DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA

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TESIS DOCTORAL

IDENTIFICACIÓN DE BIOMARCADORES URINARIOS PARA LA MEJORA DEL DIAGNÓSTICO DEL DAÑO RENAL AGUDO INDUCIDO POR GENTAMICINA

Yaremi Quiros Luis
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UNIVERSIDAD DE SALAMANCA

DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA

IDENTIFICACIÓN DE BIOMARCADORES URINARIOS PARA LA MEJORA DEL DIAGNÓSTICO DEL DAÑO RENAL AGUDO INDUCIDO POR GENTAMICINA

MEMORIA PRESENTADA POR YAREMI QUIROS LUIS PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSIDAD DE SALAMANCA

Salamanca , a 30 de octubre de 2010

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EL DOCTOR D. RAFAEL JIMÉNEZ FERNÁNDEZ, DIRECTOR DEL DEPARTAMENTO DE FISIOLOGÍA Y FARMACOLOGÍA DE LA UNIVERSIDAD DE SALAMANCA

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CERTIFICA:

Que el presente trabajo, elaborado por la Lda. en Farmacia Yaremi Quiros Luis para optar al Grado de Doctor, con el título "IDENTIFICACIÓN DE BIOMARCADORES URINARIOS PARA LA MEJORA DEL DIAGNÓSTICO DEL DAÑO RENAL AGUDO INDUCIDO POR GENTAMICINA", ha sido realizado bajo la dirección del Doctor D. José Miguel López Novoa y el Doctor D. Francisco J. López Hernández, en el Departamento de Fisiología y Farmacología de la Universidad de Salamanca.

Y para que así conste, expido y firmo el presente certificado en Salamanca, a 30 de septiembre de 2010.

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Que el presente trabajo, titulado "IDENTIFICACIÓN DE BIOMARCADORES URINARIOS PARA LA MEJORA DEL DIAGNÓSTICO DEL DAÑO RENAL AGUDO INDUCIDO POR GENTAMICINA", presentado por la Licenciada en Farmacia Yaremi Quiros Luis para optar al Grado de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Fisiología y Farmacología de la Universidad de Salamanca, consideramos que cumple las condiciones necesarias y autorizamos su presentación con el fin de que pueda ser defendido ante el tribunal correspondiente.

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Fdo.: Dr. D. José Miguel López Novoa

Fdo.: Dr. D. Francisco J. López Hernández

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INTRODUCCIÓN

La imaginación es mas importante que la cultura,
Porque el hombre que solo es culto, termina rodeado de limites,
Mientras que la imaginación puede darle la vuelta al mundo

A. Einstein

INTRODUCCIÓN

1. EL SISTEMA RENAL.

El aparato urinario humano está constituido por dos riñones, dos uréteres, una vejiga y la uretra. A partir de un filtrado inicial del plasma sanguíneo, por medio de procesos de reabsorción y secreción, los riñones producen la orina que llega a la vejiga a través de los uréteres y se elimina al exterior por la uretra.

1.1. Anatomía del riñón.

Los riñones son dos órganos de color pardo-rojizo situados retroperitonealmente en la parte dorsal del abdomen, a cada lado de la columna vertebral. Los riñones están recubiertos por una *cápsula* de tejido fibroso.

