UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

BIOLOGÍA EXPERIMENTAL

EFECTO DEL TRATAMIENTO ANTIOXIDANTE SOBRE EL ESTRÉS OXIDANTE Y EL DAÑO RENAL EN LA NEFROTOXICIDAD EXPERIMENTAL

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

DANIELA JOYCE TRUJILLO SILVA

TUTOR(A) PRINCIPAL DE TESIS: DR. JOSÉ PEDRAZA CHAVERRÍ
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MÉXICO, D.F. ABRIL, 2015.

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ASUNTO: Oficio de Jurado

Dr. Isidro Ávila Martínez
Director General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **19 de enero de 2015**, se aprobó el siguiente jurado para el examen de grado de del (la) alumno (a) **TRUJILLO SILVA DANIELA JOYCE** con número de cuenta **505012210** con la tesis titulada: **"EFECTO DEL TRATAMIENTO ANTIOXIDANTE SOBRE EL ESTRÉS OXIDANTE Y EL DAÑO RENAL EN LA NEFROTOXICIDAD EXPERIMENTAL"**, realizada bajo la dirección del (la) **DR. JOSÉ PEDRAZA CHAVERRI:**

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Sin otro particular, me es grato enviarle un cordial saludo.

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Dra. María del Coro Arizmendi Arriaga
Coordinadora del Programa

FACULTAD DE CIENCIAS
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Lalo, Olimpia; por hacer la carga más ligera.

Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica:
la voluntad

A. Einstein

CONTENIDO

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Abreviaturas

Abreviatura	Significado
1M2Fi	1-metil-2-fenilindol
$^1O_2$	Radical singulete de oxígeno
3-NT	Nitrotirosina-3
AAPH	2,2-azobis (2-amidinopropano)
ADN	Ácido desoxirribonucleico
ADNmito	ADN mitocondrial
AIF	Factor inductor de apoptosis
AJ	Unión celular adherente
ATP	Trifosfato de adenosina
AUC	Área bajo la curva
Bax	BCL2-asociadas a proteínas X
Bcl2	Linfoma células B 2
BUN	Nitrógeno de urea sanguíneo
$C_6H_5Na_3O_7 \cdot 2H_2O$	Citrato de sodio
$CaCl_2$	Cloruro de calcio
Cas	Caspasa
CAT	Catalasa
CDNB	1-cloro-2,4-dinitrobenceno
CH_3COONa	Acetato de sodio
CP	Cisplatino o cis-(diamino II dicloruro)
Ctr1	Transportador de cobre 1
Cur	Curcumina
DAPI	4', 6-diamino-2-fenilindol
DCDHF-DA	2', 7'-diacetato diclorofluoresceína
DHE	Dihidroetidio
DHR-123	Dihidrorodamina 123
DIABLO	Inhibidor directo de la proteína de unión a apoptosis
DMPK	Desmoplaquina
DMSO	Dimetil sulfóxido
DMTU	Dimetiltiourea
DPBF	1,3-difenilisobenzofurano
DPI	Difenileno de yodonio
DppD	Dipeptil peptidasa
DPPH	2,2-difenil-1-picrilhidrazilo
DTPA	Ácido dietilentriaminopentaacético

EDTA	Ácido etilendiaminotetraacético
EMT	Transición mesenquimal-epitelial
EROs	Especies reactivas de oxígeno
FDA	Food and Drug Administration
$FeCl_3$	Cloruro de hierro
Fe_2SO_4	Sulfato de hierro
FRAP	Radicales férricos
GADPH	Gliceraldehído-3-fosfato deshidrogenasa
GL	Glomérulos
GPx	Glutatión peroxidasa
GR	Glutatión reductasa
GSH	Glutatión reducido
GSSG	Glutatión disulfuro
GST	Glutatión-S-transferase
GT	Glutatión transferasa
H&E	Hematoxilina-eosina
H_2O_2	Peróxido de hidrógeno
HCl	Ácido clorhídrico
HO-1	Hemo oxigenasa-1
HOCl	Ácido hipocloroso
HRP	Peroxidasa de rábano
IF	Inmunofluorescencia
ip	Intraperitoneal
IRA	Insuficiencia renal aguda
KB	Solución de Krebs-bicarbonato
KCl	Cloruro de potasio
KDIGO	Kidney Disease Improving Global Outcomes
Keap1	Proteína asociadora de Kelch ECH 1
KH_2PO_4	Fosfato de potasio monobásico
KIM-1	Molécula de daño renal-1
L-Arg	L-arginina
MAPK	Proteínas cinasas activadas por mitogen
MCP-1	Proteína quimioatrayente de macrófagos-1
MDA	Malondialdehído
$MgSO_4$	Sulfato de magnesio
Na_2CO_3	Carbonato de sodio
Na_2HPO_4	Fosfato de sodio dibásico

NaCl	Cloruro de sodio
NaClO	Hipoclorito de sodio
NADH	β-nicotinamida adenina dinucleótido
NADPH	β-nicotinamida adenina dinucleótido fosfato
NAG	N-acetil-β-D-glucosaminidasa
$NaHCO_3$	Bicarbonato de sodio
NAMPT	Fosforibosiltransfera nicotinamida
NBT	Nitroazul tetrazolio
NF-kB	Ligeras kappa de las células B activadas
NGAL	Lipocaina asociada a la gelatinasa de neutrófilos
$NO^{\bullet}$	Óxido nítrico
NOM	Norma Oficial Mexicana
Nrf2	Factor nuclear eritroide-2-asociado al factor 2
$O_2^{\bullet -}$	Anión superóxido
OCT2	Transportador orgánico de cationes 2
$OH^{\bullet}$	Radical hidroxilo
$ONOO^-$	Anión peroxinitrito
PBS	Amortiguador de fosfatos-salina
PKC	Proteína cinasa C
PKCβ2	Proteína cinasa C beta 2
PMSF	Fluoruro de fenilmetilsulfonilo
PUMA	Modulador de apoptosis sobre-regulando p53
PVDF	Membranas de fluoruro de polivinilideno
RIPA	Ensayo de radio-immunoprecipitación
RET	Resistencia eléctrica trans-epitelial
RKB	Solución de Ringer-Krebs bicarbonato
$ROO^{\bullet}$	Radical peroxilo
SDS	Dodecil sulfato de sodio
Smac	Segundo activador de caspasas derivado de la mitocondria
SOD	Superóxido dismutasa
Succ	Succinato
TD	Túbulos distales
TGF-β	Factor de cremiento transformante-beta
Tirón	Sodio 4,5-dihidroxibenceno-1,3-disulfonato
TJ	Unión celular estrecha
TMPO	1,1,3,3-tetrametoxipropano
TNFR1	Receptor 1 del factor de necrosis tumoral

TNFR2	Receptor 2 del factor de necrosis tumoral
TNFα	Factor de necrosis tumoral alpha
TP	Túbulos proximales
TPTZ	Tripiridil-s-triazina
Trolox	Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico
UC	Unión celular
U937	Linea celular tumoral de linfoma
V	Vehículo
WB	Western blot
XAN	Xantina
ZO-1	Zonula ocluyente-1
α-SMA	Actina del músculo liso alpha

RESUMEN

Introducción: La insuficiencia renal aguda se caracteriza por la pérdida de la función renal ocasionada por múltiples causas como hipovolemia, shock séptico, deshidratación, cirugía, metales pesados, así como el uso de fármacos como antiinflamatorios, antibióticos, contrastes yodados, inmunosupresores y antineoplásicos. El cisplatino (CP) es un antineoplásico para tumores sólidos, su uso causa nefrotoxicidad; asociada al transporte del fármaco en las células del túbulo proximal renal (TP), la activación de procesos de muerte celular; así como la respuesta inflamatoria, el estrés oxidante, particularmente generación de anión superóxido ($O_2^{\bullet-}$) y la reducción en la defensa antioxidante. La curcumina, es un compuesto fenólico, considerado agente terapéutico con funciones biológicas anti-inflamatorias, anticancerígenas, neuro, cardio y renoprotectoras. Se considera un antioxidante bifuncional por reaccionar directamente con las especies reactivas e inducir proteínas citoprotectoras y antioxidantes.

Objetivo: Determinar si la mayor generación de $O_2^{\bullet-}$ y la desintegración de la unión celular participan en los mecanismos de daño renal inducido por CP; así como si la administración de un antioxidante confiere renoprotección en el daño inducido por CP.

Métodos: Se estudiaron cuatro grupos de ratas Wistar macho (n=5). El primer grupo conformado por ratas que recibieron vehículo; solución salina (V), un segundo grupo tratado con una dosis única de CP (5 mg/kg) vía i.p; el tercer grupo recibió CP+Curcumina (CP+Cur; 200 mg/kg) vía intragástrica y finalmente el cuarto grupo fue tratado con V+Cur (Cur); se administraron 3 dosis de Cur. Se evaluaron los niveles de creatinina plasmática (CrS) y de nitrógeno de urea sanguíneo (BUN), la actividad de N-acetil-β-D-glucosaminidasa (NAG), la expresión de la lipocaína asociada a la gelatinasa de neutrófilos (NGAL), la expresión y localización de la molécula de daño renal (KIM-1), la actividad de las enzimas antioxidantes catalasa, glutatión reductasa (GR) y superóxido dismutasa, los niveles de lipoperoxidación, así como la generación de $O_2^{\bullet-}$. También se midió la expresión del factor de crecimiento transformante beta (TGF-β1), de colágeno I y IV, de 3-nitrotirosina (3-NT), de proteína cinasa C beta 2 (PKCβ2) y de las subunidades de nicotinamida adenín dinucleótido fosfato (NADPH) oxidasa $gp91^{phox}$ y $p47^{phox}$. Finalmente se estudió la integridad de las células tubulares renales a través de la expresión y localización de proteínas de unión celular: ocludina, claudina-2 y caderina-E

Resultados: Las ratas administradas con CP presentaron nefrotoxicidad que se caracterizó por un incremento de ≈2.5 veces en la CrS y BUN así como por reducción en los niveles de NAG y aumento de NGAL y KIM-1. El CP indujo incremento en las citocinas profibróticas TGF-β1, colágeno I y IV; reducción del 25% y 20% en la actividad de las enzimas antioxidantes catalasa y GR, respectivamente. Esto se asoció con un incremento en los niveles de lipoperoxidación y ≈6 y 4 veces mayor generación de $O_2^{\bullet-}$ en glomérulo y TP, respectivamente. Se identificó a NADPH oxidasa como la fuente principal de $O_2^{\bullet-}$ asociado con incremento en la expresión de sus subunidades $gp91^{phox}$ y $p47^{phox}$. También se encontró pérdida de la unión celular asociada a la reducción en la expresión de las proteínas ocludina, claudina-2 y E-cadherina. En contraste la administración de Cur previno la disfunción y el daño renal; cambios asociados con la prevención del aumento de CrS, BUN, NAG, NGAL y KIM-1. También previno el aumento en la lipoperoxidación; relacionado con reducción en la generación de $O_2^{\bullet-}$ y de la las subunidades $gp91^{phox}$ y $p47^{phox}$, principalmente

en el TP. La administración de Cur también preservó la actividad de las enzimas antioxidante catalasa y GR y previno la pérdida de proteínas de unión celular.

Conclusiones:

La generación de $O_2^{\bullet -}$ particularmente en el TP; proveniente de NADPH oxidasa y la desintegración de la unión celular participan en los mecanismos de nefrotoxicidad inducida por CP. La administración de curcumina previno dichas alteraciones y, por lo tanto, redujo el daño renal inducido por CP.

ABSTRACT

Background: Acute renal failure is characterized by the loss of renal function triggered by multiple causes such as hypovolemia, shock, dehydration, surgery, heavy metals, and use of drugs including anti-inflammatory, antibiotics, iodinated contrast agents, immunosuppressive and antineoplastic. Cisplatin (CP) is an antineoplastic drugs for solid tumors; their use causes nephrotoxicity associated with drug transport in renal proximal tubular cells (PT), the activation of cell death processes and inflammatory response, oxidative stress, particularly generation of superoxide anion ($O_2^{\bullet-}$) and reduction in antioxidant defense. Curcumin is a phenolic compound, biological therapeutic agent considered anti-inflammatory, anticancer, neuro, cardio and renoprotective. It is considered a bifunctional antioxidant since it is able to react with reactive species and to induce cytoprotective responses and antioxidant proteins.

Aim: To evaluate whether the increased generation of $O_2^{\bullet-}$ and dissolution of tight junction are involved in renal damage induced by CP; and whether administration of an antioxidant, curcumin, conferring renoprotection CP induced.

Methods: Twenty male Wistar rats, divided into 4 groups, were studied. The first group received vehicle; saline solution (V), the second group was treated with CP (5 mg/kg) i.p; the third group received CP + Curcumin (CP + Cur; 200 mg/kg) p.o and finally the fourth group was treated with V + Cur (Cur); three doses of Cur were administered. The following parameters were evaluated: serum creatinine (SCr), blood urea nitrogen (BUN), activity of N-acetyl-β−D-glucosaminidase (NAG), expression of neutrophil gelatinase-associated lipocalin (NGAL), expression and location of kidney injury molecule (KIM-1), activity of the antioxidant enzymes catalase, glutathione reductase (GR) and superoxide dismutase, lipid peroxidation levels and generation of $O_2^{\bullet-}$. Expression of transforming growth factor (TGF-β1), collagen I and IV, 3-nitrotyrosine (3-NT), protein kinase C beta 2 (PKCβ2) and NADPH oxidase subunit $gp91^{phox}$ and $p47^{phox}$ were also analyzed. Finally the integrity of renal tubular cells was studied through the expression and location of the tight junctions proteins occludin, claudin-2 and E-cadherin.

Results: Rats administered with CP showed nephrotoxicity characterized by increased levels of serum SCr and BUN and expression of KIM-1 and NGAL as well as by reduced activity of NAG. The CP increased the profibrotic cytokines TGF-β1, collagen I and collagen IV and decreased activity of the antioxidant enzymes catalase and GR. These changes were associated with increased lipid peroxidation and ≈6 and 4 times greater generation of $O_2^{\bullet-}$ in glomeruli and PT, respectively. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was identified as main source of $O_2^{\bullet-}$ associated with increased expression of its subunits $gp91^{phox}$ and $p47^{phox}$. CP induced loss of integrity of cell tight junction associated with reduction in the expression of occludin, claudin-2 and E-cadherin proteins. In contrast Cur administration prevented kidney dysfunction and injury, changes that were associated with the prevention of increased SCr, BUN, NGAL and KIM-1. Moreover, Cur prevented the increase in lipid peroxidation, in the $O_2^{\bullet-}$ generation and in the expression of the NADPH subunits $gp91^{phox}$ and $p47^{phox}$, mainly in the PT. Cur also preserved the activity of antioxidant enzymes catalase and GR and prevented the loss of integrity of cell tight junctions.

Conclusions: The generation of $O_2^{\bullet-}$ particularly in PT by NADPH oxidase and loss of integrity of cell tight junctions are mechanisms involved in nephrotoxicity induced by CP. Curcumin administration prevented these changes and reduced renal damage induced by CP.

I. Introducción

El mantenimiento de un volumen de líquidos relativamente constante y de una composición estable electrolitos, es esencial para la homeostasis del cuerpo [Guyton y Hall., 2006]. Los riñones juegan un papel importante en mantener esta homeostasis mediante la regulación del equilibrio hidroelectrolítico, la regulación de la osmolaridad de los líquidos corporales y de las concentraciones de los electrólitos, la regulación del equilibrio ácido-base, la excreción de los productos de desecho del metabolismo y de las sustancias químicas extrañas, entre otras funciones que lleva a cabo a través de su unidad funcional, la nefrona; en sus diferentes segmentos: glomérulo, túbulo proximal, asa de Henle, túbulo distal, conductos conectores y colectores [Guyton y Hall., 2006].

1.1 Insuficiencia renal aguda (IRA)

La insuficiencia renal aguda (IRA) es un padecimiento con la repentina pérdida de la función renal, frecuentemente con oliguria, asociada con incremento en la morbimortalidad de los pacientes [Li *et al*., 2013]. A nivel mundial se estima que cerca de dos millones de personas mueren al año por IRA [Murugan y Kellum., 2011; Uchino *et al*., 2005] o ésta conduce al desarrollo de la enfermedad renal crónica. La prevalencia anual en países desarrollados de IRA varía en un rango de 2,147 a 4,085 por millón de habitantes [Ali *et al*., 2007; Khwaja., 2012]. La definición clínica para IRA de acuerdo al grupo de trabajo Kidney Disease Improving Global Outcomes (KDIGO), incluye un incremento de creatinina sérica ≥ 0.3 mg/dL, un incremento de

creatinina ≥ 1.5 veces respecto los valores basales o una disminución del volumen urinario < 0.5 $ml \cdot kg^{-1} \cdot h$ durante 6 h [Khwaja., 2012].

Las causas de la IRA son múltiples, entre ellas: hipovolemia (hemorragia, shock séptico, lesión, deshidratación, cirugía), sepsis, infecciones, obstrucción, toxicidad por metales pesados, así como el uso de diversos fármacos considerados agentes nefrotóxicos que están implicados como factores etiológicos en un 17-26% de la IRA que se presenta en hospitales y están asociados a las tasas de morbimortalidad [Uchino *et al*., 2005]. Entre los fármacos nefrotóxicos se encuentran antiinflamatorios no esteroideos, antibióticos aminoglucósidos, contrastes yodados, inhibidores de la enzima convertidora de angiotensina I, inmunosupresores y quimioterapéuticos [Guyton y Hall., 2006].

Los agentes quimioterapéuticos juegan un papel importante en el tratamiento de varias neoplasias; desafortunadamente su uso puede resultar en complicaciones serias, entre ellas una amplia gama de complicaciones renales que son causadas por varios de los agentes antineoplásicos: ifosfamida, MTX, pemetrexed, cetuximab, palmidronato, zoledronato y cisplatino [Pazhayattil y Shirali., 2014].

1.2 El cisplatino

El cisplatino o cis-diamino (II) dicloruro (CP) (Fig. 1), molécula formada por un catión platino 2^+ central unido a 2 iones de cloruro y 2 aminas. Es uno de los agentes antineoplásicos más efectivo

Figura 1. Estructura química de Cis-diamino (II) dicloruro; Cisplatino (CP)

y usado, en la práctica clínica para el tratamiento de tumores sólidos, principalmente cabeza, cuello, pulmón, testículos y ovarios, aprobado por la Food and Drug Administration (FDA) en 1978; sin embargo el uso de este antineoplásico causa nefrotoxicidad con una incidencia de entre el 6 al 13% de los pacientes que lo utilizan, lo que limita su uso [Dolin y Himmelfarb., 2008; Miller *et al*., 2010]. La nefrotoxicidad se caracteriza por un incremento de creatinina sérica, un incremento de nitrógeno de urea sanguíneo (BUN), una reducción del 20-40% de la tasa de filtración glomerular, hipomagnesemia, hipopotasemia, síndrome similar al de Fanconi, acidosis renal túbulo-distal, hipocalcemia e hiperuricemia. El desarrollo de la nefrotoxicidad por CP es complejo y se debe a varios factores [Miller *et al*., 2010]. Se ha reportado que en células tubulares y epiteliales la concentración de CP es aproximadamente 5 veces mayor que en el plasma [Kuhlmann *et al*., 1997], debido a la absorción peritubular que lleva a cabo de los fármacos [van Angelen *et al*., 2013]. Los efectos tóxicos ocurren principalmente en los túbulos proximales renales, particularmente en la células epiteliales tubulares del segmento 3, los glomérulos y túbulos distales también se afectan con menor grado [Werner *et al*., 1995; Trujillo *et al*., 2014b].

1.2.1 Mecanismos de la nefrotoxicidad por CP

Los mecanismos que participan en el desarrollo de la nefrotoxicidad descritos hasta ahora incluyen: el transporte del fármaco en las células tubulares renales, específicamente en el túbulo proximal, uniéndose al ácido desoxirribonucleico (ADN) nuclear y mitocondrial, activando la muerte celular por apoptosis y/o necrosis, desencadenando estrés oxidante, fibrogénesis así como la activación de una

respuesta inflamatoria y recientemente nuestro grupo describió la desintegración de las uniones celulares en el túbulo proximal [Trujillo *et al*., 2014b].

1.2.2 Sistema de transporte celular del CP

El sistema de transporte celular del CP, esta mediado por el transportador orgánico de cationes 2 (OCT2) y el transportador de cobre 1 (Ctr1). Ambos transportadores son expresados en riñón [Pabla *et al*., 2009, Ludwing *et al*., 2004]. El OCT2 se expresa abundantemente en la membrana basolateral de los túbulos proximales y participa en la acumulación de CP en esta zona [Ludwing *et al*., 2004]. A este respecto se describió que ratones doble knockout para OCT1/2 tratados con CP, presentaban menor nefrotoxicidad y menor acumulación en el tejido de fármaco en comparación con ratones normales [Ciarimboli *et al*., 2005]. Mientras que células deficientes de Ctr1 acumulan menos platino en su ADN y son más resistentes a los efectos citotóxicos [Lin *et al.*, 2002]. Como se observa en la figura 2, una vez en el entorno intracelular el CP sufre hidrólisis en la que uno o los dos iones cloruro son sustituidos por agua; el agua actua como donador de electrones y el catión platino 2^+ como aceptor de electrones formando un enlace covalente, con estos cambios el CP reacciona fácilmente con el ADN a través de enlaces covalentes con las purinas en la posición N7, permitiendo que se intercale en la hebra de ADN, resultando en la inhibición de la replicación y transcripción, arresto del ciclo celular y finalmente en apoptosis e inhibición de la proliferación celular [Wang y Lippard., 2005; Lieberthal *et al.*, 1996].

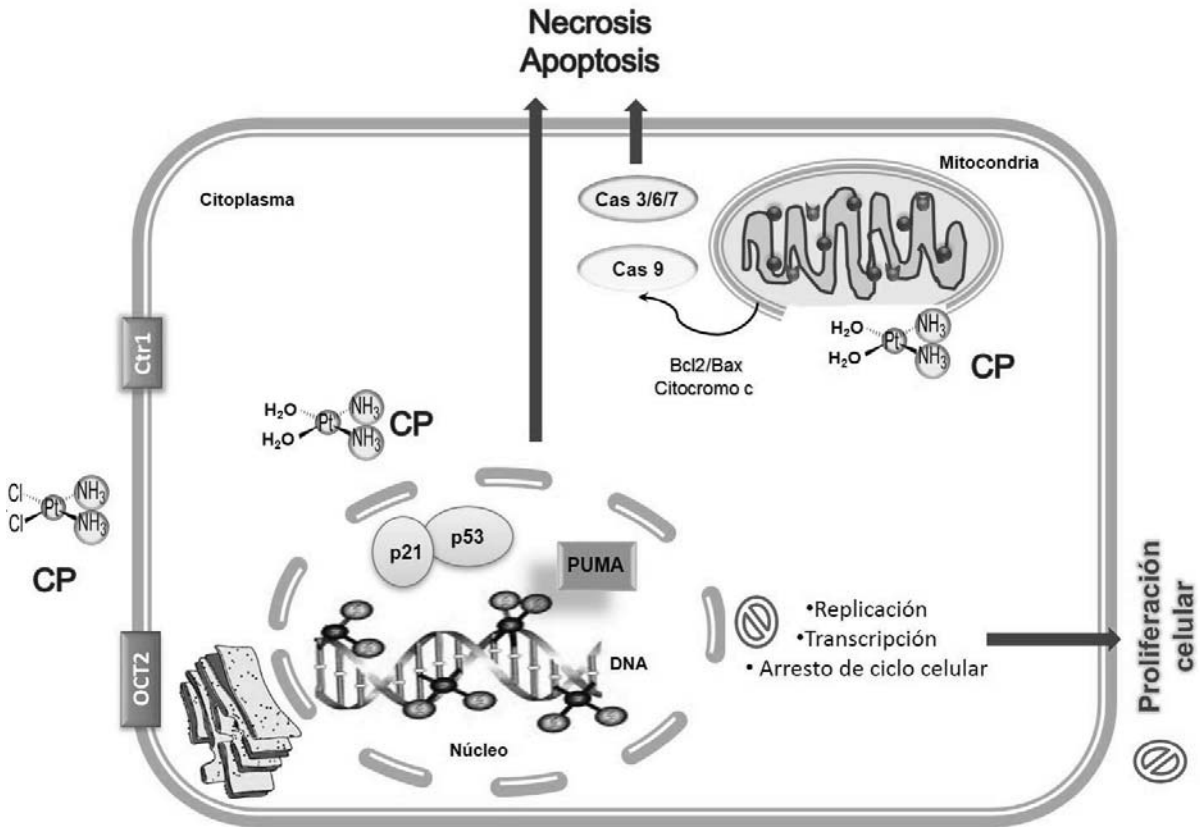

Figura 2. Mecanismo de acción del cisplatino. Transportador de cationes orgánicos (OCT2); transportador de cobre 1 (Ctr1); modulador de apoptosis sobre-regulando p53 (PUMA), caspasa (Cas).

1.2.3 Unión al ADN nuclear y mitocondrial.

El CP, al intercalarse en la hebra de ADN, además de inhibir la replicación, transcripción y proliferación celular debido al arresto del ciclo celular, activa genes involucrados en la reparación del daño como p53, p21, capaces de iniciar procesos de apoptosis y necrosis, activación del modulador de apoptosis sobre-regulando p53 (PUMA), las cuales desencadenan la activación de la vía de Bax/Bcl2, lo que favorece la liberación del citocromo c, que activa la vía de las caspasas para favorecer necrosis y/o apoptosis [Wang y Lippard., 2005; Lieberthal *et al.*, 1996] (Fig. 2). Se han llevado a cabo estudios para evaluar el efecto de CP en el ADN

mitocondrial (ADNmito) con resultados contradictorios. En un primer estudio se observó que la ausencia de ADNmito en una línea celular tumoral proveniente de un linfoma (U937) inducia un incremento en la sensibilidad a apoptosis inducida por CP [Liang y Ullyatt., 1998]. En contraste en un segundo estudio reportaron que la depleción de ADNmito en dos líneas celulares, una de hepatoma y otra de cáncer de mama, presentaban una resistencia significativa a la muerte celular inducida por quimioterapéuticos [Park *et al.*, 2004]. Finalmente en un tercer estudio demostraron la participación de la disfunción mitocondrial en la nefrotoxicidad inducida por CP; la cual depende del estado redox, integridad del ADN y las funciones bioenergéticas de la mitocondria [Marullo *et al*., 2013].

1.2.4 Muerte celular

Se conoce que el CP induce 2 tipos de muerte celular apoptosis y necrosis; anteriormente se sabía que altas concentraciones de CP inducían necrosis y bajas concentraciones comenzaban induciendo apoptosis. Interesantemente ambos mecanismos se han observado *in vivo* [Wang y Lippard., 2005, Yousef y Hussien., 2015], la contribución relativa de cada una de ellas aún no se ha descrito, la apoptosis es la que ha generado mayor atención en los últimos años; esta última es un proceso dependiente de la energía de trifosfato de adenosina (ATP), asociada relativamente con daño mitocondrial: mientras que la necrosis se asocia principalmente al daño mitocondrial severo y depleción de ATP [Wang y Lippard., 2005]. Se han descrito dos vías implicadas en la muerte celular por CP: 1) vía intrínseca, la cual se activa a través de la mitocondria y 2) la vía extrínseca que es

mediada por el factor de necrosis tumoral (TNFα) y el sistema de ligandos de muerte Fas [Ramesh y Reeves., 2002].

En la vía intrínseca, el daño mitocondrial libera factores como citocromo c y el factor inductor de apoptosis (AIF), con la consecuente activación de la vía de las caspasas (caspasa 9 y caspasa-3 en su forma activa) [Ramesh y Reeves., 2002]. Mientras que el segundo activador de caspasas derivado de la mitocondria/Inhibidor directo de la proteína de unión a apoptosis (Smac/DIABLO) interfiere con las vías de citocromo c y caspasas [Du *et al.*, 2000]. La vía extrínseca la detallaremos más adelante.

1.2.5 Estrés oxidativo y nitrosativo.

Se ha establecido, tanto *in vivo* e *in vitro,* que el estrés oxidativo/nitrosativo y la reducción en la defensa antioxidante, incluyendo superóxido dismutasa (SOD), catalasa y glutatión (GSH) [Chirino y Pedraza-Chaverri., 2009], glutatión peroxidasa (GPx), glutatión reductasa (GR), glutatión transferasa (GT) son parte de los mecanismos involucrados en el daño celular inducido por CP [Kadikoylu *et al*., 2004]. Davis y cols encontraron que el CP también induce la producción de especies reactivas de oxígeno (EROs) [Davis et al., 2001], particularmente anión superóxido ($O_2^{\bullet -}$), siendo este el de mayor relevancia [Chirino y Pedraza-Chaverri., 2009; Davis et al., 2001], peróxido de hidrogeno (H_2O_2) [Kadikoylu *et al*., 2004] y radical hidroxilo ($OH^{\bullet}$) [Shino *et al*., 2003]. Chirino y cols. [2008b] y Pan y cols [2009], demostraron un incremento en la nitración de proteínas mediante inmunohistoquimica de nitrotirosina-3 (3-NT) en ratas y ratones, respectivamente. Nosotros observamos resultados similares mediante Western blot en ratas con nefrotoxicidad por CP

[Trujillo *et al*., 2014b]. Recientemente hemos descrito que el CP induce estrés oxidante/nitrosativo a través del incremento en la producción de $O_2^{\bullet-}$, específicamente en el glomérulo y túbulo proximal de la nefrona en rata; se emplearon varios sustratos; NADH se utilizó como sustrato de la NADPH oxidasa, la L-arginina (L-Arg) se utilizó como sustrato de la sintasa de óxido nítrico, la xantina se utilizó como sustrato de la xantina oxidasa y finalmente se empleó succinato, para evaluar la participación de la producción de $O_2^{\bullet-}$ intramitocondrial. La administración de CP incrementó la producción de $O_2^{\bullet-}$ en glomérulo (Fig. 3A) y túbulo proximal de la nefrona (Fig. 3B) comparado con el grupo control; sin cambios significativos en túbulo distal, sugiriendo que el estrés oxidativo por CP, se produce mayormente en

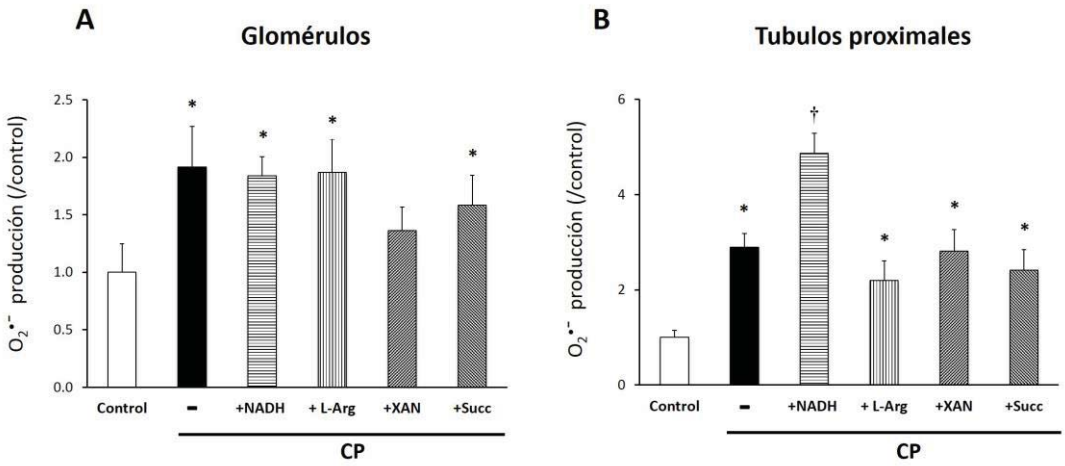

Figura 3. Producción de superóxido ($O_2^{\bullet-}$) en glomérulo (A) y túbulos proximales (B). Las muestras se incubaron en presencia de sustratos de enzimas que son fuentes de $O_2^{\bullet-}$. NADH fue usado como sustrato de NADPH oxidasa, L-arginina (L-Arg) como sustrato de la sintasa de óxido nítrico, xantina (XAN) como sustrato de xantina-oxidasa y succinato (Succ) para evaluar la reducción intramitocondrial. La fluorescencia de las ratas con CP es relativa al control, sin sustrato. Los valores son los promedios ± error estándar *P<0.05 vs control, †P <0.05 vs CP [Trujillo et al., 2014a]

glomérulo y en túbulo proximal. En el grupo tratado con CP al cual se le adiciono el sustrato de NADPH oxidasa (NADH) observamos un incremento mayor en la producción de $O_2^{\bullet-}$, específicamente en el túbulo proximal; sin cambios en glomérulo

y túbulo distal. Además no se observaron cambios significativos con la adición de los otros sustratos L-Arg, xantina y succinato, en ningún segmento. Lo que sugiere que estas vías no participan en la producción de $O_2^{\bullet-}$, en la nefrotoxicidad inducida por CP. Para corroborar la especificidad de la producción de $O_2^{\bullet-}$, se empleó un inhibidor de NAPH oxidasa DPI y se observó que efectivamente este inhibía el incremento en la producción de $O_2^{\bullet-}$ inducido por CP, en glomérulo y túbulo proximal [Trujillo *et al*., 2014a].

La NADPH oxidasa es una enzima de unión a membrana la cual es considerada la mayor fuente de EROs y tiene la habilidad de generar altos niveles de $O_2^{\bullet-}$. Esta oxidasa está compuesta por diversas subunidades: $p47^{phox}$, $p67^{phox}$, Rac, $gp91^{phox}$ y $p22^{phox}$ estas dos últimas involucradas en la transferencia de un electrón de NADPH al oxígeno, originando el $O_2^{\bullet-}$ y nicotinamida adenina dinucleótido (NADP), mientras que el resto de las subunidades son reguladoras de la actividad de la enzima [Takeya y Sumimoto., 2003]. Las subunidades Rac1/2, $p67^{phox}$, $p47^{phox}$ y $p40^{phox}$ en condiciones normales habitualmente residen en el citosol y se asocian con las subunidades $gp91^{phox}$ y

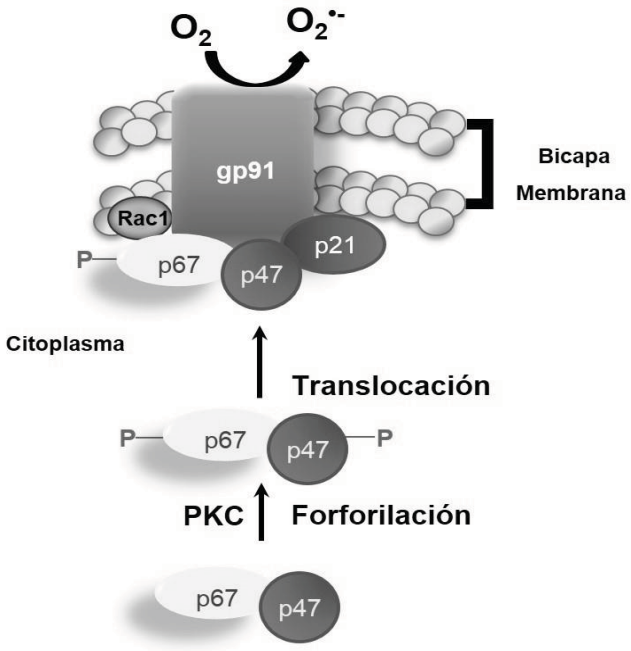

Figura 4. Activación y subunidades de NADPH oxidasa. Oxigeno (O_2); superóxido ($O_2^{\bullet-}$); proteina cinasa C (PKC)

p22^{phox} que se encuentran ancladas a la membrana; esto sólo ocurre después de su activación. Particularmente p47^{phox} interactúa con la proteína cinasa C (PKC) induciendo la fosforilación de la subunidad p47^{phox}, ésta a su vez interactúa con componentes del citoesqueleto para favorecer su correcto ensamblaje del complejo activo e iniciar la generación de $O_2^{\bullet-}$ [Sachse y Wolf., 2007] (Fig. 4). Para confirmar la activación de NADPH oxidasa por CP; nuestro grupo describió recientemente que el CP induce un incremento en la expresión de gp91^{phox} y p47^{phox} en glomérulo y túbulo proximal, determinamos la expresión de las subunidades gp91^{phox} (Fig. 5A) y p47^{phox} (Fig. 5B) de la NADPH oxidasa, las cuales forman parte del complejo de la NADPH

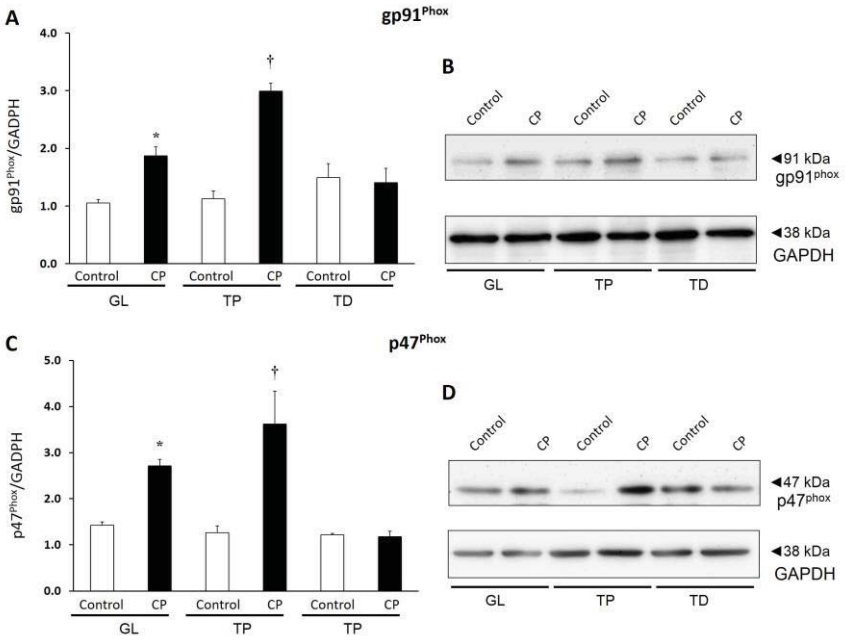

Figura 5. La expresión de las subunidades gp91^{phox} y p47^{phox} de la β-nicotinamida adenina dinucleótido fosfato (NADPH) oxidasa en los glomérulos (GL), túbulos proximales (TP) y los túbulos distales (TD) de ratas tratadas con vehículo y cisplatino (CP). Los análisis densitométricos de los Western blot (n=3) se presentan en los paneles A y C, las imágenes representativas se presentan en los paneles B y D para gp91^{phox} y p47^{phox}, respectivamente. Los datos están expresados como la densidad relativa de 5 ratas/grupo normalizado con Gliceraldehído-3-fosfato deshidrogenasa (GAPDH) como control de carga. Los valores son ± error estándar, * P <0.05 vs GL control, † P <0.05 vs control de TP [Trujillo *et al.*, 2014a].

oxidasa activa, mediante western blot en homogenado de fracciones enriquecidas con glomérulos, túbulos proximales y túbulos distales; encontramos que la administración de CP induce un incremento significativo en la expresión de $gp91^{phox}$ como lo muestra la figura 8A, B; y $p47^{phox}$ en la figura 5C, D en glomérulo y túbulo proximal pero no en túbulo distal. Sugiriendo que la administración de CP incrementa el complejo activo de NADPH oxidasa en glomérulo y túbulo proximal. (Fig. 5) [Trujillo *et al.*, 2014a].

