosome dynamics, alternative splicing, transcriptional inhibition and destruction of mRNA. Despite the vast number of noncoding RNAs, the ones considered to be the most important in epigenetic silencing include: siRNAs, which are small molecules of 21–25 nucleotides, miRNAs, which are also small molecules that originate as endogenous precursor helix loop structures, and lncRNAs, which are bigger molecules with a length of 17 kilobases. The siRNAs and miRNAs regulate gene expression by an RNA interference phenomenon causing a repression at the translational level, while the lncRNAs work by modulating chromatin states and consequently modulating gene expression.[9]

ACUTE KIDNEY INJURY

Acute kidney injury (AKI) is a syndrome characterized by a sudden decline in renal excretory function, with a consequent failure in maintaining fluid, electrolyte and acid-base balance. AKI is diagnosed by clinical and laboratory manifestations such as oliguria (not always present) and elevations in serum creatinine, urea, phosphate and potassium concentrations.[10]

This syndrome was first described in the 1940s in London, where a report of cases of renal failure related to crush injuries was published.[11] In 2004, the Acute Dialysis Quality Initiative group published the Risk, Injury, Failure, Loss, and End-stage renal disease (RIFLE) classification in which the term 'acute kidney injury' and a formal definition were established. This definition evolved further into the AKI Network (AKIN) and in Kidney Disease: Improving Global Outcomes (KDIGO) definition.[12]

KDIGO defined AKI as: an increase in serum creatinine (SCr) equal to or greater than 0.3 mg/dL (>26.5 μmol/L) within 48 h; an increase in SCr equal to or greater than 1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or a urine output volume less than 0.5 mL/kg per h for 6 h. It also classifies AKI into three different stages (summarized in Table 1).[13]

It is difficult to assess the prevalence of AKI in the community. Nevertheless, there are some epidemiological studies showing that the incidence of AKI is between 2000 to 4000 people per million-population/year.[14] AKI is more prevalent

Table 1 Acute kidney injury classifications

System	Serum creatinine criteria	Urine output criteria
RIFLE class		
Risk	Serum creatinine increase to 1.5-fold OR GFR decrease >25% from baseline	<0.5 mL/kg per h for 6 h
Injury	Serum creatinine increase to 2.0-fold OR GFR decrease >50% from baseline	<0.5 mL/kg per h for 12 h
Failure	Serum creatinine increase to 3.0-fold OR GFR decrease >75% from baseline OR serum creatinine ≥354 μmol/L (≥4 mg/dL) with an acute increase of at least 44 μmol/L (0.5 mg/dL)	Anuria for 12 h
AKIN stage		
1	Serum creatinine increase ≥26.5 μmol/L (≥0.3 mg/dL) OR increase to 1.5–2.0-fold from baseline	<0.5 mL/kg per h for 6 h
2	Serum creatinine increase >2.0–3.0-fold from baseline	<0.5 mL/kg per h for 12 h
3	Serum creatinine increase >3.0-fold from baseline OR serum creatinine ≥354 μmol/L (≥4.0 mg/dL) with an acute increase of at least 44 μmol/L (0.5 mg/dL) OR need for RRT	<0.3 mL/kg per h for 24 h OR anuria for 12 h OR need for RRT

GFR, glomerular filtration rate; RRT, renal replacement therapy.

in the inpatient population, 3.2% to 9.6% of admissions[15] and is especially common in critically ill patients, 40% to 60%.[16]

The diagnosis and the identification between the different causes of AKI can be challenging given the limited sensitivity and specificity of biochemical parameters. But, significant progress has been made in the identification of diverse biomarkers like neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (Kim-1) interleukin-18 (IL-18), fatty acid-binding protein 1 (L-FABP1), and heat shock protein 72 (Hsp72), although their widespread use has been limited for different reasons (for review[17]).

There are a myriad of causes of AKI. For didactic and clinical reasons, they are typically divided into pre-renal (hypo-perfusion of the kidney), intrinsic renal (pathologic process within the kidneys), and post-renal causes (obstruction of urine flow distal to the kidneys), although AKI is often multifactorial. It is believed that pre-renal and post-renal AKI begin as functional processes, and if they are corrected in a timely manner, any residual renal damage can be limited. Intrinsic AKI, however, represents structural damage.[18] But, many authors consider AKI to be a continuum of injury that starts with pre-renal AKI.[19]

The cellular and molecular events that take place during an event of AKI can be divided into four phases: (i) 'The initiation' phase, which takes place as a direct consequence of decreased renal blood flow (RBF) resulting in significant cellular ATP depletion and tubular epithelial cell injury with typical histologic alterations (loss or inversion of polarity and loss of adhesion to the basement membrane).[20] In this phase there are releases of cytokines and chemokines that initiate the inflammation cascade. (ii) Sustained hypoxia due to vascular endothelial cell damage and an intense inflammatory response characterizes 'the extension' phase, which is marked by renal epithelial cell injury and death by both necrosis and apoptosis. Some have argued that it is during this phase that a therapeutic intervention could be most successful by preventing the amplification of inflammation.[21] (iii) 'The maintenance' phase, where the inflammation becomes regulated and there is an attempt to re-establish and maintain cellular and tubular integrity. There is marked epithelial cell repair, migration and proliferation, and (iv) 'The recovery' phase in which cellular differentiation continues. After recuperation of the renal epithelial cell polarity, normal organ function returns.[22]

Despite the progress in the understanding of the pathophysiology of AKI, the cornerstone of treatment remains supportive care. There are some medications that offer a theoretical benefit such as loop diuretics or low doses of dopamine. However, these drugs have not shown any significant improvement in renal outcomes in different meta-analyses.[23] There is some evidence demonstrating that receptor mineralocorticoid antagonism,[24–27] anti-inflammatory,[28] and antioxidant therapies[29] could improve outcomes in AKI, although large clinical trials are lacking.

AKI TO CKD TRANSITION

In the past, the renal outcomes in patients after recovery from AKI were believed to be benign with a low probability of developing chronic kidney disease (CKD) and end-stage renal disease (ESRD). Nevertheless, in the last few years, evidence has revealed a strong association between AKI and the consequent development of CKD. Most of the evidence comes from large observational studies that have consistently shown that a significant number of patients with AKI, even those patients without prior kidney disease, after partial or complete recovery of renal function often then progress to advanced stages of CKD or even ESRD in some cases. The number of episodes, the severity of AKI and pre-existing kidney disease appear to correlate with a greater risk for progression to CKD.[30] In this regard, Bucoloiu ID *et al*.[31] showed that 6.6% of AKI patients that had a complete renal function recovery exhibited a greater risk of de novo CKD and death in the following months. Furthermore, a meta-analysis that included 13 large studies found that AKI is an independent risk factor for CKD.[30] Worldwide, it has been shown that 20% of patients with an AKI episode will develop CKD after 3 years.[32]

As was commented before, during AKI a number of processes are activated to repair the affected renal structures, but they can lead to cell proliferation, hypertrophy and exaggerated extracellular matrix production.[33] Renal vasoconstriction predominates after AKI due to an increase of endothelin-1, angiotensin II, thromboxane A2, and adenosine, as well as, by the reduction in nitric oxide (NO) synthesis.[34–37] These effects are more enhanced by increased leukocyte adhesion to the endothelium, which occludes small vessels and compromise renal vascular microcirculation.[38] In addition, the number of renal vessels decreases as a result of capillary rarefaction phenomenon.[39–41] This process seems to be facilitated by the vascular endothelial growth factor reduction.[42,43] As a result, chronic hypoxia leads to progressive deterioration of the tubular epithelium, leading to cell cycle arrest and epigenetic alterations that eventually cause the progressive development of tubulointerstitial fibrosis.[44,45]

IS EPIGENETIC REGULATION A NEW FACTOR IN THE TRANSITION FROM AKI TO CKD?

It is not surprising to find that epigenetic regulation may participate in AKI to CKD transition because of its implication in the cell adaptation to extreme circumstances, such as oxidative stress, hypoxia, and mitochondrial injury.[46]

Chromatin structure modifications

During an AKI episode, the tubular epithelial cells are subjected to a hypoxic milieu, causing modification not only in the cellular metabolism, but also in the chromatin structure and in the binding of different transcription factors.[47] It is well known that there is an increase in the expression of pro-inflammatory cytokines such as tumour necrosis factor (TNF-α) and monocyte chemoattractant protein (MCP-1) after an AKI episode, which persists until 7 days.[48,49] This effect seems to be the result of epigenetic regulation, because there is an increment in the multiprotein chromatin remodelling complex that includes the SWItch/Sucrose Non-Fermentable (SWI/SNF) factor. This complex depends on helicase-like ATPase activity and regulates chromatin structure. The ATPases of this complex are the machinery that allow dynamic changes in chromatin structure by activating or inactivating gene expression. Specifically, the human SWI/SNF complex is also able to slide nucleosomes along the DNA, promoting the transcription start sites and making them more accessible for specific genes. This complex contains the Brahma-related gene1 (BRG1), which is an ATPase catalytic chromatin remodelling subunit. In the mice, BRG1 is a regulator of the nucleosome remodelling complexes in the TNF-α gene.[49] Recent findings have shown that there is also an increase in MCP-1 independent of the causes of AKI[47] (Fig. 2A).