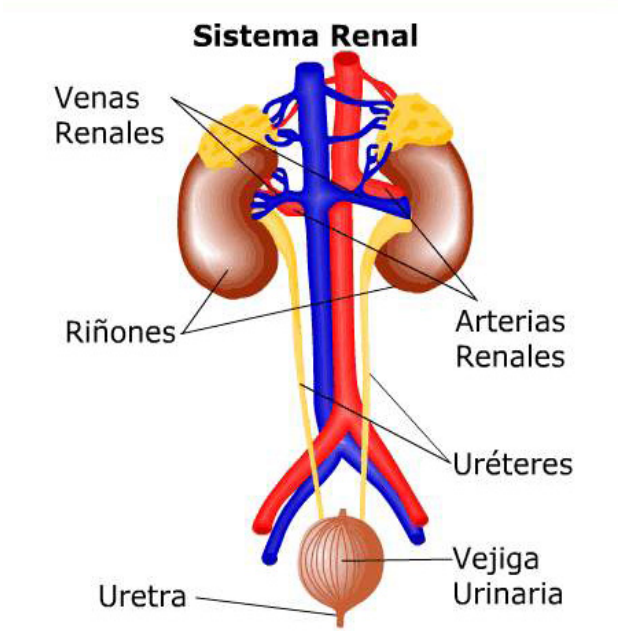

Figura 1. *Representación de las partes del aparato urinario.*

En una sección longitudinal (Fig 2) se distinguen dos regiones: la corteza en la parte externa y médula en la parte interna. La corteza cubre la base de cada una de las unidades cónicas de la médula, denominadas pirámides medulares, cuyo ápice se prolonga en la pelvis renal dando lugar a la papila. Un examen macroscópico permite distinguir que la corteza tiene un aspecto ligeramente granular que no se observa en la médula. Cada pirámide medular puede dividirse en una zona exterior, adyacente a la corteza, y una zona interior que incluye la papila (Bulger y Doyban, 1982; Tisher, 1981). Las estrías que aparecen en las pirámides corresponden con la porción tubular de las nefronas.

1.2. Anatomía de la nefrona.

La nefrona es la unidad anatómica y funcional del riñón. El riñón humano está formado por cerca de un millón y medio de nefronas. Cada una de ellas consta de un elemento filtrante denominado *corpúsculo renal*, y un *sistema tubular* que se extiende por fuera del corpúsculo renal, en el cual se realizan los procesos de secreción y reabsorción (Madsen y Tisher, 1986), que determinan la composición final de la orina. Se distinguen dos tipos de nefronas. Las *nefronas yuxtamedulares* y *nefronas corticales*.

1.2.1. El corpúsculo renal.

El corpúsculo renal o de Malpighi está compuesto por el *glomérulo capilar* y la *cápsula de Bowman* que lo recubre (figura 3). Existe un espacio dentro de la cápsula, *espacio de Bowman*, hacia donde pasa el líquido filtrado procedente del glomérulo.

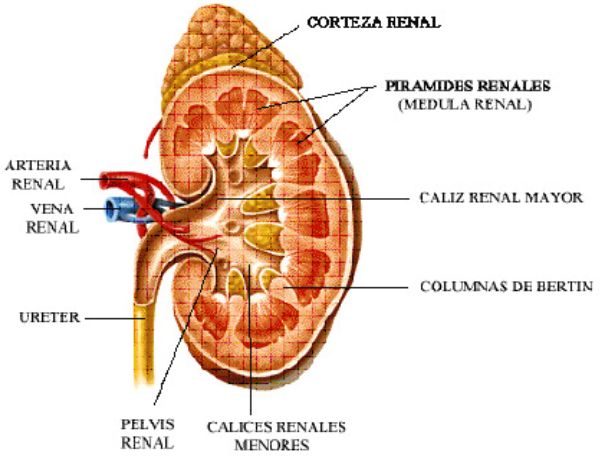

Figura 2. *Estructura general de un riñón humano (adaptada de Netter, 2003).*

La barrera de filtración del corpúsculo renal o membrana glomerular, consta de tres capas: el endotelio de los capilares glomerulares, la membrana basal y una capa de células epiteliales especializadas con fenestraciones. Las células epiteliales que descansan sobre la membrana basal son muy diferentes de las células simples y aplanadas que revisten el resto de la cápsula de Bowman y se denominan *podocitos*, tienen gran número de extensiones podálicas o *pedicelos* integradas en la membrana basal. Las hendiduras entre pedicelos adyacentes constituyen la vía de paso del filtrado, que una vez atraviesa las células endoteliales y la membrana basal, penetra en el espacio de Bowman y desde allí pasa a la primera porción del túbulo proximal (Berne y Levi, 2001).