Otro factor implicado en los mecanismos de estrés oxidativo asociado a la nefrotoxicidad por CP, es la reducción en la expresión del factor nuclear eritroide-2-asociado al factor 2 (Nrf2), el cual induce la expresión de proteínas citoprotectoras [Marullo *et al.*, 2013; Sahin *et al*., 2010b; Trujillo *et al.*, 2014b]. Está coordinado por un mecanismo molecular común que incluyen elementos de respuesta a factores antioxidantes, sensor de inductores químicos y la activación de proteína asociadora de Kelch ECH 1 (Keap-1), encargado de inhibir a Nrf2. Por lo que se ha establecido que Nrf2 es un regulador maestro contra el estrés oxidante [Marullo *et al.*, 2013; Sahin *et al.*, 2010a; Sahin *et al*., 2010b; Hayes y Dinkova-Kostova., 2014].

1.2.6 Activación de la vía de señalización de proteínas cinasas activadas por mitógenos (MAPK)

De manera interesante, se ha informado que las vías de transducción de señales relacionadas a PKC pueden modular la nefrotoxicidad por CP; esta vía se inicia con la activación de la señalización de las MAPK [Ikeda *et al*., 1999]. Se sabe que la expresión proteína cinasa beta 2 (PKCβ2) favorece condiciones de estrés

oxidativo, debido a que PKCβ estimula la actividad de la NADPH oxidasa y así aumenta la generación de $O_2^{\bullet-}$ [Kitada *et al.*, 2003].

1.2.7 Marcadores de inflamación.

El CP induce eventos inflamatorios en riñón, asociado principalmente a un aumento en la expresión de TNF-α [Ramesh y Reeves., 2002], una citocina con importantes funciones inmunológicas e inflamatorias. Está implicado en la vía extrínseca de muerte celular y su función está mediada a través de sus receptores de muerte celular receptor 1 (TNFR1) y receptor dos (TNF2), de los cuales TNFR1 induce directamente la vía de apoptosis por tener elementos de respuesta y activa a caspasa-8. El TNFR2 en cambio, se asocia con la activación de respuesta inflamatoria, indirectamente induce apoptosis y necrosis en las células epiteliales del túbulo renal a través de la activación de los ligandos de muerte celular y de la activación de caspasa-8 y finalmente caspasa-3 [Ramesh y Reeves., 2002, Tsuruya *et al*., 2003]. El TNF-α también está implicado en la respuesta inflamatoria activando citocinas pro-inflamatorias, quimiocinas y reclutando leucocitos, estado que favorece un incremento en la generación de EROs, las cuales a su vez, inducen a TNF-α, lo que amplifica y perpetúa el daño [Szlosarek y Balkwill et al., 2003]. En la respuesta inflamatoria también se ha descrito la inducción del factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-kB).

1.2.8 Desintegración de la unión celular en túbulo proximal.

En la nefrona, los diferentes segmentos tubulares juegan un papel importante en la absorción de agua y solutos [Guyton y Hall., 2006]. Uno de los mecanismos que participa en dicha función es el transporte trans-epitelial que puede ser tanto trans-celular o para-celular. La unión celular (UC), está formada por proteínas integrales y periféricas, que restringen el transporte para-celular de solutos y agua. Las proteínas integrales establecen el contacto célula-célula en el espacio intercelular, mientras que las proteínas periféricas sirven como un puente entre las proteínas integrales y el citoesqueleto de actina [González-Mariscal *et al.*, 2008]. Se han identificado muchas proteínas integrales de membrana como componentes de la UC estrecha (TJ), incluyendo claudinas y ocludinas (Fig. 6) [Furuse *et al*., 1993; Furuse *et al*., 1998]. Las claudinas son proteínas con una distribución limitada en cada segmento tubular, se han descrito 24 isoformas en mamíferos y su función es determinar la permeabilidad de los epitelios para pequeños iones, solutos y agua [Hou *et al*., 2013]. A nivel renal, la claudina-2 se

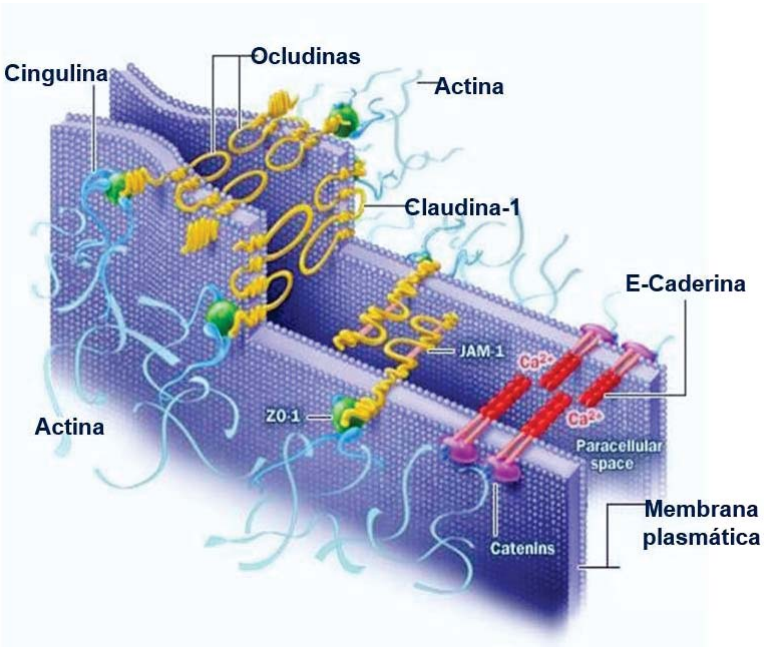

Figura 6. Proteínas de unión celular en túbulo proximal (tomado de http://biologiacelularmaestria2010.blogspot.mx/)

expresa en epitelios permeables [Enck *et al*., 2001]; en estudios *in vitro* se ha demostrado que funciona como un poro para-celular; selectivo para los cationes [Kiuchi-Saishin *et al*., 2002] y que participa en el transporte de agua en el túbulo proximal renal [Rosenthal *et al*., 2010]. Las ocludinas son proteínas integrales de membrana que están presentes en las células epiteliales y endoteliales; las ocludinas están ausentes, a nivel de ARNm y de proteína, en las células que carecen de UC tales como los fibroblastos [Saitou *et al*., 1997]. La ocludina está presente exclusivamente en el borde en cepillo de las células epiteliales del túbulo proximal y su distribución a lo largo de la nefrona. Se relaciona con la resistencia eléctrica trans-epitelial (RET), por lo tanto en regiones donde la RET es alta, la ocludina se encuentra en los límites de las células, sugiriendo una relación entre su presencia y la fuerza de la UC [González-Mariscal *et al*., 2000]. Las ocludinas se expresan de manera ubicua a lo largo de los segmentos tubulares [Kwon *et al*., 1998; Reyes *et al.*, 2002]. Mientras que la cadherina-E es la principal proteína de UC adherente (AJ) en las células epiteliales y altamente expresada en la nefrona distal y conductos colectores [Perantoni., 1999]; la pérdida de cadherina-E en las células epiteliales tubulares renales, regula la transición mesenquimal-epitelial (EMT) [Zheng *et al.*, 2009].

En estudios anteriores se ha demostrado que la estructura y la función de la UC son sensibles al estrés oxidante inducido por la presencia de metales pesados [Arreola-Mendoza *et al*., 2009; Jacquillet *et al.*, 2006] y al H_2O_2 [Meyer *et al.*, 2001]. Recientemente nuestro grupo describió la desintegración de la UC asociado a la reducción en la expresión y localización de proteínas de la TJ en el túbulo proximal. Para evaluar dichos parámetros realizamos microscopía confocal y análisis de

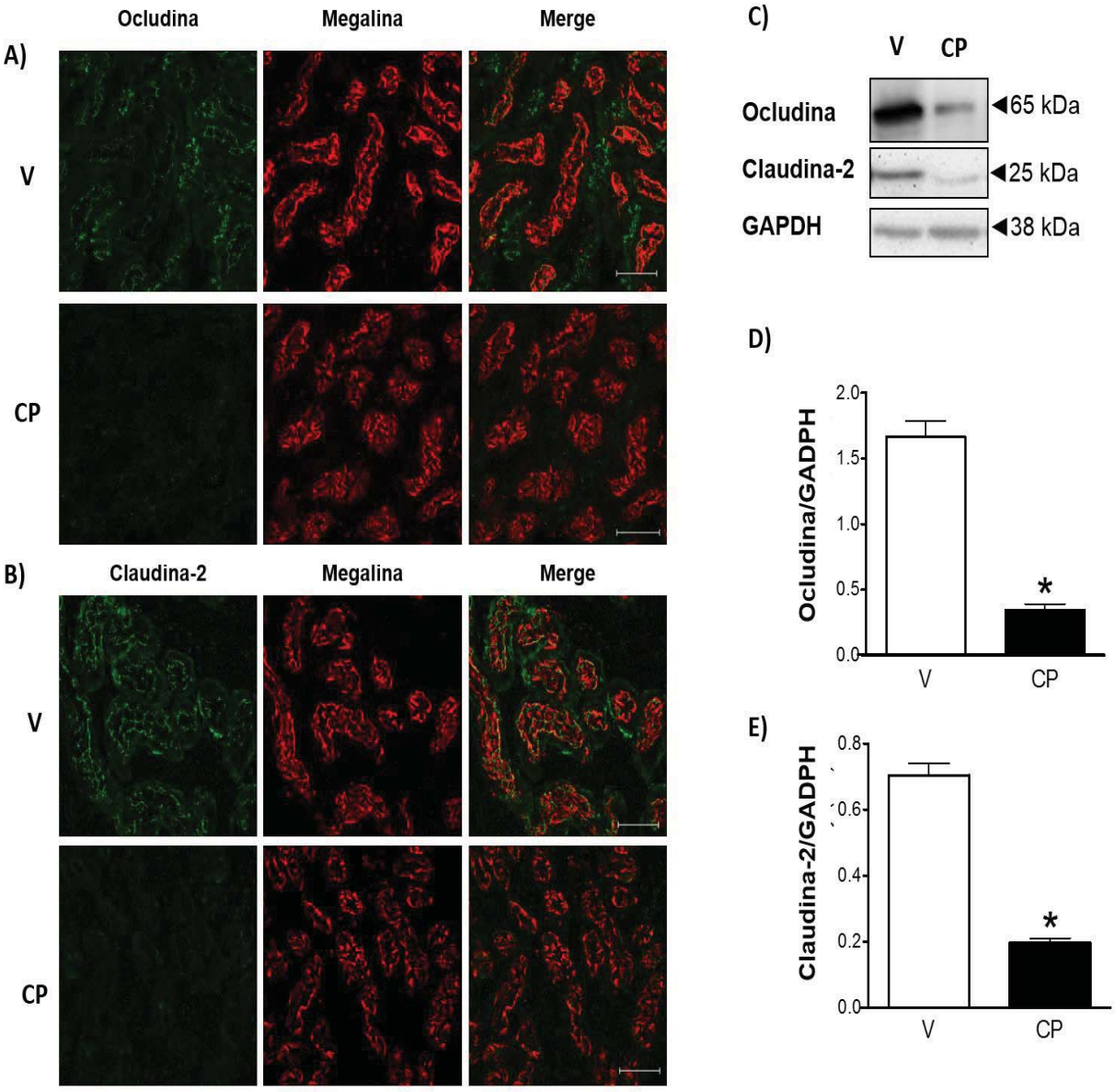

Figura 7. Reducción de la expresión de ocludina y claudina-2 inducido por CP. En los paneles A y B se presenta la localización de ocludina y claudina-2, respectivamente por microscopía confocal y la expresión por Western blot de los homogenizados de la corteza renal (C) en los dos grupos experimentales estudiados. En las microfotografías de ratas tratadas con vehículo (V) se determinó que ocludina y claudina-2 rodean el borde en cepillo de las células tubulares proximales (A, B; marca verde). En contraste las microfotografías de los animales que recibieron CP se observa una disminución de la localización (A y B) y la expresión (C) de ocludina y claudina-2. La expresión de megalina (A, B; marca roja) se empleó como un marcador de la membrana apical del túbulo proximal; en el tercer recuadro se observa la sobreposición de las imágenes. Los análisis densitométricos de los Western blot se presentan en los paneles D y E para ocludina y claudina-2, respectivamente. Los valores son los promedios ± error estándar, n= 3, *P<0.05 vs V. Barra =50 μm [Trujillo *et al.*, 2014b].

western blot en ratas que recibieron vehículo; así como en las tratadas con CP y

observamos que el CP redujo la expresión de ocludina (Fig. 5A) y claudina-2 (Fig. 5B), con respecto a las ratas vehículo como lo revelan las microfotografías de inmunofluorescencia (IF), resultado que se confirmó mediante análisis de western blot (Figs. 5C, D, E). Cabe mencionar que megalina, es un marcador de túbulos proximales, el cual se mantuvo sin cambios, indicando que la reducción en la expresión de claudina-2 y ocludina, es selectiva (Figs. 5A, B). Aunado a esto; la expresión de claudin-5 y zonula ocluyente (ZO-1) no se modificó por el tratamiento con CP [Trujillo *et al.*, 2014b].

1.3 La curcumina

La cúrcuma o *Curcuma longa* es una planta herbácea de la familia Zingiberaceae. Su cultivo se da principalmente en regiones tropicales y subtropicales (India, China, Sureste de Asia), siendo la India el mayor productor, consumidor y exportador de cúrcuma, produciendo 992,940 toneladas en el año 2011 [González-Salazar *et al*., 2011]. La cúrcuma contiene 60-70% de carbohidratos, 6-8% de proteína, del 5-10% de grasas, del 3-7% de aceites esenciales, del 2-7% de fibra y del 2-6% de curcuminoides, de

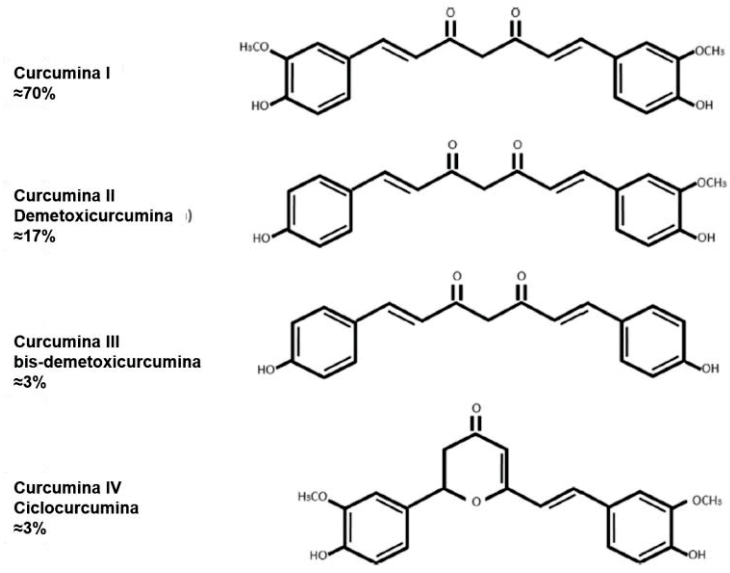

Figura 8. Estructuras químicas de la curcumina y los curcuminoides

los cuales ≈70% es curcumina (curcumina I), ≈17% demetoxicurcumina (curcumina II), ≈3% *bis*-demetoxicurcumina (curcumina III) y ciclocurcumina (curcumin IV). La curcumina, es un compuesto fenólico, extraído del rizoma de *Curcuma longa,* se emplea comúnmente en la alimentación como condimento (forma parte del curry), en la industria alimentaria como aditivo y colorante y en la industria cosmética y textil como pigmento natural (amarillo 3). En la medicina tradicional de la India y de China, consideran a la curcumina como un agente terapéutico para diversas enfermedades; por lo que se ha descrito que tiene amplias funciones biológicas, particularmente anti-inflamatorias [Ueki *et al*., 2013; Wang *et al*., 2012], antimicrobianas [De *et al.*, 2009], anticancerígenas [Sarkar et al., 2010], neuroprotector [Wang *et al*., 2010], cardioprotector [González-Salazar *et al*., 2011] y renoprotectoras [Molina-Jijón *et al*., 2011; Trujillo *et al*., 2013]

Se ha demostrado que la curcumina es un antioxidante bifuncional debido a que puede reaccionar directamente con las especies reactivas, dando estabilidad a las moléculas; así como inducir la expresión de diversas proteínas citoprotectoras y antioxidantes [Dinkova-Kostova *et al.*, 2004]. La curcumina es capaz de atrapar $O_2^{\bullet-}$ [Ak y Gulcin., 2008; Sreejayan y Rao., 1996], $OH^{\bullet}$ [Barzegar y Moosavi-Movahedi., 2011], H_2O_2 [Barzegar y Moosavi-Movahedi., 2011; Ak y Gulcin., 2008], radical singulete de oxígeno ($^1O_2$) [Das y Das., 2002], óxido nítrico (NO•) [Sreejayan y Rao., 1996; Sumanont *et al.*, 2004], peroxinitrito ($ONOO^-$) [Kim *et al*., 2003] y radicales peroxilo ($ROO^{\bullet}$) [Galati *et al*., 2002; Barzegar y Moosavi-Movahedi., 2011]. En conjunto estos efectos podrían explicar en parte algunos de los efectos citoprotectores de la curcumina; aunada su estructura química, la cual cuenta con la

presencia de grupos funcionales como el grupo β-dicetona [Masuda *et al.*, 1999; Sugiyama *et al*., 1996], dobles enlaces carbono-carbono y anillos fenol con grupos hidroxilo y metoxilo [Jovanovic *et al*., 1999]. La curcumina también es capaz de inducir la expresión de proteínas citoprotectoras como SOD, catalasa [Panchal *et al.*, 2008], GR, GPx [Yarru *et al.*, 2009], hemo oxigenasa 1 (HO-1) [Jeong *et al*., 2006; Reyes-Fermín et al., 2012], glutatión-S-transferasa (GST), NADPH:quinona, óxido reductasa 1 [Dinkova-Kostova *et al*., 2004; Ye *et al*., 2007], y Nrf2 [Calabrese *et al.*, 2008; Cuadrado *et al.*, 2009; Eggler *et al*., 2008; Rojo *et al*., 2012].

1.3.1 Efecto de la curcumina en la nefrotoxicidad por cisplatino

Llevamos a cabo una revisión de los diferentes estudios para evaluar el papel de la curcumina en la nefrotoxicidad inducida por CP [Trujillo *et al.*, 2013]. La administración de curcumina en ratas (8 mg/kg antes y después de la administración de CP) ejerce neuroprotección y renoprotección; asociado a una reducción de la creatinina sérica, un aumento en la depuración de creatinina y reducción en el estrés oxidante, evaluado mediante los niveles de malondialdehído (MDA) y glutatión oxidado [Antunes *et al.*, 2001]. Sin embargo, en otro grupo de investigadores con un diseño experimental diferente: un pre-tratamiento de curcumina de dos días y un tratamiento de 15, 30 y 60 mg/kg por 2 días en un modelo de nefrotoxicidad inducida por CP, observaron que el grupo que recibió 60 mg/kg de curcumina presentó una función renal normal asociada a niveles normales de BUN y creatinina plasmáticos; correlacionado con una reducción en los niveles de lipoperoxidación e interesantemente la administración de curcumina en ratas con nefrotoxicidad por CP aminoró de manera dosis dependiente la reducción de GSH, principal antioxidante

endógeno, SOD y catalasa [Kuhad *et al.*, 2007]. Recientemente se estudió el efecto de la administración de 100 mg/kg de curcumina en la nefrotoxicidad inducida por CP en ratón y observaron que los mecanismos inflamatorios están involucrados de manera significativa y que la administración de curcumina es capaz de prevenir la necrosis tubular y la disfunción renal [Ueki *et al*., 2013]; así como los marcadores proinflamatorios como TNF-α [Ramesh y Reeves., 2002] en suero y la proteína quimioatrayente de macrófagos (MCP-1) [Ueki *et al*., 2013]. A este respecto se demostró que el efecto de la administración de 100 mg/kg de curcumina por 10 días en la nefrotoxicidad inducida por CP en ratas promovía el mantenimiento de fosforibosiltransfera nicotinamida (NAMPT o visfantina), considerada una adipocina implicada en la resistencia al estrés y la modulación de muerte celular, ya que inhibe la apoptosis de neutrófilos. Resultados similares se observaron al evaluar la expresión de sirtuina, con actividad de ADP-ribosiltransferasa, o desacetilasa de histonas, que han sido implicadas con amplio rango de procesos celulares como envejecimiento, apoptosis e inflamación [Ugur *et al*., 2015].

Debido a la prevalencia de IRA por el uso de fármacos nefrotoxicos, al amplio uso de agentes antineoplásicos y al conjunto de mecanismos que participan en el desarrollo de la nefrotoxicidad por CP; entre los que destacan el transporte del fármaco en las células tubulares proximales renales, fibrogénesis, activación de la respuesta inflamatoria, activación de la muerte celular por apoptosis y/o necrosis; desencadenando estrés oxidante asociado al incremento de $O_2^{\bullet -}$ a través de NADPH oxidasa; principalmente en el túbulo proximal de la nefrona y finalmente la desintegración de las uniones celulares. Por lo que el presente estudio pretende

conocer si es posible que la administración de curcumina en la nefrotoxicidad por CP, disminuya el estrés oxidante y la desintegración celular.

II. Hipótesis

Si la generación de $O_2^{\bullet -}$ en el túbulo proximal por NADPH oxidasa y la desintegración de la unión celular participan en los mecanismos de nefrotoxicidad por CP, entonces es posible que la administración de un antioxidante, en conjunto con el fármaco antineoplásico, disminuya la nefrotoxicidad inducida por CP.

III. Objetivo general

Determinar si la mayor generación de $O_2^{\bullet-}$ y la desintegración de la unión celular participan en los mecanismos de daño renal inducido por CP y si la administración de un antioxidante confiere renoprotección en este modelo experimental.

IV. Objetivos particulares

En la nefrotoxicidad por CP:

1.1 Determinar cuál de los siguientes segmentos de la nefrona es el que más contribuye a la generación de $O_2^{\bullet-}$: glomérulo, túbulo proximal y túbulo distal.

1.2 Establecer cuál de las siguientes fuentes es la responsable de la producción renal de $O_2^{\bullet-}$: NADPH oxidasa, sintasa de óxido nítrico desacoplada, xantina oxidasa y mitocondria.

1.3 Determinar la expresión y localización de ocludina, claudina-2 y cadherina-E.

1.4 Establecer si la administración conjunta de curcumina, previene la disfunción renal y el daño estructural.

1.5 Determinar si la administración de curcumina, previene la mayor generación de $O_2^{\bullet-}$; atenuando la activación de NADPH oxidasa y preveniendo la pérdida de la unión celular.

V. Material y Métodos

5.1 Reactivos:

Los siguientes reactivos se obtuvieron de Sigma-Aldrich (St. Louis, MO, EUA): CP (Cis-diaminoplatino (II) dicloruro, No. Cat. 479306, Lt MKBH5984V), curcumina (No. Cat. C1386, Lt 079K1756V, 2,2-azobis (2-amidinopropano) (AAPH), xantina, xantina oxidasa, nitroazul tetrazolio (NBT), dihidroetidio (DHE), fluoresceína, DL-penicilamina, ácido dietilentriaminopentaacético (DTPA), 2,2-difenil-1-picrilhidrazilo (DPPH), ácido tereftálico, ácido ascórbico, Amplex® Red, peroxidasa de rábano (HRP), dimetiltiourea (DMTU), ácido lipoico, cloruro de hierro ($FeCl_3$), 1-cloro-2,4-dinitrobenceno (CDNB), tripiridil-s-triazina (TPTZ), dimetil sulfóxido (DMSO), fluoruro de fenilmetilsulfonilo (PMSF), 1,3-difenilisobenzofurano (DPBF), 1-metil-2-fenilindol (1M2Fi), 1,1,3,3-tetrametoxipropano (TMPO), colagenasa (de *Clostridum histoliticum*, tipo II), GSH, glutatión disulfuro (GSSG), NADPH, NADH, difenileno de yodonio (DPI), p-nitrofenil-β-D-glucosaminido, p-nitrofenol, ácido 3-cloro-4-aminobenzoico, acetato de sodio (CH_3COONa), citrato de sodio ($C_6H_5Na_3O_7{\cdot}2H_2O$), dodecil sulfato de sodio (SDS), triton X-100, desoxicolato de sodio, albúmina bovina fracción V, glucosa, Tween 20; glicerol, alanina, ADN de testículo de salmón, percoll, anticuerpos de conejo anti-PKCβ2 y anti-Nrf2. El sodio 4,5-dihidroxibenceno-1,3-disulfonato (tirón; mimético de SOD u oxidante directo de $O_2^{\bullet-}$) se obtuvo de Fluka (St. Louis, MO, EUA). El ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico (trolox; analogo de vitamina E) y el anticuerpo de conejo anti-GADPH se adquirieron de Millipore Corp. (Billerica, MA, EUA). El ácido etilendiaminotetraacético (EDTA), el cloruro de potasio (KCl), el cloruro de sodio (NaCl), el bicarbonato de sodio

($NaHCO_3$), el sulfato de magnesio ($MgSO_4$), el fosfato de potasio monobásico (KH_2PO_4), el fosfato de sodio dibásico (Na_2HPO_4), el cloruro de calcio ($CaCl_2$), el carbonato de sodio (Na_2CO_3), el hipoclorito de sodio (NaClO), el ácido clorhídrico (HCl), el acetonitrilo, el metanol y el H_2O_2 se obtuvieron de JT Baker (Xalostoc, Edo. de México, México). El pentobarbital sódico se adquirió de Holland de México (México, D.F.). El cóctel inhibidor de proteasas 1X "complete" se obtuvo de Roche Applied Science (Mannheim, Germany). La dihidrorodamina 123 (DHR-123), el 2', 7'-diacetato diclorofluoresceína (DCDHF-DA) y el anticuerpo de ratón anti-3-NT fueron de Cayman Chemical Co. (Ann Arbor, MI, EUA). Los anticuerpos de conejo anti-claudina-2, anti-ocludina, anti-conejo conjugado con peroxidasa, anti-ratón conjugado con peroxidasa, anti-cabra conjugado con peroxidasa, los Alexa-Fluor® 488 anti-conejo y Alexa Fluor® 594 anti-ratón se adquirieron de Invitrogen (Carlsbad, CA, EUA). Los anticuerpos de cabra anti-molecula de daño renal (KIM-1) se adquirieron de R & D Systems (McKinley Place, MN, EUA). Los anticuerpos de conejo anti-factor de creciemiento transformante beta (TGF-β1) y anti-cadherina, de ratón anti-lipocaina asociada a la gelatinasa de neutrófilos (NGAL), anti-colágeno I, anti-colágeno IV, anti-actina del musculo liso alpha (α-SMA), anti-caspasa, de cabra anti-$p47^{phox}$ y anti-$gp91^{phox}$ se adquirieron de Santa Cruz Biotech, Inc. (Santa Cruz, CA, EUA). El anticuerpo de ratón anti-dipeptil peptidasa (DppD) se adquirió de AbD Serotec (Raleigh, NC, EUA). El anticuerpo de ratón anti-desmoplaquina (DMPK) se adquirió de MP Biomedicals (Solon, OH, USA). El estuche de detección de Western blot Amersham ECL™ fue de General Electric Healthcare Lifesciences (Buckinghamshire, Reino Unido). Los estuches comerciales para la medición de

creatinina plasmática y BUN (Sera-pak creatinina No. Cat. 1001111 y urea No Cat. 1001325) fueron de Spinreact (Girona, España). Los reactivos fueron de grado analítico y se adquirieron comercialmente.

5.2 Ensayos de la actividad antioxidante de la curcumina in vitro

5.2.1 Ensayo de atrapamiento del anión superóxido ($O_2^{\bullet-}$).

El $O_2^{\bullet-}$ se generó mediante el sistema xantina-xantina oxidasa. La actividad de atrapamiento de la curcumina contra el anión se evaluó por su capacidad de inhibir la oxidación de DHR123. Brevemente: 25 μL de una solución de curcumina (3 mg/mL) o de amortiguador de fosfato 50 mM, pH 7.0 (0% de atrapamiento) o de tirón (compuesto de referencia) se mezclaron con 200 μL de la mezcla de reacción (xantina 0.09 mM, EDTA 0.09 mM, Na_2CO_3 36.6 mM, DHR123 45 μM) en amortiguador de fosfato 20 mM, pH 7.0. La reacción comenzó mediante la adición de 10 μL de xantina oxidasa (2.52 U/mL) y la fluorescencia se midió después de 3 min a 505 nm de excitación y 528 nm de emisión [Yanagisawa *et al*., 2009] utilizando un lector de microplacas multimodo Synergy HT (Biotek Instruments, Winooski, VT, EUA).

5.2.2 Ensayo de atrapamiento del oxígeno singulete ($^1O_2$).

El $^1O_2$ se generó a partir de NaClO y H_2O_2 de la manera descrita previamente [Cervantes *et al*., 2013]. El $^1O_2$ causa una reducción en la fluorescencia de DPBF que se determinó a 410 nm de excitación y 455 nm de emisión. La reacción se llevó a cabo con 25 μL de curcumina o de amortiguador de fosfatos 45 mM, pH 7.0 (0% de atrapamiento) o de ácido lipoico (compuesto de referencia) a los cuales se le

adicionaron 225 μL de la mezcla de reacción (H_2O_2 1 mM, NaClO 30 mM y DPBF 1 μM).

5.2.3 Ensayo de atrapamiento de peróxido de hidrógeno (H_2O_2).

La capacidad de la curcumina para secuestrar H_2O_2 se realizó utilizando Amplex® Red. Este compuesto se oxida en presencia de H_2O_2 y se produce resorufina, un compuesto fluorescente que se mide usando filtros de excitación y de emisión de 530/25 y 590/35, respectivamente [Cervantes *et al*., 2013]. El ensayo se realizó de la siguiente manera: se incubaron 25 μL de curcumina o de amortiguador de fosfatos 50 mM, pH 7.4 (0% de atrapamiento) o de piruvato (compuesto de referencia) con 25 μL de H_2O_2 200 μM por 20 min. El H_2O_2 restante se detectó mediante la adición de 100 μL de la mezcla de reacción (Amplex® Red 0.1 mM, HRP 0.2 U/mL).

5.2.4 Ensayo de atrapamiento de radical hidroxilo ($OH^{\bullet}$)

El $OH^{\bullet}$ se generó por la reacción de Fenton [Gaona-Gaona *et al*., 2011]. Brevemente a una solución de 20 μL de curcumina o de agua destilada (0% de atrapamiento) o DMTU (compuesto de referencia) se añadieron 180 μL de la mezcla de reacción (ácido ascórbico 0.2 mM, $FeCl_3$ 0.2 mM, EDTA 0.208 mM, H_2O_2 1 mM, ácido tereftálico 1.4 mM y amortiguador de fosfatos 20 mM, pH 7.4). La mezcla se incubó durante 30 min a temperatura ambiente para obtener un producto fluorescente que se mide en longitudes de onda de excitación y emisión de 326 nm y 432 nm, respectivamente.

5.2.5 Ensayo de atrapamiento del anión peroxinitrito ($ONOO^-$)

Este anión se sintetizó de acuerdo al método descrito por Cervantes *et al*. [2013] y su concentración se determinó usando ε302 nm= 1670 M^{-1} cm^{-1}. El DCDHF-DA se utilizó como un indicador de la presencia del anión. Brevemente, a una solución de 20 µL de curcumina o de amortiguador de fosfatos 0.1 M, pH 7.4 (0% de atrapamiento) o de penicilamina (compuesto de referencia) se le añadieron 180 µL de la mezcla de reacción (DTPA 0.041 mM, DCDHF-DA 145 µM y $ONOO^-$ 0.026 µM). En ausencia de un antioxidante, el DCDHF-DA se oxida a diclorofluoresceína, compuesto fluorescente que se mide en longitudes de onda de excitación y emisión de 502 y 523 nm, respectivamente.

5.2.6 Ensayo de atrapamiento de ácido hipocloroso (HOCl)

Se determinó utilizando ácido para-aminobenzoico, el cual reacciona con HOCl para producir el compuesto fluorescente ácido 3-cloro-4-aminobenzoico [Van Antwerpen y Nève., 2004]. Brevemente, 25 µL de curcumina o de ácido ascórbico (compuesto de referencia) o de amortiguador de fosfatos 50 mM, pH 7.4 (0% de atrapamiento) se mezclaron con 225 µL de la mezcla de reacción (HOCl 0.05 mM y ácido para-aminobenzoico 0.1 mM en amortiguador de fosfatos de potasio 50 mM, pH 7.4) y la fluorescencia se determinó a longitudes de onda de excitación y emisión de 280 nm y 340 nm, respectivamente

5.2.7 Ensayo de atrapamiento del radical peroxilo ($ROO^{\bullet}$)

La actividad de atrapamiento de la curcumina se determinó por la estabilidad de la fluorescencia de la fluoresceína por $ROO^{\bullet}$ [Huang *et al*., 2002]. Brevemente, se añadió una solución de 25 µL de curcumina o de amortiguador de fosfatos 75 mM, pH 7.4 (0% de atrapamiento) o de trolox (compuesto de referencia) a una solución compuesta por 150 µL de fluoresceína 30 nM y 25 µL de AAPH 153 mM. La fluorescencia se midió a longitudes de onda de excitación y emisión de 485 nm y 520 nm, respectivamente durante 1.5 h a 37°C. Al final del ensayo el área bajo la curva (AUC) se obtuvo por Gen 5 software (Biotek Instruments).

5.2.8 Ensayo de atrapamiento de radicales férricos (FRAP)

Se utilizó para estimar la actividad de atrapamiento de la curcumina [Benzie y Strain., 1996]. Brevemente, a una solución de 30 µL de curcumina o de amortiguador de acetatos 300 mM, pH 3.6 (0% de atrapamiento) se le adicionó 300 µL de una mezcla de reacción (TPTZ 10 mM en HCl 40 mM, $FeCl_3$ 20 mM y amortiguador de acetatos 300 mM, pH 3.6). La mezcla se incubó por 15 min a temperatura ambiente, la reducción de hierro a pH bajo produce un complejo ferroso con TPTZ color azul y se midió a una longitud de onda de 593 nm, la actividad antioxidante se expresó en términos de µmoles equivamentes sulfato de hierro (Fe_2SO_4)/g de curcumina.

Finalmente se determinaron los IC_{50} de cada uno de los ensayos; excepto el ensayo de FRAP.