Histone epigenetic modifications during AKI

Adequate cholesterol synthesis helps to preserve the epithelial cells during an ischaemic insult because cholesterol regulates both plasma membrane integrity and mitochondrial function. Naito M *et al*.[50] found that after 3-days of ischaemia, there was an increase in the RNA polymerase II recruitment (Pol II) and sterol regulatory element binding proteins-1 and 2 (SREBP-1 and SREBP-2) to co-enzyme A reductase (HMGCR), which results in enhanced cholesterol synthesis – this recruitment is possible because, after renal ischaemia/reperfusion (I/R), the trimethylation of histone 3-lysine 4 (H3K4m3) and the acetylation of histone 3-lysine 9 (H3K9) occur. These coordinated events led to greater epithelial cell survival during an ischaemic event (Fig. 2B).

ATF3 belongs to the activating transcription factor/cAMP responsive element-binding protein (ATF/CREB) family and has been identified as a transcriptional repressor. During I/R injury there is an induction of ATF3 in the kidney. Accordingly, ATF3-deficient mice exhibited greater renal I/R-induced mortality, kidney dysfunction, inflammation and proximal tubular apoptosis compared with wild-type mice. When ATF3 was re-established in the kidney, rescue of the renal I/R-induced injury was observed, suggesting that this factor increases the expression of cytoprotective molecules.[51] In addition, it has been proven that ATF3 interacts with histone deacetylase-1 (HDAC1) after I/R injury, which results in the condensation of chromatin, the interference of nuclear factor-κB (NF-κB) binding to the DNA, and the inhibition of inflammatory gene transcription such as, IL-2b and P-selectin (Fig. 2B). Unfortunately, the increase in cholesterol synthesis and ATF3 activation are not enough to prevent renal injury, but both are important mechanisms triggered by epithelial cells to reduce renal injury.

DNA epigenetic modifications during AKI

Another epigenetic modification is DNA methylation/demethylation. Pratt JR *et al*.[52] show the upregulation of complement C3 that was due to an increase in the demethylation of a cytosine residue in the interferon-γ (IFN-γ) responsive element within the C3 promoter in the rat kidney that had undergone I/R. Accordingly, Huang N *et al*.[53] confirmed these findings in C57BL/6 mice that underwent renal I/R. Moreover, they demonstrated that this epigenetic modification promoted a decrease in Tet1 and Tet2, which catalyzes the oxidation of 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine. This reduced oxidation was associated with an increase in the expression of IL-10 and IFN-γ receptor-2. Another interesting finding of this study was that the demethylation of the C3 promoter persisted for at least 6 months in transplanted rat kidneys (Fig. 2B). In a recent study, DNA methylation was evaluated in two groups of patients with CKD: with rapid decline in kidney function and with stable kidney function. Interestingly, the stable kidney function group exhibited a greater hypemethylation in

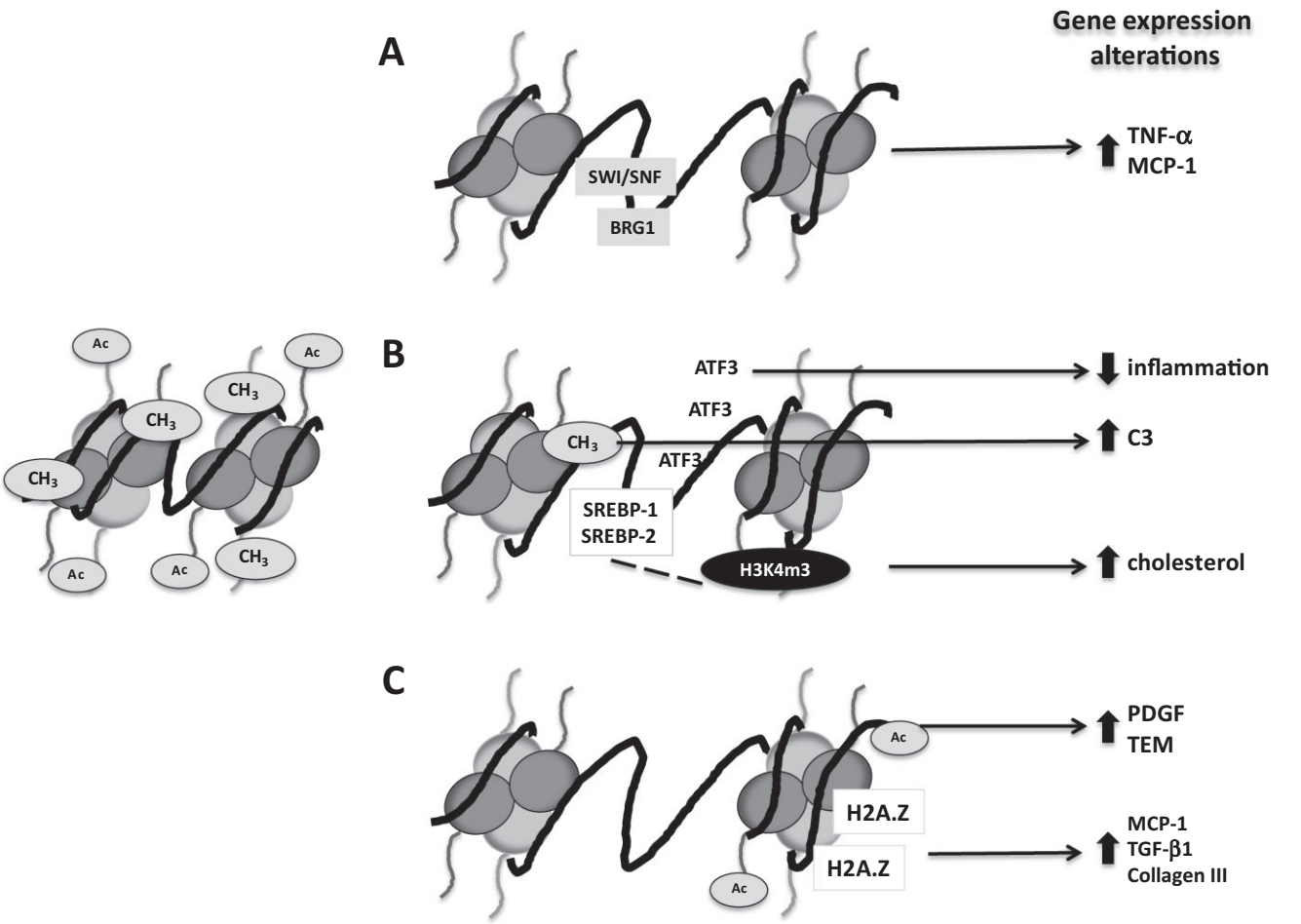

Fig. 2 Epigenetic modifications that occur after an episode of acute kidney injury. (A) Relaxation of chromatine which is mediated by SWItch/Sucrose Non Fermentable (SWI/SNF) factor, sliding the nucleosomes along the DNA and promoting the transcription start sites more accessible for specific genes. This complex contains the Brahma-related gene 1 (BRG1), a regulator of the nucleosome remodelling complexes in tumour necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) genes. (B) The DNA demethylation allows the recruitment of RNA polymerase II (Pol II) and sterol regulatory element binding proteins 1 and 2 (SREBP-1 and SREBP-2) to co-enzyme A reductase (HMGCR), which results in enhanced cholesterol synthesis that leads to a greater survival of the epithelial cell during an ischaemic event. Also, there is an induction of the transcriptional repressor ATF3 in the kidney, which helps to reduce the kidney dysfunction, inflammation and proximal tubular apoptosis. In addition, complement C3 upregulation is observed as a result of an increase in demethylation of a cytosine residue in the interferon-γ responsive element within the C3 promoter. This demethylation of the C3 promoter has been reported to persist at least 6-months in transplanted rat kidneys. (C) Histones deacetylation promotes myofibroblast proliferation and epithelial-to-mesenchymal transition (EMT). In addition, the increased expression of histone 2 variant (H2A.Z) promotes the increase n of fibrotic and inflammatory genes such as MCP-1, transforming growth factor-β (TGF-β1), collagen III.

nephronophthisis 4 (NPHP4), IQ motif, Sec7 domain (IQSEC1), GEP100, and transcription factor 3 (TCF3), genes involved in the epithelial-to-mesenchymal transition (EMT), suggesting that this epigenetic modification confers certain renoprotection in these patients with lower rate of renal progression.[54]

Histone changes during AKI

The inhibition of HDAC activity by trichostatin A (TSA) decreased the proliferation induced by platelet-derived growth factor (PDGF) of NIH-3T3 skin fibroblasts. In addition, Pang M *et al*. used an in vivo model of unilateral urethral obstruction to demonstrate that TSA blocks the EMT induced by transforming growth factor-β (TGF-β) in renal tubular epithelial cells, suppresses the expression of α-smooth muscle actin (α-SMA) and fibronectin and attenuates the accumulation of renal interstitial fibroblasts in the kidney (Fig. 2C).[55] Additionally, it has been reported that pharmacological HDAC inhibition promotes anti-inflammatory and antifibrotic effects in other diseases such as Alzheimer's, Parkinson's and multiple sclerosis.[56]

In 2008, Marumo T *et al*.[57] reported a transient decrease in histone acetylation in the proximal tubular cells of mice that

was appreciated immediately following severe unilateral I/R. This effect was recovered after 24 h due to a decrease in HDAC5. In a second study, these authors reproduced these findings and showed that HDAC5 knockdown by RNAi significantly increased histone acetylation and upregulated BMP7 expression promoting the tubular epithelium recovery.[58] In contrast, Zager *et al*.[59] used an enzyme-linked immune-absorbent assay (ELISA) of renal cortexes and found an increase in renal acetylated histone H3 levels after I/R; these changes were seen 24 h after injury and persisted for 3 weeks. This discrepancy could be explained through a different temporal response following I/R.