Además de las células endoteliales capilares y de los podocitos hay un tercer tipo de células en el corpúsculo renal, las células mesangiales, que se encuentran en la parte central del glomérulo entre las asas capilares, a veces penetrando en ellas, de forma que hacen contacto con las células endoteliales. Las células mesangiales tienen las siguientes funciones:

- Contracción. La capacidad de contracción debida a los miofilamentos de actina y miosina es la principal función de estas células. De esta manera se regula el coeficiente de ultrafiltración, en respuesta a distintos agonistas.
- Capacidad fagocítica.
- Capacidad de síntesis y degradación de su propia matriz extracelular.

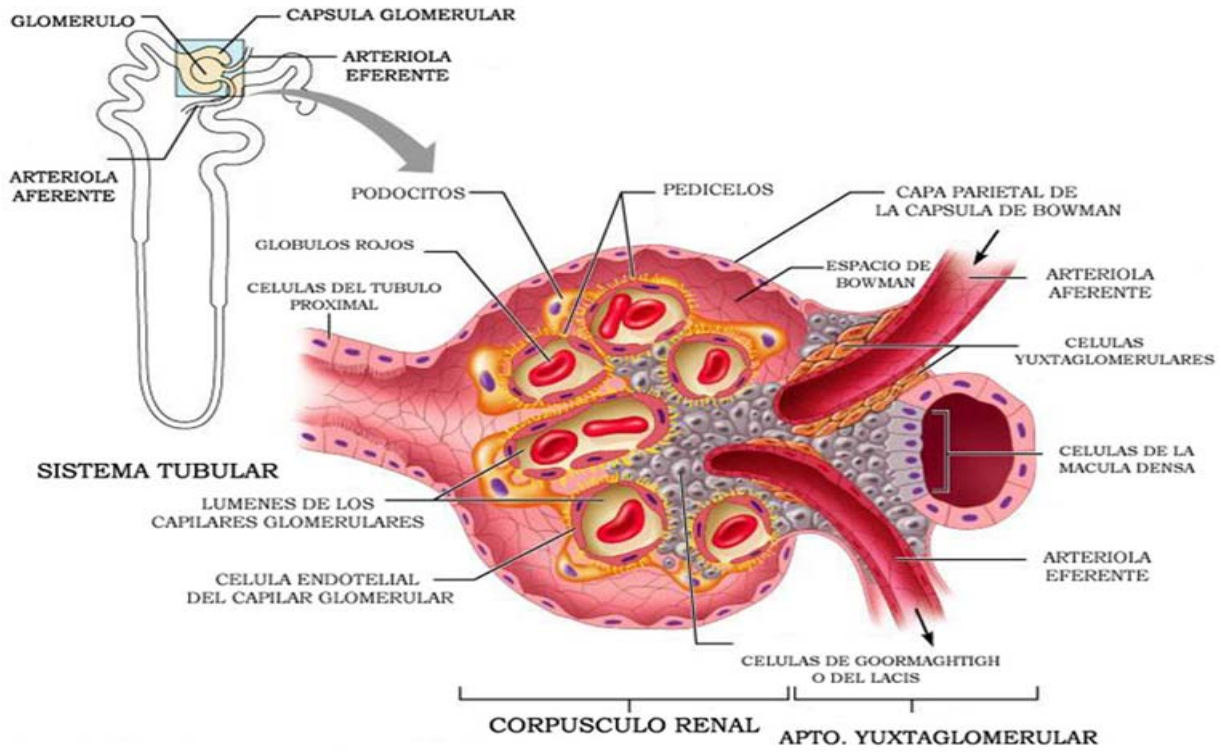

Figura 3. *Representación esquemática de una nefrona y de un corpúsculo (adaptada de Netter, 2003).*

- Síntesis de determinados autacoides y factores de crecimiento que pueden actuar de forma autocrina o paracrina.