5.3 Inducción del modelo experimental in vivo:

Se incluyeron 40 ratas Wistar macho (200-250 g) que se alimentaron con una dieta estándar [De *et al.*, 2009] y agua *ad libitum*, bajo la Norma Oficial Mexicana (NOM-062-ZOO-1999) y con la aprobación del comité de ética local (FQ/CICUAL/069/13). Las ratas se distribuyeron aleatoriamente en cuatro grupos: el primer grupo integrado por 5 ratas que recibieron vehículo (V, solución salina isotónica) por inyección intraperitoneal (ip); el segundo grupo compuesto de 5 ratas que recibieron 5 mg/kg de cisplatino Aldrich® (CP; ip); el tercero lo formaron 5 ratas que recibieron CP (5 mg/kg) + curcumina (Cur; 200 mg/kg), el último grupo de 5 ratas recibieron solo Cur (200 mg/kg). Se administró una sola dosis de CP y 3 dosis de curcumina via oral 30 min previos a la administración del CP, 24 h y 48 h posteriores a CP. Un día previo al sacrificio se recolectó la orina de 24 h en jaulas metabólicas; setenta y dos horas después de la administración de CP las ratas se anestesiaron con pentobarbital sódico (90 mg/kg, ip), se recolectó una muestra de sangre de la aorta en tubos heparinizados, la cual se utilizó para los procedimientos analíticos. Posteriormente, las ratas se sacrificaron y el riñón izquierdo se congeló inmediatamente en nitrógeno líquido para análisis de Western blot y para medir la actividad de N-acetil-β-D-glucosaminidasa (NAG) y las enzimas antioxidantes. El riñón derecho se fijó en formalina al 10%, se realizaron tinciones de hematoxilina-eosina (H&E), para el análisis histológico. Se incluyeron grupos adicionales de los 4 grupos para obtener muestras del riñón derecho y llevar a cabo estudios de IF; con el resto de la masa renal se obtuvieron homogenados de las fracciones enriquecidas con glomérulo, túbulo proximal y túbulo distal.

5.4 Estudios fisiológicos:

Para evaluar el daño renal se midieron los niveles de creatinina en plasma y orina [Myers *et al*., 2006] así como BUN [Guerrero-Beltran *et al*., 2010, Young y Friedman., 2001] en plasma mediante estuches comerciales basados en ensayos colorimétricos. La actividad de NAG renal, el cual es un marcador sensible al daño tubular proximal, se midió mediante un ensayo colorimétrico [Charlton *et al.*, 2014; Skrha *et al*., 1987].

5.5 Estudios bioquímicos:

5.5.1 Niveles de MDA en homogenados renales

Este ensayo se basa en la reacción entre el MDA con 1M2Fi que produce un cromóforo de alto coeficiente de extinción molar y un máximo de absorción de longitud de onda de 586 nm. Esta reacción es específica en presencia de ácido clorhídrico. El procedimiento se realizó de acuerdo a la metodología descrita previamente por Gérard-Monnier y cols (1998). Al inicio del ensayo se preparó una solución 15.4 mM de 1M2Fi en una mezcla de acetonitrilo: metanol (3:1). Para la curva estándar se utilizó la solución de TMPO 0.0739 mM y el stock de TMPO en concentraciones: 0, 10, 20, 40, 80, 160 y 320 µL. Se mezclaron 200 µL de homogenado renal con 650 µL de la solución de 1M2Fi con acetonitrilo/metanol (3:1) a una concentración final de 10 mM. La reacción se inició por la adición de 150 µL de HCl al 37%. Posteriormente, se incubaron los puntos de la curva estándar y las muestras a 48°C por 50 min. Transcurrido el tiempo, se centrifugaron los tubos a 20,000 x g durante 5 minutos.

5.5.2 Actividad de las enzimas antioxidantes

Se determinó en homogenados renales [Molina-Jijón *et al*., 2011; Pérez-Rojas *et al*., 2009]. La catalasa se analizó mediante la desaparición de H_2O_2 30 mM a 240 nm y los datos se expresaron como κ/mg de proteína. La actividad de la GR se midió utilizando GSSG como sustrato y midiendo la desaparición de NADPH a 340 nm. Una unidad de GR se define como la cantidad de enzima que oxida 1 mol de NADPH/min, los datos se expresaron como U/mg de proteína.

5.6 La extracción de proteínas a partir de la corteza renal.

Se disecó la corteza de una sección del riñón izquierdo, la cual se colocó en solución de Krebs-bicarbonato (KB) [Ali y Sultana., 2012] sobre hielo (mM): NaCl 110, $NaHCO_3$ 25, KCl 3, $CaCl_2$ 1.2, $MgSO_4$ 0.7, KH_2PO_4 2, acetato de sodio 10, glucosa 5.5, alanina 5 y albúmina bovina 0.5 g/L; pH 7.4. Se lavaron tres veces con KB y se resuspendieron en 10 mL de la misma solución con 0.2 g/100 mL de colagenasa. Las muestras se incubaron en un baño María con agitación a 37ºC durante 30 min. Posteriormente se colocaron 10 mL de KB frío; el cual contenía un cóctel de inhibidores de proteasas (Complete, Roche) y PMSF (20 mg/ml). Esta solución se filtró para eliminar fibras de colágeno y se centrifugó (18 x g/30 s) por 3 veces, el precipitado se resuspendió en amortiguador ensayo de radio-immunoprecipitación (RIPA; mM): Tris-HCl 40, NaCl 150, EDTA 2, glicerol 10%, Tritón X-100 1%, deoxicolato de sodio 0.5% y SDS 0.2%; pH 7.6. Las muestras se incubaron durante 30 min a 4°C. Posteriormente, las muestras se sonicaron 3 veces 30 segundos, a 1.5 de intensidad sobre hielo, y se centrifugaron a 14,000 x g, a 4°C por 40 min. Se obtuvo el sobrenadante y se cuantifico la proteína total mediante un

estuche comercial (Micro BCA Assay Reagent Kit; Laboratorio BioRad, Hercules, CA, EUA).

5.7 Aislamiento de glomérulos y túbulos proximales.

En el riñón derecho la corteza renal se separó macroscópicamente. Los glomérulos se obtuvieron mediante el método descrito por Gauthier y Mannik [1988]. Después de la obtención de la corteza renal, se hacen cortes finos sobre el tejido, los cuales se filtran a través de un tamiz con un poro de 117 μm (Thomas Scientific, Swedesboro, NJ, EUA), bajo una suave presión, el tejido tamizado contiene una preparación enriquecida en los glomérulos y se centrifugó a 20,000 x g por 20 min. Se recuperó el sobrenadante en donde se tiene una fracción enriquecida con glomérulos [Bautista-Garcia *et al*., 2013].

El aislamiento de túbulos proximales se llevó a cabo de acuerdo con el protocolo descrito por Vinay cols [1981]. En el riñón izquierdo la corteza renal se separó macroscópicamente, posteriormente se hacen cortes finos sobre el tejido, se incuba con una solución de Ringer-Krebs bicarbonato (RKB) que contiene 0.15% de colagenasa y 5% de albúmina sérica bovina a 37°C durante 25 min, se agitó, se filtró y se centrifugó, el botón resultante se resuspendió en solución de Percoll y se centrifugó a 20,000 x g por 40 min; estableciendo un gradiente de densidad mediante el cual se separan los diferentes componentes del extracto de corteza renal. Se obtuvieron 4 secciones; la primera es la fracción enriquecida con glomérulos, la siguiente fracción es rica en túbulos distales, la sección 3 contiene una fracción enriquecida en túbulos proximales y finalmente la sección 4 contiene

restos celulares mixtos. El contenido de cada fracción empleada fue confirmado por la observación de microscopía de luz.

5.8 Análisis de Western blot

Las muestras de corteza renal se desnaturalizaron a 95°C durante 12 min, después se diluyeron 1:5 en amortiguador 5x Laemli con urea (5 M). Posteriormente se llevó a cabo una electroforesis en geles de SDS-poliacrilamida al 12% [Pedraza-Chaverri *et al*., 1996]. Las proteínas se transfirieron a membranas de fluoruro de polivinilideno (PVDF; Amersham Biosci, Uppsala, Suecia). Las membranas se bloquearon para unión no específica, mediante incubación con leche libre de grasa al 5% en amortiguador de fosfatos-salina (PBS) 1x (Tween 20 0.4%) durante 1 h, a temperatura ambiente. Las membranas se incubaron durante toda la noche a 4°C con los anticuerpos primarios de nuestro interés: NGAL, KIM-1, caspasa 3 activa, pro-caspasa, TGF-β1, colágeno I y IV, α-SMA, Nrf2, 3-NT, gp91^{phox}, p47^{phox}, PKCβ2, ocludina claudina-2, cadherina-E y GAPDH, este último como control de carga (1:1,000). Los anticuerpos secundarios de todas estas proteínas se incubaron durante 1 h a temperatura ambiente (1:20,000, excepto gp91^{phox}, p47^{phox} 1:10,000). La quimioluminiscencia se reveló a través de ECL™ (Amersham; GE Healthcare, Buckinghamshire, Reino Unido) y las imágenes se capturaron mediante un sistema de EC3 (UVP Biolmaging Systems, Cambridge, UK). La densidad de cada banda de proteína se cuantificó por densitometría (Software UVP Biolmaging Systems, Cambridge, Reino Unido).

5.9 Producción de $O_2^{\bullet-}$ en el glomérulo y el túbulo proximal.

La producción de $O_2^{\bullet-}$ se determinó en homogenado de las fracciones renales enriquecidas con glomérulos y túbulo proximal. Los homogenados de las fracciones se centrifugaron a 800 x g, a 4°C por 10 min para remover células rotas y procesarlas inmediatamente. Para determinar la producción de $O_2^{\bullet-}$ y su fuente, se empleó el método fluorométrico descrito por Satoh y cols [2005] el cual se basa en la oxidación del compuesto fluorescente DHE a etidio y se mide a una longitud de onda de excitación de 480 nm y de emisión de 610 nm, cada 2 min por 16 min. Brevemente al homogenado se le adiciona la mezcla de reacción que contiene DHE 400 μM, 10 mg/mL ADN de salmón, el inhibidor (DPI 1.125 mM), el NADH se utilizó como sustrato de la NADPH oxidasa. Los resultados se corrigen por mg de proteína, la cual se determina por el método de Lowry.

5.10 Análisis histológico

Los riñones derechos se seccionaron transversalmente y se fijaron en solución de formalina al 10% durante 12 h y se embebieron en parafina. Se llevaron a cabo cortes histológicos de 4-5 micras de espesor y se tiñeron con H&E, utilizando procedimientos estándar. Los cortes teñidos con H&E se evaluaron por un patólogo experto de forma doble ciego, con una cámara digital incorporada a un microscopio óptico Zeiss Axiophot 2. A través del software, AxioVision 4.8. El grado de la lesión tubular se calculó de forma semicuantitativa en 8 campos aleatorios subcorticales y periglomerular (aumento x200) por cada rata para la apoptosis, necrosis tubular aguda y la formación de cilindros, utilizando una escala de 0 a 3: 0 (ausencia); 1+ (leve o <5%); 2+ (moderada o 5 a 25%); 3+ (grave o >25%).

5.11 Inmunofluorescencia (IF)

Los riñones de los grupos adicionales se prepararon para la IF de la manera descrita anteriormente por Arrreola-Mendoza y cols. [2009]. Los cortes renales se incubaron durante toda la noche a 4°C con los anticuerpos primarios anti-KIM-1, anti-claudina-2, anti-ocludina, anti-DppD, (1:100). Este último empleado como marcador de la membrana apical del borde en cepillo de los túbulos proximales, mientras que los anticuerpos secundarios Alexa Fluor ® 488 y Alexa Fluor ® 594 fueron incubados (1:300) por 2h a temperatura ambiente en oscuridad. La IF se evaluó utilizando un microscopio invertido confocal (TCS-SP2, Leica, Heidelberg, Alemania). Los experimentos de IF se realizaron por triplicado, la señal inespecífica de IF se estimó mediante la omisión de los anticuerpos primarios en la incubación.

5.12 Análisis estadístico

Todos los valores se expresaron como el promedio ± el error estándar. Se utilizó la prueba t no pareada para comparar los datos de los dos grupos. Para comparar los datos de producción de $O_2^{\bullet -}$ se utilizó ANOVA de una vía y las diferencias entre grupos se determinaron con la prueba de Bonferroni. Los datos se analizaron con el software Graph Pad Prism 5.0 (GraphPad Software, Inc., La Joya, CA, EUA). Los valores de $P < 0.05$ se consideraron significativos.

VI. Resultados

6.1 Evaluación de la actividad antioxidante de la curcumina "in vitro".

En la figura 9 se presenta la actividad antioxidante de la curcumina contra los principales ERO: $O_2^{\bullet-}$, $^1O_2$, H_2O_2, $OH^{\bullet}$, $ONOO^-$, HOCl y $ROO^{\bullet}$. Se observa que la curcumina atrapa a las ERO de manera dependiente a su concentración.

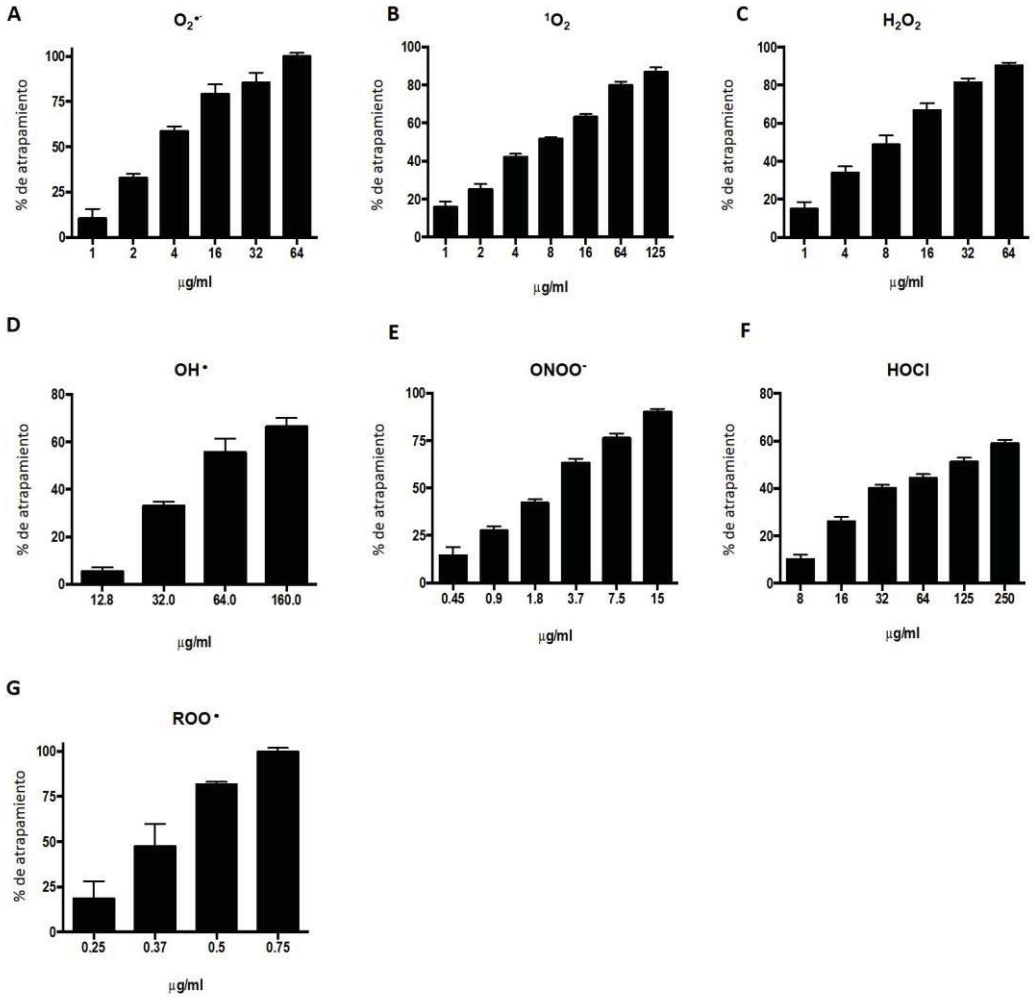

Figure 9. Actividad antioxidante de la curcumina. **A:** superóxido; $O_2^{\bullet-}$, **B:** oxígeno singulete; $^1O_2$, **C:** peróxido de hidrógeno; H_2O_2, **D:** radical hidroxilo; $OH^{\bullet}$, **E:** anión peroxinitrito; $ONOO^-$, **F:** ácido hipocloroso; HOCl, **G:** radical peroxilo; $ROO^{\bullet}$. Los valores se presentan como promedios ± error estándar; n=3.

En la tabla 1 se presentan los valores de IC_{50}. El orden de eficiencia de curcumina para secuestrar la ERO fue la siguiente: $HOCl > {}^1O_2 > O_2^{\bullet-} > H_2O_2 > ONOO^-$. En el caso de radicales férricos observamos que la curcumina fue capaz de atraparlos a una concentración de 4548.2 ± 109.4 μmoles equivalentes Fe_2SO_4/g de curcumina. En conjunto estos datos demuestran que la curcumina utilizada en este trabajo es un antioxidante directo eficaz.

Tabla 1. Actividad antioxidante de la curcumina y compuestos de referencia. Los datos son expresados como IC50 (μg/mL).

	$O_2^{\bullet-}$	$^1O_2$	H_2O_2	$OH^{\bullet}$	$ONOO^-$	HOCl	$ROO^{\bullet}$
Curcumina	3.9±0.5	8.6±0.4	7.4±0.3	56.2±7.8	2.3±0.2	106±5	0.35±0.03
Compuesto referencia	3.7±0.3 (1)	576±6* (2)	136.5±9.5* (3)	67.0±7.2 (4)	9.9±1.4* (5)	75±3* (6)	1.2±0.08* (7)
p	0.748	0.0016	0.0002	0.7254	0.0058	0.006	0.0006

(1) Sodio 4,5-dihidroxibenceno-1,3-disulfonato (Tiron); (2) Ácido lipoico; (3) Piruvato; (4) Dimetiltiurea; (5) Penicilamina; (6) Ácido ascórbico; (7) Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico (Trolox). Los datos son expresados como el promedio ± error estándar resultado de 3 experimentos. Los asteriscos indican la significancia estadística, el valor de p en la parte baja de la tabla.

6.2 Evaluación del daño renal inducido por CP en ratas.

La administración de CP, como era de esperarse, indujo disfunción renal que fue evidenciado por un incremento significativo en la creatinina plasmática (Fig. 10A) y el BUN (Fig. 10B) en comparación con los animales tratados con V. También la administración de CP provocó lesión tubular, determinado por la disminución de la actividad renal de NAG (Fig. 10C), un marcador sensible del daño en túbulo proximal; así como por un incremento en la expresión de NGAL (Figs. 10D, E). En la Fig. 10D se presenta el análisis densitométrico y en la Fig. 10E se presenta una imagen representativa de un Western blot de NGAL. La administración de curcumina previno la disfunción renal (Figs. 10A y 10B) y protegió al riñón del daño tubular por CP (Figs. 10C-E). En el grupo de animales tratados sólo con curcumina, no se observaron cambios funcionales ni daño renal.

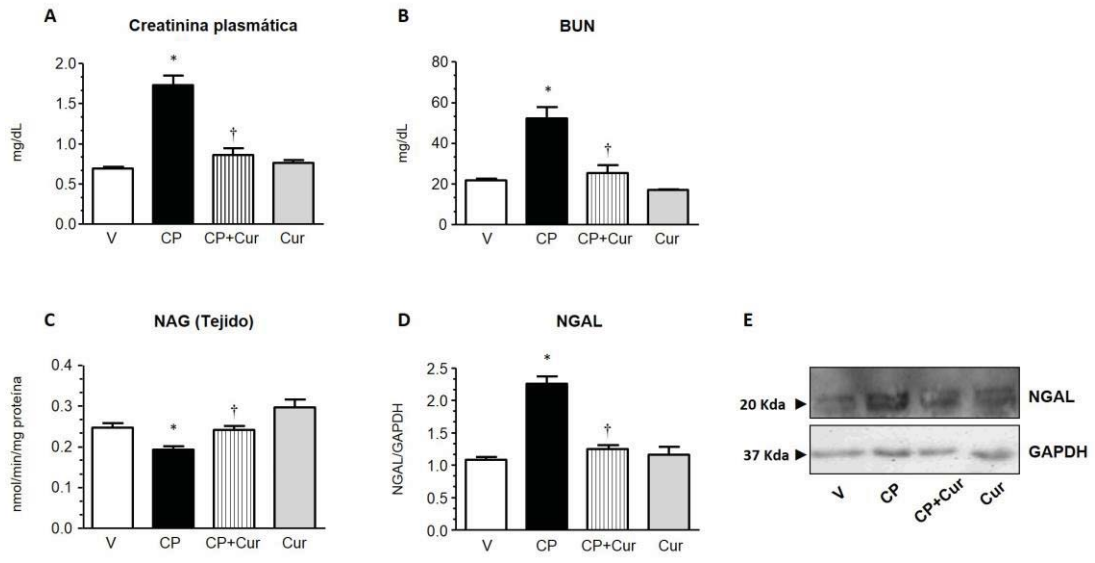

Figura 10. Marcadores de daño renal en ratas con nefrotoxicidad inducida por CP. Niveles de creatinina plasmática (A), nitrógeno de urea sanguíneo (BUN; B), N-acetil-β-D-glucosaminidasa (NAG; C), lipocaina asociada a la gelatinasa de neutrófilos (NGAL; D). En el análisis densitométrico del ensayo de Western blot, los datos están expresados como la densidad relativa de 4 ratas por grupo y normalizados con gliceraldehido-3-fosfato (GADPH) como control de carga. Una imagen representativa del Western blot de NGAL se presenta en el panel E. Los valores son los promedios ± error estándar. (Vehículo; V; n=5), 5 mg/kg de cisplatino (CP; n=9), 5 mg/kg de CP + 200 mg/kg de curcumina (CP+Cur; n=9) y 200 mg/kg de curcumina (Cur; n=5). *P<0.05 vs. V; † p<0.05 vs CP.

La nefrotoxicidad inducida por CP se caracteriza por daño en el túbulo proximal, por lo que se evaluaron los niveles de KIM-1, proteína transmembrana que en condiciones fisiológicas normales no se detecta en el tejido renal, pero se sobre-expresa en células epiteliales en humanos y roedores después de la isquemia-reperfusión o de la administración de tóxicos [Charlton *et al*., 2014; Han *et al*., 2002; Ichimura *et al.*, 2004]. La administración de CP aumentó de manera significativa la presencia en túbulo proximal (Fig. 11B) y aproximadamente 5 veces la expresión de KIM-1 (Figs. 11E y 11F) en comparación con el grupo V (Fig. 11A). Cuando a los animales con nefrotoxicidad por CP se les administró conjuntamente curcumina, se

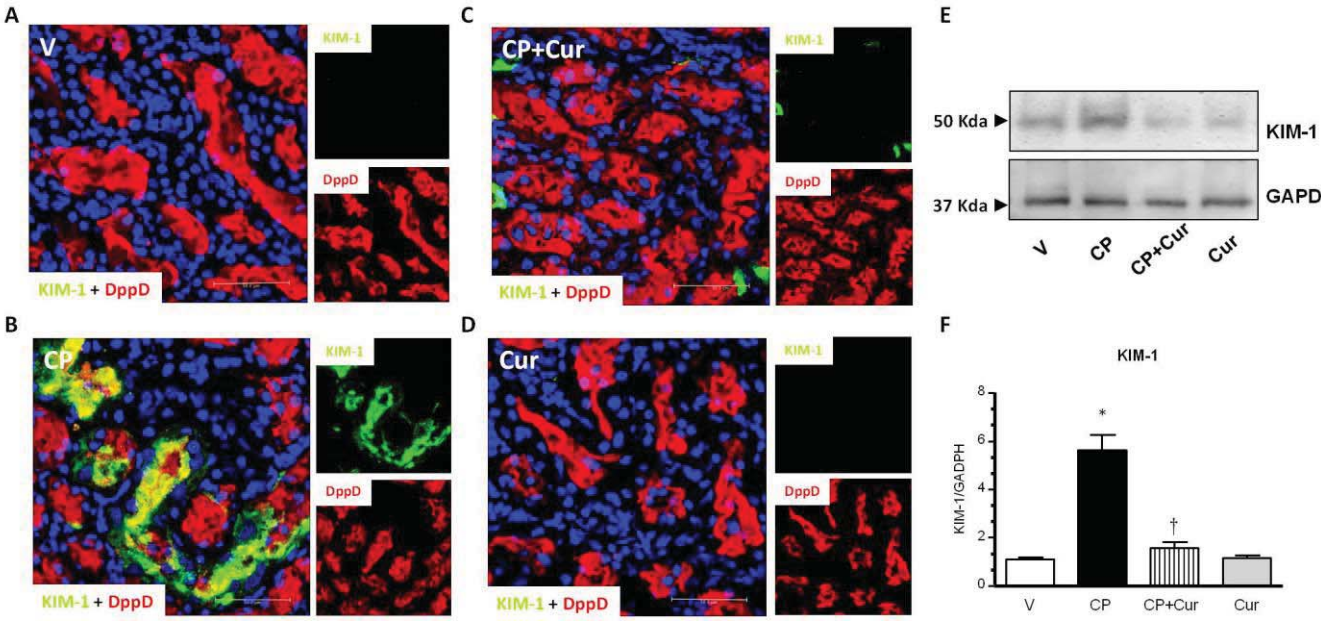

Figure 11. La localización de la molécula de daño renal (KIM-1) por microscopia confocal (A-D; marca en verde) en los cuatro grupos experimentales, la expresión de dipeptil peptidasa (DppD; marca en rojo) se usó como marcador de la membrana apical del túbulo proximal y 4', 6-diamino-2-fenilindol (DAPI; marca en azul) se usó como marcador de núcleos celulares. La sobreposición de las diferentes marcas se presenta en los paneles mayores. Una imagen representativa y el análisis densitométrico del Western blot de KIM-1 se presenta en los paneles E y F, respectivamente; los datos están expresados como la densidad relativa de 3 ratas por grupo en los estudios de inmunoflouroscencia y 4 ratas por grupo en el Western blot y normalizados con GADPH como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP. Barra=50 μm.

previno el aumento en la expresión de KIM-1 (Fig. 11C). Este marcador de daño renal no se modificó en los animales tratados sólo con curcumina (Fig. 11D).

6.3 Daño histológico y la expresión de caspasa 3-activa inducidos por CP.

El estudio del daño histopatológico de las ratas tratadas con 5 mg/kg de CP reveló que las alteraciones características en el tejido renal después de la administración de este antineoplásico se caracteriza por desprendimiento de células epiteliales, apoptosis (↑), necrosis tubular (*) y por la formación de cilindros (★) en el lumen tubular (Fig. 12B, Tabla 2), también se observa un mayor aumento (x400) del tejido renal dañado. En la Fig. 12C se observa una imagen representativa de animales tratados con CP+Cur la cual presenta menor daño. En el grupo de animales tratados con vehículo, no se observaron cambios morfológicos (Fig. 12A, Tabla 2). Para confirmar la activación de apoptosis inducida por CP evaluamos la expresión de caspasa 3-activa por Western blot, y observamos un incremento significativo de ≈2.5 veces en los animales tratados con CP e interesantemente la administración de curcumina junto con CP, previno la activación de apoptosis (Fig. 12E y 12F).

Tabla 2. Cambios morfológicos inducidos en la nefrotoxicidad por CP.

Lesión	V	CP	CP+Cur	Cur
Apoptosis	-	+++	++	-
Necrosis tubular aguda	-	+	-	-
Formación de cilindros	-	+++	++	-
Desprendimiento celular	+	+++	+++	+

La severidad del daño tubular se calculó mediante un análisis semi-cuantitativo en 8 campos continuos subcorticales (ampliación x200) por cada rata para apoptosis, necrosis tubular aguda, formación de cilindros y desprendimiento celular usando una escala 0 to 3: -, ausencia de daño; 1+ (medio o <5 % del tejido dañado); 2+ (moderado o 5 al 25%); 3+ (severo o >25%) de los túbulos juxtamedulares.

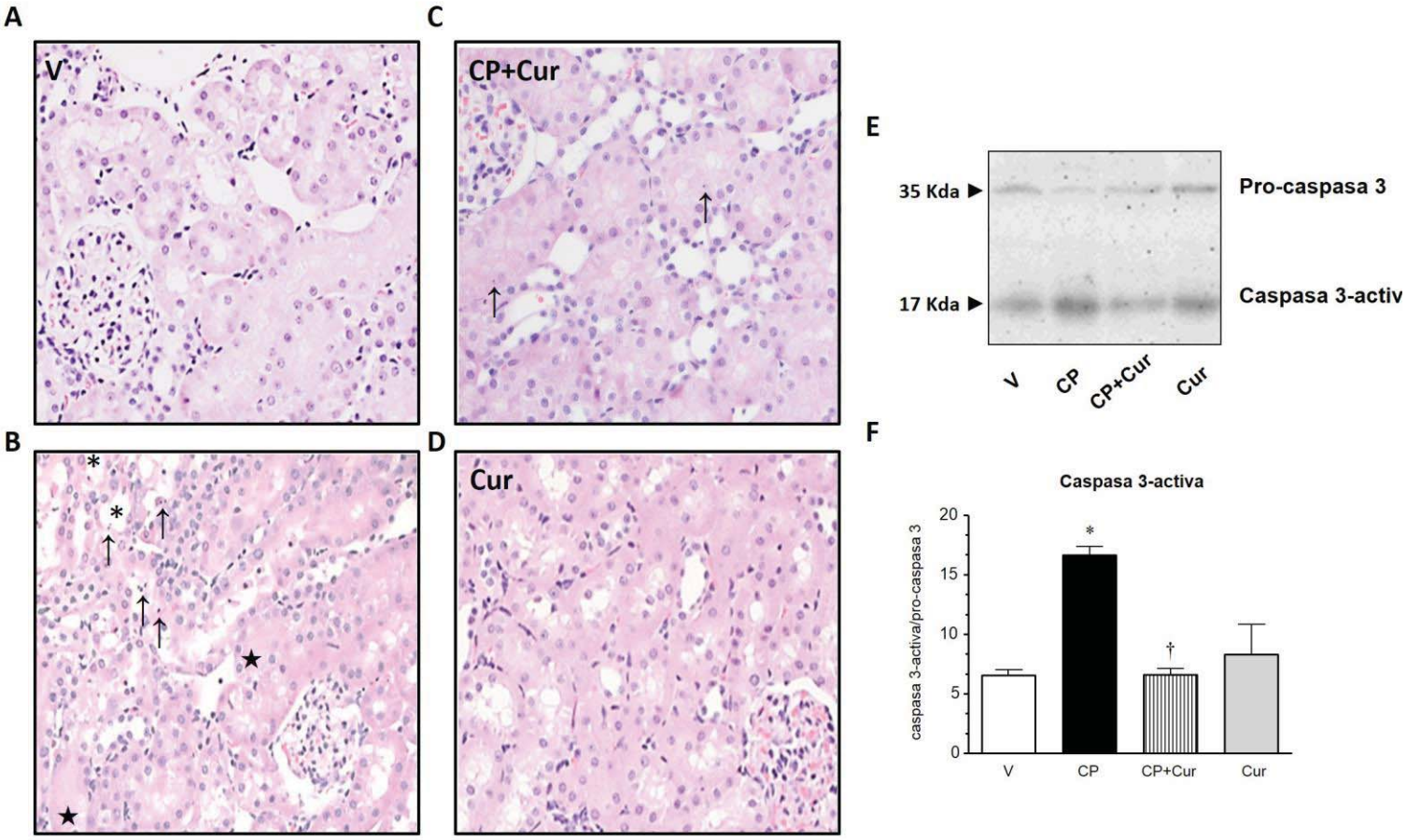

Figure 12. El CP indujo cambios morfológicos a nivel renal y la administración de curcumina evita la aparición del daño morfológico. Se presenta la microscopia de luz, de cortes teñidos con hematoxilina-eosina (H&E) a las 72 h en los cuatro grupos estudiados. En el panel superior izquierdo (A) se presenta una imagen representativa de animales tratados con vehículo los cuales no presentan lesiones morfológicas, en el panel inferior izquierdo (B) se presenta el grupo que recibió CP con células apoptóticas (↑), necrosis tubular aguda (*) y formación de cilindros (★), en el panel superior derecho (C) se presenta el grupo que recibió CP+Cur, en el panel inferior derecho (D) se presenta el grupo tratado solo con curcumina sin lesión renal. La ampliación original es x200. Una imagen representativa y el análisis densitométrico del Western blot de caspasa 3 activa, forma activa de apoptosis, se presenta en los paneles E y F, respectivamente. Los datos están expresados como la densidad relativa de 4 ratas por grupo y normalizados con pro-caspasa como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP

Los cambios morfológicos inducidos por CP, como apoptosis, se han asociado con la inducción de factores profibróticos como TGF-β. En este estudio evaluamos la expresión de TGF-β, colágeno I y IV y α-SMA por Western blot (Fig. 13); además esta última proteína se usa como marcador de EMT. La administración de CP incrementa la expresión de TGF-β (Fig. 13A), colágeno I (Fig. 13B), colágeno IV (Fig. 13C) y α-SMA (Fig. 13D) en comparación con el grupo V. La administración con curcumina previno el incremento de TGF-β y colágeno y redujo significativamente la expresión de colágeno IV y α-SMA; lo que sugiere que la curcumina previene el estado profibrótico en los animales que reciben CP.

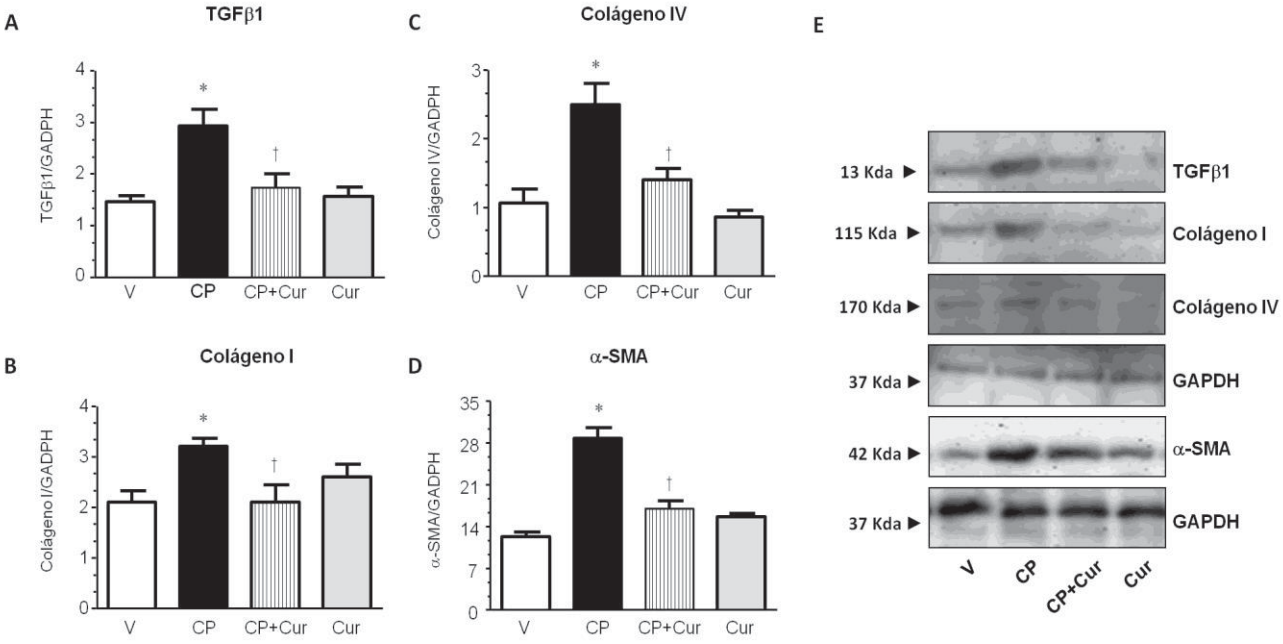

Figure 13. La curcumina previno el incremento de factores pro-fibróticos inducidos por la administración con CP. Los análisis densitométricos del Western blot del factor de crecimiento transformante beta 1 (TGFβ1; panel A), colágeno I (panel B), colágeno IV (panel C) y actina del músculo liso alpha (α-SMA; panel D). Las imágenes representativas de los Western blots (Panel E) de TGFβ1, colágeno I, colágeno IV, α-SMA y GAPDH se presentan el panel E. Los datos están expresados como la densidad relativa de 4 ratas por grupo y normalizados con GADPH como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP.

6.4 Evaluación del estrés oxidante y la actividad de las enzimas antioxidantes en ratas con nefrotoxicidad por CP.

El daño renal por CP indujo estrés oxidante que se evidenció por un incremento significativo del 60% de los niveles de MDA (un índice de la lipoperoxidación, Fig. 14A) y una reducción del 25 y 20% en la actividad de catalasa (Fig. 14B) y GR (Fig. 14C), respectivamente con respecto al V. Con el fin de explorar la participación de la vía de Nrf2, la cual promueve una respuesta antioxidante, evaluamos la expresión de este factor por Western blot en el daño renal ejercido por CP y como se observa en las Figuras 14D y 14F, los niveles de Nrf2 se reducen significativamente después de 72 h a partir de la dosis unica de CP versus V. Interesantemente la administración de curcumina previene la reducción de Nrf2 (Fig. 14D y 14E). Previamente se ha descrito que el estrés nitrosativo, evaluado por la presencia de 3-NT, la cual está implicada en el daño por CP. En este trabajo se evaluaron los valores de 3-NT mediante Western blot en homogenado renal y se observó un incremento significativo en los valores de 3-NT asociado a proteínas de pesos moleculares diferentes (Fig. 14E y 14F). Mientras que en los animales tratados con CP en conjunto con curcumina también se presentó una reducción en el estrés nitrosativo.