Although histone acetylation modifications appear to be involved in the transition of AKI to CKD, the story is more complicated, because it has been demonstrated in mice that underwent unilateral I/R that two gene-activating histone alterations also occur: histone 3, lysine 4 trimethylation (H3K4m3) and increased expression of histone 2 variant (H2A.Z). Both changes promote an increase in the expression of fibrotic and inflammatory genes such as MCP-1, TGF-β1 and collagen III[48] (Fig. 2C). Moreover, Marumo T *et al*.[58] explored the role of HDAC in tubulointerstitial injury by using the model UUO, in which HDAC1 and HDAC2 are activated and are responsible for reducing histone acetylation in the injured kidney. As expected, TSA treatment attenuated macrophage infiltration and tubule-interstitial fibrosis. The induction of colony-stimulating factor-1 (CSF-1), a chemokine known to be involved in macrophage infiltration in tubulointerstitial injury, was also reduced. Accordingly, the knockdown of HDAC1 or HDAC2 significantly reduced CSF-1 induced by TNF-α in renal tubular cells.

Another epigenetic modification described in cancer and diabetes is the dysfunction of histone acetyl-transferases (HATs), which also have been seen as potential targets for the design of new therapies. In renal I/R injury, the administration of curcumin (diferuloylmethane), which is a specific inhibitor of HAT (p300/CREB-binding protein), reduced oxidative stress and improved renal function, suggesting the participation of HAT in promoting renal injury.[62]

After AKI, the surviving epithelial cells proliferate to re-establish the normal tubular structure, although some of these cells may remain arrested in the G2/M cell cycle phase, delaying renal structure recovery and promoting the development of chronic fibrosis.[44,45] Thus, it is reasonable to hypothesize that drugs able to stimulate the cell cycle may have a beneficial effect on this pathology. Accordingly, m4PTB, a histone deacetylase inhibitor, was able to promote renal progenitor cell proliferation, accelerate the recovery of AKI induced by gentamicin in zebrafish, and reduce renal ischaemic injury in mice. The protective effect of m4PTB was associated with increased proliferation of the tubular cells mediated by both inducing the expression of genes involved in the cell cycle and with a higher number of cells in the S-phase. Long-term kidney fibrosis was also reduced by m4PTB.[60] Similarly, Novitskaya T *et al*.[61] recently showed that the administration of phenylthiobutanoic acids (PTBAs), a new class of histone deacetylase (HDAC) inhibitor, was also able to accelerate the AKI recovery and reduce fibrosis in a progressive model of AKI induced by aristolochic acid, due to increased tubular proliferation and decreased G2/M cell cycle arrest. In addition, Richard A Zager *et al*.[59] showed in mice that the progressive renal disease observed throughout the 3 weeks after ischaemia was associated with a progressive increase from 5% (at baseline) to 75% (at 3 weeks) in pro-inflammatory cytokine/chemokine genes such as: MCP-1, TNF-α, and TGF-β1. These changes were in accord with a progressive gene-activating H3 acetylation.

All of these studies together suggest that epigenetic changes that occur after an ischaemic insult can persist despite the resolution of the AKI episode and seem to be partially responsible for the persistent inflammation, pro-fibrotic milieu and EMT that have been shown to contribute to CKD development. Further research is still needed regarding these findings, but these are promising findings that provide opportunities to find and develop new targets to prevent AKI and the progression to CKD.

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DISCLOSURE

The authors declare no financial conflict of interest.

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AT1 receptor antagonism before ischemia prevents the transition of acute kidney injury to chronic kidney disease

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Despite clinical recovery of patients from an episode of acute kidney injury (AKI), progression to chronic kidney disease (CKD) is possible on long-term follow-up. However, mechanisms of this are poorly understood. Here, we determine whether activation of angiotensin-II type 1 receptors during AKI triggers maladaptive mechanisms that lead to CKD. Nine months after AKI, male Wistar rats develop CKD characterized by renal dysfunction, proteinuria, renal hypertrophy, glomerulosclerosis, tubular atrophy, and tubulointerstitial fibrosis. Renal injury was associated with increased oxidative stress, inflammation, α-smooth muscle actin expression, and activation of transforming growth factor β; the latter mainly found in epithelial cells. Although administration of losartan prior to the initial ischemic insult did not prevent or reduce AKI severity, it effectively prevented eventual CKD. Three days after AKI, renal dysfunction, tubular structural injury, and elevation of urinary biomarkers were present. While the losartan group had similar early renal injury, renal perfusion was completely restored as early as day 3 postischemia. Further, there was increased vascular endothelial growth factor expression and an early activation of hypoxia-inducible factor 1 α, a transcription factor that regulates expression of many genes that help reduce renal injury. Thus, AT1 receptor antagonism prior to ischemia prevented AKI to CKD transition by improving early renal blood flow recovery, lesser inflammation, and increased hypoxia-inducible factor 1 α activity.

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KEYWORDS: glomerular hypertrophy; HIF-1α; inflammation; proximal tubule; renal fibrosis; renal hypoperfusion; VEGF

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Acute kidney injury (AKI) is a common complication in patients who are undergoing major cardiac surgery, experiencing hemorrhage, dehydration, septic shock, diabetes mellitus, or receiving nephrotoxic drugs or contrast media (see review, by Bonventre).[1] The hallmark of ischemic AKI is the reduction in renal blood flow (RBF),[2] provoking endothelial and tubular epithelial injury.[3,4] For many years, it was believed that patients recovering from an AKI episode had no further consequences in the kidney function. However, it is now known that AKI constitutes a risk factor for the development of chronic kidney disease (CKD) and the transition from CKD to end-stage renal disease (ESRD).[5–8] A recent meta-analysis that included 13 major studies with patients who experienced an AKI episode concluded that AKI is an independent risk factor for CKD development.[9] Moreover, from a large number of patients who may suffer an AKI episode throughout their life, it is estimated that 20% would develop CKD in the next 3 years, meaning an estimated 0.3 million patients in higher-income countries and 1.8 million patients in lower-income countries.[10]

During AKI, a number of signaling pathways are activated to repair the affected structures. However, for reasons that are not completely understood, this can lead to cell proliferation, hypertrophy, and disproportionate extracellular matrix production.[4] In the postischemic kidney, renal vasoconstriction is enhanced because of an imbalance of vasoactive substances.[11–13] In addition, the number of vessels in the outer medulla decreases as a result of capillary rarefaction.[14–16] This process can be facilitated by the downregulation of vascular endothelial growth factor.[17] Therefore, the reduction in the number of vessels generates chronic hypoxia that leads to progressive deterioration of the tubular epithelium, leading to cell cycle arrest and epigenetic alterations that eventually cause the progressive development of tubulointerstitial fibrosis.[18,19] Thus, it is essential to delve deeply into the mechanisms by which an AKI episode can trigger an inadequate renal response.

We have recently characterized a model of CKD induced by a single ischemic process in the rat. After recovering from the AKI episode, the animals exhibited progressive

proteinuria, renal dysfunction, and significant histological alterations. Mineralocorticoid receptor blockade before the ischemic insult completely prevented the development of AKI and thus the progression to CKD. Interestingly, spironolactone administration 1–3 h after ischemia also prevented transition from AKI to CKD, implying that activation of the rennin–angiotensin–aldosterone system is involved in the progression to CKD after an AKI episode. We also observed that renal function was completely recovered 10 days after the AKI episode, but signs of inflammation in the kidney persisted that were associated with progression from AKI to CKD along the following months.[20] We previously showed that angiotensin II receptor blockade (ARB) did not prevent AKI induced by unilateral ischemia and because of the cross-talk between vascular and inflammatory effects of angiotensin II, in the present study, we analyzed to what extent ARB with losartan before the ischemic insult was effective in abrogating the severity of the AKI episode and/or the progression to CKD after the AKI episode was resolved.

RESULTS

Figure 1 shows the renal function, structural findings, and biochemical parameters after 24 h of ischemia. Mean arterial pressure was similar among the groups (Figure 1a). Renal injury induced by 45 min of bilateral ischemia was characterized by a significant reduction in the RBF (Figure 1b), reduction in creatinine clearance (Figure 1c), increased serum aldosterone (Figure 1d), elevation of proteinuria (Figure 1e), and severe tubular injury (Figure 1f and 1h). Renal injury was also evidenced by the significant elevation of urinary biomarkers Hsp72 and Kim-1 (Figure 1i and j, respectively). Ischemic renal damage was associated by nitric oxide reduction (Figure 1k) and elevation of oxidative stress (Figure 1l) as we previously reported.[21,22] All of these alterations were not modified by losartan pretreatment, including the elevation of serum aldosterone. Thus, the development of AKI after a single ischemic insult was neither prevented nor reduced by losartan administration.