1.2.2. El sistema tubular.

La pared tubular está constituida por una sola capa de células epiteliales que descansan sobre una membrana basal. La estructura y función de esas células epiteliales varía mucho de un segmento a otro del túbulo, pero tienen una característica común: la presencia de uniones estrechas entre células adyacentes. El segmento de túbulo donde drena la cápsula de Bowman se denomina *túbulo contorneado proximal*, el cual inicialmente forma varias espiras, y es el segmento más largo y grueso de la nefrona. Esta

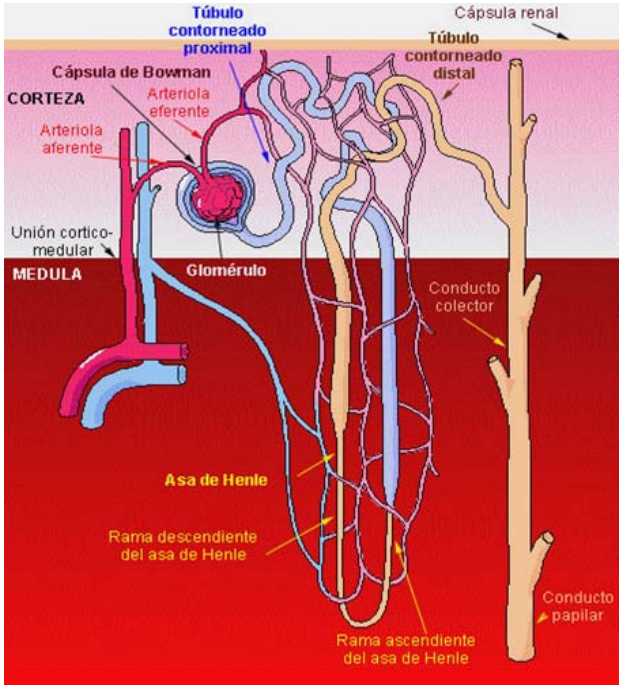

Figura 4. *Esquema del sistema tubular y de la red vascular asociada.*

tapizado por un epitelio cúbico simple, con células que presentan un borde en cepillo muy desarrollado en su superficie luminar, numerosas invaginaciones en la membrana basolateral, un gran aparato endolisosómico, y numerosos peroxisomas y mitocondrias, que va seguido por un segmento recto que desciende hacia la médula, el tubulo proximal recto, o S3, que se continua con la *rama estrecha descendente del asa de Henle*. Esta rama termina en horquilla y entonces comienza un ascenso paralelo a la rama descendente, formando la rama ascendente estrecha del asa de Henle, que se continua con la *rama ascendente gruesa del asa de Henle*, seguido del *túbulo contorneado distal*. Varios tubulos distalesdan lugar a un *túbulo colector*, el cual vacía su contenido en cada uno de los cálices de la pelvis renal. Los distintos segmentos del túbulo están representados en la figura 4. La pelvis se continúa con el uréter, y éste con la vejiga urinaria, donde la orina se almacena de manera transitoria y de la cual se elimina en forma intermitente. Después de penetrar a un cáliz, la orina ya no sufre alteraciones. A partir de ese punto, el resto del sistema urinario sirve sólo como conductor hacia el exterior (Bulger y Doblan, 1982, Tisher, 1981).

1.2.3. El aparato yuxtaglomerular.

El aparato yuxtaglomerular está situado entre la primera porción del túbulo contorneado distal y la arteriola aferente y eferente pertenecientes al corpúsculo renal de su propia nefrona (Barajas, 1979) (Figura 3). Está formado por tres tipos de células:

-Las *células yuxtaglomerulares*: son células mioepiteliales que rodean el final de la arteriola aferente, ricas en gránulos de secreción que producen el 90% de la renina.

-Las *células de la mácula densa*: son células epiteliales diferenciadas de la pared del túbulo recto distal ascendente, que controlan la secreción de renina y la velocidad de filtración glomerular.

-Las *células de Goormaghtigh o del lacis:* son células mesangiales extraglomerulares, que responden a múltiples mediadores y controlan la superficie de filtrado.

2. FUNCIONES DE LOS RIÑONES.

2.1. Regulación del equilibrio hidroelectrolítico.

La función primaria de los riñones es lograr un equilibrio entre la injesta y la eliminación del agua corporal y los iones inorgánicos para mantener estable la cantidad y la concentración de esas sustancias en el líquido extracelular. Dicho equilibrio se logra mediante cambios en la composición de la orina excretada. Algunas de las sustancias del medio interno reguladas en gran parte por los riñones son el agua, sodio, potasio, cloro, calcio, magnesio, sulfato, fosfato y el ión hidrógenión. No obstante, los riñones no son los únicos reguladores de las sustancias inorgánicas esenciales. De igual manera, los riñones también participan en la regulación de algunos nutrientes orgánicos, como son la glucosa, aminoácidos, proteínas, urea, ácido úrico y otros (Vander, 1993).