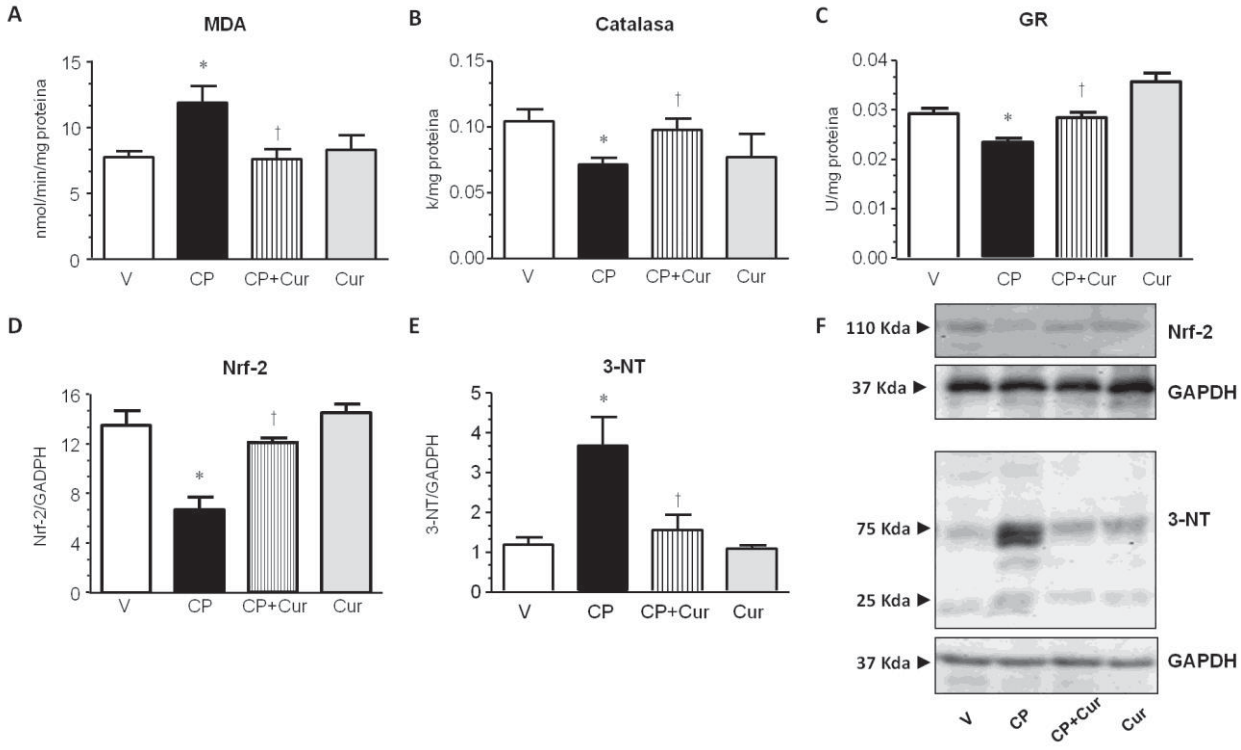

Figure 14. Estrés oxidativo y nitrosativo en animales con nefrotoxicidad por CP. Los niveles de malondialdehído (MDA; Panel A) son un índice de lipoperoxidación, la actividad de las enzimas antioxidantes en homogenados renales (catalasa; panel B y glutatión reductasa (GR; panel C). Los análisis densitométricos de la expresión del factor nuclear eritroide-2-asociado al factor 2 (Nrf-2; panel D) y de los niveles de 3-nitrotirosina (3-NT; paneles E). Los Western blots representativos de Nrf2 y 3-NT se presentan en el panel E. Los datos están expresados como la densidad relativa de 4 ratas por grupo y normalizados con GADPH como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP.

6.5 Estimación de la producción de $O_2^{\bullet-}$ en glomérulo y túbulo proximal inducida por CP.

Debido a nuestros resultados y estudios previos en nuestro laboratorio, inferimos la participación del estrés oxidativo en la nefrotoxicidad inducida por CP y previamente establecimos que el glomérulo y el túbulo proximal son los segmentos de la nefrona que participan de manera más activa en la generación de $O_2^{\bullet-}$ [Trujillo *et al*., 2014a], analizamos la producción de $O_2^{\bullet-}$ mediante la oxidación de DHE a

Etidio. Como se observa en la Figura 15 la administración de CP incrementa la producción de $O_2^{\bullet-}$ 6 veces en glomérulo (Fig. 15A) y 4 veces en túbulo proximal (Fig. 15B), comparado con el grupo V. La curcumina redujo significativamente la producción de $O_2^{\bullet-}$ inducida por CP. Como previamente describimos que la NADPH oxidasa es la principal fuente de $O_2^{\bullet-}$ en la nefrotoxicidad por CP [Trujillo et al., 2014a], en este estudio, evaluamos la activación de NADPH oxidasa para inducir la generación de $O_2^{\bullet-}$, a través del empleo DPI, un inhibidor de NADPH oxidasa y se ratifico que inhibía el incremento en la producción de $O_2^{\bullet-}$ inducido por CP en glomérulo y túbulo proximal (Figs. 15A y 15B, respectivamente); indicando que la NADPH oxidasa es una fuente potencialmente importante de $O_2^{\bullet-}$ en la nefrotoxicidad inducida por CP.

6.6 Evaluación de la expresión de gp91^{phox} y p47^{phox} inducido por CP.

Para corroborar la activación de NADPH oxidasa por CP se determinó, mediante Western blot en homogenado renal, la expresión de las subunidades gp91^{phox} y p47^{phox} de la NADPH oxidasa, las cuales forman parte del complejo de la NADPH oxidasa activa, y se encontró que la administración de CP induce un incremento significativo en la expresión de p47^{phox} en comparación con el grupo V (Figs. 15C y 15F) y de gp91^{phox} (Figs. 15D y 15F). La administración de curcumina previene la activación del complejo activo de NADPH oxidasa, inducido por CP en la subunidades p47^{phox} (Fig. 15C y 15F) y gp91^{phox} (Fig. 15D y 15F).

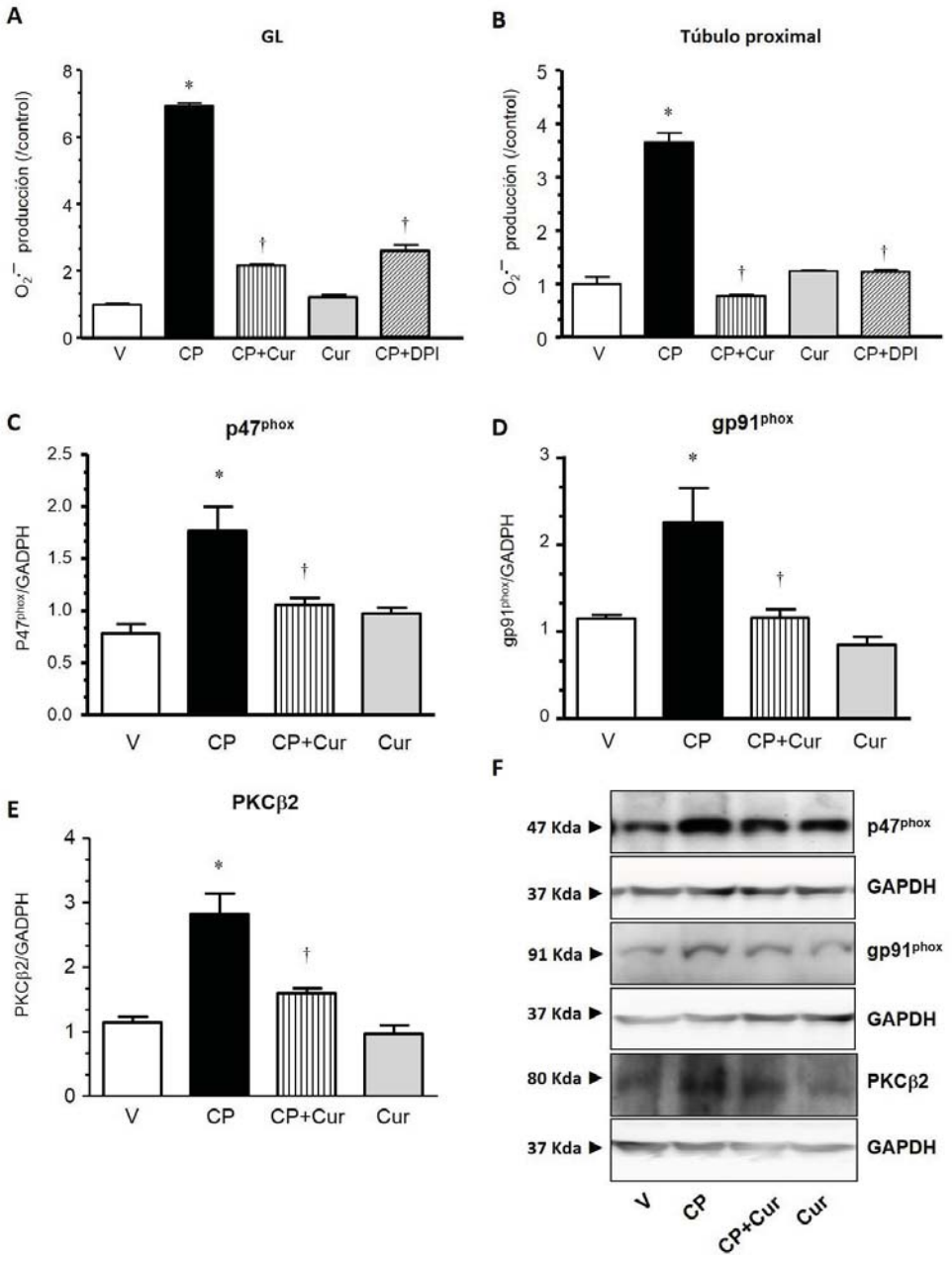

Figure 15. Producción de superóxido ($O_2^{\bullet-}$) en glomérulos aislados (GL; panel A) y túbulo proximal (TP; panel B), se evaluó la producción de $O_2^{\bullet-}$ en presencia de un inhibidor de NADPH oxidasa, difenileno de yodonio (DPI; barra con líneas diagonales paneles A y B), mediante un ensayo de dihidroetidio a etidio y medido por fluorescencia, los resultados se expresan como la producción de $O_2^{\bullet-}$ relativa al vehículo. Tambien se evaluaron los valores de expresión de las subunidades de NADPH oxidasa: $p47^{phox}$ (panel C) y $gp91^{phox}$ (panel D); asi como la proteína cinasa C beta 2 (PKCβ2; panel E) en los cuatro grupos estudiados, se presentan los análisis densitométrico del Western blot (n=3) para cada una de las proteínas. Los Western blots representativos de $p47^{phox}$, $gp91^{phox}$ y PKCβ2 se presentan en los paneles F y los datos están expresados como la densidad relativa de 4 ratas por grupo y normalizados con GADPH como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP.

6.7 Evaluación de la expresión de la PKCβ2 inducido por CP.

Otro factor implicado en la nefrotoxicidad por CP es la PKC que pueden modular el daño por estrés oxidativo [Charlton *et al.*, 2014; Han *et al.*, 2002; Ikeda *et al.*, 1999]. En nuestro estudio se observó que un incremento significativo de la expresión de PKCβ2 (Figs. 15E y 15F), en comparación con el grupo V, el cual se previnó por la administración de curcumina.

6.8 Proteínas de UC; ocludina, claudina-2 y cadherina-E a nivel renal inducidas por la administración de CP.

Se ha demostrado que la administración de CP ocasiona la pérdida de los contactos entre célula y célula, específicamente el CP induce la pérdida de los contactos entre las células epiteliales renales proximales, al modificar la localización de las uniones adherentes asociadas a β-catenina [Imamdi *et al.*, 2004]. De ahí el interés por analizar si el CP afecta la distribución y la expresión de las proteínas de UC (ocludina, claudina-2 y cadherina-E) y si el uso de un antioxidante como curcumina es capaz de prevenirlo. Para evaluar dichos parámetros realizamos microscopía confocal y análisis de Western blot en los cuatro grupo de ratas que estudiamos. Se observó que el CP redujo la presencia en túbulo proximal de claudina-2 (Fig. 16A-D), ocludina (Fig. 16E-H) y cadherina-E (Fig. 17A-D) como lo revelan las microfotografías de IF. Así como su expresión a nivel renal de claudina-2 (Fig. 16I), ocludina (Fig. 16J) y cadherina-E (Fig. 17E) con respecto a las ratas del grupo V. Cabe mencionar que DppD y DMPK son marcadores de túbulo proximal y

túbulo distal, respectivamente; indicando que la reducción en la expresión de claudina-2, ocludina y cadherina-E es selectiva.

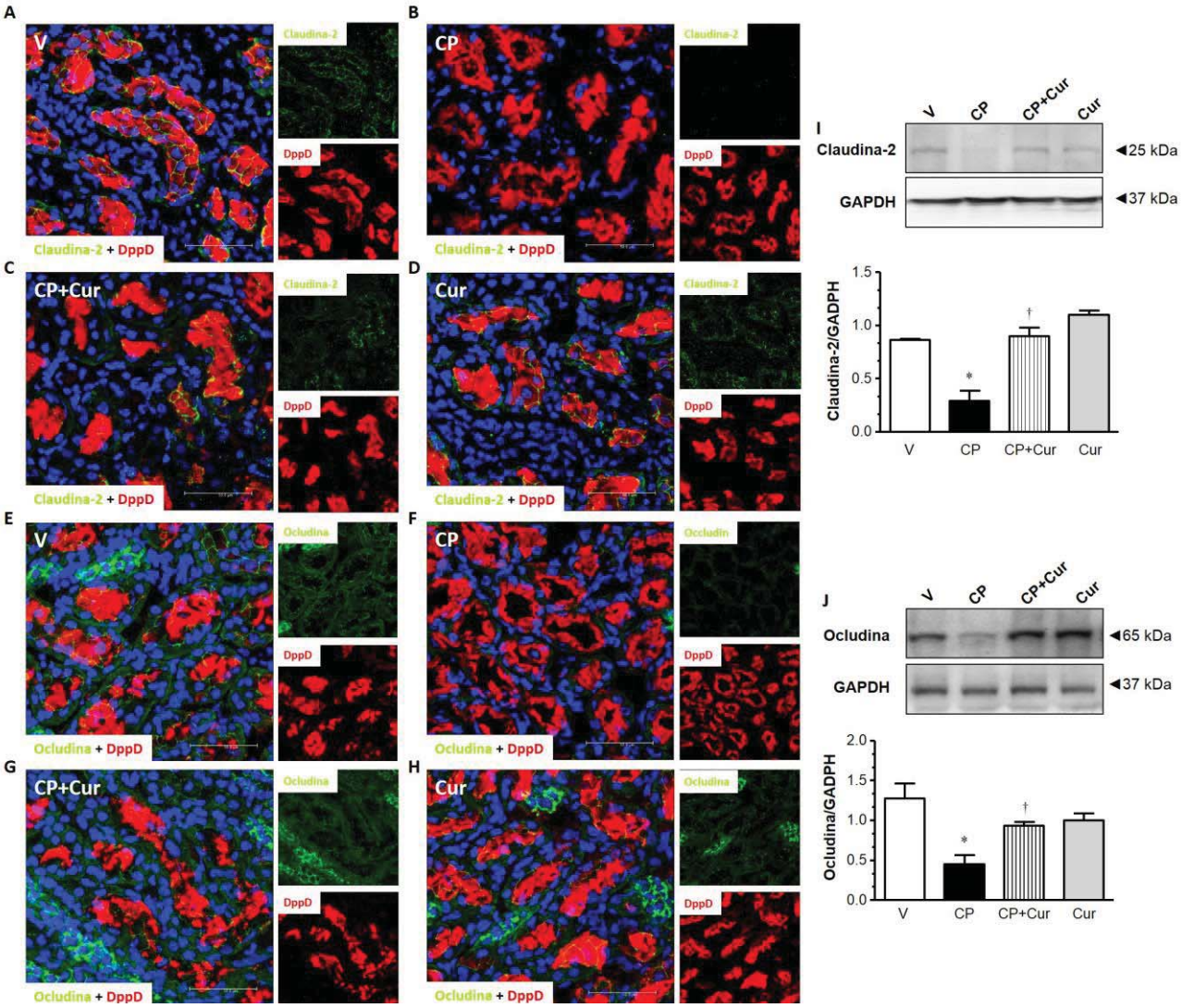

Figure 16. El cisplatino disminuye la presencia y expresión de proteínas de la integridad de la unión celular: claudina-2 y ocludina. La localización de claudina-2 y ocludina se realizó por inmunofluorescencia (IF) mediante microscopía confocal (paneles A-H; marca en verde para claudina-2 y ocludina), medida en los cuatro grupos experimentales. La claudina-2 y la ocludina se detectaron en el borde en cepillo de las células tubulares proximales ayudados por la expresión de dipeptil peptidasa (DppD; marca en rojo) que se usó como marcador de la membrana apical del túbulo proximal mientras que la tinción con 4', 6-diamino-2-fenilindol (DAPI; marca en azul) se usó como marcador de núcleos celulares. La sobreposición claudina-2 y ocludina con DppD se observa en los paneles mayores de A-H. Los Western blots (WB) representativos y su respectivo análisis densitométrico de claudina-2 y ocludina se presentan en los paneles I y J, respectivamente. Los datos para IF son experimentos con 3 muestras aleatorias, los datos para WB están expresados como la densidad relativa de 3 ratas por grupo y normalizados con GADPH como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP. Barra=50 μm

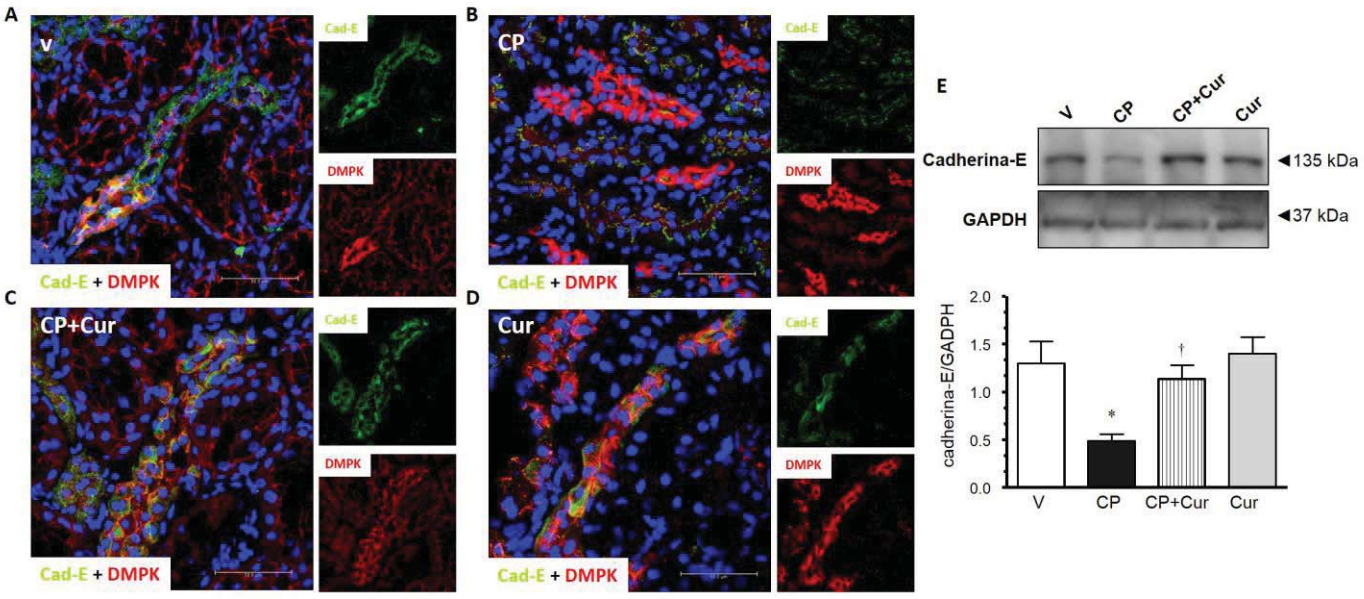

Figure 17. Presencia y expresión de cadherina-E, la localización de cadherina-E se realizó por inmunofluorescencia (IF) mediante microscopía confocal (paneles A-D; marca en verde) en los cuatro grupos experimentales. La cadherina-E se detectó en la membrana basolateral de las células tubulares distales ayudados por la expresión de desmoplaquina (DMPK; marca en rojo) que se usó como marcador, mientras que la tinción con 4', 6-diamino-2-fenilindol (DAPI; marca en azul) se usó como marcador de núcleos celulares. La sobreposición cadherina-E con DMPK se observa en los paneles mayores de A-D. El Western blot (WB) representativo y su respectivo análisis densitométrico de cadherina-E se presentan en el paneles E. Los datos para IF son experimentos con 3 muestras aleatorias, los datos para WB están expresados como la densidad relativa de 3 ratas por grupo y normalizados con GADPH como control de carga. Los valores son los promedios ± error estándar. *P<0.05 vs. V; † p<0.05 vs CP. Barra=50 μm

VII. Discusión

El CP es un fármaco quimioterapéutico con varios efectos secundarios como ototoxicidad, hepatotoxicidad, neurotoxicidad y nefrotoxicidad, lo que limita su uso; las células epiteliales tubulares proximales son blanco del CP [Miller *et al.*, 2010].

De acuerdo con nuestros resultados los animales tratados con CP desarrollaron IRA, asociado a un incremento en la expresión de KIM-1 e incremento en la expresión de NGAL, ambos son biomarcadores sensibles y precoces del daño renal en túbulo proximal y distal [Bonventre., 2009; Kramer *et al*., 2009; Mishra *et al.*, 2004]. También observamos disminución en la actividad renal de NAG, aumento en creatinina plasmática y BUN; así como daño renal histológico evidenciado por la presencia de daño vascular y tubular; así como necrosis tubular aguda, apoptosis y formación de cilindros en la luz tubular; interesantemente la administración de curcumina previno el desarrollo de daño funcional y renal inducido por CP.

El CP, al intercalarse en la hebra de ADN, induce el arresto del ciclo celular que activa genes involucrados en la reparación del daño, generando una cascada de señalización capaz de iniciar procesos de apoptosis y/o necrosis, básicamente a través de la vía de las caspasas [Wang y Lippard., 2005; Lieberthal *et al*., 1996]. Se conoce que el CP induce 2 tipos de muerte celular apoptosis y necrosis, ambos mecanismos se han observado *in vivo* [Wang y Lippard., 2005, Yousef y Hussien., 2015]. Para comprobar la asociación de muerte celular por apoptosis con el daño histológico característico de nuestro modelo, evaluamos citocinas profibróticas como TGF-β y colágeno I y IV [Park *et al*., 2013; Pérez-Rojas *et al.*, 2011; Ramesh y

Reeves., 2002], las cuales incrementaron significativamente; asociado con un incremento en la expresión de caspasa-3 activa, mostrando la activación del proceso profibrótico y apoptosis; procesos que fueron prevenidos por la administración de curcumina. En apoyo a estos datos, recientemente se demostró que la administración de curcumina en ratas con nefrotoxicidad inducida por CP promovió el mantenimiento de NAMPT y sirtuina, proteínas implicadas en la resistencia al estrés y la modulación de muerte celular, ya que inhibe la apoptosis [Ugur *et al*., 2015].

Se ha establecido tanto *in vivo* e *in vitro* que el estrés oxidativo/nitrosativo y la reducción en la defensa antioxidante, incluyendo SOD, catalasa y GSH, GST, GPx, GR, GT; algunos de estos estudios asociados con una reducción en la expresión del factor de transcripción Nrf2 [Chirino y Pedraza-Chaverrí., 2009; Chirino *et al.,* 2008b; Enck *et al*., 2001] son parte de los mecanismos involucrados en el daño celular inducido por CP [Kadikoyluy *et al.*, 2004]. En nuestro estudio observamos la participación del estrés oxidativo en la nefrotoxicidad por CP; asociado al incremento en los niveles de MDA, reducción en la actividad de catalasa, GR y Nrf2 y nitración de proteínas (3-NT). La nitración de proteínas es una modificación post-traduccional a las proteínas inducida por $ONOO^-$ bajo condiciones de estrés oxidativo [Radi*.,* 2013], lo que puede modificar la estructura y función de algunas proteínas, esto podría explicar la reducción en los niveles de Nrf2 después de la administración CP. El estrés oxidativo y nitrosativo se inhibió con la administración de un antioxidante; lo que demostró la participación de este mecanismo de daño renal por CP.

En 2001 se demostró que el CP induce la producción de EROs, particularmente $O_2^{\bullet-}$ [Davis *et al.*, 2001]. En apoyo nuestro grupo recientemente

describió que el CP induce un incremento en la producción de $O_2^{\bullet-}$, de manera interesante este aumento fue específicamente en el glomérulo y el túbulo proximal de la nefrona en ratas [Trujillo *et al*., 2014a] y que la fuente responsable de la producción de este anión es la NADPH oxidasa [Kawai *et al*., 2006; Rashed *et al.*, 2011; Deng *et al*., 2012; Gang *et al*., 2013; Trujillo *et al*., 2014a].

Otro grupo demostró que la producción de $O_2^{\bullet-}$ se desencadena principalmente por el aumento en la expresión de la NADPH oxidasa y la sintasa de óxido nítrico [Pan *et al.,* 2009]. Recientemente se demostró que la NADPH oxidasa está involucrada en la nefrotoxicidad inducida por el CP [El-Naga., 2014]. En apoyo a esto, Chirino y cols. [Chirino et al, 2008a] demostraron que la administración de apocinina, un inhibidor de la NADPH oxidasa, previene el desarrollo de nefrotoxicidad inducida por CP, sugiriendo que esta enzima prooxidante es fundamental en el daño renal inducido por CP. Recientemente en nuestro laboratorio observamos un incremento en las subunidades $gp91^{phox}$ y $p47^{phox}$ de la NADPH oxidasa mediante Western blot, en homogenizados de riñones de ratas con nefrotoxicidad inducida por CP [Gomez-Sierra *et al*., 2014; Trujillo *et al*., 2014a], dicho incremento puede explicar el aumento en la actividad de la NADPH oxidasa en este modelo. Además se sabe que la expresión PKCβ2 favorece condiciones de estrés oxidativo, debido a PKCβ2 estimula la actividad de NADPH oxidasa y así aumenta la generación de $O_2^{\bullet-}$ [Kitada *et al*., 2003]. Resultados similares se observaron en nuestro estudio con respecto al incremento en la producción de $O_2^{\bullet-}$, la expresión de las subunidades $gp91^{phox}$ y $p47^{phox}$ y PKCβ2 sugiriendo su participación como mecanismo de daño renal; de manera interesante la curcumina

es capaz de prevenir la generación de $O_2^{\bullet-}$ a través de la inhibición de la activación de la NADPH.

Por otro lado se ha informado que el CP induce la desintegración de las UC; principalmente de las células epiteliales tubulares renales. En estudios anteriores se ha demostrado que la estructura y la función de la UC son sensibles al estrés oxidante inducido por la presencia de metales pesados [Arreola-Mendoza et al., 2009; Jacquillet *et al.*, 2006]. El CP también induce alteraciones en la localización de proteínas de UC como la beta catenina, la cual es una proteína de unión adherente asociada a la inducción de apoptosis en las células tubulares proximales [Imamdi *et a*l., 2004]. La claudina-2 se expresa principalmente en el túbulo proximal [Enck *et al*., 2001] y la ocludina y la cadherina-E se expresan principalmente en los segmentos distales y conductos colectores [González-Mariscal *et al*., 2000; Perantoni*.,* 1999]. Nosotros observaron que en la nefrotoxicidad por CP ocurre una desintegración de la UC por pérdida de claudina-2 y ocludina, sin cambios en presencia de claudina-5 y ZO-1, mediante estudios de microscopía confocal [Trujillo *et al.,* 2014b]. Estos resultados tienen relevancia fisiológica dado que claudina-2 es una proteína fundamental para la reabsorción de sodio y el mantenimiento de los contactos célula-célula en esta porción de la nefrona [Enck *et al*., 2001]. Lo anterior sugiere que la ausencia o reducción en la claudina-2 por CP podría estar relacionado con la disfunción tubular; también se ha informado que la expresión de ocludina incrementa la RET, con un aumento en la cantidad de fibras en la UC [McCarthy *et al.,* 1996]. De manera interesante en nuestro estudio observamos una alteración en la UC por reducción en la expresión de claudina-2, ocludina y E-cadherina en ratas tratados con CP. Además, el aumento de la actividad de las isoformas de PKC

convencionales, como PKCβ, participan en la fosforilación de componentes y desintegración de la TJ [González-Mariscal *et al.*, 2008], por lo tanto la activación de PKCβ2 inducida por CP podría explicar la pérdida de claudina-2 y ocludina en el túbulo proximal. Aunado a esto se ha reportado la co-inmunoprecipitacion de PKCβ2 con claudina-2 en nefropatía diabética temprana. Mientras que la administración de ácido trans-retinoico, un compuesto antioxidante que juega un papel fundamental en el desarrollo y mantenimiento renal, fue capaz de atenuar la pérdida de claudina-2; debido a la disminución en la interacción de PKCβ2 y claudina-2 [Molina-Jijón *et al.*, 2015]. Aunado a esto se conoce que la pérdida de cadherina-E en las células, regula la transición EMT; proceso asociado con remodelación y apoptosis en las células epiteliales tubulares renales [Zheng *et al*., 1999]. La administración de CP reduce la presencia de cadherina-E, principal componente de las uniones adherentes, que reduce la permeabilidad paracelular de la nefrona distal [Perantoni., 1999]. En el proceso de la nefrotoxicidad por CP marcadores epiteliales como la cadherina-E y claudina se pierden, mientras que se induce el aumento de expresión de factores como TGF-β, colágenos y α-SMA implicados en procesos de EMT. En nuestro estudio la administración de curcumina redujo y/o previno procesos como EMT; fibrosis y pérdida de la integridad de la UC, lo que podría estar relacionado con las propiedades antioxidantes de la curcumina.

VIII. Conclusión

En este estudio observamos que la administración de CP induce incremento en el estrés oxidativo/nitrosativo asociado a la pérdida de la actividad de enzimas antioxidantes e inducción de la NADPH oxidasa que conlleva a una mayor generación de $O_2^{\bullet-}$, principalmente en túbulo proximal de la nefrona; así como la desintegración de las UC; esencialmente de las células epiteliales tubulares renales.

Por lo que nuestros resultados sugieren que la vía a través de la cual la curcumina previene el daño funcional y estructural en la nefrotoxicidad por CP; participan varios factores: prevención en la activacion de muerte celular por apoptosis, reducción en el estrés oxidativo asociado a la preservación de algunas de las enzimas antioxidantes e inhibición en la producción de $O_2^{\bullet-}$ en túbulo proximal; asociado a una menor expresión de las subunidades de NADPH oxidasa y PKCβ2; cinasa que también esta implicada en la preservación de la integridad de las UC; asociado con la preservación en la presencia de claudina-2, ocludina y cadherina-E en las células epiteliales tubulares renales; dichos factores podrían estar asociados a las propiedades antioxidantes de la curcumina

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RESEARCH ARTICLE

Renal tight junction proteins are decreased in cisplatin-induced nephrotoxicity in rats

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Abstract

Cisplatin (CP) is an antineoplastic agent that induces nephrotoxicity and oxidative stress. It is unknown whether renal tight junction (TJ) proteins expression and localization are modified in CP-induced nephrotoxicity.

Objective: To study if the expression of the TJ proteins occludin, claudin-2, claudin-5 and zonula occludens-1 (ZO-1) is modified in rats with CP-induced nephrotoxicity.

Materials and methods: Male Wistar rats ($n = 5$/group) were injected with saline solution (V group), and the other group (CP group) was injected with a single dose of saline solution and CP (7.5 mg/kg i.p.). Rats were sacrificed 72 h after CP injection and blood, and 24-h urine samples were collected. Several plasma and urinary injury biomarkers as well as renal histopathology lesions, oxidative and nitrosative stress markers were evaluated, and protein levels of ocludin, claudin-2, claudin-5, ZO-1 were measured by Western blot. Statistically significant changes noted with different $p < 0.05$ versus V.

Results: Nephrotoxicity was evident by histological alterations, glycosuria, decrease in creatinine clearance, increase in fractional excretion of sodium, serum creatinine and kidney injury molecule-1. These changes were associated with oxidative/nitrosative stress (increased renal abundance of 3-nitrotyrosine and protein kinase Cβ2 and decreased renal expression of nuclear factor-erythroid-2-related factor 2) and decreased activity of antioxidant enzymes. Finally, it was found that CP-induced renal damage was associated with decreased renal expression of occludin and claudin-2.

Discussion and conclusion: CP altered the TJ proteins expression and localization in the proximal tubule that was associated with oxidative/nitrosative stress.

Keywords

Cisplatin, claudins, nitrotyrosine, occludin, oxidative stress, tight junction proteins

History

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Introduction

Cisplatin (CP) is an effective anticancer drug used against lung and ovarian cancer and some lymphomas, however, renal damage has limited its use (Guerrero-Beltran et al., 2012). CP concentration is about five times more in proximal tubular and epithelial cells than in serum (Kuhlmann et al., 1997) and in kidney tissue is accumulated by peritubular uptake in the proximal and distal tubules (van Angelen et al., 2013). The mechanisms of CP-induced nephrotoxicity are complex, comprising oxidative/nitrosative stress, apoptosis, inflammation and fibrogenesis. It has been described that oxidative/nitrosative stress (Chirino et al., 2008) and decrease of antioxidant defense, including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) (Kuhad et al., 2007) are involved in the development of renal damage induced by CP. Interestingly, the renal nuclear factor-erythroid-2-related factor 2 (Nrf2) is decreased during CP-induced nephrotoxicity (Sahin et al., 2010). In contrast CP increases renal mRNA levels of the fibrotic marker transforming growth factor beta (TGF-β) (Ramesh & Reeves, 2002).

Renal tubule plays a major role in the absorption of water and solutes, one of the most important mechanisms is known as transepithelial transport it can be either transcellular or paracellular. Tight junction (TJ) that is formed by integral and peripheral proteins restricts paracellular transport of solutes and water. The integral proteins establish cell–cell contact in

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the intercellular space, while the peripheral ones serve as a bridge between the integral proteins and the actin cytoskeleton (Gonzalez-Mariscal et al., 2008). Many types of integral membrane proteins have been identified as components of TJ strands, some of them include occludin and claudins (Furuse et al., 1993, 1998). Zonula occludens (ZO-1 and ZO-2) proteins are peripheral proteins of the TJ; both proteins bind to actin filaments, functioning as a cross-linker between TJ fibrils and the cytoskeleton (Fanning et al., 1998). The mammalian nephron displays a wide spectrum of claudins, whose distribution varies in each tubular segment that determines the permeability properties of the renal epithelia. Claudins are TJ membrane proteins that regulate cellular permeability of renal epithelia to small ions, solutes and water (Hou et al., 2013). In the kidney, occludin and ZO proteins are expressed along the nephron tubular segments (Kwon et al., 1998; Reyes et al., 2002), and claudin-2 is expressed in leaky epithelia (Enck et al., 2001). In vitro studies have shown that claudin-2 functions as a cation-selective paracellular pore (Kiuchi-Saishin et al., 2002) which mediates water transport in the renal proximal tubule (Rosenthal et al., 2010). Claudin-5, the most predominant claudin in endothelial cells but is also found in podocytes (Koda et al., 2011). Occludin is an integral membrane protein found at the TJ that has been located by freeze-fracture immunolabeling at the junctional strands (Saitou et al., 1997). It is present in epithelial and endothelial cells, and absent, both at the mRNA and protein levels, in cells that lack TJs such as fibroblasts (Saitou et al., 1997). In tubular renal cells, occludin is exclusively present at the cell borders, and its distribution follows the increase in TJ complexity and function displayed by the nephron. The overall distribution of occludin along the nephron agrees with the transepithelial electrical resistance (TER) reported in renal segments. Thus, in regions where TER is high, occludin fluorescent signal is located at cell borders, suggesting an intimate relationship between its presence and the strength of the TJ (Gonzalez-Mariscal et al., 2000).

Previous studies reported that TJ structure and function are sensitive to oxidative stress damage induced by presence of heavy metals (Arreola-Mendoza et al., 2009; Jacquillet et al., 2006) and hydrogen peroxide (H_2O_2) (Meyer et al., 2001). It is well known that CP-induced nephrotoxicity occurs mainly at the proximal tubule and that oxidative stress is one of the most important mechanisms involved in renal dysfunction. However, it is unknown if CP alters the TJ expression and localization in the nephron and if these changes are related to oxidative stress. In this work was studied if the TJ proteins occludin, claudin 2, claudin 5 and ZO-1 are modified in rats after CP-induced nephrotoxicity. It was found that the CP administration produced renal damage and oxidative/nitrosative stress associated with the decreased renal expression of occludin and claudin 2.