In another set of experiments, the animals were followed for 9 months after the ischemic insult with and without losartan pretreatment and compared with their respective control groups. As Figure 2a shows in the first 90 days after AKI recovery none of the groups exhibited proteinuria. However, a progressive increase was observed in the untreated ischemic group (UTxI) compared with the sham and losartan control groups. The increased proteinuria was not observed in the animals exposed to ischemia, but previously treated with losartan (Los-Pre), in spite of similar AKI degree (Figure 1a). None of the rats developed hypertension (Figure 2b), as we previously reported.[20] Therefore, all functional and structural alterations were associated with the ischemic process. At the end of the experimental period, UTxI group exhibited a significant reduction in creatinine clearance (Figure 2c), which was accompanied by a slight reduction in RBF. These renal

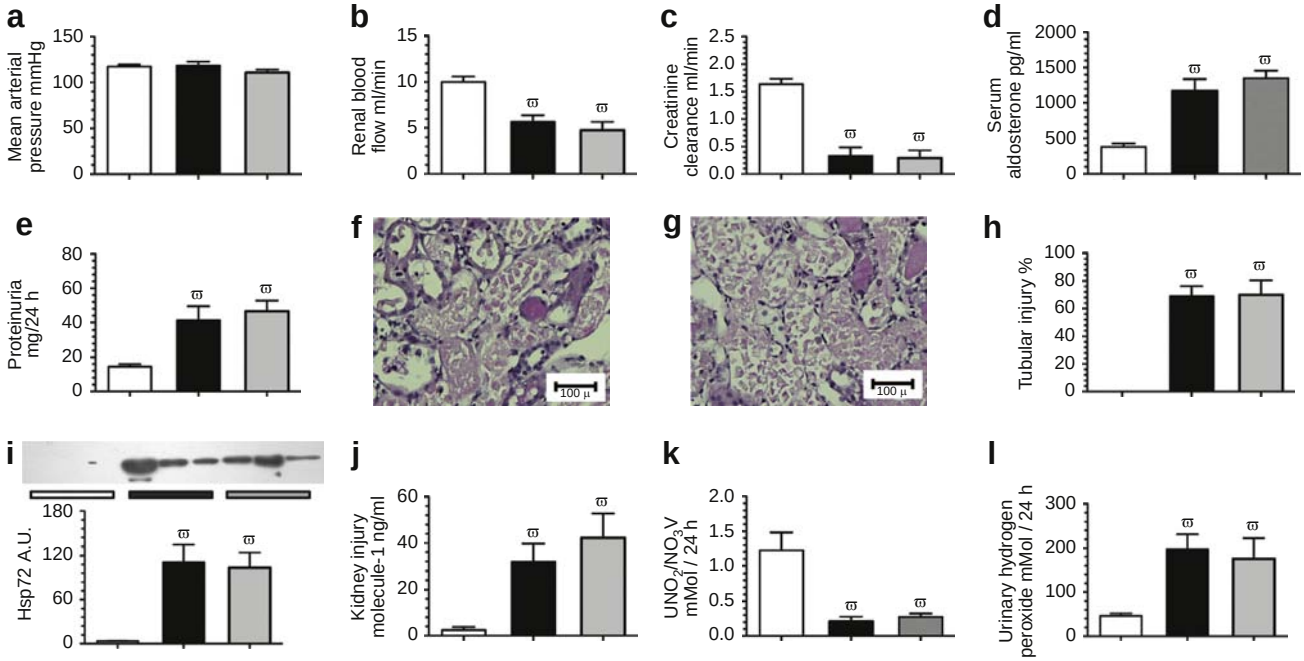

Figure 1 | The prophylactic administration of losartan did not prevent renal injury induced by ischemia/reperfusion. (a) Mean arterial pressure, **(b)** renal blood flow, **(c)** creatinine clearance, **(d)** serum aldosterone, **(e)** proteinuria. Sham n = at least 7, UTxI n = at least 6, and Los-Pre n = at least 8 rats; each rat represents one experiment. **(f–g)** Periodic acid–Schiff-stained kidney slides from an UTxI and Los-Pre groups, respectively, **(h)** tubular injury percentage, total tubules taken like 100%, and was determined in at least 5 rats per group. **(i)** Urinary Hsp72 levels by western blot ($n = 6$ rats per group, one assay), **(j)** urinary Kim-1 excretion ($n = 8$, one assay), **(k)** urinary NO_2/NO_3 excretion ($n = 5$, one assay), and **(l)** urinary H_2O_2 excretion (in at least 6 per group, one assay) in sham (white bars), ischemic (black bars), and Los-Pre (gray bars). All parameters were analyzed 24 h after ischemia, and data are shown like mean ± s.e. $^{\varpi}P < 0.05$ versus sham group by analysis of variance and the Bonferroni test.

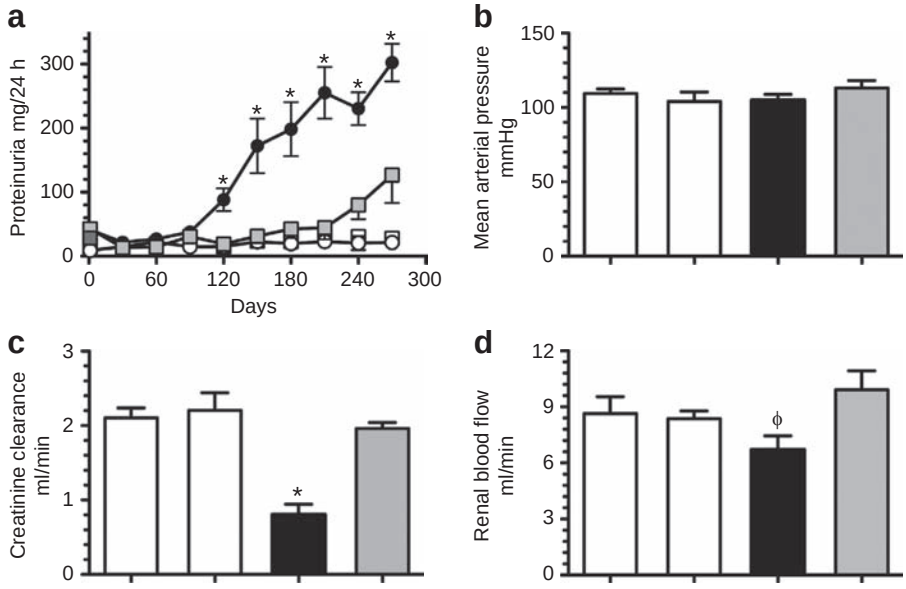

Figure 2 | The prophylactic administration of losartan prevents the progression to chronic kidney disease after an acute kidney injury episode. (a) Urinary protein excretion measured every 30 days during follow-up: open circles represent sham-operated rats (n = at least 6); open squares represent rats that received losartan (50 mg/kg per day) 3 days before sham surgery (n = at least 6); black circles represent rats that underwent renal bilateral ischemia (n = at least 9); gray squares represent rats that received losartan 3 days before renal bilateral ischemia ($n = 8$). **(b)** Mean arterial pressure (n = at least 6 per group), **(c)** creatinine clearance (n = at least 7 per group), and **(d)** renal blood flow (n = at least 6 per group). All parameters were determined after 9 months in sham (white bars), Los (second white bars), ischemic (black bars), and Los-Pre groups (gray bars). $^{*}P < 0.05$ versus all groups, $^{\Phi}P < 0.05$ versus Los-Pre group, both by analysis of variance and the Bonferroni test.

functional changes were not observed in the ischemic group that received losartan.

Nine months after AKI episode was recovered, histopathological analysis revealed that the UTxI group developed severe structural damage, such as glomerular hypertrophy, tubular atrophy, and cast formation (Figure 3c), compared with control groups (Figure 3a and b). These changes were absent in the Los-Pre group (Figure 3d). Glomerular injury in the UTxI group was confirmed by the glomerulosclerosis percentage (18%), whereas the Los-Pre group was safeguarded from glomerulosclerosis (Figure 3e). A strong correlation between glomerulosclerosis and proteinuria was found (Figure 3f, $P = 0.0001$).

The UTxI group developed significant renal hypertrophy, as their kidney weight was 74% heavier than that of the control group (Supplementary Figure S1A online). The

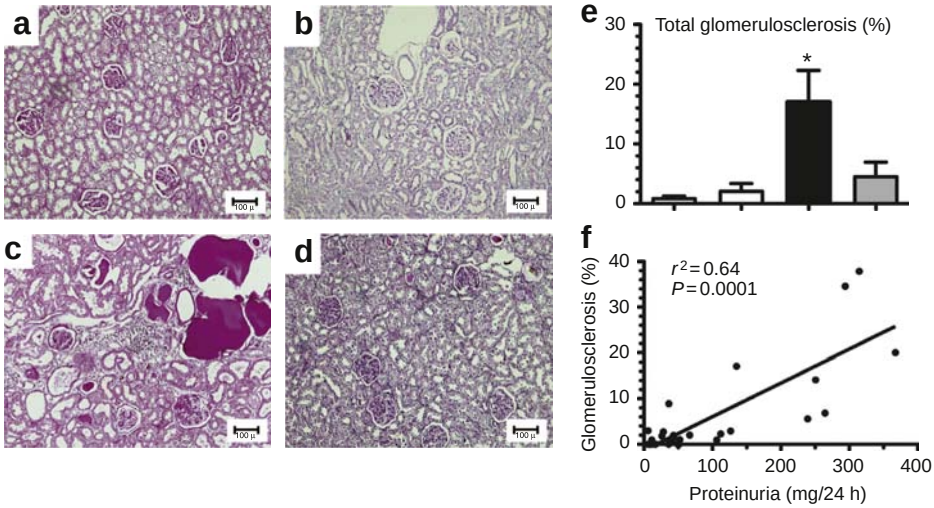

Figure 3 | An acute kidney injury episode leads to extensive structural alterations and prevention by losartan. Representative images of periodic acid–Schiff-stained kidney sections from **(a)** sham-operated, **(b)** Los, **(c)** UTxI, and **(d)** Los-Pre (magnification x100). **(e)** The percentage of glomerulosclerosis was quantified by counting at least 50 glomeruli, which were considered 100% in sham, $n = 7$ (white bars), Los, $n = 8$ (second white bars), UTxI, $n = 7$ (black bars), and Los-Pre, $n = 8$ (gray bars). **(f)** Pearson correlation between glomerulosclerosis % and urinary protein excretion. $^{*}P < 0.05$ versus all groups by analysis of variance and the Bonferroni test.