2.2. Excreción y concentración de catabolitos y sustancias tóxicas.

Consiste en retirar los productos metabólicos de la sangre y excretarlos por la orina. Entre estos productos de desecho que son eliminados se encuentran la *urea* (resultante del catabolismo de proteínas), el ácido úrico (resultante del catabolismo de los ácidos nucleicos), la creatinina (resultante del metabolismo del músculo), la bilirrubina (procedente del metabolismo de la hemoglobina), metabolitos de hormonas y otros. Algunos de ellos son relativamente inocuos, pero la acumulación de otros cuando la función renal está dañada causa algunos de los trastornos de las funciones corporales observadas en las enfermedades renales graves (Vander, 1993). Los riñones tienen otra función excretora general: la eliminación de sustancias químicas extrañas, fármacos, pesticidas, aditivos de alimentos, etc. (Vander, 1993).

2.3. Regulación de la presión arterial.

Los riñones participan estrechamente en la regulación de la presión arterial mediante varios mecanismos que actúan a corto, medio o largo plazo. A continuación se describen brevemente los principales.

2.3.1. Regulación de la excreción de sodio.

Se trata del principal mecanismo de regulación arterial a largo plazo del organismo (López-Hernández y López-Novoa, 2006). El equilibrio del sodio es un factor determinante crítico del gasto cardiaco. El aumento del balance positivo de sodio aumenta el volumen extracelular. Cuando se acumula mucho líquido extracelular en el organismo la presión arterial aumenta debido al mayor volumen sanguíneo, que eleva la presión media de llenado circulatorio y el retorno venoso de sangre hacia el corazón. En consecuencia aumenta el gasto cardiaco y a su vez la presión arterial. De esta manera, cuando hay exceso de sal en el cuerpo, se incrementa la osmolaridad de los líquidos corporales, lo que conlleva a la ingesta de agua adicional para diluir la sal extracelular hasta su concentración normal. Así se incrementa el volumen de líquido extracelular y con él la presión arterial. El aumento de la osmolaridad del líquido extracelular estimula también la secreción de hormona antidiurética, que hace que el riñón reabsorba cantidades elevadas de agua de la orina, lo que contribuye también a aumentar el líquido extracelular. Además, el equilibrio del sodio tal vez sea también un factor determinante de la resistencia arteriolar durante periodos prolongados (Knox y Granger, 1987; Guyton, 1992; Vander, 1993).

2.3.2. Sistema renina-angiotensina (SRA).

El riñón funciona como una glándula endocrina del SRA, un complejo hormonal de enzimas, proteínas y péptidos importantes en la regulación de la presión arterial. La renina es una enzima proteolítica segregada a la sangre por los

riñones, específicamente por las células granulares del aparato yuxtaglomerular. Una vez en la corriente sanguínea la renina cataliza el desdoblamiento de la angiotensina I (Ag I), derivada de una proteína plasmática denominada angiotensinógeno, a su vez segregada por el hígado y casi siempre presente en el plasma en concentración elevada. La enzima convertidora de angiotensina (ECA) separa los dos aminoácidos terminales de la poco activa Ag I para producir la angiotensina II (Ag II), muy activa. En el plasma se encuentra una cierta cantidad de ECA, pero la mayor parte está en el endotelio de los vasos sanguíneos, en particular, en los capilares pulmonares. No obstante, como los riñones producen renina y el tejido renal también contiene angiotensinógeno y ECA, la reacción generadora de Ag I y II ocurre en cierta medida dentro de los riñones.

La Ag II ejerce muchos efectos sobre diversos tejidos, pero el objetivo final de gran parte de ellos es incrementar la presión arterial. Habitualmente el angiotensinógeno y la ECA están presentes en el suero en concentración casi constante, por tanto, el determinante primario de la velocidad de formación de Ag II es la concentración plasmática de renina, fisiológicamente regulada por su secreción (Dzau y cols., 1988; Vander, 1993).