Materials and methods

Reagents

CP (Cis-Diamineplatinum (II) dichloride, Cat. No. 479306, Lt MKBH5984V), xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), collagenase (from *Clostridium histolyticum*, type II), glutathione (GSH), disulfide glutathione (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), p-nitrofenil-β-D-glucosaminido, p-nitrofenol, sodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), sodium dodecyl sulfate (SDS), sodium deoxycholate, rabbit anti-protein kinase C β2 (PKCβ2) and rabbit anti-Nrf2 antibodies were from Sigma-Aldrich (St. Louis, MO). Ethylene diamine tetraacetic acid (EDTA), monobasic potassium phosphate (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium hypochlorite and hydrogen peroxide (H_2O_2) were obtained from JT Baker (Xalostoc, Edo. Mexico, Mexico), and sodium pentobarbital was purchased from Holland of México (Mexico City). The rabbit anti-claudin-2, rabbit anti-occludin, rabbit anti-ZO-1, mouse anti-claudin-5, mouse anti-megalin, peroxidase-conjugated anti-rabbit, peroxidase-conjugated anti-mouse, Alexa Fluor® 488 donkey anti-rabbit and Alexa Fluor® 594 donkey anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA). Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mouse anti-3-nitrotyrosine (3-NT) antibodies were purchased from Millipore Corp. (Billerica, MA). Goat anti-kidney injury molecule (KIM-1) was purchased from R&D Systems (McKinley Place, MN). Rabbit anti-TGFβ1 was purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Protease inhibitor cocktail Complete 1X was from Roche Applied Science (Mannheim, Germany). Commercial kits for the measurement of blood urea nitrogen (BUN) and plasma creatinine concentration (Sera-pak plus creatinine Cat. No. 1001111 and urea Cat. No. 1001325) were from Spinreact (Girona, Spain). Micro bicinchoninic acid (BCA)™ Protein Assay Reagent Kit was bought from Pierce (Rockford, IL). All other reagents were of analytical grade and commercially available.

In vivo experimental model

Ten male Wistar rats (200–250 g) were fed with standard chow diet and water *ad libitum*. They were randomly distributed in two groups: the first group composed of five rats received only vehicle (V, isotonic saline) by intraperitoneal (i.p.) injection; the second group composed of five rats received a single dose of 7.5 mg/kg CP i.p. It has been previously reported that this dose (7.5 mg/kg) is able to induce both functional and proximal tubule damage, which was evaluated by measuring urinary levels of KIM-1 (Vaidya et al., 2006). Seventy-two hour after CP administration, rats were anesthetized with sodium pentobarbital (90 mg/kg, i.p.), blood was collected from the aorta in heparinized tubes and used for analytical procedures. Subsequently, rats were sacrificed and the left kidney was frozen immediately in liquid nitrogen for Western blot analyses and to measure activity of both N-acetyl-beta-D-glucosaminidase (NAG) and antioxidant enzymes. Right kidney was fixed in 10% neutral buffered formalin solution for histological analysis. Additional V and CP groups ($n = 5$ for each group) were used to obtain kidney samples for immunofluorescence studies. The guidelines of the Official Mexican Standard Care and Use of Laboratory Animals (NOM-062-ZOO-1999) were followed and the Local Ethics Committee (FQ/CICUAL/069/13) approved the protocol.

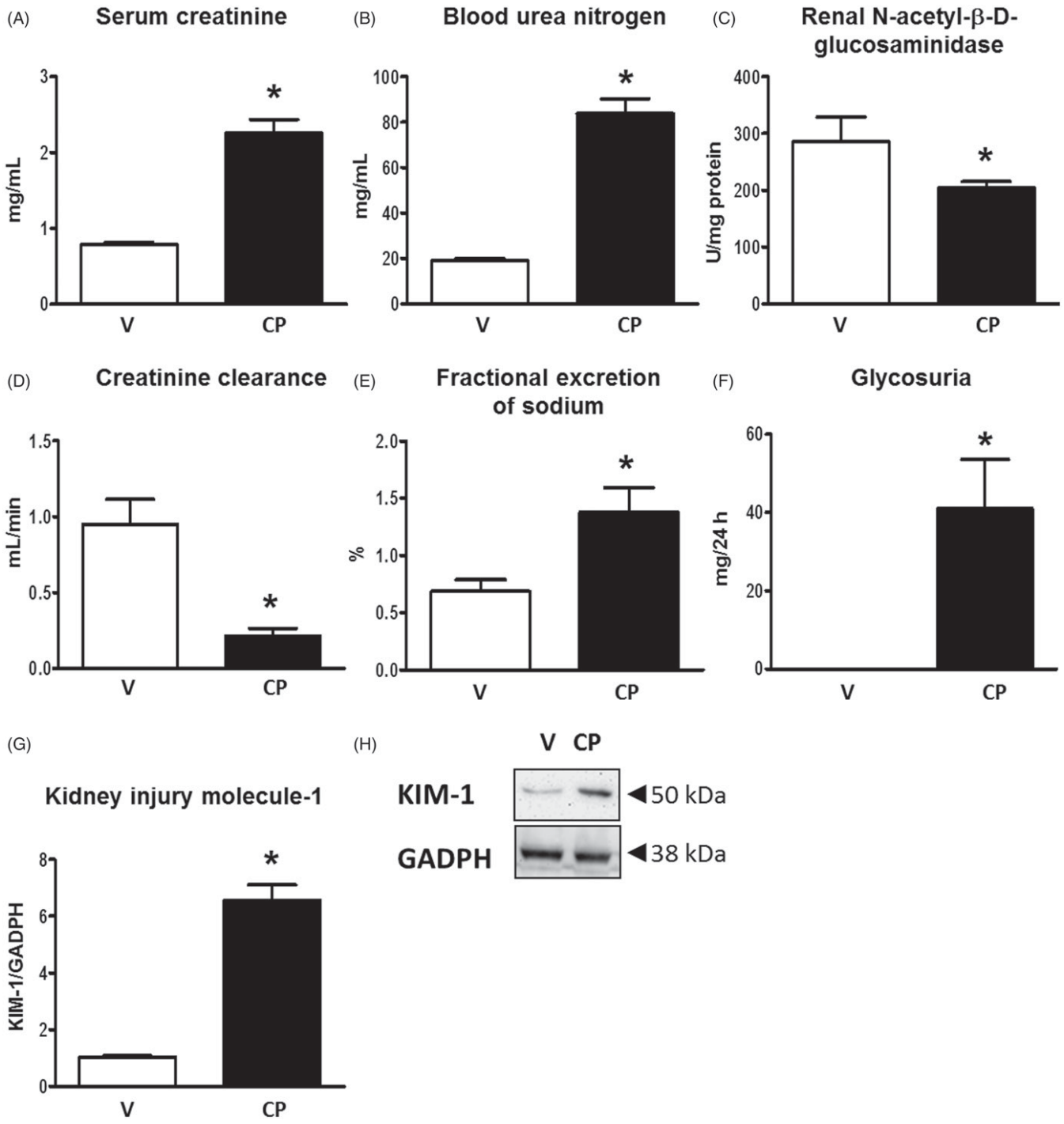

Figure 1. Renal injury markers in rats treated with vehicle (V; $n = 5$) and 7.5 mg/k of cisplatin (CP; $n = 5$). Renal injury in rats that received CP was evinced by a significant increase in serum creatinine (A) and blood urea nitrogen (B) and by significant decrease in renal activity of N-acetyl-β-D-glucosaminidase (C) and in creatinine clearance (D). This renal damage was also evident by glycosuria (F) and an increase of the fractional excretion of sodium (E) and of protein levels of kidney injury molecule-1 (KIM-1; G). A representative image of KIM-1 Western blot is shown (H). Data are expressed as relative density from four rats/group normalized with GADPH as loading control. Values are mean ± SEM. *$p < 0.05$ versus V.

Renal injury markers

Plasma and urine creatinine (Cr) was measured with a commercial kit based on the reaction with the alkaline picrate reagent described by Jaffe (Charlton et al., 2014), and BUN was determined with a commercial kit based on a colorimetric assay. NAG is a sensitive marker for proximal tubule injury with loss of lysosomal integrity; this parameter was measured in kidney tissue by a colorimetric assay, as previously described (Charlton et al., 2014). Cr clearance (Ccr) was calculated by the standard formula ($Cr_{urine} \times$ Urine flow rate/Cr_{plasma}). Plasma and urine sodium concentrations were measured by flame photometry using the ILab300 Plus clinical chemistry analyser (Instrumentation Laboratory, Bedford, MA). FeNa was calculated as part of the evaluation of acute renal failure, and the values of fractional excretion of sodium (%FeNa) were calculated using the standard formula (Na clearance/Ccr) × 100. At the end of the protocol,

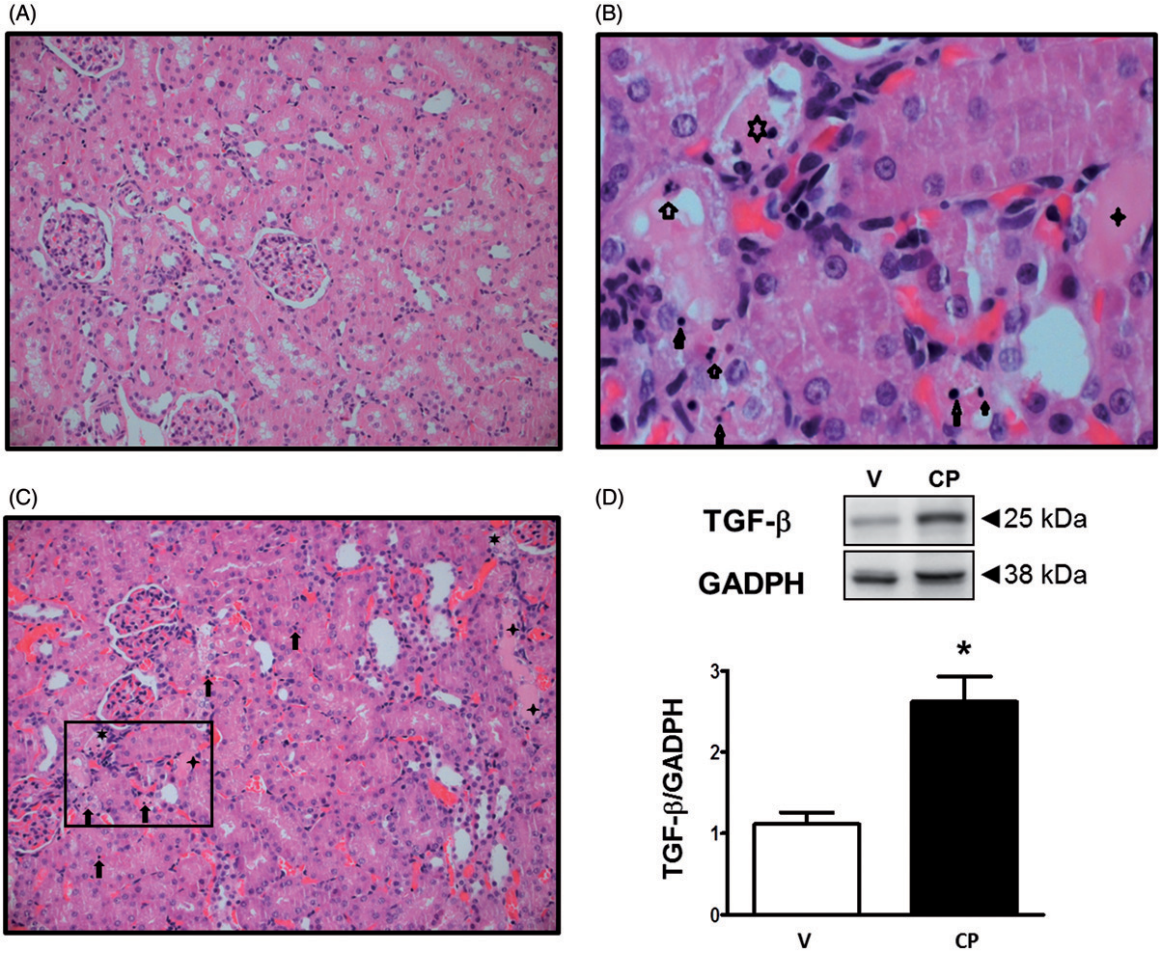

Figure 2. Morphological changes in kidney induced by cisplatin. Panel (A) shows control group with no morphological lesion, panel (B) and panel (C) show CP group with apoptotic cells (open and closed arrows), acute tubular necrosis (six peaks star) and cast formation (four peaks star) in the corticomedullary junction; panel (B) is a magnification ×400 of panel C (square). H&E-staining, original magnification ×200. CP-induced transforming growth factor beta (TGF-β) expression (D). A representative image (upper panel, D) and the densitometric analysis (lower panel, D) are shown. Data are expressed as relative density of three rats/group normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Values are mean ± SEM. *$p < 0.05$.

urinary glucose concentration (glycosuria) was determined enzymatically using the IL Test™ GLUC kit (Glucose oxidase) from Instrumentation Laboratory SpA (Milano, Italy). Glycosuria and enhanced FeNa have been found in CP-treated rats (Vaidya et al., 2006).

Activity of antioxidant enzymes

The activity of antioxidants enzymes was assayed in kidney homogenates (Perez-Rojas et al., 2009). CAT was analyzed by a method based on the disappearance of 30 mM H_2O_2 at 240 nm, and the data were expressed as κ/mg protein. Glutathione reductase (GR) activity was measured using GSSG as substrate and measuring the disappearance of NADPH at 340 nm, one unit of GR was defined as the amount of enzyme that oxidizes 1 µmol of NADPH/min; data were expressed as U/mg protein. Glutathione S transferase (GST) activity was assayed in a mixture containing GSH and CDNB as previously described, one unit of GST was defined as the amount of enzyme that conjugates 1 µmol of CDNB with GSH per min, data were expressed as U/mg protein.

Table 1. Morphological changes induced by cisplatin in kidney.

Lesion	Control	Cisplatin (7.5 mg/kg)
Acute tubular necrosis	0	0/+
Apoptosis	0	++
Tubular cast	0	+

The severity of tubular injury was calculated semi-quantitatively in eight random subcortical periglomerular fields (magnification ×200) per each rat for apoptosis, acute tubular necrosis and cast formation, using a 0–3 scale: 0, absence; 1+ (mild or <5%); 2+ (moderate or 5–25%); 3+ (severe or >25%) of juxtamedullary proximal tubules

Extraction of proteins from renal cortex for Western blot

Kidneys were excised and decapsulated, and the cortex was dissected out. Pieces of renal cortex were placed in ice-cold Krebs-bicarbonate solution (KB, mM): 110 NaCl, 25 $NaHCO_3$, 3 KCl, 1.2 $CaCl_2$, 0.7 $MgSO_4$, 2 KH_2PO_4, 10 sodium acetate, 5.5 glucose, 5 alanine, and 0.5 g/l bovine albumin and pH 7.4. Pieces of renal cortex were placed in ice-cold KB, washed three times and resuspended in 10 mL of KB containing 0.2 g/100 ml collagenase. Samples were gassed

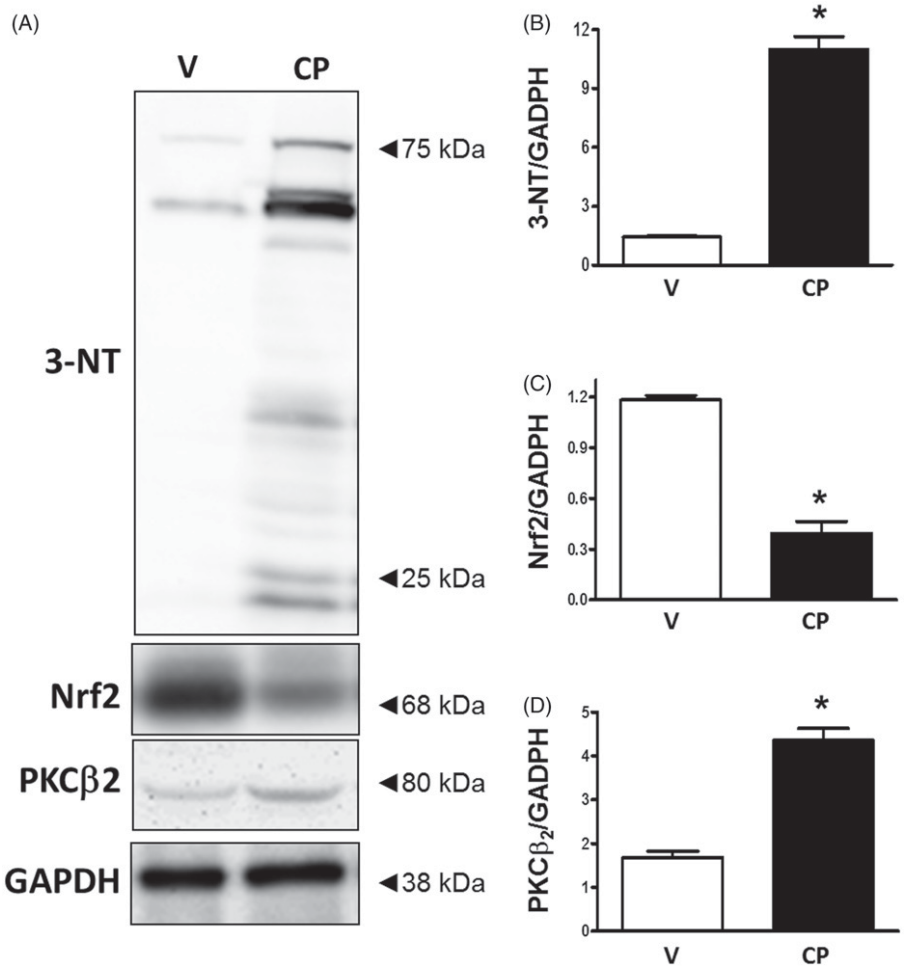

Figure 3. CP-induced nitrosative stress, decrease in Nrf2 and increase in protein kinase C β2 (PKCβ2). Representative Western blots of 3-nitrotyrosine (3-NT), nuclear factor-erythroid-2-related factor 2 (Nrf2), PKCβ2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in panel A. Densitometric analyses of Western blots for 3-NT (B), Nrf2 (C) and PKCβ2 (D) are shown. Data are expressed as relative density from four rats/group normalized with GAPDH as loading control. Values are mean ± SEM. *$p < 0.05$.

with 95% O_2/5% CO_2 in a shaking water bath at 37 °C for 30 min. After digestion, approximately 10 ml of ice-cold KB with a protease inhibitor cocktail (Complete, Roche) and PMSF (20 µg/ml) were added, and suspension was gently shaken to disperse tissue fragments. Collagen fibers were removed by filtration, and tissue suspension was gently centrifuged ($18 \times g$/30 s). This washing procedure was repeated three times. Pellet was resuspended in RIPA buffer (mM): 40 Tris–HCl, 150 NaCl, 2 EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate and 0.2% SDS, pH 7.6. Samples were incubated for 30 min at 4 °C. Thereafter, samples were sonicated three times for 30 s each at low intensity in an ultrasonic processor. After that, samples were centrifuged at $14\,000 \times g$, at 4 °C, for 40 min, and supernatants were collected. Total protein was quantified using the Micro BCA Protein Assay Reagent Kit (BioRad lab).

Western blot analyses

Western blot analysis was performed as previously described (Arreola-Mendoza et al., 2009). Briefly, samples were denatured by boiling for 12 min and then diluted 1:5 in 5X Laemli buffer with urea (5 M). Proteins were loaded on SDS-polyacrilamide gel electrophoresis (PAGE) 12% gels. Molecular weight standards (Amersham Pharmacia Biotech, Piscataway, NJ) were run in parallel. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosci, Uppsala, Sweden). Non-specific protein binding was blocked by incubation with 5% non-fat dry milk in PBS 1X containing 0.4% Tween 20, for 1 h, at room temperature. Membranes were incubated overnight at 4 °C with the appropriate primary antibodies against claudin-2, claudin-5, occludin, ZO-1, KIM-1, Nrf2, PKCβ2, TGF-β, 3-NT and GAPDH (1:1000). Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase were incubated for 1 h at room temperature (1:20 000). Immunoblots were developed using the ECL™ prime Western blotting detection reagent (Amersham™, GE Healthcare, Buckinghamshire, UK). Chemiluminescence was detected in an EC3 Imaging System (UVP Biolmaging Systems, Cambridge, UK). Protein band density was quantified by transmittance densitometry (UVP Biolmaging Systems software, Cambridge, UK).

Histological analysis

The right kidneys were transversely sectioned and fixed in 10% neutral buffered formalin solution for 12 h and paraffin-embedded. Kidney sections were cut to 4–5 µm thickness and stained with hematoxylin-eosin (H and E) using standard procedures. HandE-stained paraffin section were assessed by an expert pathologist in a blind manner to experimental groups, with a digital camera incorporated to a Zeiss Axiophot 2 light microscope by means of an imaging software, AxioVision 4.8. The severity of tubular injury was calculated semi-quantitatively in eight random subcortical

periglomerular fields (magnification ×200) per each rat for apoptosis, acute tubular necrosis and cast formation, using a 0–scale: 0 (absence); 1+ (mild or <5%); 2+ (moderate or 5 to 25%); 3+ (severe or >25%) of juxtamedullary proximal tubules.

Immunofluorescence

The right kidneys samples were prepared for immunofluorescence as previously described by Arrreola-Mendoza et al. (Arreola-Mendoza et al., 2009). Kidney sections were incubated overnight at 4 °C with primary antibodies anti-claudin-2, anti-occludin and anti-megalin (1:100). Megalin was used as a marker of proximal tubules brush border. Secondary antibodies Alexa Fluor® 488 donkey anti-rabbit, and Alexa Fluor® 594 donkey anti-mouse were used at a 1:300 dilution. Immunofluorescence was evaluated using a confocal inverted microscope (TCS-SP2, Leica, Heidelberg, Germany). Immunofluorescence experiments were performed at least three times in samples from three different animals per group. Non-specific labeling was estimated by omission of the primary antibodies.

Statistical analysis

All the values were expressed as mean ± standard error of the mean (SEM). Unpaired *t* test was used to compare the *in vivo* data of the two groups. To know the associated between oxidative stress and TJ expression and kidney function, Pearson correlation coefficient between 3-NT and claudin 2 and between 3-NT and renal NAG activity and creatinine clearance was calculated. The data were analyzed using the software GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). $p < 0.05$ was considered significant.

Results

CP-induced renal injury

CP administration induced an increase in serum creatinine (Figure 1A) and BUN (Figure 1B). To evaluate whether CP treatment caused tubular injury, the activity of NAG, a sensitive marker for proximal tubule injury, was assessed and it was found that the administration of CP induces a decrease in the renal activity of NAG (Figure 1C). Ccr was also decreased in rats with CP-induced nephrotoxicity (Figure 1D). Furthermore, it was observed that CP-induced an increase of FeNa and urinary glucose excretion (Figure 1E and F, respectively). Finally, it was observed a significant increase in the renal expression of KIM-1 (Figure 1G and H), a type 1 transmembrane protein that is not detectable in normal kidney tissue but is overexpressed in epithelial cells in human and rodent after toxic or ischemic injury (Charlton et al., 2014). All these results confirm the nephrotoxic effect of CP at 72 h after its administration.

CP-induced histological damage and TGF-β expression

Histopathological examination of renal slides from rats treated with 7.5 mg/kg of CP showed the characteristic alterations in renal tissue after acute injury as detaching of epithelial cells, apoptosis, tubular necrosis and cast formation in the tubular lumen (Figure 2B; Table 1). No morphological changes were observed in the control group (Figure 2A; Table 1). In addition, to better represent the histological changes found, Figure 2(C) shows higher magnification (×400) of the damaged tissue. These histopathological changes were associated with enhanced renal expression of TGF-β in CP-treated animals (Figure 2D) suggesting a profibrotic state.

CP-induced protein tyrosine nitration, decreased Nrf2 and increased PKCβ_2

It is well known that increased oxidative/nitrosative stress is involved in CP-induced nephrotoxicity. 3-NT was quantified by Western blot in renal cortex homogenates. The abundance of 3-NT, observed by several bands at different molecular weights (Figure 3A), is significantly increased 3 days after CP treatment (Figure 3B). In order to explore the involvement of Nrf2 in the renal damage exerted by CP, this protein was measured by Western blot. As shown in Figure 3 (A and C), the Nrf2 levels were significantly decreased 3

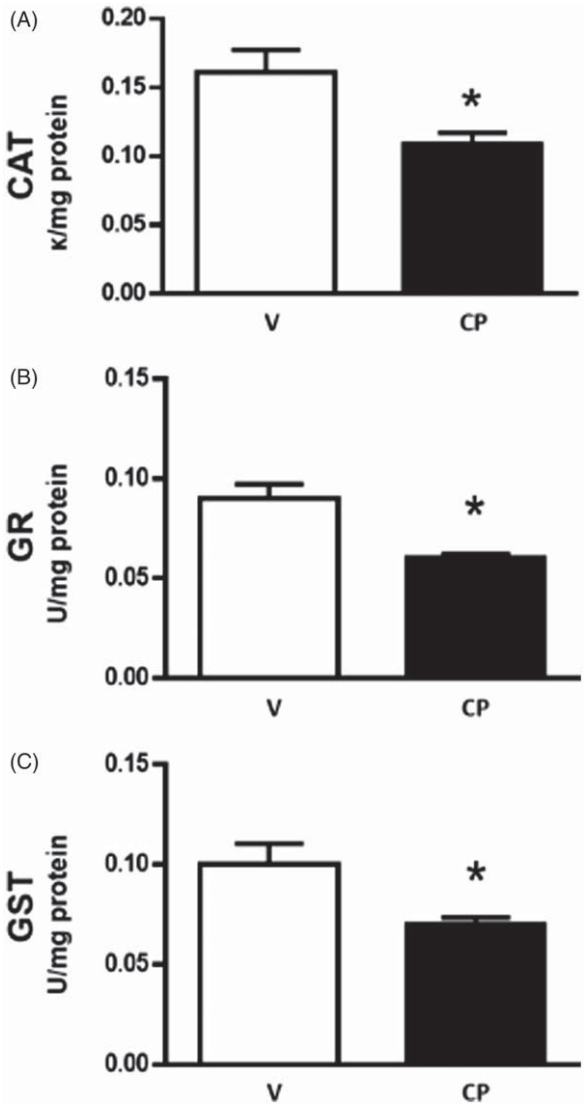

Figure 4. Activity of antioxidant enzymes in renal tissue of rats treated with V ($n = 5$) and CP ($n = 5$). The rats that received CP showed a significant decrease in catalase (CAT; A), glutathione reductase (GR; B) and glutathione S transferase (GST; C). *$p < 0.05$ versus V.

days after CP exposure. In addition, CP significantly induced PKCβ2 expression in renal cortex (Figure 3A and D). Interestingly, it has been reported that PKC-related signal transduction pathways may modulate CP nephrotoxicity (Ikeda et al., 1999).

CP-reduced activity of antioxidants enzymes

Renal damage was associated with a reduction of 38, 33 and 33% in the kidney activity of CAT (Figure 4A), GR (Figure 4B) and GST (Figure 4C), respectively.

CP-induced loss of renal TJ proteins occludin and claudin-2

Sufficient evidence shows that CP induces loss of cell–cell contacts of renal proximal epithelial cells by modifying the localization of the adherents junction associated to beta-catenin (Imamdi et al., 2004). Therefore, it was analyzed whether CP affects the distribution and expression levels of renal TJ proteins occludin, claudin 2, 5 and ZO-1. Through confocal microscopy and Western blots analyses, we evaluate the distribution and expression levels of TJ proteins in control and CP-treated rats. The amount of occludin (Figure 5A) and claudin-2 (Figure 5B) in CP-treated rats was decreased, with respect to vehicle rats as shown by immunofluorescence, which was confirmed by Western blot analysis (Figure 5C–E). Megalin, a marker of proximal tubules, was unchanged, indicating that the reduction in claudin-2 or occludin expression was selective (Figure 5A and B). No changes were observed in the expression of claudin-5 (Figure 6A and B) and ZO-1 (Figure 6A and C).

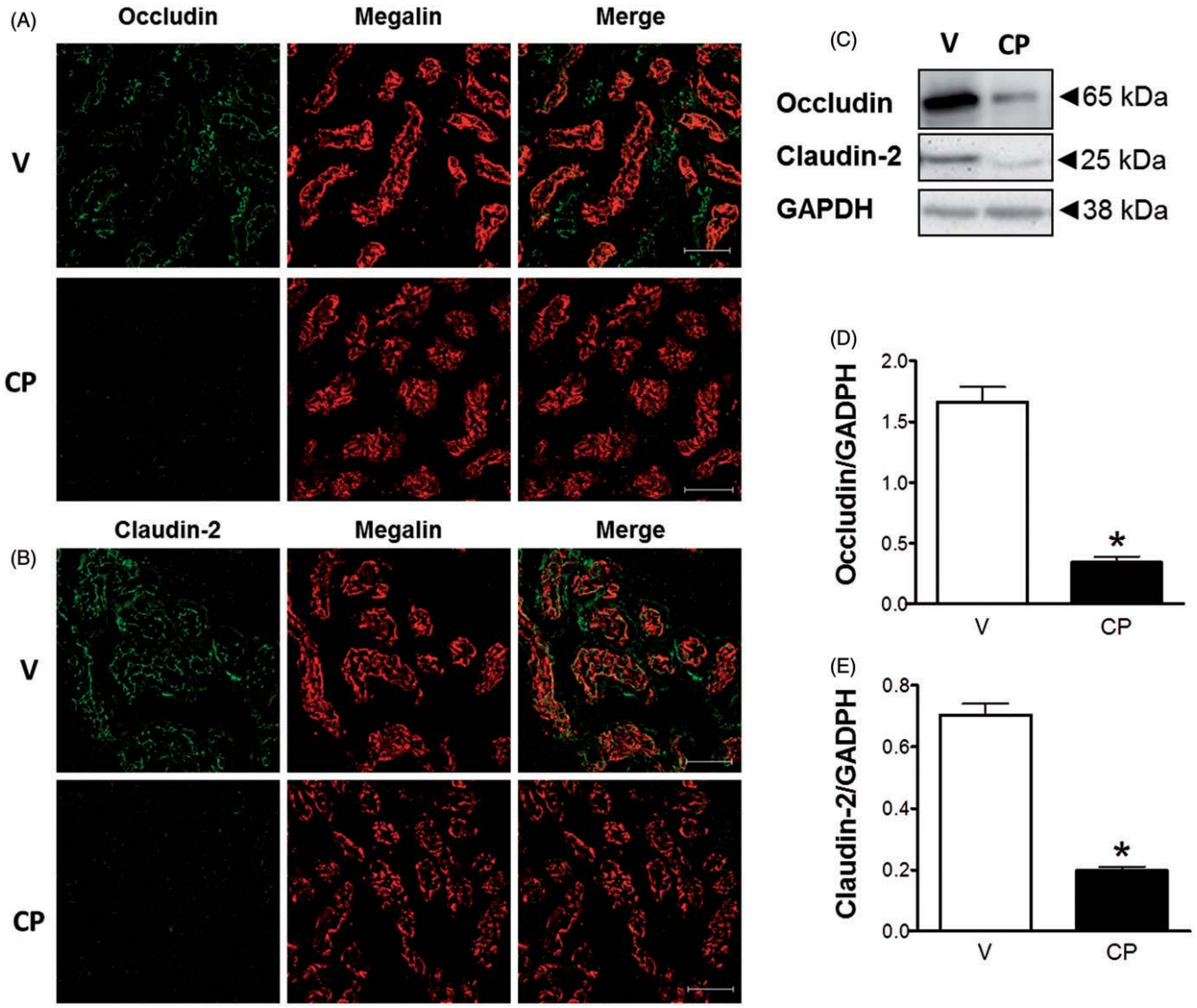

Figure 5. CP-induced decrease in occludin and claudin-2 expressions. The localization (confocal microscopy, panels A and B, respectively) and expression (Western blot from renal cortex homogenates, panel C) of occludin and claudin-2 in the two experimental groups studied were assessed. In renal cryosections of vehicle-treated rats, occludin and claudin-2 were detected surrounding the brush border of proximal tubular cells (panels A and B). In contrast, CP-treatment decreased the localization (A and B) and expressions (C) of occludin and claudin-2. Expression of megalin (panels A and B) was used as a marker of the proximal tubule apical membrane. Merge image of both proteins is shown (panels A and B). Densitometric analyses of Western blots are shown in panels D and E for occludin and claudin-2, respectively. Data are expressed as relative density from three rats/group normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Values are mean ± SEM. *$p < 0.05$. Bar = 50 μm.

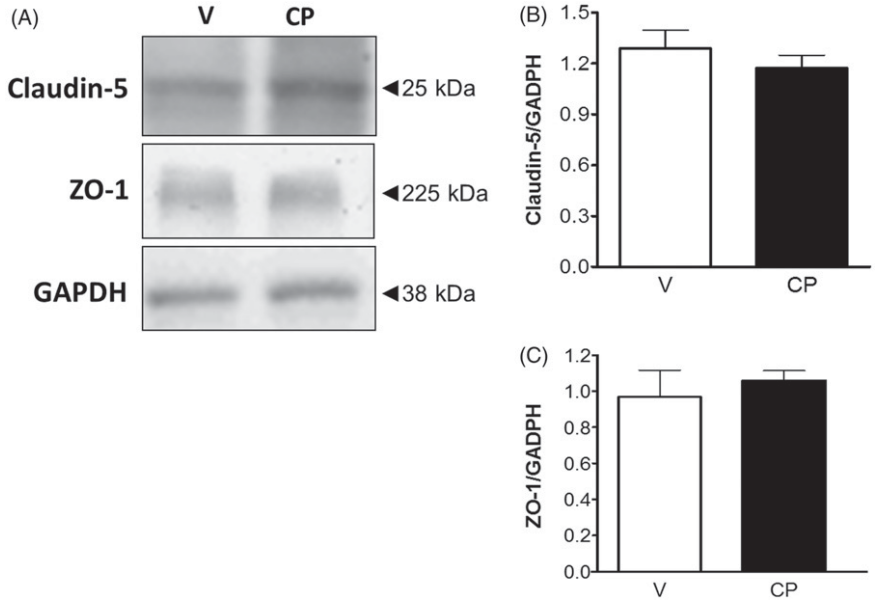

Figure 6. Expression of claudin-5 and ZO-1 in renal cortex is not affected by CP treatment. No changes were found in the expression levels of claudin-5 and ZO-1 (A) in the two experimental groups studied. Densitometric analyses of Western blot for claudin-5 (B) and ZO-1 (C) are shown. Data are expressed as relative density from three rats/group normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. Values are mean ± SEM.

Correlation analyses

Pearson correlation coefficient between 3-NT and claudin 2 was of -0.978 ($p < 0.0007$) and between 3-NT and renal NAG activity was of -0.923 ($p < 0.0085$), and 3-NT and creatinine clearance was of -0.883 ($p < 0.019$). These data suggest that 3-NT, a marker of nitrosative stress, is associated with the loss of claudin 2 levels as well as with the loss of renal function.

Discussion

CP is one of the most potent and effective anticancer drugs. However, its use is limited by its serious side effects including nephrotoxicity, with proximal tubular epithelial cells as the primary target. Renal injury induced by CP was evident by glycosuria, decrease in renal activity of NAG and of Ccr and by the increase in FeNa, renal KIM-1 and TGF-β expression and plasma creatinine and BUN. Our data suggest a profibrotic state and are consistent with previous findings showing the increase of TGF-β mRNA levels during CP nephrotoxicity (Perez-Rojas et al., 2009; Ramesh & Reeves, 2002). It is well documented that CP attenuates the antioxidant defense in the kidney by decreasing expression of the transcription factor Nrf2 (Sahin et al., 2010) leading to a failure of the antioxidant defense against ROS. It was previously shown that CP reduces the kidney activity of the antioxidant enzymes CAT, glutathione peroxidase and GST (Guerrero-Beltran et al., 2010), which was confirmed in the present study. KIM-1 expression has been used as an early and sensitive biomarker of renal damage because this protein is overexpressed under proteinuric, toxic and ischemic kidney disease (Bonventre, 2009). This could be associated with characteristic histological renal damage; in fact it was found vascular and tubular damage that was evident by acute tubular necrosis, apoptosis and cast formation in the tubular lumen. On the other hand, protein tyrosine nitration is a post-translational modification induced by peroxynitrite under oxidative stress conditions; an increase of protein nitration may modify the structure and function of target protein. Consistently with previous findings (Pan et al., 2009) in which it has been shown increased 3-NT abundance by immunohistochemistry in rats and mice, respectively, in our study, Western blot analysis revealed that CP induces 3-NT abundance in renal cortex homogenates. In addition, it has been reported that CP induces the loss of the cell–cell contact of renal epithelial tubular cells, as well as apoptosis, by a mechanism dependent of PKC activation (Imamdi et al., 2004). CP induces also alterations in the localization of beta catenin, an adherent junction-associated protein (Imamdi et al., 2004) leading to apoptosis of the proximal tubular cells. In this study, our main aim was to explore whether renal TJ proteins are altered in CP-induced nephrotoxicity. A set of TJ proteins expressed in different sections of the nephron were analyzed: claudin-5 located mainly in endothelia and glomerulus (Wen et al., 2004); claudin-2, located at the proximal tubules (Enck et al., 2001) and occludin and ZO-1 which are expressed mainly in the distal segment (Gonzalez-Mariscal et al., 2000). Confocal microscopy revealed that CP treatment reduced the expression of claudin-2 and occludin in the proximal tubule cells. This result has an important physiological relevance because claudin-2 is a protein fundamental for sodium reabsorption and maintenance of cell–cell contacts in this nephron portion (Enck et al., 2001). These findings suggest that claudin-2 absence or decreased expression induced by CP might be related to tubular dysfunction. It has been reported that occludin expression increased TER, with a concomitant increase in the mean number and complexity of TJ strands (McCarthy et al., 1996). A decreased expression of occludin in proximal tubules of CP-treated rats was found in our study, which suggests an altered structure and function of TJ fibrils that might be associated with tubular dysfunction observed in CP-induced nephrotoxicity.