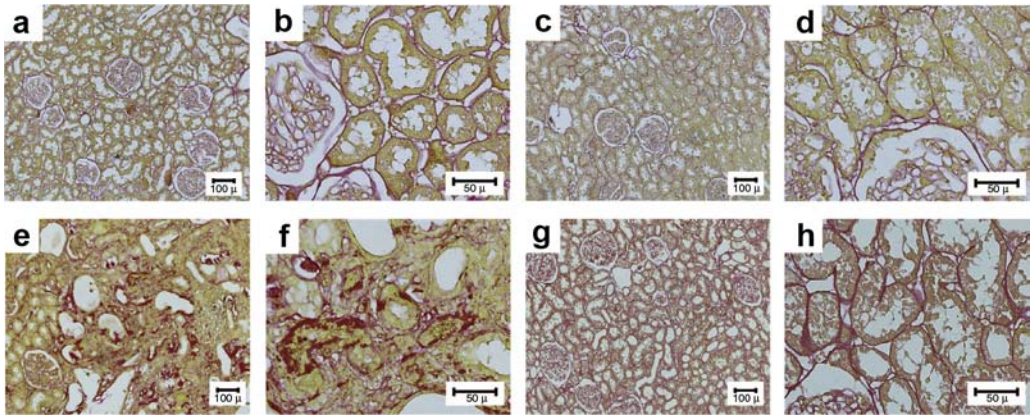

Figure 4 | Chronic kidney disease induced by an acute kidney injury episode was associated with tubulointerstitial injury and prevented by losartan pretreatment. Representative light microphotographs of kidney slides stained with Sirius red from (**a**, **b**) sham-operated, (**c**, **d**) Los, (**e**, **f**) UTxI, and (**g**, **h**) Los-Pre group (magnification ×100 or ×400, respectively).

animals that developed CKD had a higher percentage of glomeruli with diameters greater than 151 µm, compared with the sham or the Los-Pre group (Supplementary Figure S1 online).

An extensive area was affected by tubulointerstitial fibrosis in the UTxI group (Figure 4e and f) compared with the control groups (Figure 4a–d). The Los-Pre group exhibited little staining for Sirus red (Figure 4g and h). These observations were confirmed by the morphometric analysis presented in Figure 5a. Tubulointerstitial fibrosis exhibited a strong correlation with proteinuria (Figure 5b, $P = 0.0001$). Tubular dilation was observed in the UTxI group, exhibiting a 16.7% greater width than the sham-operated group or the Los-Pre group (54.3 ± 1.4 vs. 47.4 ± 1.1, or 49.2 ± 1.6, respectively, $P < 0.05$). In addition, the UTxI group showed higher renal smooth muscle actin (α-SMA) protein levels than the control groups (Figure 5c). In contrast, the ischemic group receiving losartan treatment did not

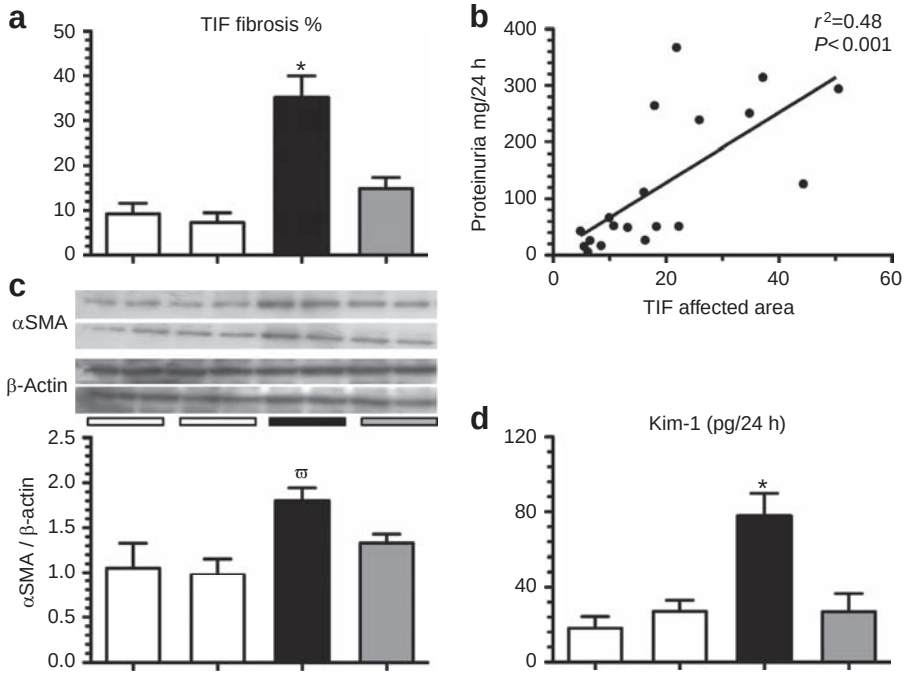

Figure 5 | Involvement of tubule-interstitial fibrosis, proteinuria, and α-smooth muscle actin (SMA) in the acute kidney injury (AKI) transition to chronic kidney disease (CKD). (a) Percentage of tubulointerstitial area affected by fibrosis (n = at least 6 per group), evaluated in 10 fields per each rat. **(b)** Pearson correlation between proteinuria and tubulointerstitial fibrosis. **(c)** Insets and densitometric analysis of the western blot of α-SMA and β-actin, respectively ($n = 4$ per group by duplicate). **(d)** Urinary Kim-1 levels (S and Los: $n = 4$, UTxI and Los-Pre: 6 per group, one assay) for sham-operated (white bars), Los (second white bars), UTxI (black bars), and Los-Pre (gray bars). *$P < 0.05$ versus all groups and $^{\varpi}P < 0.05$ versus sham-operated rats by analysis of variance and the Bonferroni test. TIF, tubulointerstital fibrosis.

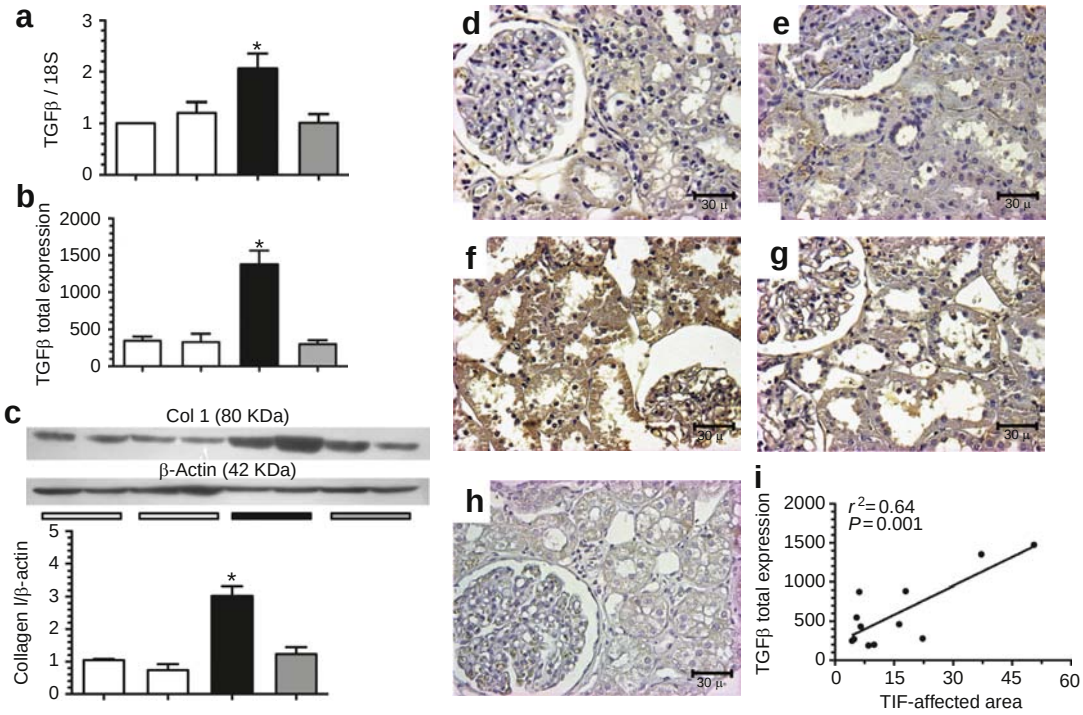

Figure 6 | Pro-fibrotic transforming growth factor-β (TGF-β) cytokine contribution to chronic kidney disease progression. (a) TGF-β mRNA levels were quantified by real-time reverse transcription-polymerase chain reaction. S, $n = 7$; Los, $n = 8$; UTxI, $n = 6$; and Los-Pre group, $n = 7$, by duplicate. **(b)** Renal cortex TGF-β protein levels were assessed by tissue microarray immunohistochemistry and digital image analysis per triplicate in at least 4 rats per group; the protein levels were quantified as total density expression of TGF-β, three different sections per rat. **(c)** Insets and densitometric analysis of the western blot of Col1AI and β-actin, respectively; $n = 4$ per group by duplicate. Representative images of kidney tissue microarray and immunohistochemistry for TGF-β in **(d)** sham-operated, **(e)** Los, **(f)** UTxI, **(g)** Los-Pre (magnification ×400), and **(h)** isotype control. **(i)** Pearson correlation between TGF-β total expression and tubulointerstitial fibrosis. Sham-operated (white bars), Los (second white bars), UTxI (black bars), and Los-Pre (gray bars). *$P < 0.05$ versus all groups by analysis of variance and the Bonferroni test. TIF, tubulointerstital fibrosis.

show this renal α-SMA upregulation. Renal structural damage was also confirmed by urinary kidney injury molecule-1 levels (uKim-1).[23,24] The UTxI group displayed a fourfold increase in uKim-1 (Figure 5d). The renoprotection conferred by prophylactic losartan administration was also demonstrated by the normalization of tubular atrophy and uKim-1.