2.3.3. Otras sustancias vasoactivas.

Es muy probable que los riñones segreguen a la sangre o retiren de ella otras sustancias vasoactivas diferentes de la renina. Así, los riñones pueden sintetizar varios eicosanoides de acción vasodilatadora o vasoconstrictora. De la misma manera se segregan lípidos vasodilatadores (Vander, 1993).

2.4. Síntesis y secreción de otras sustancias.

2.4.1. Secreción de eritropoyetina.

Los riñones producen eritropoyetina (EPO), esta hormona relacionada con el control de la producción de eritrocitos en la médula ósea, y por tanto con el mantenimiento de niveles de oxígeno adecuados para el funcionamiento del organismo. Las enfermedades renales pueden reducir la secreción de EPO, con la consecuente producción de anemia grave (Vander, 1993).

2.4.2. Secreción de 1,25-dihidroxivitamina D3.

Los riñones producen 1,25-dihidroxivitamina D3, la forma activa de la vitamina D, la cual interviene en el metabolismo del calcio aumentando la absorción del calcio intestinal y la movilización del calcio óseo (Vander, 1993).

2.4.3. Gluconeogénesis.

Durante el ayuno prolongado los riñones sintetizan glucosa a partir de aminoácidos y otros precursores y la liberan a la sangre. Por tanto, el riñón es un órgano gluconeogénico (Vander, 1993).

3. PROCESOS RENALES BÁSICOS.

El riñón lleva a cabo sus funciones generales (secciones 2.1. y 2.2.) mediante dos procesos fundamentales que tienen lugar en cada una de las nefronas: el **filtrado glomerular**, que origina un gran volumen diario de filtrado de líquido extracelular (180 litros), y el **transporte tubular (reabsorción** y **secreción tubular)**. A continuación se describen estos procesos.

3.1. Filtración glomerular.

La formación de la orina comienza por la filtración en el glomerulo hacia la capsula de Bowman de unos 125 ml de plasma por minuto, lo que corresponde aproximadamente a un 20% del plasma que pasa por el riñón. Este proceso de formación de ultrafiltrado a través de las membranas capilares glomerulares recibe el nombre de ultrafiltración glomerular. No obstante, a pesar de su gran permeabilidad, la membrana glomerular tiene una cierta selectividad por moléculas relativamente

grandesen función del diámetro, configuración molecular y carga eléctrica.

La permeabilidad de la membrana glomerular está condicionada en parte por los pesos moleculares de las sustancias, siendo prácticamente impermeable a las proteínas plasmáticas, pero muy permeable al resto de las sustancias de menor peso molecular disueltas en el plasma normal. Por otra parte, los poros de la membrana son suficientemente grandes como para permitir el paso de moléculas con diámetro de hasta 8 nm. La membrana basal glomerular está recubierta por una red de proteoglicanos cargados negativamente. Así pues, las proteínas que presentan carga negativa producen una repulsión electrostática con las paredes de los poros, esto evita prácticamente que todas las proteínas pasen a su través. Otro factor que influye es la presión de perfusión, sobre todo cuando atendiendo a los factores anteriores la molécula estuviera en el límite de paso (Guyton, 1992).

El filtrado glomerular tiene casi la misma composición que el plasma, desde el punto de vista electrolítico, excepto por la ausencia de celulas y proteínas de alto y medio peso molecular (Guyton, 1992). Su concentración de proteínas es del orden de 0.03%, es decir, unas 240 veces menor que el contenido proteico del plasma. La relación entre el filtrado glomerular y el flujo plasmático renal se denomina *fracción de filtración.*