It is known that PKCβ2 expression favors oxidative stress conditions, because PKCβ stimulates NADPH oxidase activity and increases superoxide anion production (Kitada et al., 2003). Increases in the activity of conventional PKC isoforms, such as PKCβ2, are involved in the phosphorylation of TJ components and also has been reported that PKC isoforms

modulate the disassembly of the TJ (Gonzalez-Mariscal et al., 2008). In this work, we observed that CP increased PKCβ2 levels, which could modify their activity favoring the phosphorylation of TJ that would explain the loss of claudin-2 and occludin in the proximal tubule. In contrast, changes in expression ZO-1 and claudin-5 were not observed, confirming that the nephrotoxic effects of CP are mainly in the proximal tubule.

In conclusion, CP administration increases the oxidative stress that leads to down-regulation of some TJ proteins, which could affect sodium reabsorption and structure of proximal tubule cells thus perpetuating the damage.

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Declaration of interest

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Superoxide Anion Production and Expression of gp91^{phox} and p47^{phox} Are Increased in Glomeruli and Proximal Tubules of Cisplatin-Treated Rats

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ABSTRACT: The chemotherapeutic drug cisplatin has some side effects including nephrotoxicity that has been associated with reactive oxygen species production, particularly superoxide anion. The major source of superoxide anion is nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase. However, the specific segment of the nephron in which superoxide anion is produced has not been identified. Rats were sacrificed 72 h after cisplatin injection (7.5 mg/kg), and kidneys were obtained to isolate glomeruli and proximal and distal tubules. Cisplatin induced superoxide anion production in glomeruli and proximal tubules but not in distal tubules. This enhanced superoxide anion production was prevented by diphenylene iodonium, an inhibitor of NADPH oxidase. Consistently, this effect was associated with the increased expression of gp91^{phox} and p47^{phox}, subunits of NADPH oxidase. The enhanced superoxide anion production in glomeruli and proximal tubules, associated with the increased expression of gp91^{phox} and p47^{phox}, is involved in the oxidative stress in cisplatin-induced nephrotoxicity. © 2014 Wiley Periodicals, Inc.

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KEYWORDS: **Cisplatin; Superoxide Anion; NADPH Oxidase; Proximal Tubules**

INTRODUCTION

Cisplatin (CP; *cis*-diammine dichloro platinum II) is an effective anticancer agent in clinical practice for treatment of solid tumors, especially head, neck, lung, testis, ovary, and some lymphomas; however, the use of this antineoplastic has been limited because of it causes nephrotoxicity with an incidence of between 6% and 13% of patients [1;2]. The development of CP-induced nephrotoxicity is complex, and several factors are involved, mainly drug transport in the renal tubular cells, specifically in proximal tubules [2]. Kuhlmann et al. reported that the CP concentration in tubular epithelial cells is approximately five times higher than in plasma [3] due to peritubular absorption [4]. Several authors have described that oxidative/nitrosative stress [5–8] and a decrease of antioxidant defense [5-7; 9] are involved in the development of CP-induced renal damage. Davis et al. found that CP also induces the production of reactive oxygen species (ROS) [10]; particularly superoxide anion ($O_2^{\bullet -}$) [8;10], hydrogen peroxide (H_2O_2) [11], and hydroxyl radical ($^{\bullet}OH$) [12]. ROS damage biological molecules including deoxyribonucleic acid (DNA), proteins, and lipids, causing changes in the structural

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integrity of cell. Some enzymes are involved in $O_2^{\bullet -}$ production: mitochondrial oxidases, xanthine oxidase, cyclooxygenase, cytochrome P-450, and uncoupled nitric oxide synthase (NOS) among others [13]. Based on studies on both neuronal NOS (nNOS) and endothelial NOS (eNOS), it has been described that in the absence of the substrate L-arginine or cofactor tetrahydrobiopterin (BH_4), heme reduction in the enzyme results in the uncoupling of eNOS and consequently leads to the production of $O_2^{\bullet -}$ rather than NO [14]. However, a major source of this ROS is nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, a membrane-bound enzyme composed of several subunits: $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac. $gp91^{phox}$, and $p22^{phox}$ are involved in transfer of an electron from NADPH to oxygen, originating $O_2^{\bullet -}$ and $NADP^+$, whereas $p47^{phox}$, $p67^{phox}$, and Rac are regulators of the enzyme activity [15]. Moreover, at least five isoforms of $gp91^{phox}$, named Nox1 through 5, have been characterized. All components of the neutrophil NADPH oxidase including Nox2 are present in endothelial cells of kidney. In addition, Nox4 is widely expressed in renal cells and $p47^{phox}$ is strongly expressed in glomeruli [16]. Rac1/2, p67phox, p47phox, and p40phox subunits reside under normal conditions in cytosol, and they are associated with the membrane-bound Nox(gp91phox)/p22phox subunits only after activation. $p47^{phox}$ interacts with components of the cytoskeleton, leading to the correct assembly of the active complex and $O_2^{\bullet -}$ production from oxygen [16]. This enzyme was found specifically in neutrophils with a physiological role in host defense and cellular signaling and differentiation. NADPH oxidase was found to be involved in many pathological conditions such as immunity disorders, cardiovascular [17] and neurodegenerative diseases [18], and CP-induced nephrotoxicity [19–21]. However, the specific segment of the nephron in which $O_2^{\bullet -}$ is produced rats with CP-induced nephrotoxicity has not been described. The purpose of this study was to measure the $O_2^{\bullet -}$ production in isolated glomeruli (GL), proximal tubules (PT), and distal tubules (DT) of kidneys from CP treated rats. Furthermore, the role of several enzymes in $O_2^{\bullet -}$ production was evaluated using specific substrates. Nicotinamide adenine dinucleotide hydrogen (NADH) was used as a substrate for NADPH oxidase, L-arginine as a substrate for NOS, xanthine as a substrate for xanthine oxidase (XO) and succinate to assess the involvement of the intramitochondrial $O_2^{\bullet -}$ production.

MATERIALS AND METHODS

Reagents

Collagenase (from *Clostridium histolyticum*, type II), CP (cat. no. 479306, Lt MKBH5984V), NADH, L-arginine, xanthine, succinate, diphenylene iodonium (DPI), sodium dodecyl sulfate (SDS), sodium acetate (CH_3COONa), Triton X-100, sodium deoxycholate, bovine serum albumin fraction V (BSA), glucose, Tween 20, glycerol, alanine, phenylmethylsulfonyl fluoride (PMSF), salmon testes DNA, percoll and dihydroethidium (DHE) were purchased from Sigma-Aldrich (St. Louis, MO). Potassium chloride (KCl), sodium chloride (NaCl), monobasic potassium phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), magnesium sulfate ($MgSO_4$), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and calcium chloride ($CaCl_2$) were obtained from JT Baker (Xalostoc, Edo. Mexico, Mexico), and sodium pentobarbital was purchased from Holland of México (Mexico City, Mexico). Protease inhibitor cocktail Complete 1X (Roche Applied Science, Mannheim, Germany). Mouse anti-$gp91^{phox}$, mouse anti-$p47^{phox}$, and peroxidase-conjugated antimouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antig-lyceraldehyde 3 phosphate dehydrogenase (GAPDH) was purchased from Millipore (Billerica, MA). The Amersham ECL™ prime Western blotting detection reagent was procured from General Electric Healthcare (Buckinghamshire, UK). Commercial kits for the measurement of blood urea nitrogen (BUN) and plasma creatinine concentration (Sera-pak plus creatinine, cat. no. 1001111; urea, cat. no. 1001325) were from Spinreact (Girona, Spain). The micro bicinchoninic acid (BCA) protein assay reagent kit was obtained from BioRad Labs (Hercules, CA). Molecular weight standards were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents were of analytical grade and commercially available.

In Vivo Experimental Model

Ten male Wistar rats (200 – 250 g) were fed with standard chow diet and water ad libitum. They were randomly distributed in two groups: The first group of five control rats received only vehicle (control, isotonic saline) by an intraperitoneal (i.p.) injection; the second group of five rats received a single dose of 7.5 mg/kg of CP i.p [22; 23]. Seventy-two hours after CP administration, rats were anesthetized with sodium pentobarbital (90 mg/kg, i.p.), blood was collected from aorta in heparinized tubes and kidneys were used to prepare a whole renal homogenate and to obtain fractions enriched with GL, PT, and DT for $O_2^{\bullet -}$ production assay and quantification of $gp91^{phox}$ and $p47^{phox}$ subunits by Western blot. The guidelines from Official Mexican Standard Care and Use of Laboratory Animals (NOM-062-ZOO-1999) were followed, and the Local Ethics Committee (FQ/CICUAL/069/13) approved the protocol.

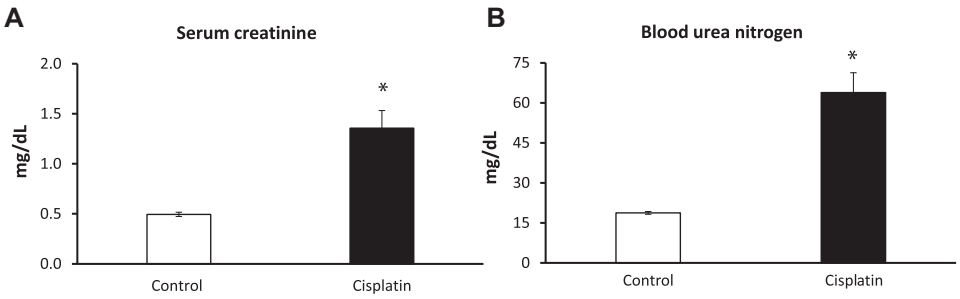

FIGURE 1. Serum creatinine (A) and blood urea nitrogen (B) in control and CP-treated rats. Values are means ± SEM, $n = 5$, $^*P < 0.05$ versus control.

Renal Injury Markers

Plasma creatinine was measured with a commercial kit based on the reaction with an alkaline picrate reagent described by Jaffe [24; 25], and BUN was determined with a commercial kit based on a colorimetric assay described by Young and Friedman [26].

Preparation of a Renal Homogenate and Isolation of Glomeruli, Proximal Tubules, and Distal Tubules

For studies in the renal homogenates, kidney was homogenized in with Krebs-bicarbonate solution (KBS) and centrifuged (14,000×g/30 min); the determinations were carried out in the supernatant. GL were isolated by the method described by Gauthier and Mannik [27], as previously described [28]. Briefly, capsules were removed by macroscopic dissection and kidney cortex was separated from medulla and minced on a glass dish. The homogenized tissue was pushed through a stainless sieve with a pore size of 117 μm (cat. no. 8321A44; Thomas Scientific, Swedesboro, NJ) applying gentle pressure with the bottom of a glass flask. The sieve was rinsed several times with KBS (mM): 110 NaCl, 25 $NaHCO_3$, 3 KCl, 1.2 $CaCl_2$, 0.7 $MgSO_4$, 2 KH_2PO_4, 10 sodium acetate, 5.5 glucose, 5 alanine, and 0.5 g/L BSA, pH 7.4 and osmolarity 290 mOsm/kg H_2O. The sieved tissue containing a preparation enriched in GL was washed and collected with cold KBS, and centrifuged for 10 min at 20,000×g. The supernatant was decanted, and the pellet containing GL was resuspended in KBS. While renal tubules were isolated by percoll gradients [29] as previously described [28]. Briefly, renal cortex from five rats were placed in 15 mL ice-cold KBS, washed three times, and resuspended in 10 mL of KBS containing 15 mg of collagenase and 0.5 mL of 10% BSA. Samples were incubated in a water bath for 20 min at 37°C. After digestion, tissue suspension was gently agitated to disperse tissue fragments. Suspension was filtered to remove collagen fibers, and tissue suspension was gently centrifuged (18×g/30 s). The pellet was resuspended in 10 mL ice-cold KBS with a protease inhibitor cocktail. This washing procedure was repeated three times. The tissue pellet was then resuspended in 5% BSA with protease inhibitors for 5 min, at 4°C and centrifuged for 1 min. Tissue pellets were suspended in 30 mL of a freshly prepared mixture of ice-cold percoll and KBS (1:1, v/v). Thereafter, the suspension was centrifuged (1,071×g/30 min), resulting in separation of three bands. The first one was enriched with GL, the second one with DT, and the third one with PT. The content of each fraction was confirmed by light microscopy observation (see Supplementary Figure 1 in the Supporting Information).

Superoxide Anion Production Assay

Fluorescent detection of $O_2^{\bullet -}$ production in tissue was performed by the method of Satoh and co-workers [30] as described by Maldonado et al. [18]. Fluorescence was detected by conversion of DHE to ethidium (Eth). Briefly, isolated GL, PT, and DT were homogenized in 500 μL of a phosphate-buffered saline (PBS) solution. Homogenates were subjected to a low-speed centrifugation (800×g/10 min, 4°C) to remove the unbroken cells and debris, and aliquots were used immediately. Tissue homogenates (20 μg) were incubated with DHE (0.02 mmol/L), salmon testes DNA (0.5 mg/mL) and the corresponding substrate (1.125 mM NADH, 11.25 mM L-arginine, 1.125 mM xanthine, 56.25 mM succinate) or inhibitor (1.125 mM, DPI). The concentrations of the above-described substrates and inhibitor were selected based on previous reported assays where $O_2^{\bullet -}$ production was assessed in isolated glomeruli [30]. The assay was performed in a microtiter plate placed away from direct light at 37°C for 30 min. Eth-DNA fluorescence was measured at an excitation of 480 nm and an emission of 610 nm by using the Synergy HT multimode microplate reader (Biotek Instruments, Winooski, VT). The fluorescence intensity of each sample was normalized relative to the control. The protein content was measured using the Lowry method.

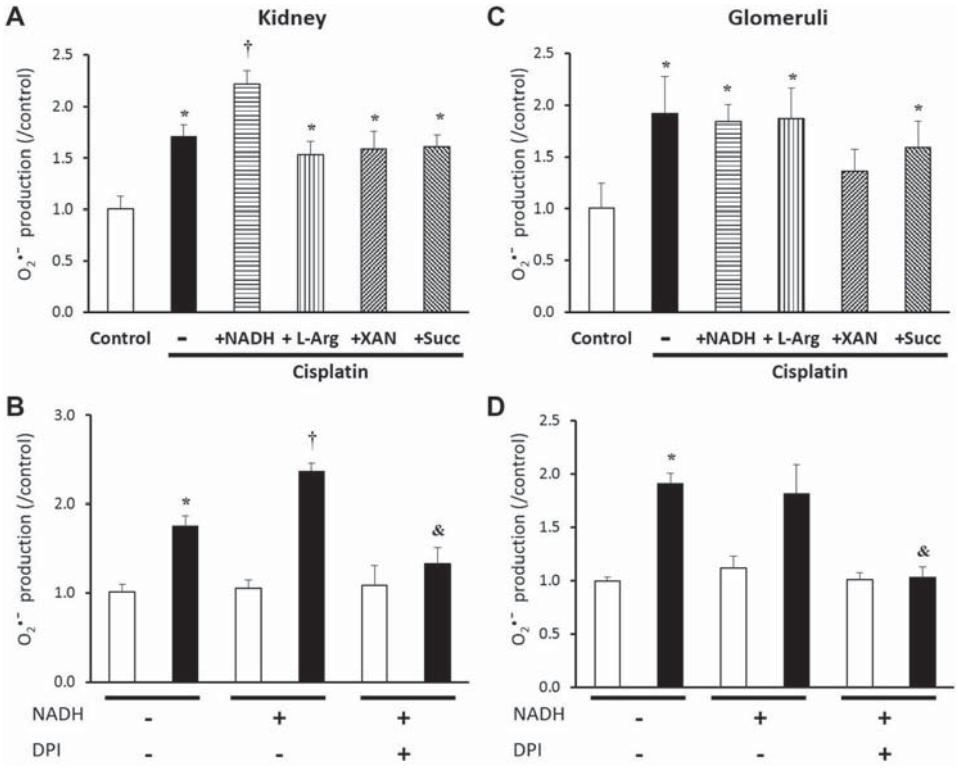

FIGURE 2. Superoxide anion ($O_2^{\bullet-}$) production in kidney homogenates (A) and in isolated glomeruli (GL) (C) of control and CP-treated rats. $O_2^{\bullet-}$ production in samples from CP-treated rats was also evaluated in the presence of NADH, L-arginine (L-Arg), xanthine (XAN), and succinate (Succ). Diphenylene iodonium (DPI; B and D in kidney and glomeruli, respectively) was used as an inhibitor of NADPH oxidase. Data are expressed as $O_2^{\bullet-}$ production/control. Values are means ± SEM, $n = 3$, $^{*}P < 0.05$ versus control, $^{\dagger}P < 0.05$ versus CP, $^{\&}P < 0.05$ versus CP + NADH.

Extraction of Proteins from GL, PT, And DT for Western Blot

Suspension of GL, PT, and DT was centrifuged (1,071×g/10 min), and the pellet was resuspended in radioimmunoprecipitation buffer (mM): 40 Tris–HCl, 150 NaCl, 2 EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.2% SDS, pH 7.6. Samples were incubated for 30 min at 4°C. Thereafter, samples were sonicated three times for 30 s at low intensity in an ultrasonic dismembrator. After that, samples were centrifuged (14,000×g/40 min, 4°C) and supernatants were collected. The total protein was quantified using a micro BCA protein assay reagent kit.

Western Blot Analysis

Western blot was performed as previously described [28]. Briefly, samples were denatured by boiling for 12 min and then diluted 1:5 in 5× Laemli buffer with 5 M urea. Proteins were loaded on SDS-polyacrylamide gel electrophoresis 12% gels. Molecular weight standards were run in parallel. Proteins were transferred to polyvinylidene fluoride membranes (Amersham Biosci, Uppsala, Sweden). Nonspecific protein binding was blocked by incubation with 5% nonfat dry milk in PBS 1× containing 0.4% Tween 20 for 1 h, at room temperature. Membranes were incubated overnight at 4°C with the appropriate primary antibodies against $gp91^{phox}$, $p47^{phox}$, and GAPDH (1:1,000). Thereafter, membranes were incubated with peroxidase-conjugated anti-mouse (1:10,000) for 1 h, after washing, immunoblots were developed using the Amersham ECL™ prime Western blotting detection reagent. Chemiluminescence was detected in an EC3 Imaging System (UVP BioImaging Systems, Cambridge, UK). Protein band density was quantified by transmittance densitometry (UVP BioImaging Systems software, Cambridge, UK).

Data Analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between means from two groups were evaluated by the Student's t test. Analysis of variance and Bonferroni post hoc test were used to compare more than three groups in the $O_2^{\bullet-}$ assay. $P < 0.05$ was considered to be statistically significant.

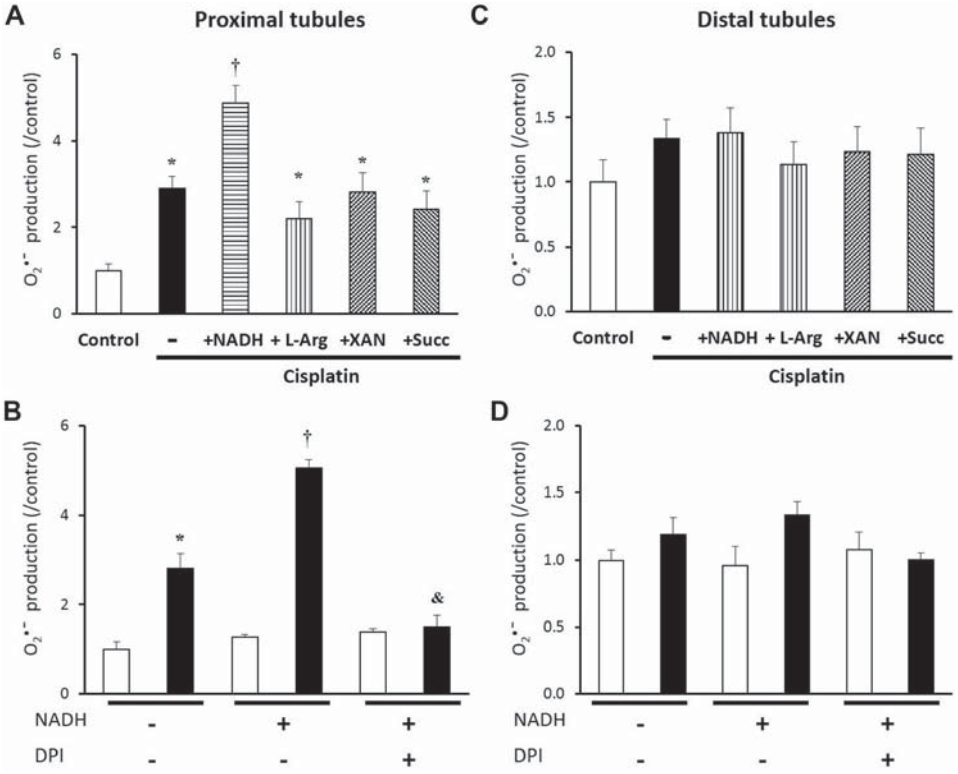

FIGURE 3. Superoxide anion ($O_2^{\bullet-}$) production in proximal (PT) (A) and distal tubules (DT) (C) of control and CP-treated rats. $O_2^{\bullet-}$ production in samples from CP-treated rats was also evaluated in the presence of NADH, L-arginine, xanthine, and succinate. DPI (B and D in PT and DT, respectively) was used as an inhibitor of NADPH oxidase. Data are expressed as $O_2^{\bullet-}$ production/control. Values are means ± SEM, $n = 3$, $^{*}P < 0.05$ versus control, $^{\dagger}P < 0.05$ versus CP, $^{\&}P < 0.05$ versus CP + NADH.

RESULTS

CP -Induced Renal Injury

CP administration induced an increase in plasma levels of creatinine (Figure 1A) and BUN (Figure 1B). These data confirm the nephrotoxic effect of CP 72 h after its administration.

CP -Induced $O_2^{\bullet-}$ Production in Total Kidney, GL, and PT, but Not in DT

As shown in Figures 2 and 3, under basal conditions without addition of any substrate, $O_2^{\bullet-}$ production was increased in total kidney (Figure 2A), GL (Figure 2C) and PT (Figure 3A) of CP-treated rats. The increase in $O_2^{\bullet-}$ production in DT was not significant (Figure 3C). These data suggest that renal $O_2^{\bullet-}$ production in CP-treated rats is produced mostly in GL and PT and to a lesser extent in DT. When NADH was added, an increased $O_2^{\bullet-}$ production was found in total kidney (Figure 2A) and PT (Figure 3A) sections, suggesting the involvement of NADPH oxidase in $O_2^{\bullet-}$ production. No changes were found when L-arginine, xanthine and succinate were added, suggesting a negligible role of NOS, XO, and mitochondria in $O_2^{\bullet-}$ production. To confirm the source of $O_2^{\bullet-}$ production, DPI, an inhibitor of NADPH oxidase activity, was used. A decreased $O_2^{\bullet-}$ production was observed when DPI was added to homogenates from total kidney (Figure 2B), GL (Figure 2D), and PT (Figure 3B) but not in DT (Figure 3D) of CP-treated rats. Inhibition of CP-induced $O_2^{\bullet-}$ generation by adding DPI suggests that NADPH oxidase is a source of $O_2^{\bullet-}$ in CP-treated rats. It is noteworthy that $O_2^{\bullet-}$ production was not induced when the substrates and the corresponding inhibitor were added to homogenates isolated from control rats. These data suggest that NADH induced $O_2^{\bullet-}$ production only in CP-treated rats but not in control rats. In conclusion, CP increased oxidative stress in a NADPH oxidase–dependent pathway in GL and PT nephron sections.

CP Treatment Increases NADPH Oxidase $gp91^{phox}$ and $p47^{phox}$ Subunits

Western blot analyses of $gp91^{phox}$ and $p47^{phox}$ were performed in isolated GL, PT, and DT. It was found that CP significantly increases expression of $gp91^{phox}$ (Figures 4A and 4B) and $p47^{phox}$ (Figures 4C and 4D)

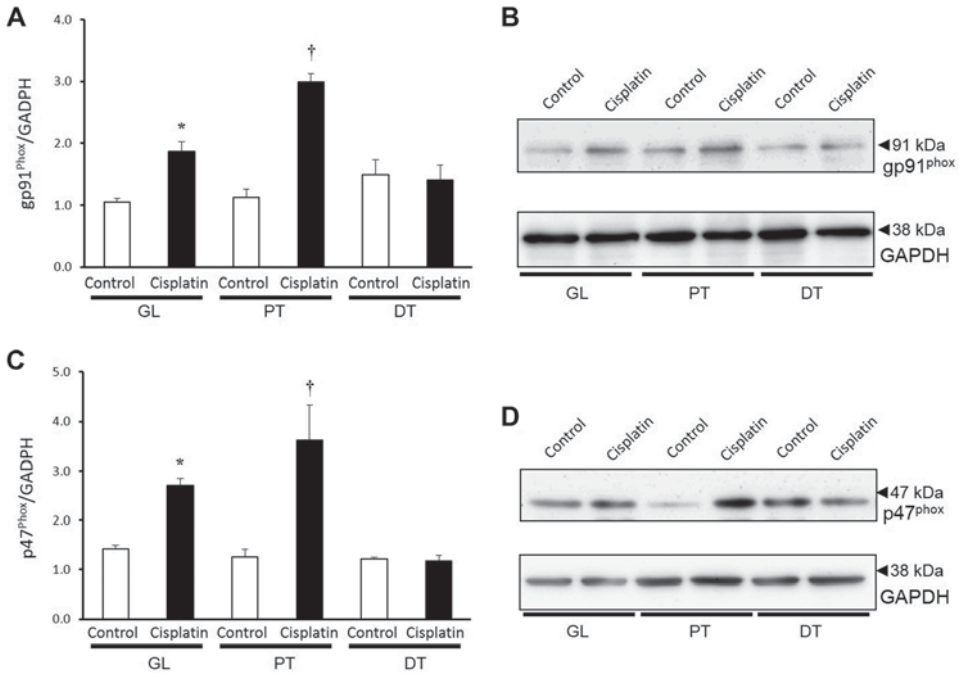

FIGURE 4. Expression of NADPH oxidase gp91^{phox} and p47^{phox} subunits in GL, PT, and DT of control and CP-treated rats. Densitometric analyses of western blots ($n = 3$) are shown in panels A and C and representative images are shown in B and D for gp91^{phox} and p47^{phox}, respectively. Data are expressed as relative density from five rats per group normalized with GAPDH as a loading control. Values are means ± SEM, *$P < 0.05$ versus control GL, $^{\dagger}P < 0.05$ versus control PT.

in GL and PT but not in DT. This suggests that CP increases NADPH oxidase active complex in GL and PT.

DISCUSSION

CP is a chemotherapeutic drug; it has a number of side effects including ototoxicity, neurotoxicity, and nephrotoxicity that limit its use. Proximal tubular epithelial cells are a primary target of CP [2]. It was previously shown that CP induces ROS production [5–8]. Currently, little is known about the mechanism involved in ROS generation; however, Marullo et al. [31] showed that CP-induced intracellular ROS levels, as a consequence of its direct effect on mitochondrial DNA that leads to decreased synthesis of electron transport chain proteins and impairment of mitochondrial redox status, DNA integrity and bioenergetic functionality [31]. CP also induces a reduction in the activity of antioxidant enzymes [5–7, 9] in kidney. Several studies have shown that a single dose of CP (7.5 mg/kg) induces acute renal failure in rats, characterized by high levels of plasma creatinine and BUN; these data were confirmed in our CP-treated rats. In addition, our data show that CP treatment increases the activity of NADPH oxidase, which is consistent with previous data that have shown that NADPH oxidase is highly expressed in cultured renal epithelial cells [32] and in animals treated with CP [33–35]. Pan et al. [19] reported that this $O_2\bullet^-$ production is triggered mainly by increased NADPH oxidase and inducible NOS expression. Recently, El-Naga [20] reported that upregulation of NADPH oxidase was found to be involved in CP-induced nephrotoxicity. Several potential sources of $O_2\bullet^-$ production (including GL, PT, and DT) were analyzed in the present study. It was found that only NADPH oxidase is the main source of $O_2\bullet^-$ in GL and PT but not in DT. Chirino et al. [36] showed that administration of apocynin, an inhibitor of NADPH oxidase, prevents the development of CP-induced nephrotoxicity, which suggests the role of this prooxidant enzyme in this experimental model. Gomez-Sierra and co-workers [21] showed that gp91^{phox} and p47^{phox} expression is increased in kidney homogenates of rats with CP-induced nephrotoxicity, which may explain the increase of NADPH oxidase activity in this model. The present study confirms and expands these data; in fact western blot analyses revealed that CP induces expression of both subunits (gp91^{phox} and p47^{phox}) in GL and PT but not in DT, which suggest that CP increases NADPH oxidase active complex in GL and PT.

In conclusion, CP administration increases $O_2\bullet^-$ production through NADPH oxidase in GL and PT, which was associated with increased expression of gp91^{phox} and p47^{phox} subunits of NADPH oxidase.

SUPPORTING INFORMATION

Supplementary Figure 1. A representative image of morphological structures of isolated glomeruli (GL), proximal tubules (PT), and distal tubules (DT). Bright field image indicates isolated glomeruli (A), proximal segment displays the characteristic brush border structure (B, arrow), and distal segment shows the cobblestone pattern with defined cell boundaries (C, arrows). 1000×.

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Review Article

Mitochondria as a Target in the Therapeutic Properties of Curcumin

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Curcumin, a phenolic compound extracted from *Curcuma longa*, is commonly used in Asia as a spice and pigment and has several biological functions, particularly antioxidant properties. It has been reported that curcumin exhibits bifunctional antioxidant properties related to its capability to react directly with reactive oxygen species (ROS) and also to its ability to induce the expression of cytoprotective and antioxidant proteins through the transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2). Recently, it has been postulated that the mitochondrial function and metabolism are associated with Nrf2 and that curcumin has shown activities against mitochondrial dysfunction. The damage in mitochondria has been implicated in the pathogenesis of diseases like diabetes, cancer, aging, and neurodegenerative disorders. This review focuses on some of the most recent findings of curcumin properties that suggest a close relationship of this antioxidant with the mitochondrial function.

Keywords: Anticancer agent / Antioxidant enzymes / Apoptosis / Mitochondria / Nrf2

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Abbreviations: ADP, adenosine diphosphate; **AIF,** apoptosis inducing factor; **Apaf-1,** apoptotic protease activating factor 1; **APE1/Ref-1,** apurinic/apyrimidinic endonuclease1/redox factor-1; **ARE,** antioxidant response element; **ASPC,** pancreatic carcinoma cell line; **ATP,** adenosine triphosphate; **Bak,** Bcl2-antagonist/killer; **Bax,** Bcl2-associated X protein; **Bcl2,** B-cell lymphoma 2; **Bcl2L1,** Bcl2-like 1; **BH3,** interacting domain; **Bid,** BH3 interacting-domain death agonist; **BxPC-3,** pancreatic carcinoma cell line; **Capan-1,** pancreatic carcinoma cell line-1; **Capan-2,** pancreatic carcinoma cell line-2; **CAT,** catalase; **CrVI,** hexavalent chromium; **CUR,** curcumin; **Cyt c,** cytochrome c; **Diablo,** direct inhibitor of apoptosis protein (IAP)-binding protein; **DNA,** deoxyribonucleic acid; **EpRE,** electrophile response element; **ERK,** extracellular signal-regulated kinases; **ETC,** mitochondrial electron transport chain; **γGCL,** γ-glutamylcysteine ligase; **GPx,** glutathione peroxidase; **GR,** glutathione reductase; **GSH,** glutathione; **GST,** glutathione-*S*-transferase; **H_2O_2,** hydrogen peroxide; **HepG2,** human hepatoma G2 cell line; **HO-1,** heme oxygenase 1; **HS766-T,** pancreatic carcinoma cell line; **IAP,** inhibitor of apoptosis; **IκBα,** nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; **JNK,** c-Jun N-terminal kinases; **Keap1,** Kelch ECH associating protein 1; **ΔΨm,** mitochondrial membrane potential; **MCR,** mitochondrial respiratory chain; **MDA,** malondialdehyde; **MDA-MB-231,** *Homo sapiens* breast epithelial adenocarcinoma cell line; **mDNA,** mitochondrial DNA; **MnSOD,** mitochondrial superoxide dismutase; **N2a,** mouse neuro 2A cell line; **NADH,** nicotinamide adenine dinucleotide reduced form; **NADPH,** nicotinamide adenine dinucleotide phosphate reduced form; **NF-κB,** nuclear factor kappa-light-chain-enhancer of activated B cells; **NIH3T3,** mouse embryonic fibroblast cell line; **$NO^{\bullet}$,** nitric oxide; **NOS,** nitric oxide synthase; **NQO1,** nicotinamide adenine dinucleotide phosphate (NADPH):quinone oxidoreductase 1; **NRF1,** nuclear respiratory factor 1; **Nrf2,** nuclear factor erythroid 2-related factor 2; **$^1O_2$,** singlet oxygen; **$O_2^{\bullet-}$,** superoxide anion; **$^{\bullet}OH$,** hydroxyl radicals; **$ONOO^-$,** peroxynitrite; **p38,** p38 mitogen-activated protein kinases; **PC12,** pheochromocytoma of rat adrenal medulla cell line; **PON1,** paraoxonase 1; **Prxs,** peroxiredoxin; **PTP,** permeability transition pore; **RNS,** reactive nitrogen species; **$ROO^{\bullet}$,** peroxyl radicals; **ROS,** reactive oxygen species; **SK-N-SH,** human neuroblastoma cell line; **Smac,** second mitochondria-derived activator of caspases; **SOD,** superoxide dismutase; **SOH,** *S*-hydroxycysteine; **Srxn1,** sulfiredoxin 1; **T98G,** *Homo sapiens* brain glioblastoma cell line; **tBHP,** *tert*-butyl hydroperoxide; **tBid,** truncated BH3 interacting-domain death agonist; **Tfam,** mitochondrial transcription factor A.

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Introduction

Curcumin, considered an important therapeutic agent in Indian and Chinese traditional medicine from long time ago, has multiple biological and pleiotropic properties including antioxidant [1, 2], antibacterial [3, 4], anti-inflammatory [5–7], antineoplastic [8], and antiproliferative activities [9]. Furthermore, curcumin has therapeutic potential against neurodegenerative [7], cardiovascular [10], hepatic [11, 12], and renal diseases [13–15].

Concerning safety and toxicity, curcumin was approved by the Food and Drug Administration and the Joint FAO/WHO Expert Committee on Food Additives [16]. It was evaluated for acute oral toxicity studies on animals (rats, mice, guinea pigs, and monkey) and it was found an LD_{50} of 3.0 g/kg [16]. Moreover human clinical trials also have reported minimal toxicity of curcumin. Lao et al. [17] found that curcumin treatment caused diarrhea, skin rash, headaches, and yellow-colored feces in about 30% of human beings studied [17]. Chemically, curcumin is a β-diketone bis-α-β or diferuloyl methane molecule (1,7-bis(4-hydroxy-3-methoxyphenol)-1,6-heptadiene-3,5-dione) that contains two ferulic acid residues linked by a methylene and has two hydrophobic phenyl regions (Fig. 1). Curcumin can adopt many different conformations for maximizing its hydrophobic zone; the phenyl ring of curcumin favors van der Waals interactions with the side chains of aromatic amino acids. This hydrophobic structure with phenolic and carbonyl functional groups, which are located at the both farthest sites and at the center of the molecule, are able to participate in hydrogen bonding with a target macromolecule [18]. Curcumin binds to deoxyribonucleic acid (DNA) by hydrogen bond interactions with the minor groove in AT-rich regions [19, 20]. Curcumin has keto-enol tautomers; keto form is predominant in acid and neutral solutions and enol form in alkaline solutions, which allows the midsection of the molecule to both donate and accept hydrogen bonds. It is also an ideal chelator of positively charged metals, which are often found in the active sites of some target proteins [21, 22].

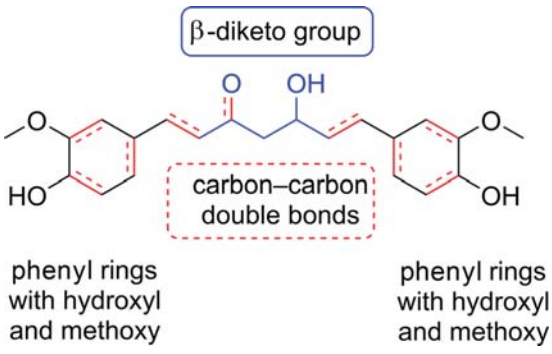

Figure 1. Functional groups of curcumin. (1) β-Diketo group (blue solid line), (2) phenyl rings with hydroxyl and methoxy (red dotted lines), and (3) carbon–carbon double bonds (black solid lines).

The discovery of the antioxidant properties of curcumin explains many of its wide range of pharmacological activities. As shown in Table 1, curcumin is a bifunctional antioxidant because of its properties to react directly with reactive oxygen (ROS) and nitrogen (RNS) species and also to indirectly induce an up-regulation of several cytoprotective and antioxidant proteins [23]. In addition to these effects, it has been found that curcumin has beneficial effects in both humans and rats with type 2 diabetes, which is partially due to a decrease in free fatty acids concentration that was associated with an increase in fatty acid oxidation [24, 25].