Figure 6a shows that the renal cortex transforming growth factor-β (TGF-β) mRNA levels were significantly enhanced in the UTxI group, an effect that was reversed by losartan treatment. This finding was corroborated by TGF-β immunohistochemistry from renal microarrays derived from the different studied groups (Figure 6d–g). The UTxI group exhibited greater TGF-β staining, mostly in the tubular epithelium (Figure 6f), compared with the control groups (Figure 6d and e). This staining was not observed in the Los-Pre group (Figure 6g). Accordingly, digital image analysis from these microphotographs revealed a significant upregulation of TGF-β (Figure 6b) in the UTxI group, which was reversed by losartan administration preischemia. As a result of TGF-β activation, collagen I protein levels were significantly enhanced; this effect was not seen in the Los-Pre group (Figure 6c). The influence of TGF-β on renal fibrosis was indicated by the significant correlation between the total expression of TGF-β and tubulointerstitial fibrosis ($P = 0.001$, Figure 6i).

Tubular epithelial proliferation was assessed by immunostaining of proliferating cell nuclear antigen and Ki67. Proliferation was very low in the control groups, as shown by representative microphotographs and by counting positive tubular epithelial cells (Figure 7a–d). In contrast, a significant increase in proliferation was observed in the UTxI group (Figure 7e and g, respectively). In the Los-Pre group, the observed proliferation was similar to the control group (Figure 7f and h, respectively).

All functional and structural alterations observed in the UTxI group were associated with greater oxidative stress, which was assessed by the urinary excretion of H_2O_2 (Supplementary Figure S2A online), in spite of increasing intra-renal G6PD mRNA levels (Supplementary Figure S2B online). Interestingly, oxidative stress enhancement was not observed in the losartan-treated group. Another event involved in the progression to CKD is the activation of inflammation. Consequently, monocyte chemoattractant protein 1 and interleukin-6 mRNA levels were upregulated in the UTxI group (Supplementary Figure S2C–D online). This

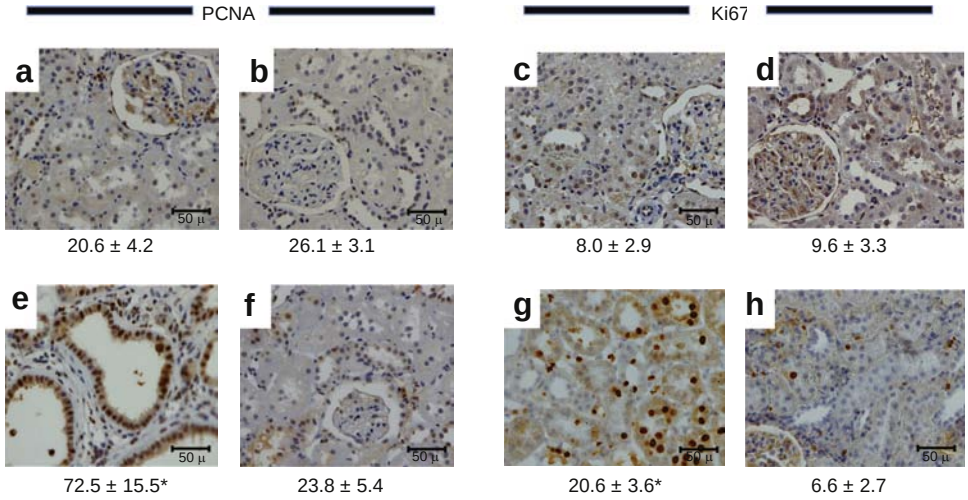

Figure 7 | Tubular atrophy observed in rats with chronic kidney disease was associated with epithelial cell proliferation and preserved by losartan. Tubular proliferation was assessed by proliferating cell nuclear antigen (PCNA) and Ki67 immunohistochemistry as shown in the representative microphotographs from kidney slides (magnification ×400). **(a, c)** Sham-operated rats. **(b, d)** Los. **(e, g)** UTxI. **(f, h)** Los pre. The mean ± SD of PCNA and Ki67-positive epithelial cells is shown under the corresponding image. *$P < 0.05$ versus all groups by analysis of variance and the Bonferroni test.

pattern was not observed in the Los-Pre group, showing a state of minor inflammation.

To determine the mechanisms by which losartan prevented the AKI to CKD transition, although AKI development was not prevented, we studied a set of rats in an early stage postischemia.

In Figure 8 appears the physiological and biochemical results at 3 days postischemia. We found that, in the UTxI group, the renal hypoperfusion and dysfunction persisted (Figure 8b and c) without proteinuria (Figure 8d). At the structural level, tubular injury was evident (Figure 8e) and correlated with the elevation of urinary Hsp72 and Kim-1 (Figure 8g and h, respectively). All these alterations were similarly observed in the Los-Pre group, except in the early recovery of RBF (Figure 8b).

Figure 9 shows the physiological, biochemical, and molecular findings at 5 days postischemia. We found that, in the UTxI group, the renal dysfunction lasted (Figure 9b and 9c) without proteinuria (Figure 9d), but with urinary Kim-1 that persisted elevated (Figure 9e). All these abnormalities were not seen in Los-Pre group. Although urinary H_2O_2 elevation in the UTxI group did not reach statistical difference

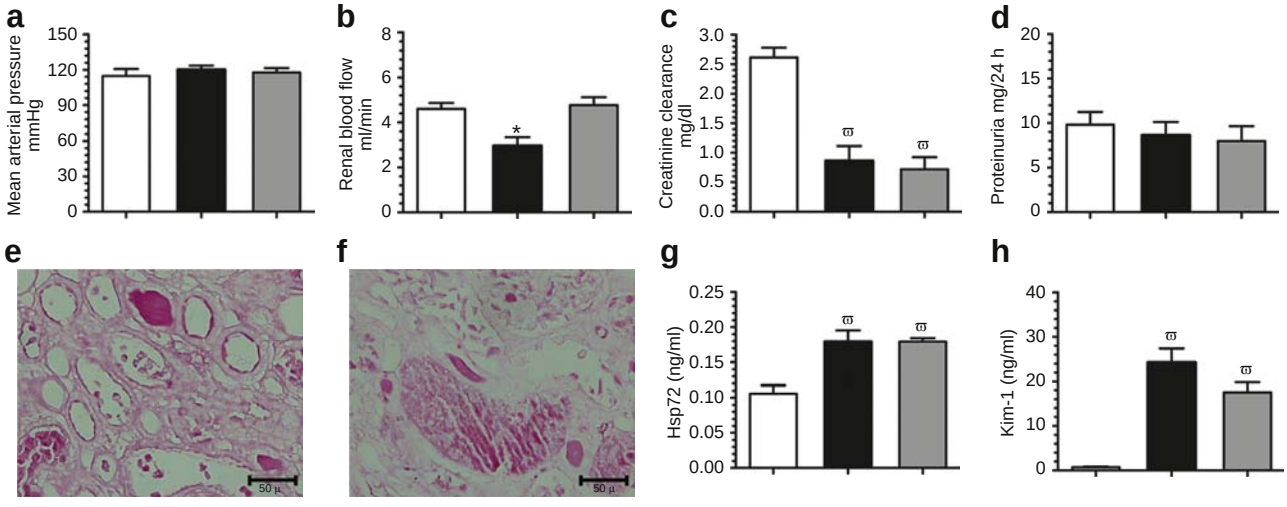

Figure 8 | Renal dysfunction and structural injury persist after 3 days of ischemia, and losartan only prevented renal hypoperfusion. (a) Mean arterial pressure, **(b)** renal blood flow, **(c)** creatinine clearance, **(d)** proteinuria, **(e)** periodic acid–Schiff-stained kidney slides from an UTxI and **(f)** Los-Pre groups, respectively; **(g)** urinary Hsp72 levels and **(h)** urinary Kim-1 excretion. Sham operated is represented by white bars, $n =$ at least 5; UTxI is represented by black bars, $n =$ at least 6 rats; and Los-Pre is represented by gray bars, $n =$ at least 5. *$P < 0.05$ versus all groups, $^{\varpi}P < 0.05$ versus sham, by analysis of variance and the Bonferroni test.