Curcumin as a direct ROS scavenger: Role of its functional groups

Curcumin is an effective antioxidant and has the ability to scavenge superoxide anion ($O_2^{\bullet -}$) [26, 27], hydroxyl radical ($^{\bullet}OH$) [28], hydrogen peroxide (H_2O_2) [26, 28], singlet oxygen ($^1O_2$) [29], nitric oxide ($NO^{\bullet}$) [30, 31], peroxynitrite ($ONOO^-$) [32], and peroxyl radical [28]. The free radical scavenging activity of curcumin has been associated with the following functional groups: (i) the β-diketone group (blue solid line in Fig. 1) [33, 34], (ii) carbon–carbon double bonds

Table 1. Direct and indirect effects of curcumin.

	Reactive specie/free radical	Refs.
Direct antioxidant	Superoxide anion ($O2^{\bullet -}$)	[26, 27, 30]
	Hydroxyl radical ($^{\bullet}OH$)	[28]
	Hydrogen peroxide (H_2O_2)	[26, 28]
	Singlet oxygen ($^1O_2$)	[29]
	Nitric oxide ($NO^{\bullet}$)	[30, 31]
	Peroxynitrite ($ONOO^-$)	[115]
	Peroxyl radical ($ROO^{\bullet}$)	[28]
	3-Ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$)	[26]
	N,*N*-Dimethyl-*p*-phenylenediamine dihydrochloride ($DMPD^+$)	[26]
	2,2-Dipheny-ι-picrylhydrazyl (DPPH)	[126]
	Inductor	
Indirect antioxidant	Superoxide dismutase (SOD)	[46]
	Catalase (CAT)	[46]
	Glutathione reductase (GR)	[47]
	Glutathione peroxidase (GPx)	[47]
	Heme oxygenase-1 (HO-1)	[48, 49]
	Glutathione-*S*-transferase (GST)	[51]
	NADPH: quinone oxidoreductase-1 (NQO1)	[51]
	γ-Glutamylcysteine ligase (γGCL)	[52]
	Thioredoxin reductase (TrxR)	[127]

(red dotted line in Fig. 1), (iii) phenyl rings containing varying amounts of hydroxyl and methoxy substituents (black solid lines in Fig. 1) [35], and (iv) keto and enol form in aqueous solutions [36].

The differences between its groups have led studies to determine, which functional groups are playing an important role in the free radical trapping activity. Some authors have postulated that its properties are mainly derived from its phenolic structure, but scavenging properties also have been observed in compounds where its phenolic group was modified by methyl groups [37, 38]. Various authors concluded that curcumin is an H-atom donor by giving H-atom from the phenolic group, which is the central methylene or the methoxy group [35, 39, 40]. The methylene group of the β-diketone is a potent $O_2^{\bullet -}$ scavenger [41]. Jovanovic et al. [42] showed that the only presence of the β-diketone was not sufficient for the radical-scavenging activity of curcumin. On the other hand, Litwinienko and Ingold [43] showed that the central hydrogen atom of the methyl and the phenolic hydroxyl group is involved in the formation of phenoxyl, which is more stable at *para* position of the hydroxyl group conferring a stable conformation [38]. The phenolic hydroxyl group is important due to be an electron donor, so it plays a major role in the antioxidant activity of curcumin in both enol and keto forms [26, 39, 44]. The radical scavenging activity is enhanced with the number and the substitution pattern of the hydroxyl group on the benzene ring; radical-scavenging properties have also been determined for synthetic curcumin analogs, where the two aromatic rings are bridged by seven carbon atoms (such as curcumin), or those with three and five carbon atoms [45]. Phenolic analogs of curcumin are more potent inhibitors of lipid peroxidation [42] and show more potent free-radical-scavenging activity against 1,1′-diphenyl-2-picrylhydrazyl and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) than the non-phenolic analogs [37].

Curcumin and its indirect antioxidant properties: Role of the Nrf2 pathway

Curcumin induces endogenous antioxidant defense mechanisms through gene regulatory mechanisms; it induces the expression of cytoprotective proteins such as superoxide dismutase (SOD), catalase (CAT) [46], glutathione reductase (GR), glutathione peroxidase (GPx) [47], heme oxygenase-1 (HO-1) [48, 49], glutathione-*S*-transferase (GST), nicotinamide adenine dinucleotide phosphate (NADPH):quinone oxidoreductase-1 (NQO1) [50, 51], γ-glutamylcysteine ligase (γGCL) [52], and apurinic/apyrimidinic endonuclease1/redox factor-1 (Ape1/Ref-1) DNA repair protein [53] (Fig. 2).

The transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) pathway is the major regulator of the mentioned antioxidant enzymes and cytoprotective responses; together with its repressor protein: Kelch ECH associating protein-1 (Keap1) a sensitive protein to ROS [54]. The key signaling proteins within the pathway are: (i) in redox homeostasis conditions Keap1 binds to Nrf2, inhibiting its translocation to the nucleus and promoting its degradation by the ubiquitin proteasome pathway, (ii) when stress stimuli are sensed, the Keap1/Nrf2 complex is disrupted allowing the translocation of Nrf2 into the nucleus, Nrf2, together with small Maf proteins, binds to antioxidant response element (ARE), thus leading to the antioxidant genes transcription.

Nrf2 can be released from Keap1 through diverse kinase-dependent signaling pathways, such as p38 mitogen-activated protein kinases, extracellular signal-regulated kinases (ERK), or cJun NH2 terminal kinase (JNK) [55]. Moreover, Keap1 is a cysteine-rich protein with thiol groups; most of them can be modified in vitro by different oxidants and electrophiles. This alters the conformation of Keap1 hence this promotes the Nrf2 stabilization and its nuclear translocation to finally target gene expression of enzymes with antioxidant activity such as: NQO1, HO-1, γGCL [56], GST, and sulfiredoxin-1 (Srxn1) [57]. NQO1 is critical for cytoprotection against many highly reactive and potentially damaging quinones [50]. On primary cultured cortical neurons damaged by ischemia [58] and astrocytes exposed to metmyoglobin, an oxygen restrainer [59], it was observed an up-regulation of the cell antioxidant defenses (NQO1 and HO-1) through activation of the Nrf2.

Recently, Gao et al. [60] showed that curcumin attenuates arsenic-induced hepatic injuries and oxidative stress in experimental mice, this observation was associated with NQO1 and HO-1 induction, which were consistently up-regulated in curcumin-treated mice, through activation of the Nrf2 pathway [60]. Also, it was shown that the induction of HO-1 in curcumin-treated renal epithelial cells confers protection to counteract the characteristic damage of organ transplantation; the induction of HO-1 was mediated by the activation of Nrf2 [61, 62]. Mouse embryonic fibroblast cell line (NIH3T3) treated with curcumin showed a significant dose-dependent increase in HO-1 mRNA and protein levels compared to untreated cells. HO-1, which catalyzes the heme group degradation, is an inducible enzyme with antioxidant and anti-inflammatory effects [63]. Although, curcumin contains two ferulic acid residues, the treatment with ferulic acid did not induce HO-1, suggesting that the cytoprotective effects of curcumin may not be mediated only by its functional groups.

Curcumin is also capable of inhibiting scavenger receptor activity and expression in macrophages and monocytes. Those receptors recognize oxidized low-density lipoprotein and acetylated lipoprotein, which are associated with the upregulation of HO-1 and the modification of γGCL subunits [64].

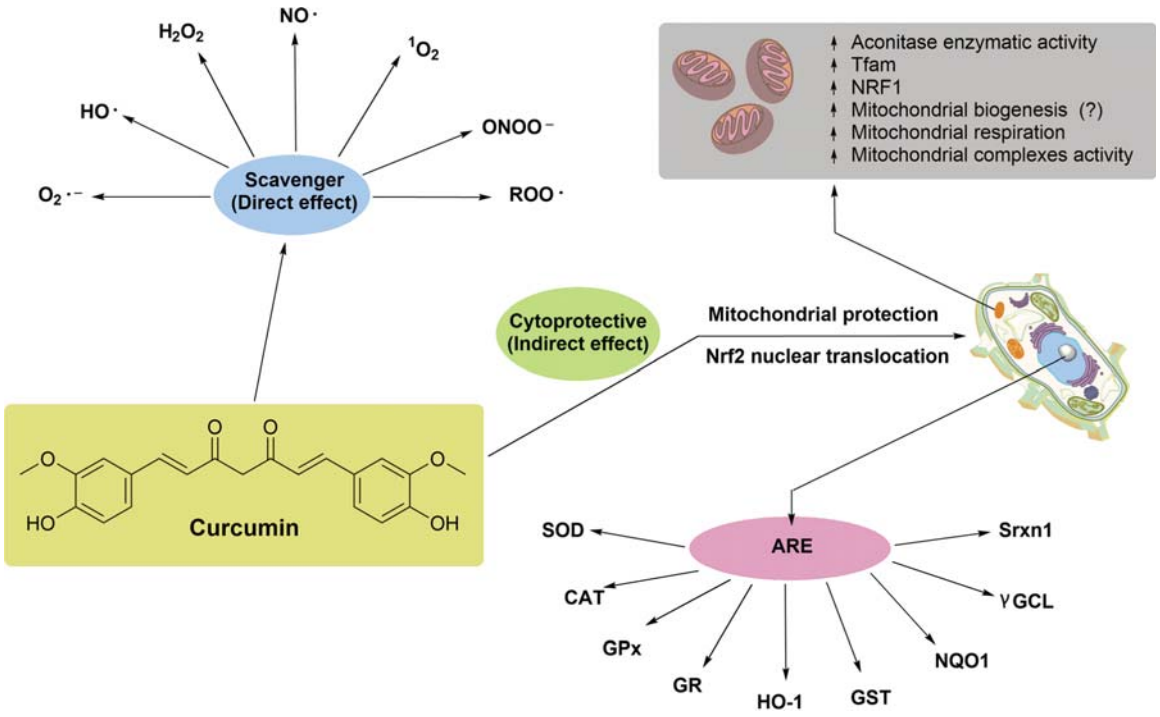

Figure 2. The bifunctional antioxidant properties of curcumin and the mitochondrial protection are illustrated. $O_2^{\bullet-}$, superoxide anion; $OH^{\bullet}$, hydroxyl radicals; H_2O_2, hydrogen peroxide; $NO^{\bullet}$, nitric oxide; $^1O_2$, singlet oxygen; $ONOO^-$, peroxynitrite; $ROO^{\bullet}$, peroxyl radicals; ARE, antioxidant response element; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; HO-1, heme oxygenase 1; GST, glutathione-*S*-transferase; NQO1, nicotinamide adenine dinucleotide phosphate oxidase:quinone oxidoreductase 1; γGCL, γ-glutamylcysteine ligase; Srxn1, sulfiredoxin 1; Tfam, mitochondrial transcription factor A; NRF1, nuclear respiratory factor 1; Nrf2, nuclear factor erythroid 2-related factor 2.

Furthermore, curcumin was able to protect human neuroblastoma cells (SK-N-SH) against acrolein toxicity, this protection was mediated through restoration of the expression of γGCL, but it had no effect on decreased glutathione (GSH) levels and elevation of carbonylated proteins [65]. Furthermore, biosynthesis of GSH, the most important cytosolic antioxidant, is also induced by curcumin [66]. The key enzyme in GSH biosynthesis is γGCL, which is in turn under the transcriptional control of Nrf2. The induction of GSH was not observed with ferulic acid [63]. Garg et al. [67] showed that curcumin modulates transcriptional regulators of phase I and phase II enzymes in benzo[*a*]pyrene-treated mice, through the induction of enzymatic activity, protein content, and mRNA levels of GST and NQO1 enzymes. It was demonstrated in a transgenic mouse model, encoding luciferase under control of electrophile response element (EpRE) from the thioredoxin promoter, that curcumin increased transactivation of Nrf2 in a time-dependent manner after an intraperitoneal injection [68]. While that Srxn1 catalyzes the reduction of peroxiredoxin (Prxs) to form Prxn-(*S*-hydroxycysteine) (SOH), which catalyzes the reduction of H_2O_2. Prxs-SOH has a Nrf2-functional ARE in its promoter region, so it was shown that the administration of the Nrf2 activator (CDDO-trifluoroethylamide) can induce a quick synthesis of Prxs-SOH (20-fold) in the liver *in vivo* [69]. Therefore, an administration of an Nrf2 inductor could induce the synthesis of Srnx1. Curcumin also induces the antioxidant enzyme paraoxonase 1 (PON1) that exerts anti-atherogenic effects [70]. PON1 is primarily synthesized in the liver and circulates in the blood bound to lipoproteins to prevent or slow down its oxidation.

Mitochondria: A critical source of ROS and an important target in disease state

ROS production can be a consequence of endogenous and/or exogenous stimuli, including ultraviolet radiation, chemotherapy, environmental toxins, and exercise [71]. The principal ROS sources within the cell include NADPH oxidase, nitric oxide synthase (NOS), xanthine oxidase, and the mitochondrial electron transport chain (ETC) [72, 73]. Interestingly, it has been recently postulated that mitochondrial function and metabolism is associated to Nrf2 [74–76].

Alterations in the ETC and oxidative phosphorylation lead to an increase in ROS production both in cytoplasm and mitochondria [77]. Mitochondrial ROS are generated during the univalent reduction of molecular oxygen to water. The addition of one electron to molecular oxygen generates $O_2^{\bullet -}$, whereas further reductions generate H_2O_2 and $^{\bullet}OH$ [78]. ROS produced in the mitochondria, unless adequately neutralized, causes mitochondrial oxidative stress and, by reactions with polyunsaturated fatty acids, form lipid hydroperoxides and unsaturated aldehydes that propagate among cellular compartments and also can react with proteins and nucleic acids. The oxidative stress cascade impacts on several mitochondrial functions like biogenesis, ionic homeostasis, and antioxidant defense mechanisms leading to diminished cellular energetic efficiency, altered bioenergetics, apoptosis, and degradation [79]. Therefore, mitochondrial dysfunction is related with the pathogenesis of many diseases such as diabetes, cancer, aging, neurodegenerative disorders, among many others [80, 81]. Besides, it has been described that increased ROS has been implicated in numerous chronic degenerative processes such as cardiovascular diseases [10, 81, 82], hypertension [83], cancer [84], type 2 diabetes [85, 86], neurodegenerative diseases [87, 88], and obesity [89].

Mitochondria possess redox components: GSH, thioredoxin system, reduced nicotinamide adenine dinucleotide (NADH), and vitamins with concentrations in the mM range, which may act in a catalytic manner, rapidly activate cytosolic signaling pathways that ultimately alter nuclear gene expression, oxidative phosphorylation, heme synthesis, in mitochondrial DNA replication, and transcription [90], through activation of nuclear respiratory factor 1 (NRF1) and NRF-2. Such an effect may be due to hormesis or mitohormesis, which is defined as any adaptive response to a mild oxidative stress, and counteracting the protective mechanisms capable of neutralizing higher-level oxidative stress [91]. Studies in yeast show that the exact response depends in part on the specific mitochondrial perturbation. In general, this mitochondrial perturbation included cytoplasmic signaling pathways and subsequent nuclear transcriptional changes, resulting in a reconfiguration of metabolism. These adaptive responses include mitochondrial biogenesis, mitochondrial protein import, alterations in mitochondrial metabolism, increased antioxidant defenses, and augmented protein chaperone expression [92].

Because it has not been reported that curcumin can reach inside to mitochondria, studies have been conducted for development of mitochondria-targeted antioxidants. Effort has been made to seek preferential accumulation into mitochondria, effect that is known as "mitochondriotropic" that is achieved by conversion of a pro-drug to an active specie by specific mitochondrial enzymes, binding to cardiolipin, a mitochondria-specific component and use of transgenes [93]. Mitochondriotropic quercetin and resveratrol derivates have been built by linkage to phosphonium cations, in which they represent the electrophoretic "engine" insuring import into the mitochondrial matrix [94–96]. However, so far there are no studies done with curcumin.

Curcumin treatment in *in vivo* and *in vitro* models: Mitochondrial dysfunction observations

Direct as well as nuclear-indirect protective effects of curcumin have been shown primarily in *in vivo* models (Table 2). Curcumin prevented the nephrotoxicity induced by hexavalent chromium (CrVI) by induction of Nrf2, the reduction of oxidant stress and the maintenance of the activity of antioxidant enzymes. In that work, Molina-Jijón et al. [97] showed that curcumin preserved the following mitochondrial parameters altered by the administration of potassium dichromate: respiratory control rate, adenosine triphosphate content, calcium retention, mitochondrial membrane potential ($\Delta\Psi m$), enzymatic activity of aconitase, activity in complexes I–IV of the respiratory chain, and ATP production. These data show a close relation between curcumin and mitochondrial function. Our group also reported that curcumin pretreatment prevents oxidative stress and mitochondrial dysfunction in potassium dichromate-induced hepatotoxicity, curcumin prevented the increase in the enzymatic activity of alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase in plasma; curcumin was also able to decrease the malondialdehyde and protein carbonyl levels. Besides, it prevented the decrease in the activity of hepatic antioxidant enzymes (SOD, CAT, GPx, GR, and GST). Interestingly, this effect was also observed in isolated mitochondria; which was associated with prevention in the reduction in mitochondrial respiration, evaluated by the state 3 of respiration and the respiratory control index. Potassium dichromate induced decrease in the activity of respiratory complex I, induced permeability transition pore (PTP) opening and cytochrome c (Cyt c) release; these alterations were also prevented with curcumin treatment. Histological analysis was consistent with the biochemical results already observed [98]. Furthermore, several parameters evaluated in animals treated only with curcumin were not altered [97, 98]. In a chronic kidney model (nephrectomy 5/6), curcumin also has direct effects maintaining cardiac mitochondrial function by reducing ROS production and end-products of lipid peroxidation (MDA and 4-hydroxynonenal), also by nuclear indirect effects increasing the activity of heart antioxidant enzymes associated with increased protein kinase C, that favors Nrf2 phosphorylation (serine 40) and promotes the

Table 2. *In vivo* studies of the beneficial effects of curcumin in mitochondrial functions.

Model	ROS inductor	Curcumin concentration	Observations	Refs.
Wistar rats	Hexavalent chromium Cr(VI)	100–400 mg/kg 2 weeks	Curcumin prevents aconitase, CAT, GR, GPx, GST, and SOD activities decrease, attenuates lipid peroxidation and protein carbonyl increase as well as GSH levels decrease, prevents both the state 3 diminution and state 4 increase, prevents RC, ADP/O, ATP content and activities of complexes I, II, II–III, and V diminution; prevents Ca^{2+} release and loss of $\Delta\Psi m$	[22, 97]
Wistar rats	5/6 Nx	120 mg/kg 9.5 weeks	Curcumin prevents RC diminution and maintains transmembrane potential and aconitase activity	[82]
C57BL/6J ob/ob mice	Liver steatosis	8 weeks	The expression of mitochondrial biogenesis genes NRF1 and Tfam is increased	[100]
Rats	Indomethacin	40 mg/kg	Mitochondrial respiration was increased; protein carbonyl, MDA, and myeloperoxidase were decreased	[99]
C57BL6 mice	High-fat diet	0.5–1% diet 14 weeks	Oxygen consumption from liver and kidney mitochondria was increased	[102]

ADP/O, Adenosine diphosphate/oxygen ratios; ATP, adenosine triphosphate; Ca^{2+}, calcium; CAT, catalase; Cr, chromium; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-*S*-transferase; MDA, malondialdehyde; $\Delta\Psi m$, mitochondrial membrane potential; Tfam, mitochondrial transcription factor A; Nx, nephrectomized; NRF1, nuclear respiratory factor 1; RC, respiratory control rate; SOD, superoxide dismutase.

dissociation of Nrf2 from Keap1 and its subsequent nuclear translocation. In this study, it was also observed the mitochondrial integrity in subtotally nephrectomized rats treated with curcumin by measuring respiratory rates in isolated cardiac mitochondria, an increase in aconitase activity, a marker of mitochondrial superoxide presence, as well as an increase in mitochondrial respiration produced by curcumin treatment were observed [82]. It has been reported that curcumin directly up-regulated aconitase activity, an enzyme of the Krebs cycle that functions as a stress marker in mitochondria, and enhanced oxidative phosphorylation capacities in heart-mitochondria isolated from rats treated with curcumin [10, 82]. Sivalingam et al. [99] observed that rats administered with indomethacin, a non-steroidal anti-inflammatory and a potent ROS producer, showed a reduction in the colon mitochondrial respiration and reduced GSH content, also an increase in the content of protein carbonyl, MDA and myeloperoxidase were observed; curcumin (40 mg/kg administered before indomethacin treatment) prevented all these alterations in colon mitochondria, suggesting again its potent direct therapeutic activity related to mitochondrial functions [99]. Furthermore, it was shown that curcumin increased indirectly the expression of mitochondrial biogenesis genes in obese mice with liver steatosis, such genes were NRF1 and mitochondrial transcription factor A (Tfam), which are responsible for the lower mitochondrial respiratory chain (MRC) complex I activity and the ATP production [100]. In high fat diet-fed mice, the liver ROS production was attenuated directly by curcumin addition, showing that the increase in mitochondrial respiration was directly mediated by curcumin and was not triggered by ROS production [101]. In another study, it has been shown mitochondrial dysfunction in liver and kidney of mice with obesity induced with a high-fat diet by 14 weeks. Interestingly, the respiration of kidney mitochondria was improved with doses of 0.5 and 1% of curcumin [102].

Curcumin also has showed protective direct effects against ROS production *in vitro* (Table 3). In primary cortical neurons cultured from embryonic rats (16–18 days) treated with *tert*-butyl-hydroperoxide (tBHP), an inductor of ROS production, it was observed that the treatment with 20 μM curcumin for 18 h attenuated ROS generation, apoptosis, and reduction in the membrane potential and glutathione content [103]. Also, a diminution (~20 to 30%) in the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is associated to oxidative stress and inflammation [104], was observed in human pancreatic cells treated with curcumin [105].

Therefore, the mitochondrial function has been evaluated in several *in vivo* (Table 2) and *in vitro* (Table 3) studies and is associated with the oxidative stress in a variety of tissues [10, 97, 103, 106–113].

Curcumin and mitochondria: Apoptosis in cancer models

As we have mentioned, curcumin has also demonstrated indirect anti-cancer properties. As it is known, apoptosis can occur by two main pathways: (i) the mitochondria-independent pathway: wherein caspase-8 is the initiator, producing

Table 3. Effect of curcumin on mitochondrial function in *in vitro* studies.

Normal cells				
Model	**Inductor ROS**	**Curcumin concentration (μM)**	**Observations**	**Refs.**
Primary cortical neurons	tBHP	20	ROS generation, Cyt c release, apoptosis, decrease in $\Delta\Psi$m and GSH content are attenuated by curcumin	[103]
Astrocytes	H_2O_2	10	The intracellular H_2O_2 production is decreased and cell viability and HO-1 activity are increased	[110]
Dopaminergic neurons (N27)	Normal cell	0.5	Derivatives of curcumin attenuate reduction in $\Delta\Psi$m and the viability of dopaminergic cells was increased	[128]
Embryonic stem cells (ESC-B5)	Mgx	20	Apoptosis, ROS production and caspase-3 activation were decreased and viability was increased	[129]

Cyt c, Cytochrome c; GSH, glutathione; HO-1, heme oxygenase 1; H_2O_2, hydrogen peroxide; Mgx, methyglyoxal; $\Delta\Psi$m, mitochondrial membrane potential; ROS, reactive oxygen spices; tBHP, *tert*-butyl hydroperoxide.

the truncated BH3 interacting-domain death agonist (tBid) that subsequently interacts with the pro-apoptotic Bcl2-associated X protein (Bax), leading to the release of Cyt c and triggering apoptosis [114] and (ii) the mitochondrial-mediated pathway, in which mitochondria release many pro-apoptotic factors such as Cyt c, second mitochondria-derived activator of caspases (Smac)/direct inhibitors of apoptosis-binding protein (Diablo) and apoptosis-inducing factor (AIF) into the cytoplasm, activating caspase-3 and caspase-9 and triggering apoptosis [115]. As shown in Table 4, in several studies it has been found that curcumin inhibits proliferation and augments apoptosis index in cancer models such as glioblastoma, skin, mammary, colon, liver, and pancreas [105]. In cancer cells, derived from a pheochromocytoma of rat adrenal medulla [116], that were treated with 25 μM curcumin for 16 h, a cellular antioxidant response was observed by a reduction of ROS. Interestingly, the activity of Cyt c oxidase, a MRC enzyme, was markedly increased (30–40%) after curcumin treatment. This may suggest that curcumin is able to induce mechanisms to protect the mitochondrial respiratory functions and maintain the redox homeostasis by the stimulation of cellular energy production [116]; for example, *Homo sapiens* brain glioblastoma cells treated with 25 and 50 μM curcumin for 24 h showed a decrease in cell viability, release of mitochondrial Cyt c, and increase in AIF levels [111]. To determine what apoptosis pathway was triggered by curcumin, Gogada et al. [112] treated *H. sapiens* breast epithelial adenocarcinoma (MDA-MB231) cells with 15 μM curcumin and observed increased expression of apoptosis protease-activating factor-1 [112], activity of caspase-9, release of Cyt c, Bim, and Bcl2-antagonist/killer (Bak) proteins in a time-dependent manner [111]. On the other hand, caspase-8 deficient cells showed no effect in the cell death activation during curcumin treatment, whereas Apaf-1 deficiency inhibited cell death and induced upregulation of caspase-9, Bim, and Bak, indicating that curcumin may regulate the apoptosis by mitochondrial pathway. In an *in vitro* model of rat liver mitochondria it was demonstrated that curcumin might induce PTP opening [106]. Morin et al. [106] showed that mitochondrial swelling was due to

Table 4. Effect of curcumin on mitochondrial function in *in vitro* studies.

Model	**Curcumin concentration**	**Observations**	**Refs.**
Rat pheochromocytoma cells (PC12)	25 μM	ROS production was reduced and mitochondrial SOD activity and oxygen consumption were increased	[116]
Human pancreatic carcinoma cells	IC_{50} of NF-kB binding on EMSA	NF-κB expression was decreased, proliferation was inhibited, and apoptosis was induced	[105]
Human brain glioblastoma cells (T98G)	25–50 μM	The release of Cyt c into the cytoplasm and the AIF levels were increased	[111]
Epithelial adenocarcinoma cells (MDA-MB231)	15 μM	The release of Cyt c into the cytoplasm and the AIF levels were increased	[112]

IC_{50}, 50% inhibitory concentration; AIF, apoptosis-inducing factor; Cyt c, cytochrome c; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen spices; SOD, superoxide dismutase.

a membrane protein thiol group oxidation through a direct interaction and also in a low intramitochondrial Ca^{2+} concentration. However, it was not mediated by an ROS overproduction to provide a mechanism for apoptotic factor release. While in five pancreatic carcinoma cells lines (BxPC-3, Capan-1, Capan-2, ASPC, and HS766-T) treated with curcumin, the induction of apoptosis and cell growth inhibition is associated with a down-regulation of NF-κB with its respective growth control molecules (inhibitor of nuclear factor kappa-B kinase subunit alpha and beta) [105].

Curcumin indirect anti-cancer properties may rely on apoptosis induction; thereby its use as a drug or as an adjuvant to traditional chemotherapy has been proposed [117]. In this context, it is very interesting that curcumin concentrations used in some of these experiments are similar to those used in experiments with non-cancerous cells in which apoptosis is not detectable.

Curcumin effect on mitochondria from tumoral cell lines

As it was mentioned, altered mitochondrial oxygen utilization may lead to an increase in ROS production [118]. Oxidative phosphorylation is a main mitochondrial function and maintenance of the $\Delta\Psi m$ is essential to couple ETC activity to ATP synthesis [119]. When the membrane is damaged, proton leak increases and $\Delta\Psi m$ decays; resulting in increased respiration rates, which may favor undesired reaction between electrons and O_2 forming $O_2^{\bullet -}$ on both sides of the inner mitochondrial membrane [120]; furthermore, loss of $\Delta\Psi m$ is an early event during apoptosis in some systems [90]. Particularly, in mouse Neuro 2A (N2a) cells, it has been shown that curcumin reduces oxidative-induced mitochondrial damage, preserves $\Delta\Psi m$ and prevents Cyt c release; these changes were increased pervasively in the neuroblastoma cell [121]. Interestingly, Cao et al. [90] showed that curcumin exerts an early change in $\Delta\Psi m$ in human hepatoma G2 cell line (HepG2). This compound induces an early hyperpolarization (1 h), followed by a constant depolarization and a $\Delta\Psi m$ collapse [90], and finally these events lead to apoptosis and cell death. Cao et al. [122] also observed that an increase in ROS generation induces oxidative damage to mtDNA in HepG2 cells. As curcumin induces the production of ROS in many tumoral cell lines and mtDNA oxidative damage can trigger cell apoptosis, this could be another pathway by which curcumin exerts its apoptotic action [123].

Even though the biochemical mechanisms associated with such responses in cancer and normal cell lines are not known yet, we described the most important biochemical properties of curcumin that may explain the anticancer and therapeutic effects of this natural compound.

Impact of curcumin on other pathways

Yoon et al. [124] showed that curcumin is preferentially cytotoxic to malignant breast cancer cells compared with normal breast cells and that cell death is due to induction of paraptosis, not apoptosis or autophagy. The paraptosis is characterized by a process of vacuolation that begins with physical enlargement of mitochondria and endoplasmic reticulum, independent of caspases pathway. It has been shown that curcumin induces paraptosis accompanied by swelling and fusion of mitochondria or ER in malignant breast cancer cells [124]. Recently, Eckert et al. [125] have studied the effect of curcumin in a model designed to study age-dependent changes in mitochondrial function. The protective effect of curcumin was associated to the improved mitochondrial function, including enhanced mitochondrial fusion and reduced fission, associated with changes in markers protein of fusion and fission (fission-1, optic atrophy-1, mitofusin-1 and dynamin related protein-1). In turn, this mitochondrial function improvement was secondary probably to the up-regulating of the nuclear factor peroxisome proliferator-activated receptor gamma co-activador alpha, which is a regulator of mitochondrial biogenesis [125].

Concluding remarks

Overwhelming evidence indicates that the mitochondria are involved in the therapeutic properties of curcumin, as it can modulate many mechanisms in which mitochondria are entangled, mainly apoptosis (mitochondrial-mediated pathway) and respiratory function.

In vivo studies have shown that curcumin attenuates oxidant stress in models in which ROS generation was triggered by one or more factors such as a pathological state, the use of drugs, chemicals, and the diet quality. The oxidant stress curtailment by curcumin was associated with nuclear translocation of Nrf2 and preservation of mitochondrial function; these beneficial effects also have been observed in *in vitro* studies (Fig. 3A). Presently, curcumin attenuates alterations by decreasing oxygen consumption, respiratory control rate, ATP production, calcium retention, $\Delta\Psi m$, membrane integrity, aconitase activity, and inhibiting the opening of the mitochondrial transition pore, thus avoiding the cell death. In several cancer models, curcumin induces apoptosis involving various pathways (Fig. 3B).

There is still a lot left to learn concerning the dual role of curcumin in cancer and normal cell, but it is important to emphasize that mitochondria are a relevant target for the protection provided by curcumin under different pathologic scenarios. However, further research must be done, as there are still unresolved questions to answer in this field; the

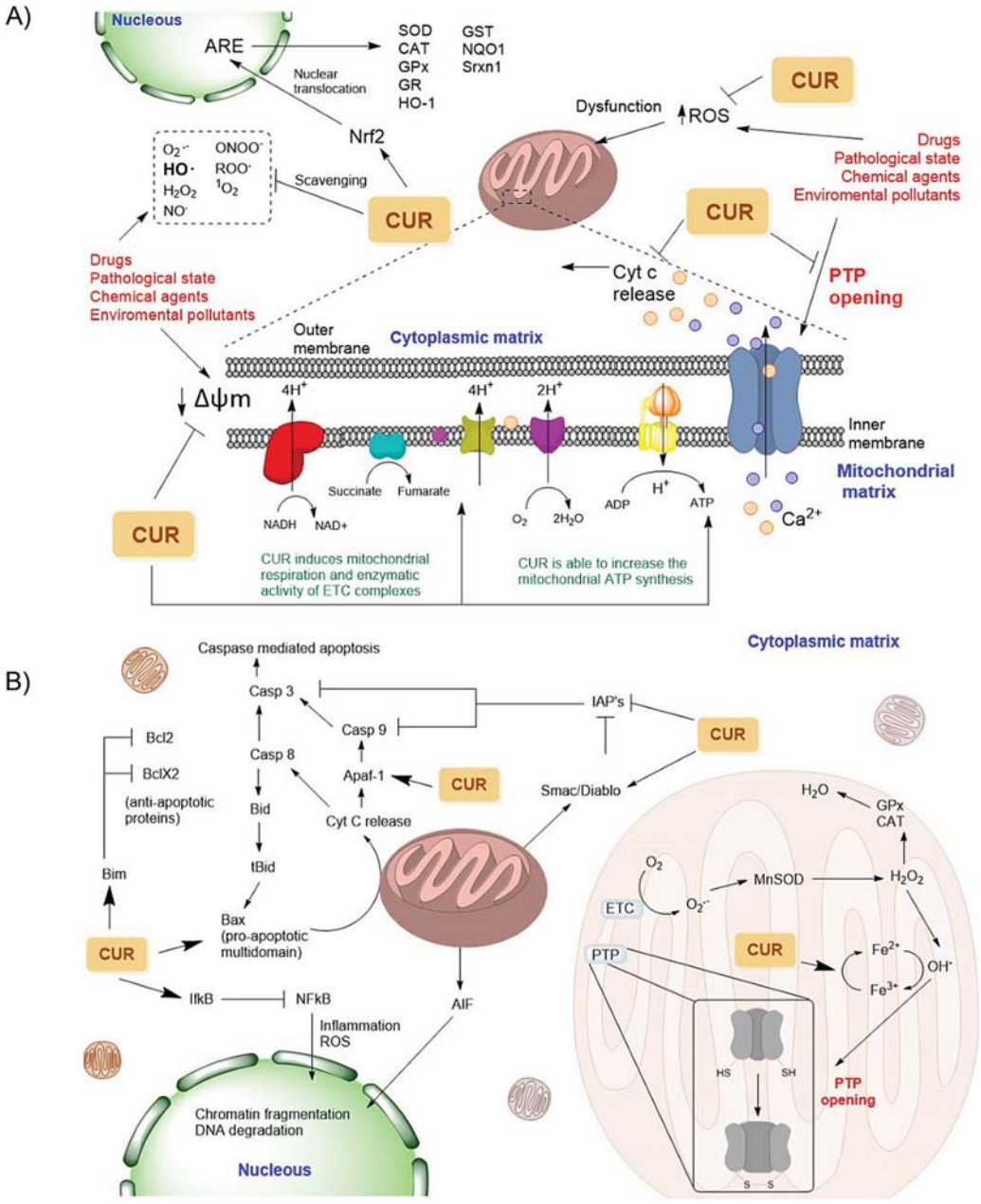

Figure 3. (A) Biochemical signaling produced by curcumin in physiological conditions. Curcumin is able to induce cytoprotective enzymes, reactive oxygen spices (ROS) diminishing, anti-apoptotic signaling, and mitochondrial respiration. (B) Biochemical signaling triggered by curcumin in cancer state. Contrary as in panel (a), curcumin is able to induce apoptosis signaling (mitochondrial-mediated pathway), permeability transition pore (PTP) opening and ROS generation. ARE, Antioxidant response elements; SOD, superoxide dismutase; MnSOD, mitochondrial superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; HO-1, heme oxygenase 1; GST, glutathione-*S*-transferase; NQO1, nicotinamide adenine dinucleotide phosphate oxidase: quinone oxidoreductase 1; Srxn1, sulfiredoxin 1; Nrf2, nuclear factor erythroid 2-related factor 2; $O_2^{\bullet -}$, superoxide anion; $OH^{\bullet}$, hydroxyl radicals; H_2O_2, hydrogen peroxide; $NO^{\bullet}$, nitric oxide; $ONOO^-$, peroxynitrite; $ROO^{\bullet}$, peroxyl radicals; $^1O_2$, singlet oxygen; CUR, curcumin; ROS, reactive oxygen species; Cyt c, cytochrome c; $\Delta\Psi m$, mitochondrial membrane potential; NADH, reduced nicotinamide adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; O_2, oxygen; H_2O, water; Ca^{2+}, calcium; Fe^{2+}, Fe^{3+}, iron; Bcl2, B-cell lymphoma 2; BclX2, Bcl2-like 1; Bid, BH3 interacting-domain death agonist; tBid, truncated BH3 interacting-domain death agonist; Bax, Bcl2-associated X protein; NF-κB, nuclear factor kappa B; casp, caspase; Apaf-1, apoptotic protease activating factor 1; IAP, inhibitor of apoptosis; AIF, apoptosis-inducing factor; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; Smac, second mitochondria-derived activator of caspases; Diablo, direct IAP-binding protein.

mitochondrial function is not yet analyzed in many models in which curcumin has conferred protection against many toxic agents, this information would help raise knowledge about how curcumin protects mitochondrial function. The biochemical pathway by which curcumin exerts its dual role in normal and cancer cells is still unknown and yet to be described.