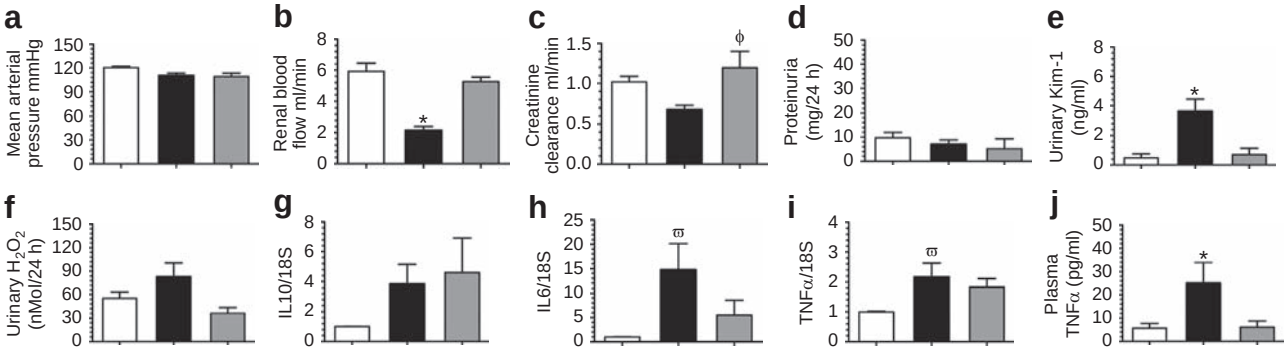

Figure 9 | The renal dysfunction and inflammation persist after 5 days of ischemia but not in losartan pretreated rats. (a) Mean arterial pressure, **(b)** renal blood flow, **(c)** creatinine clearance, **(d)** proteinuria, **(e)** urinary Kim-1 excretion, **(f)** urinary H_2O_2 excretion, **(g)** interleukin (IL)-10 mRNA levels, **(h)** IL-6 mRNA levels, **(i)** tumor necrosis factor (TNF)-α mRNA levels, and **(j)** plasma TNF-α levels. Physiological parameters and plasma TNF-α levels were determined once, whereas mRNA levels were determined at least by duplicate. Sham operated is represented by white bars, $n = 5$; UTxI is represented by black bars, $n = 5$; and Los-Pre is represented by gray bars, $n = 4$. All parameters were analyzed 5 days after ischemia. $^{*}P < 0.05$ versus all groups, $^{\varpi}P < 0.05$ versus sham, and $^{\Phi}P < 0.05$ versus Los-Pre by analysis of variance and the Bonferroni test.

(Figure 9f), interlukin-6 and tumor necrosis factor-α were significantly elevated (Figure 9h–j). Interestingly, the faster recovery of the renal dysfunction in the Los-Pre group was associated with normalization of urinary H_2O_2 excretion and inflammatory cytokines expression.

HIF-1α is a transcription factor that promotes the transcription of genes necessary for the survival of the cell when there is a drop in the oxygen supply. Renal HIF-1α mRNA levels were similar among the groups and were not modified after 1, 5 (Supplementary Figure S3 online), or 15 days post-ischemia (Figure 10g). In contrast, the total and nuclear HIF-1α protein levels, measured by tissue microarray in the kidney cortex and medulla, increased significantly after 15 days in the Los-Pre group (Figure 10e–f, respectively, and 10i–k, respectively), an effect that was not observed in the UTxI group (Figure 10c, d and k). Most of the expression was localized into the tubular epithelium. To evaluate the HIF-1α nuclear transcriptional activity, the protein levels of vascular

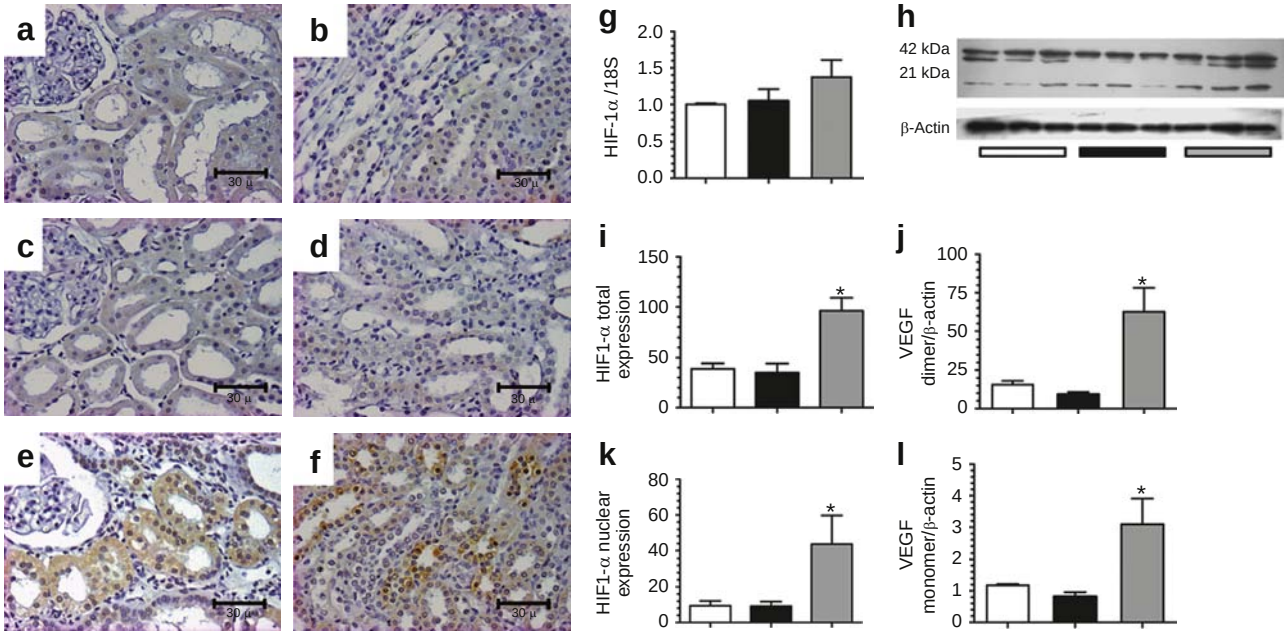

Figure 10 | HIF-1α and VEGF in the renal cortex after an ischemic event. HIF-1α/protein was assessed after 15 days of ischemia by tissue microarray immunohistochemistry and digital image analysis per triplicate in at least 4 rats per group in renal cortex **(a, c,** and **e)** and in renal medulla **(b, d,** and **f)**. **(a, b)** Representative microphotographs of the sham group, **(c** and **d)** the UTx group, and the **(e** and **f)** Los-Pre group. **(g)** HIF-1α mRNA levels after 15 days of ischemia, **(i)** total density expression of HIF-1α, three different sections per rat tissue microarray, and **(k)** nuclear HIF-1α/protein. **(h)** Insets of the western blot of VEGF and β-actin, respectively; $n = 4$ per group by duplicate. **(j** and **l)** VEGF/β-actin densitometric analysis for dimer and monomer VEGF conformation, respectively. Sham operated is represented by white bars; UTxI is represented by black bars; and Los-Pre is represented by gray bars. $^{*}P < 0.05$ versus all groups by analysis of variance and the Bonferroni test. VEGF, vascular endothelial growth factor. HIF, hypoxia-inducible factor.

endothelial growth factor (VEGF) were assessed after 15 days postischemia. Western blot analysis revealed two bands corresponding to the monomer and dimer VEGF conformation (Figure 10h). The densitometric analysis of both dimer and monomer (Figure 10j and l, respectively) shows a slight reduction in the UTx group, but the differences were not significant by analysis of variance. According to the greater nuclear HIF-1α observed in the Los-Pre group, monomer and dimer VEGF was significantly enhanced (Figure 10h, j and l).

DISCUSSION

In this study, we have investigated the mechanisms that lead to CKD induced by a single AKI episode, and we have also provided evidence of the importance of an early intervention, the prophylactic administration of losartan, for stopping or slowing down CKD progression. Our data show that, although losartan pretreatment did not protect the rats against AKI, it was effective to prevent the transition to CKD. The UTxI group developed CKD characterized by renal hypertrophy, renal dysfunction, glomerular hypertrophy, glomerulosclerosis, tubular atrophy, and tubulointerstitial fibrosis. These functional and structural alterations were associated with increased α-SMA protein levels, oxidative stress, inflammation, and activation of TGF-β.

Previously, we showed that angiotensin II blockade with a low dose of losartan (8 mg/K) did not prevent renal injury induced by unilateral ischemia, and only a higher dose (80 mg/K) had a minor effect.[25] Here, we showed that prophylactic losartan administration did not prevent AKI induced by severe bilateral renal ischemia. However, after 9 months, the Los-Pre group preserved an adequate renal function, as well as glomerular and tubular architecture.