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Mini Review

Renoprotective effect of the antioxidant curcumin: Recent findings

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ABSTRACT

For years, there have been studies based on the use of natural compounds plant-derived as potential therapeutic agents for various diseases in humans. Curcumin is a phenolic compound extracted from *Curcuma longa* rhizome commonly used in Asia as a spice, pigment and additive. In traditional medicine of India and China, curcumin is considered as a therapeutic agent used in several foods. Numerous studies have shown that curcumin has broad biological functions particularly antioxidant and antiinflammatory. In fact, it has been established that curcumin is a bifunctional antioxidant; it exerts antioxidant activity in a direct and an indirect way by scavenging reactive oxygen species and inducing an antioxidant response, respectively. The renoprotective effect of curcumin has been evaluated in several experimental models including diabetic nephropathy, chronic renal failure, ischemia and reperfusion and nephrotoxicity induced by compounds such as gentamicin, adriamycin, chloroquine, iron nitrilotriacetate, sodium fluoride, hexavalent chromium and cisplatin. It has been shown recently in a model of chronic renal failure that curcumin exerts a therapeutic effect; in fact it reverts not only systemic alterations but also glomerular hemodynamic changes. Another recent finding shows that the renoprotective effect of curcumin is associated to preservation of function and redox balance of mitochondria. Taking together, these studies attribute the protective effect of curcumin in the kidney to the induction of the master regulator of antioxidant response nuclear factor erythroid-derived 2 (Nrf2), inhibition of mitochondrial dysfunction, attenuation of inflammatory response, preservation of antioxidant enzymes and prevention of oxidative stress. The information presented in this paper identifies curcumin as a promising renoprotective molecule against renal injury.

Contents

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Introduction

History and cultivation of Curcuma longa

Curcuma longa (turmeric or curcuma) is a rhizomatus monocotyledonous perennial herbaceous plant member of the ginger family (Zingiberaceae), endemic and prevalent in tropical and subtropical regions including India, China and South East Asia. India is the most important producer, consumer and exporter of turmeric. Its Latin name Curcuma, is derived from the Arabic word, Kourkoum, the original name for saffron [16]. *C. longa* and its growth requires a hot, humid climate with temperatures between 20 and 30 °C and great amounts of water [29]. Turmeric has long been known as a spice, remedy and dye, and since 1280, Marco Polo mentioned turmeric in his travel around China and India. In the 13th century, Arabian merchants brought turmeric to the European market from India. During the British settlement of India in the 15th century, turmeric was combined with several other spices to form curry powder.

Curcuminoids and curcumin

Curcuma contains 60–70% carbohydrate, 8.6% protein, 5–10% fat, 2–7% fiber, 3–5% curcuminoids (50–70% curcumin) and up to 5% essential oils and resins. The curcuminoid content in turmeric may vary between 2 and 9%, depending on geographical conditions [29]. The composition of curcuminoids is approximately 70% curcumin (curcumin I), 17% demethoxycurcumin (curcumin II), 3% *bis*-demethoxycurcumin (curcumin III) and the rest (10%) is called cyclocurcumin (curcumin IV) [5,8,36] (Fig. 1). However, the last compound has been associated with poor or non-biological activity [61]. The most active component of turmeric is curcumin [67]; Vogel first isolated it in 1815 [29]. Curcumin is an orange–yellow crystalline powder practically insoluble in water. The structure of curcumin (Fig. 1) was first described in 1910 by Lampe and Milobedeska and proved to be diferuloylmethane [29]. Studies indicate that functional groups associated to curcumin chemical structure including *bis*-α,β – unsaturated β-diketone, two methoxy groups, two phenolic hydroxy groups and two double-conjugated

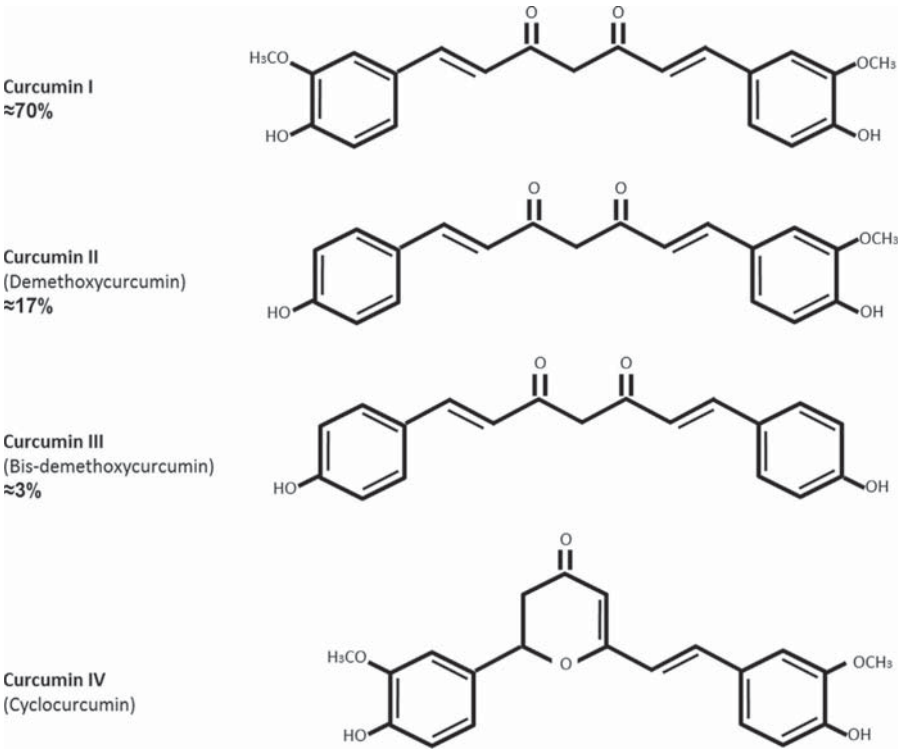

Fig. 1. Chemical structures and abundance of curcuminoids in turmeric that have terapeutic effects.

bonds might play an essential role in antiproliferative and anti-inflammatory activities assigned to curcumin [3]. Curcumin has keto-enol tautomers, of which keto form predominates in acid and neutral solutions and enol form in alkaline solutions.

Curcumin as a food additive

Turmeric is an ingredient of spice blends, mainly curry powder, which generally consists of turmeric, clove, paprika, ginger, cardamom, coriander, cumin, mace, pepper and cinnamon. Turmeric is commonly used as natural pigment (yellow 3) in the cosmetic and textile production but it is widely used in food industry. Indeed, turmeric is classified as an additive in E100 category and is used as food color additive in mustard, pastries, daily products and canned fish [40]. Furthermore, according to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) the admissible daily intake (ADI) is 0–3 mg/kg body weight and it was established at the 61st JECFA in 2003 [29].

Curcumin: Traditional uses in folk medicine and biological properties

Curcuminoids have been consumed as therapeutic infusions over the centuries worldwide. In Ayurvedic medicine, curcumin is a well-documented treatment for various respiratory conditions such as, asthma, bronchial hyperactivity and allergy, as well as for liver disorders, anorexia, rheumatism, diabetic wounds, runny nose, cough and sinusitis [10]. In traditional Chinese medicine curcumin has been used to treat diseases associated with abdominal pain [35]. In ancient Hindu medicine, it was used to treat sprains and swelling [10]. In Oriental cultures, it has traditionally been used as good therapeutic alternative, particularly as an anti-inflammatory, antioxidant, anticarcinogenic and antimicrobial reagent. In fact, it has scientifically proven that curcumin is indeed antioxidant [11,31,26,17], anti-inflammatory [2,82,86], and antibacterial [52]. Moreover, it has also been used because of its hepatoprotective [56,32,86], thrombosuppressive, neuroprotective [85,18,63], cardioprotective [37,23,73], antineoplasic [4,17,68], antiproliferative [12], hypoglycemic and antiarthritic effect [35]. Curcumin has also been used for the treatment of intestinal parasites and as a remedy for poisoning, snakebites and various other complaints [49]. In this review we are focused on the renoprotective effects of curcumin and the mechanisms involved in this effect.

Antioxidant properties of curcumin

Oxidative stress plays a major role in the pathogenesis of various diseases including myocardial ischemia, cerebral brain ischemia-reperfusion injury, hemorrhage and shock, neuronal cell injury, hypoxia and cancer. Curcuminoids exhibit a differential antioxidant activity in several *in vitro* and *in vivo* models, for example, preventing lipid peroxidation in a variety of cells such as erythrocytes, rat brain homogenates, rat liver microsomes, liposomes and macrophages, where peroxidation is induced by Fenton's reagent, as well as for metals, hydrogen peroxide (H_2O_2) and 2,2′-azo-*bis*(2-amidino-propane) hydrochloride (AAPH) [62].

Furthermore, it has been reported that curcumin is a bifunctional antioxidant [26] because of its ability to react directly with reactive species and to induce an up-regulation of various cytoprotective and antioxidant proteins. Curcumin is able to scavenge superoxide anion ($O_2^{\bullet}$) [6,76], hydroxyl radicals ($^{\bullet}OH$) [13], H_2O_2, [6,13], singlet oxygen [25], nitric oxide [77,78], peroxynitrite [44] and peroxyl radicals ($ROO^{\bullet}$) [13]. Together, these mechanisms might explain, at least in part, some of the cytoprotective effects of this compound. Features as the presence of phenolic groups in the structure of curcumin (Fig. 1) explains its ability to react with reactive oxygen species (ROS) and reactive nitrogen species (RNS) and might probably be one of the mechanisms through which curcumin treatment protects the epithelial cells of renal tubules (LLC-PK1) from oxidative damage induced by H_2O_2 [22].

The indirect antioxidant capacity of curcumin is defined by its ability to induce the expression of cytoprotective proteins such as superoxide dismutase (SOD), catalase (CAT) [59], glutathione reductase (GR), glutathione peroxidase (GPx) [87], heme oxygenase 1 (HO-1) [42,63], glutathione-*S*-transferase (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1) [88] and γ-glutamylcysteine ligase (γGCL) [65]. Furthermore, it has been reported that curcumin can increase the synthesis and concentration of reduced glutathione (GSH) in astrocytes and neurons by induction of γGCL [47]. The cytoprotective proteins induced by curcumin are regulated by the nuclear factor erythroid-derived 2 (Nrf2, [24,64]), which in turns is also activated by curcumin [17,28]. On the other hand, it is well known that encoding genes for cytoprotective proteins are induced coordinately by a common molecular mechanism in which the inductors highly modify reactive thiol groups of cysteine in the Kelch-Like ECH-Associated Protein 1 (Keap1) [27]. Keap1 protein is a zinc metalloprotein cysteine-rich bound to Nrf2 and normally associated with the protein complex cullin 3 (Cul3), which promotes the ubiquitination and subsequent proteosomal degradation, preventing Nrf2 translocation into the nucleus. Also, it was established that the gene expression of cytoprotective proteins is regulated by three cellular components: (i) Antioxidant response element (ARE) sequence, a specific sequence present in regulatory regions of the genes of cytoprotective proteins, (ii) Nrf2, a transcription factor consisting of a basic leucine zipper that regulates basal and inducible expression of cytoprotective genes, and (iii) Keap1, the chemical sensor for inductors. In general, the cysteine residues interaction of protein-Keap1 with some compounds induces conformational changes that abrogate the ability of Keap1 to repress Nrf2; this transcription factor migrates to the nucleus where it is combined with small Maf transcription factors. This complex binds to ARE facilitating the transcription of cytoprotective gene. By this reason, Nrf2 is considered a master regulator of the antioxidant response against oxidative stress.

Renal diseases epidemiology

The homeostasis of body extracellular electrolyte composition and fluid volume is essential for all animals and humans to survive. The kidney plays a fundamental role in maintaining precise body and/or extracellular electrolyte, fluid balance and blood pressure homeostasis primarily through the actions of its proximal and distal tubular segments of nephrons [38]. Under renal insufficiency conditions, deregulation of extracellular electrolytes or overall fluids volume may lead to disturbance of the circulation, including cardiac output and blood pressure [39]. Prevalence of chronic kidney disease is estimated to be 8–16% worldwide [43] and it is expected that the number of patients with chronic kidney disease increase at a fastest rate in the poorest parts of the world. According to the 2010 Global Burden of Disease Study, chronic kidney disease was ranked 27th in the list of causes of total number of global deaths in 1990, but rose to 18th in 2010 [48]. Patients with chronic kidney disease are at an increase risk of acute kidney injury (AKI). Acute kidney injury might occur with the use of several drugs, such as non-esteroidal anti-inflammatory drugs, antibiotics, antineoplastic drugs, and angiotensin-converting-enzyme inhibitors. A meta-analysis of 13 cohort studies confirmed that AKI is an important risk factor for chronic and end-stage renal disease [21]. Severe, long, and repeated episodes of acute kidney injury increase the risk of progression of chronic kidney disease. Acute and chronic renal failures are global public health issues with different features to take into account in different parts of the world, renal complications, which involve most organ systems, can be treated and prevented, by using different therapeutic strategies.

Renoprotective effect of curcumin

Renal injury induced by diabetes

Diabetic nephropathy (DN) is one of the main causes of end-stage renal disease. DN is characterized by the presence of hyperfiltration, glomerular hypertrophy, tubular albuminuria, mesangial matrix expansion, and increased expression of extracellular matrix proteins that involves several profibrotic factors such as transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF). The effect of curcumin on diabetic nephropathy has been studied [75]. Sharma et al. [69] have been found that curcumin administration (15 and 30 mg/kg/day for two weeks) protects against streptozotocin-induced diabetic nephropathy and oxidative stress. Furthermore, Soetikno et al. [72] evaluated in a diabetic nephropathy model the effect of oral curcumin administration (100 mg/kg/day for 8 weeks) showing that curcumin prevents progression of renal disease.

Curcumin treatment attenuates proteinuria and improves creatinine clearance after 3 weeks of streptozotocin injection. In addition, it decreased oxidative stress by reducing levels of subunits of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase Nox4 and p67phox, which catalyzes the synthesis of $O_2^{-\bullet}$. Moreover, it also increased the activity of the antioxidant enzyme GPx.

The renoprotective effect of curcumin was related to the down-regulation of the profibrotic cytokines vascular endothelial growth factor (VEGF), TGF-β, CTGF and osteopontin as well as in extracellular matrix proteins fibronectin and collagen IV. Moreover, it was observed a reduction in the development of structural damage evidenced by lower glomerulosclerosis index (GS), tubulointerstitial (IT) fibrosis and arteriolopathy. These effects might be in part mediated by inhibition of protein kinase C-β (PKC-β), kinase responsible for the phosphorylation of a wide diversity of proteins [72]. Cellular events such as inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [20,71] and decrease of macrophage infiltration [71], histone acetyltransferase p300 protein and oxidative stress [20], also have been involved in the mechanisms through curcumin protects against diabetic nephropathy. Furthermore, the protective effect of curcumin in diabetic nephropathy has also been associated to the prevention of kidney triglycerides buildup [74]. Interestingly, curcumin also mitigates cardiac [73] and cerebral [46] complications in streptozotocin-induced diabetes.

Curcumin derivatives have also been proved to be effective in ameliorating diabetic nephropathy. Pan et al. [58,57] studied the effect of curcumin B06 and C66 analogues in diabetic rats (0.2, 1 and 5 mg/kg/day for 6 weeks) [57,58]. B06 treatment reduced the inflammatory kidney response by the attenuation of (a) renal macrophage infiltration, (b) expression of the profibrotic cytokine TGF-β, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) and (c) the proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). The anti-inflammatory effect of B06 was associated with the inhibition of c-Jun N-terminal kinase (JNK)/NF-κB activation [58,57]. Similarly, the oral administration (80 mg/kg/day for 45 days) of tetrahydrocurcumin (THU), another curcumin derivative, attenuated the renal and hepatic dysfunction found in rats with diabetes induced by streptozotocin and nicotinamide [53].

Renal injury induced by 5/6 nephrectomy

The study of chronic progressive renal injury in rats as the 5/6 nephrectomy (5/6NX), which involves the removal of 5/6 of the renal mass, is useful in the evaluation of strategies to reduce renal injury. This model is characterized by proteinuria, hypertension, proliferation of smooth muscle cells of the glomerular arterioles (arteriolopathy), IT inflammation and hemodynamic alterations in individual nephrons [79].

In 5/6NX renal injury model, Ghosh et al. [33] found that curcumin (75 mg/kg/day for 8 weeks) exerted a renoprotective effect associated with the attenuation of inflammation, macrophage infiltration and the high levels of TNF-α in plasma and kidney and also of renal NF-κB activation [33]. Further studies in this model by Tapia et al. [79] evaluated the renoprotective effect of curcumin (60 mg/kg for 37 days). Nephrectomized animals developed hypertension, proteinuria, increase in serum creatinine and blood urea nitrogen (BUN) and glomerular hemodynamic alterations including hyperfiltration (high single nephron glomerular filtration rate and single nephron glomerular plasma flow), glomerular hypertension (high glomerular capillary pressure) and decrease in the afferent and efferent resistances as well as renal injury characterized by GS and IT fibrosis and inflammation. Interestingly, after curcumin administration, all the above systemic and glomerular alterations were attenuated. This was the first work showing that curcumin is able to prevent glomerular hemodynamic alterations secondary to 5/6NX (Fig. 2). The protective effect of curcumin in this experimental model was associated to an increased activity of the antioxidant enzymes CAT, GPx, GR, GST and SOD and a decrease in oxidative stress. Additionally, Soetikno et al. [70] found that curcumin administration (75 mg/kg/day for 8 weeks) in 5/6NX rats could reduce proteinuria, systolic blood pressure, GS, IT damage and inflammatory markers as TGF-β, TNF-α, NF-κB and COX-2. They also found that curcumin attenuated malondialdehyde (MDA) levels (a lipid peroxidation marker) which was associated with lower expression of p67phox and p22phox, essential subunits of NADPH oxidase [70]. Both studies conducted by Tapia et al. [79,80] established that the protective mechanism achieved by curcumin in the kidney was mainly due to the nuclear translocation of Nrf2. Later, Tapia et al. [80] showed that curcumin postreatment (120 mg/kg for 30 days), given 30 days after the 5/6NX induction, was able to revert renal damage and oxidative stress. These experiments show that curcumin is a therapeutic agent in experimental chronic renal failure. It is important to highlight that tissue injury throwback effect of curcumin turns this molecule into a promising therapeutic agent. In this regard, it is also remarkable that curcumin can protect not only against renal injury but also against adverse effects derived from 5/6NX. For example, Correa et al. [23] showed that curcumin

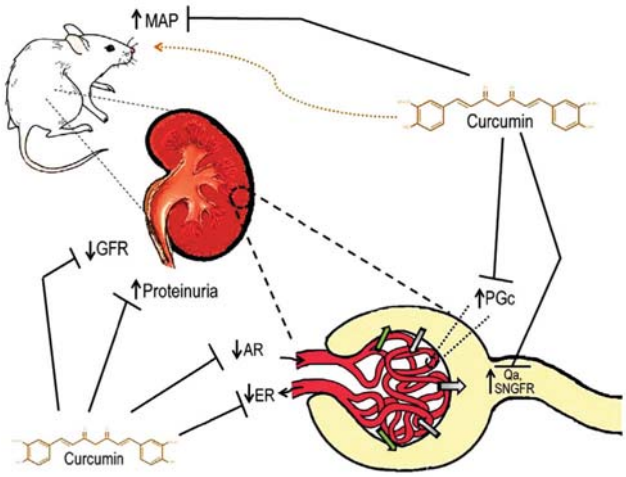

Fig. 2. Curcumin prevents renal hemodynamic alterations. Curcumin ameliorates 5/6NX-induced alterations in mean arterial pressure (MAP), proteinuria and glomerular filtration rate (GFR) and in the following parameters of renal hemodynamics: single-nephron glomerular filtration rate (SNGFR), single-nephron plasma flow (Qa), glomerular capillary pressure (PGc), afferent resistance (AR) and efferent resistance (ER).

(120 mg/kg/day for 67 days) protects against cardiovascular disorders and cardiac tissue remodeling associated to the development of chronic renal failure in rats with 5/6NX. The cardioprotective effect of curcumin in 5/6NX model was associated with reduction in ROS production and oxidative stress markers, an increased antioxidant response and preservation of mitochondria function. Finally, Ghosh et al. [34] reported in this 5/6NX model that curcumin (75 mg/kg/day for 8 weeks) blocks overexpression of inflammatory mediators such as TNF-α and interleukin 1β (IL-1β) through activation of phospholipase 2 (PLP2) and COX-2, both key regulators of inflammation and oxidative stress inductors.

Renal injury induced by ischemia and reperfusion (I/R) or by glomerulonephritis

The effect of curcumin on acute kidney injury induced by I/R also has been studied. This renal injury may be consequence of several factors as renal transplantation and involves vascular factors and tubular damage associated with high significant morbidity and mortality. Curcumin was administered orally to rats (200 mg/kg/day for 7 days) subjected to bilateral renal ischemia for 45 min followed by 24 h reperfusion [15]. Curcumin significantly attenuated the reduction of serum GPx and the levels of urea, cystatin C, and MDA in serum and the increase of the MDA concentration, nitric oxide and protein carbonyl content in kidney of rats with I/R [15]. On the other hand, Jacob et al. [41] observed, in mice with deficient immune response and glomerulonephritis injury, that curcumin administration reduces GS and improves renal function (evaluated by BUN and albuminuria), which was associated to a decrease in inflammatory markers (TGF-β and MCP-1) and matrix proteins (fibronectin, laminin and collagen).

Shock-wave lithotripsy (SWL)

SWL is commonly used for treatment of renal stones and ROS are involved in the pathophysiology of renal injury due to SWL. The protective effect of curcumin (75 mg/kg/day for 35 days) against renal injury induced by SWL was studied in Sprague-Dawley rats [14]. Curcumin prevented interstitial, glomerular, tubular epithelial and endothelial cellular injuries by decreasing of iNOS and p65 (the active subunit of NF-κB) expression and serum nitric oxide levels. This protective effect was also associated with increased levels of GSH and attenuation of high levels of MDA in kidney [14].

Triiodothyronine (T3)-induced renal injury

One of the most important effects of thyroid hormones (T3 and T4) is the elevation of mitochondrial respiration, producing a hypermetabolic state with excess generation of free radicals, Thyroxine has been reported to induce renal hypertrophy with a rise in the DNA content. However, there is a paucity of information on T3-induced oxidative damage to mammalian kidney in general and with respect to antioxidant treatment in particular [66].The effect of curcumin treatment (30 mg/kg/day for 15 days) was evaluated on renal damage and oxidative stress induced by T3 administration. It was found that curcumin was able to attenuate the mitochondrial lipid peroxidation, the enhanced SOD activity and the histopathological changes secondary to T3-administration [66].

Renal injury induced by drugs

Cisplatin and oxaliplatin

Cisplatin is an effective anticancer drug used against lung, ovarian cancer and some lymphomas, however renal damage has limited its use. It has been well established that oxidative stress is one of the mechanisms involved in cell damage induced by cisplatin. Indeed, a decrease of antioxidant defense is clearly observed *in vivo* and *in vitro* experimental models [19]. Antunes et al. [9] reported curcumin administration (8 mg/kg before and after cisplatin injection) provided protection against cisplatin induced neurotoxicity, ototoxicity and nephrotoxicity (evaluated by serum creatinine and creatinine clearance) and oxidant stress (evaluated by MDA and GSH levels) in rats. Moreover, Kuhad et al. [45] designed a two-day curcumin pretreatment and in parallel treatment of 15, 30 and 60 mg/kg of curcumin in a model of cisplatin-induced nephrotoxicity. The cisplatin-treated group that received 60 mg/kg of curcumin showed normal renal function (evaluated by measuring urea levels and creatinine clearance), which correlated with lipid peroxidation reduction. Interestingly, curcumin administration in cisplatin-treated animals attenuated, in a dose dependent manner, the cisplatin-induced decrease in GSH, SOD and CAT [45]. In addition, Ueki et al. [82] studied the effect of curcumin administration (100 mg/kg ip) on the inflammatory mechanisms involved in the pathogenesis of cisplatin-induced renal injury in mice. Curcumin prevented cisplatin-induce tubular necrosis, decreased renal dysfunction and the increase of pro inflammatory markers including of TNF-α in serum, and TNF-α and MCP-1 in renal tissue, and a rising of intracellular adhesion molecule 1 (ICAM-1) mRNA in kidney. Oxaliplatin, another platinum-based chemotherapeutic agent can induce renal damage and oxidant stress. *In vitro* studies performed by Waly et al. [84] showed that oxaliplatin or cisplatin induced oxidative stress in human embryonic kidney cells (HEK 293). These cells also showed a decrease in total antioxidant capacity (TAC) and inhibition of the activity of SOD, CAT and GPx. Interestingly, curcumin added to these cell cultures significantly restored TAC and activity of the above mentioned antioxidant enzymes. Together, these studies clear up the ability of curcumin to decrease oxidative stress through modulation of those enzymes.

Gentamicin

Gentamicin is an aminoglycoside used in the treatment of infections caused by Gram-negative bacteria that induces renal injury as a side effect. Curcumin treatment (200 mg/kg/day for 10 days) ameliorated the gentamicin-induced nephrotoxicity in rats [7]. Moreover, Manikandan et al. [50] observed a renoprotective effect after curcumin administration (200 mg/kg/day for 7, 15 and 30 days) in gentamicin-treated animals. Nephrotoxicity was evidenced by increased serum creatinine and BUN. An increase in ROS and renal lipoperoxidation and a reduction in GSH and in the antioxidant enzymes GPx, GST, SOD and CAT were related to the impaired glomerular filtration. The degree of these alterations was diminished in animals treated with curcumin. Furthermore, curcumin modulated the inflammatory response in gentamicin-treated rats through NF-κB expression in a time-dependent manner [50].

Cyclosporin A (CsA)

CsA is widely used as immunosuppressant drug in organ transplantation to prevent rejection. This therapeutic agent induces renal damage as side effect. Tirkey et al. [81] showed that curcumin was effective by protecting CsA-induced damage, since animals that received the phenolic compound plus CsA did not show renal function deterioration or depletion of antioxidant enzymes. CsA increased the thiobarbituric acid reactive substances (TBARS), decreased renal endogenous antioxidant enzymes and deteriorated the renal function as assessed by increased serum creatinine, BUN and decreased creatinine and urea clearance. Curcumin reduced

elevated levels of TBARS, attenuated renal dysfunction, increased the levels of antioxidant enzymes and normalized the altered renal morphology in CsA treated rats.

Adriamycin (doxorubicin)

Adriamycin is a chemotherapeutic drug that may induce nephrotoxicity as a side effect. Treatment with curcumin (200 mg/kg/day in 1% gum acacia for 30 days) markedly protected against adriamycin-induced proteinuria, albuminuria, hypoalbuminaemia and hyperlipidemia. Furthermore, curcumin also reduced urinary levels of the enzyme *N*-acetyl-β-D-glucosaminidase (NAG), a marker of tubular damage [83].

Chloroquine

Chloroquine is a drug used in the malaria treatment and induces renal injury and oxidant stress as secondary effect. It was found that THU (80 mg/kg/day for 15 days) and curcumin treatment prevents chloroquine-induced nephrotoxicity and lipid peroxidation and also the decrease in the antioxidants vitamin C, vitamin E, SOD, CAT and GPx in kidneys of these rats [60].

Renal injury induced by chemicals

Sodium fluoride (NaF)

Renal damage induced by chemicals also has drawn attention. Nabavi et al. [54] reported the renoprotective effect of curcumin (10 and 20 mg/kg) in rats with nephrotoxicity induced by NaF. Renal damage in these rats relies on an impairment of renal function evidenced by increased serum creatinine and BUN and structural impairment due to interstitial edema, inflammation and fibrosis which was associated with increased nitric oxide, peroxides, ROS and MDA. On the other hand, curcumin treated animals showed an improvement in renal function and showed less histological damage associated to restoration of SOD and CAT activities and increased GSH levels.

Heavy metals

Studies evaluating the effect of curcumin in experimental models of nephrotoxicity by heavy metals such as chromium (Cr) [51], cadmium [30] and mercury (Hg) [1] have been conducted. Curcumin treatment attenuated renal dysfunction, oxidative stress and the decrease in antioxidant enzymes induced by metals. Interestingly, Molina-Jijón et al. [51] demonstrated that curcumin has a protective effect against nephrotoxicity induced by hexavalent chromium (Cr VI), and this property was related to the nuclear translocation of Nrf2, prevention of oxidant stress and preservation of the activity of antioxidant enzymes and of mitochondrial function in the kidney. In this study, a pretreatment of 10-day with 400 mg/kg of curcumin attenuated the structural and functional damage to the kidney which was associated with the prevention of mitochondrial oxidant stress and of the decrease in the following mitochondrial determinations: oxygen consumption (state 3), respiratory control, ATP content, calcium retention and membrane potential. Also curcumin prevented the decrease in the following enzymatic activities: aconitase, antioxidant enzymes and mitochondrial respiratory complexes I, II, II–III and V [51], (Fig. 3). This was the first demonstration that the prevention of renal injury was associated to the preservation of mitochondrial function.

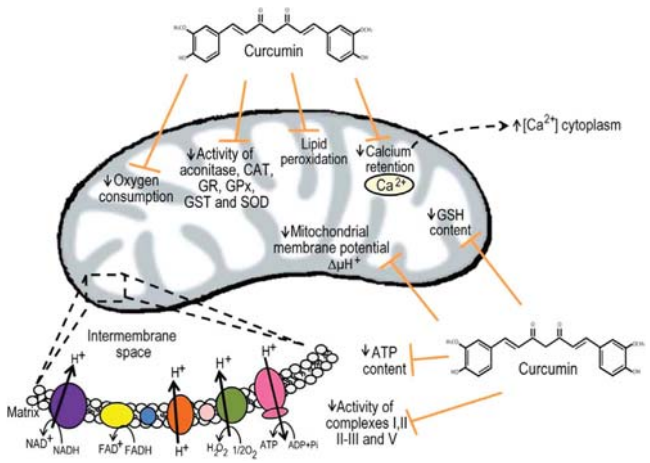

Fig. 3. Curcumin is able to prevent mitochondrial dysfunction associated to renal injury. Curcumin is able to prevent lipid peroxidation and the decrease in the following mitochondrial determinations: oxygen consumption, activity of complexes I, II, II-III and V, activity of aconitase and antioxidant enzymes, GSH content, membrane potential, calcium retention and ATP content [51]. GSH (Glutathione), SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), GST (glutathione-*S*-transferase), GR (glutathione reductase), NAD^+ (nicotinamide adenine dinucleotide), NADH (nicotinamide adenine dinucleotide, reduced form), FAD^+ (flavin adenine dinucleotide), $FADH_2$ (flavin adenine dinucleotide, reduced form), ATP (adenosine triphosphate), ADP (adenosine diphosphate).

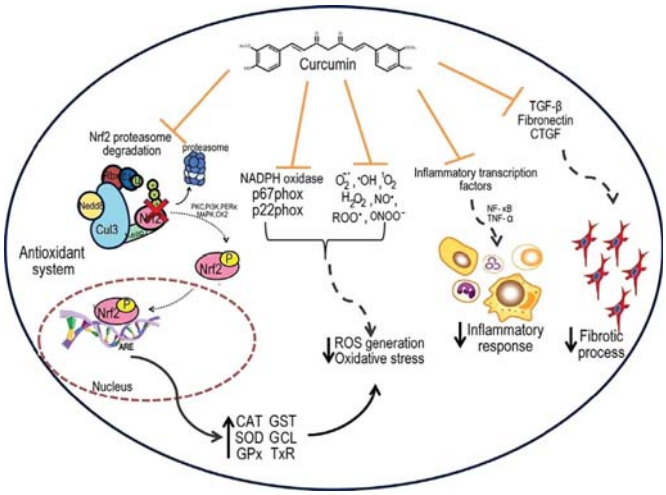

Fig. 4. Curcumin is able to prevent several mechanisms leading to renal injury. Curcumin renoprotective effects have been associated with the prevention of three main factors, first the reduction of oxidative stress by (a) preventing the generation of $O_2^{-\bullet}$ and scavenging different reactive oxygen species, and (b) by preventing the Nrf2 degradation by ubiquitin proteosoma pathway, thus an increase of many antioxidant enzymes. Curcumin has been shown also to be able to reduce inflammatory process by reducing the inflammatory transcription factors such as NF- κB and TNF-α. On the other hand the reduction of cytokines such as TGF-β or CTGF eventually prevents a fibrotic process. ROS (reactive oxygen species), $O_2^{-\bullet}$ (superoxide),•OH (hydroxyl radical), H_2O_2 (hydrogen peroxide), $ONOO^-$ (Peroxynitrite), $^1O^2$ (Singlet oxygen), $NO^\bullet$ (Nitric oxide), $ROO^\bullet$ (peroxyl radical), Nrf2 (translocation of nuclear factor erythroid-derived 2), ARE (Antioxidant responsive elements), Keap1 (Kelch-like ECH-associated protein 1), Cul3 (cullin 3), Nedd8 (Neural precursor cell expressed developmentally down-regulated 8), u (ubiquitin), SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), GST (glutathione-*S*-transferase), GCL (glutathione-cystein-ligase), TrX (thioredoxin), TGF-β (factor transforming growth beta), CTGF (connective tissue growth factor), NF- κB (nuclear factor kappa-light-chain-enhancer of activated B cells), TNF-α (tumor necrosis factor), PKC (protein kinase C), PI3K (phosphoinositol 3-kinase), PERK (protein kinase RNA-like endoplasmic reticulum kinase) MAPK (mitogen-activated protein kinase), CK2 (Casein kinase2).

Table 1
Factors associated to renoprotection by curcumin.

	Studied targets	Effect of curcumin treatment	Renal injury models
Transcription factors	Nrf2	Promotes the Nrf2 translocation to the nucleus, the major regulator of the antioxidant response	5/6 NX, HM (Cr VI)
Pro-oxidant enzymes	NADPH oxidase subunits: Nox4, p67phox, p22phox	Attenuates oxidative stress by reducing levels of subunits of NADHP oxidase	Diabetic nephropathy
Antioxidants	GPx CAT GR GST SOD NQO1 GSH levels	Increases the activity of antioxidant enzymes Increases the synthesis and concentration of GSH	Diabetic nephropathy, 5/6 NX, I/R, SWL,T3, Cisplatin, Gentamicin, CsA, Chlr, NaF, HM (CrVI), FNT
Profibrotic cytokines	VEGF TGF-β CTGF Osteopontin	Attenuates the expression of the cytokines promoting a decrease in the inflammatory response	Diabetic nephropathy, I/R
Extracellular matrix protein	Fibronectin Collagen IV Laminin	Promotes a decrease in matrix proteins	I/R
Pro-inflammatory mediators	TNF-α MCP-1 NF-κB p65 (NF-κB subunit) JNK/NF-κB	Reduces the inflammatory response	Diabetic nephropathy, 5/6 NX, I/R, SWL, Cisplatin, Gentamicin
	COX-2 iNOS IL-1β PLP2 TGF-β	Decreases the inflammatory markers by blocking its overexpression	
Mitochondrial function markers	Oxygen consumption ATP content Calcium retention	Prevents the decrease of mitochondrial parameters	HM (Cr VI)
	Mitochondrial membrane potential Activity of mitochondrial respiratory complexes	Protective effect associated with the preservation of mitochondrial function	

5/6NX:5/6 nephrectomy, I/R:ischemia and reperfusion, SWL: shock-wave lithotripsy, T3: triiodothyronine, CsA: cyclosporine, Chlr: chloroquine, NaF: sodium fluoride, HM: heavy metals, FNT: ferric nitrilotriacetate.

Ferric nitrilotriacetate

Ferric nitrilotriacetate is a carcinogen and strong inductor of renal oxidative stress. The effect of curcumin and THU on ferric nitrilotriacetate induced oxidant stress in male ddY mice was studied [55]. Animals were fed along four weeks with 0.5% curcumin or 0.5% THU before the administration of ferric nitrilotriacetate. Curcumin inhibited 4-hydroxy-2-nonenal-modified protein formation and THU inhibited lipid peroxidation and renal abundance of 4-hydroxy-2-nonenal (4-HNE, a marker of lipid peroxidation)-modified proteins and 8-hydroxy-2′-deoxyguanosine (a marker of DNA damage). THU induced GPx, GST and NQO1, as well as or better than curcumin.

Final remarks

According to epidemiological evidence, acute renal injury is a serious health and economical problem across the world with increasing cases since this disease can have its own etiology but also can be a complication from other diseases or can be a side effect from several medical treatments. The urgency to develop renoprotective strategies sets the eyes in compounds as curcumin, which has been used in the traditional medicine, specifically because its protective effects against renal damage. In this context, the experiments of Tapia et al. [80], in which curcumin was able to revert established renal injury and systemic alterations in rats with 5/6NX, are promising. At cellular and molecular levels, recent studies have demonstrated that this compound attenuates ROS generation and activates signaling pathways that involve the release of Nrf2 from Keap1, promoting transcription of genes that induce the expression of antioxidant system (GPx, GST, CAT, and SOD). Also, recent evidence shows that improvement of mitochondrial dysfunction induced during nephrotoxicity seems to be a key mechanism in curcumin protection and indirect reduction of ROS production through mechanisms including a decrease of $O_2^{-\bullet}$ through the down regulation of expression of some NADPH oxidase subunits such as p67phox, p22phox and NOX4 essential for $O_2^{-\bullet}$ generation, which is useful against inflammation, a common process during kidney injury. In this regard, curcumin also diminishes the expression of NF-κB and TNF-α, TGF-β, extracellular matrix proteins collagen type IV, fibronectin and growth factors such as CTGF. These proteins are closely involved not only in inflammation but also, in the remodeling tissue which leads to renal fibrosis. Fig. 4 and Table 1 summarize some cell protection mechanisms of curcumin in renal damage. The information presented in this paper identifies curcumin as a promising renoprotective molecule against renal injury.

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