Under hypoxic conditions, the transcription factor HIF-1α has a crucial role in regulating the expression of more than 100 target genes involved in cell proliferation, angiogenesis, glucose metabolism, and apoptosis, among others (see review by Shoji *et al.*[26]). HIF-1α expression is regulated by proteosomal degradation mediated by prolyl hydroxylase domain (PHD) containing protein enzymes. Indeed, PHD inhibitors not only induced HIF-1α activation but also reduced renal injury induced by ischemia/reperfusion,[27] subtotal nephrectomy,[28] allogenic kidney transplant,[29] or nephritis.[30] These data suggest that HIF-1α exerts a protective role under AKI. In this study, we found that HIF-1α mRNA levels were not different among the groups after 1, 5, or 15 days postischemia, but the total and nuclear HIF-1α expression assessed by tissue microarray was significantly enhanced in the Los-Pre group after 15 days of ischemia. This activation could result from AT1 receptor inhibition by losartan, because it has been proposed that PHD inhibition induces AT1 receptor downregulation and a lesser perivascular fibrosis in coronary arteries.[31] Our data also suggest that after an ischemic insult there is an ineffective HIF-1α activation to face the renal hypoperfusion, vascular rarefaction, and inflammation. In contrast, losartan treatment was able to induce HIF-1α nuclear translocation and activation, which was detected by inducing VEGF transcription. Our data suggest that VEGF could diminish capillary rarefaction, improve renal perfusion, and reduce chronic hypoxia. Although we cannot explain how ARB induced HIF-1α after 15 days of ischemia, it could result by inducing one of the following: lesser HIF-1α degradation, greater HIF-1α/HIF-1β dimerization, or enhanced nuclear translocation. Our results show that the HIF-1α activation after ischemia was associated with the prevention of AKI to CKD transition, however, the mechanism by which ARB activates HIF-1α remained to be explored.

After 9 months, the persistent proteinuria in UTxI group correlated with glomerulosclerosis and tubulointerstitial fibrosis, suggesting that abnormal proteinuria is due to gradual damage to both the glomerular filtration barrier, by diffuse podocyte effacement and reduction in nephrin expression (data not shown), and tubular epithelium atrophy and dilation after an AKI episode. This picture is very similar to that observed in the recently characterized Mesoamerican nephropathy, in which affected patients were normotensive but exhibited glomerular enlargement, glomerulosclerosis, chronic tubulointerstitial injury, tubular atrophy, and interstitial fibrosis.[32] Thus, our findings support the hypothesis that this nephropathy is related with one or several episodes of AKI.[32]

Renal ischemic injury has been associated with atubular nephrons.[33] Therefore, it is likely that, in the first weeks postischemia, a significant number of nephrons are lost. The nephron reduction means that the remaining functional nephrons must compensate for the lost function through hyperfiltration and hypertrophy. Indeed, renal hypertrophy was evident in the UTxI group and avoided by prophylactic losartan administration. A recent study showed that renal hypertrophy induced by ischemia depends on the extent of tubular epithelium death mediated by tumor necrosis factor-α signaling pathway activation.[34] These abnormalities certainly accelerate the deterioration of the functional nephrons. Thus, it feasible that the Los-Pre group had lesser atubular nephrons, which is reflected by better renal function and glomerular structure preservation. Indeed, Pagtalunan *et al.*[35] showed that losartan postischemia was able to reduce the number of atubular nephrons.

Proximal tubular epithelial cells suffer death by necrosis or apoptosis, and others lose their polarity and slough off after an AKI episode. Consequently, epithelial dedifferentiation and proliferation are triggered with the purpose of restoring the tubular epithelium. Tubular repair, however, is altered by processes such as cell arrest[18] and epigenetic changes[19] that instead of improving renal architecture switch on fibrogenesis. Our study showed that the UTxI group exhibited an atrophic tubular epithelium that was associated with increased proliferation, assessed by Ki67 and proliferating cell nuclear antigen. Similar results have been observed in renal biopsies from patients suffering AKI.[36] These results indicate that tubular proliferation is

perpetuated as a maladaptive phenomenon, which was not seen in the Los-Pre group.

Progressive tubulointerstitial fibrosis is a key player in chronic renal injury and involves chronic peritubular inflammation, tubular cell dedifferentiation, and myofibroblast activation.[37,38] Furthermore, sustained leukocyte accumulation and activation inside the kidney could extend periods of ischemia due to vascular congestion and may induce direct tubular and endothelial cell damage by the release of inflammatory mediators.[39] In our study, we observed that an ischemic insult promoted an extensive tubulointerstitial area affected by fibrosis. This alteration was, in part, mediated by TGF-β upregulation. Yang *et al.*[18] proposed that, after a severe ischemic insult, the tubular epithelium produces large amounts of TGF-β. Our results with tissue microarray confirmed these previous findings; TGF-β was upregulated, and most of the TGF-β immunostaining was found in the tubular epithelium. The fibrotic response was not observed in Los-Pre group. Bechtel *et al.*[19] have shown that injured epithelial cells trigger phenotypical modulation of fibroblasts to activated myofibroblasts. Accordingly, we observed that renal α-SMA levels, a marker of fibroblast transdifferentiation, were increased in the UTxI group, and this effect was prevented in the Los-Pre group.

Our data suggest that AT_1 receptors must be blocked at the moment of the ischemic insult to avoid angiotensin-II involvement in the ineffective endothelial and tubular reparation, which occurred in long term. In this regard, and because AKI occurs in 30% of patients undergoing cardiac surgery, several clinical studies have examined the effect of angiotensin-converting enzyme inhibitors (ACEis) or ARB on AKI incidence. However, inconclusive results have been published on AKI incidence in cardiovascular surgery: increased,[40] or no change,[41,42] or even reduced.[43,44] Recently, Coca *et al.*[45] studied the effect of held or continued ACEi/ARB treatment in patients undergoing cardiac surgery compared with patients not treated with ACEi/ARB and demonstrated similar levels of urinary biomarkers such as NGAL, interlukin-18, Kim-1, and liver-type fatty acid binding protein among the patients and a reduction in the glomerular filtration rate in the group that continued with ACEi/ARB therapy. Furthermore, glomerular filtration rate reduction has not been attributed as an adverse effect because these drugs improve peritubular capillary perfusion, which in turn could reduce tubular ischemia and necrosis.[46–48] Unfortunately, long-term evaluation of patients suffering from AKI and receiving ACEi or ARB therapy has not been reported.

Previously, we showed that prophylactic administration of spironolactone completely prevented AKI and the transition to CKD.[20] In this study, losartan pretreatment before ischemia did not prevent either aldosterone elevation or AKI severity within 24-h postinsult, demonstrating that ARB protection was independent of MR receptor antagonism and suggesting that aldosterone and angiotensin II differentially regulate both renal hemodynamics and the involvement of signal pathways for endothelial and tubular reparation.

Our findings not only reinforce the great importance of an AKI episode as a risk factor for the development of CKD but also show the deleterious effect of the activation of AT1 receptors during an ischemic insult and its impact on the lasting renal function and structure. This study also shows the potentiality of ARB in preventing the AKI transition to CKD by a mechanism related with the early recovery of RBF, prevention of an inflammatory process, enhanced HIF-1α nuclear translocation, and early induction of VEGF after the ischemic insult. Timely identification of AKI, together with an effective prophylactic intervention, would have a marked impact in slowing down CKD progression.

MATERIALS AND METHODS

All experiments involving animals were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf) and were approved by the Animal Care and Use Committee at our Institutions.

Thirty-four male Wistar rats weighing 250–300 g were divided into four groups—(i) sham-operated rats, $n = 8$ (S); (ii) sham-operated rats plus losartan (50 mg/kg/day by gastric gavage) 3 days before surgery, $n = 8$ (Los); (iii) rats who underwent bilateral renal ischemia for 45 min, $n = 9$(UTxI), and (iv) group that received losartan 3 days before bilateral renal ischemia, $n = 9$ (Los-Pre)—and were observed for 9 months. In another set of experiments, 21 rats from S, 26 from UtxI, and 25 from Los-Pre groups were studied and divided into four different periods: 1, 3, 5, or 15 days after ischemia. All animals were kept in a 12:12 h day–night cycle and had free access to water and food.

Ischemia/reperfusion model

Rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital (30 mg/kg) and placed on a heating pad to maintain core body temperature at 37 °C. Renal pedicles were isolated, and bilateral renal ischemia was induced using a non-traumatic clamp on each renal artery for 45 min. Ischemia was verified visually by a change in the kidney color. Reperfusion was achieved by release of the clips and confirmed by return of oxygenated blood to the kidney. The incision in the muscle and the skin was closed with 3-0 vicryl and silk sutures, respectively. For sham surgery, laparotomy and renal pedicle dissection, without clamping, were performed in anesthetized rats.

Statistical analysis

The results are presented as the mean ± s.e. The significance of the differences between groups was assessed by analysis of variance using the Bonferroni correction for multiple comparisons. All comparisons passed the normality test. The differences in the ranks of glomerular diameters among the groups were evaluated by contingency analysis, and the differences were assessed using the χ^2 test with the Yates correction. The correlation among the data was evaluated by Pearson's test. Statistical significance was defined when the P value was < 0.05.

More detailed Methods appear as Supplementary Material.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTION

RRR and NAB: conceived and design the study. RRR, KB, JBC, RPV, AG, and NAB: performed the experiments, molecular, biochemical, and histopathological analysis. NU and DA: histology and immunohistochemistry. SH and JFRS: immunohistochemistry by microarrays. RRR and NAB: analyzed the data. NAB: contributed reagents or analysis tools. RRR, GG, and NAB: wrote the article.

SUPPLEMENTARY MATERIAL

Figure S1. Renal and glomerular hypertrophy induced by an AKI episode was prevented by losartan administration.

Figure S2. Oxidative stress and inflammation responses in CKD group were inhibited with losartan administration before ischemic insult.

Figure S3. HIF-1α mRNA and protein levels in the renal cortex after 1 and 5 days of ischemia.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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