El proceso de filtración glomerular está condicionado por la suma neta de las diferentes fuerzas que se originan en los capilares glomerulares y en la cápsula de Bowman (Vander, 1993). Favorecen la filtración la presión hidrostática de los capilares glomerulares (Pg), que es la presión media en los capilares glomerulares y tiene un valor de 60 torr, y la presión oncótica de la cápsula de Bowman (πi), sin embargo como la concentración de proteínas en la cápsula de Bowman es muy pequeña, este factor es despreciable y se considera cero. Se oponen a la filtración la presión oncótica de los capilares glomerulares (πg), que es la presión que ejercen las proteínas del plasma y tiene un valor de 32 torr, y la presión hidrostática de la cápsula de Bowman (Pi), que es la presión que ejerce el líquido filtrado en la cápsula de Bowman y tiene un valor de 18 torr. La *presión efectiva de ultrafiltración* (PEF), se define como la diferencia entre la presión que favorece la filtración, es decir Pg, y las que se oponen, que son Pi y πg. Su valor aproximado es de 10 torr.

$$PEF = Pg - (Pi + \pi g)$$

El *coeficiente de filtración* (Kf), es el producto de la permeabilidad hidráulica de la membrana por el área disponible para la filtración. En función de Kf y PEF, se define la *tasa filtración glomerular* (TFG):

$$TFG = Kf \times PEF$$

La *tasa filtración glomerular* (TFG) o cantidad de filtrado glomerular que se forma por minuto en todas las nefronas de ambos riñones, es un índice de la función renal y es esencial para evaluar la gravedad y evolución de los trastornos renales. Más del 95% del filtrado se reabsorbe normalmente en los túbulos y el resto se elimina en forma de orina (Vander, 1993).

3.2. Reabsorción y secreción tubular.

A medida que el filtrado glomerular recorre los túbulos renales, sufre procesos de reabsorción y secreción de distintas sustancias de forma selectiva, de tal forma que la composición final de la orina depende del balance neto de los procesos de filtración, reabsorción y secreción; regulándose de esta forma, el volumen y la composición de los líquidos biológicos. La reabsorción es cuantitativamente mayor que la secreción, pero esta última determina las cantidades finales urinarias de potasio, hidrogeniones y otras sustancias (Guyton, 1992).

4. FRACASO RENAL AGUDO.

El fracaso renal agudo (FRA) es un tipo de lesión de gravedad clínica en la que la función excretora renal se reduce súbitamente tanto que los riñones son incapaces de depurar la sangre de los fármacos, tóxicos y productos nocivos de deshecho procedentes del metabolismo, y de conseguir el equilibrio electrolítico. Como consecuencia de ello, la función de muchos otros órganos y tejidos y, con ella, la vida del paciente se ven seriamente comprometidas. El FRA se caracteriza por una disfunción renal aguda (que surge horas o pocos días después del inicio del daño) derivada de un estímulo patológico rápido e intenso (Esteller y Cordero, 1998; Rivero y cols., 2000).

EL FRA se puede producir como consecuencia de cualquier proceso que disminuya la función renal (medida por la tasa de filtración glomerular). Supone un deterioro brusco de la función renal excretora con aparición de uremia, oliguria, anuria o diuresis renal. La función renal puede normalizarse si se descubre y se trata satisfactoriamente la causa subyacente del problema. El pronóstico depende fundamentalmente de la intensidad y el tipo de lesión. En general, una lesión tisular leve o una disfunción moderada desaparecen con la retirada del agente nefrotóxico, mientras que la destrucción extensa de uno o varios compartimentos renales (p.e. la necrosis tubular aguda) puede originar una deficiente reparación e incluso un deterioro progresivo y crónico.

4.1 Incidencia y morbimortalidad.

El FRA presenta todavía una alta morbi-mortalidad con consecuencias humanas y sociales muy importantes. Se calcula que aproximadamente del 1 al 7% de los pacientes que ingresan en los hospitales presentan FRA (Chertow y cols., 2001; Liangos y cols., 2006), el 25% en unidad de cuidados intensivos (UCI) desarrollan FRA en algún momento (Mendonca y cols., 2000) y que aproximadamente un 15% de los pacientes sometidos a *bypass* y de las mujeres embarazadas sufren algún grado de FRA. La tasa de mortalidad debido al FRA se mantiene alarmantemente constante alrededor del 50% de los casos, que asciende al 80% entre los pacientes que desarrollan fallo multiorgánico como consecuencia de el FRA (Rivero Sánchez y cols., 2000) y en la mayoría de los casos es necesario aplicar diálisis, lo que supone un gran coste humano y socioeconómica.

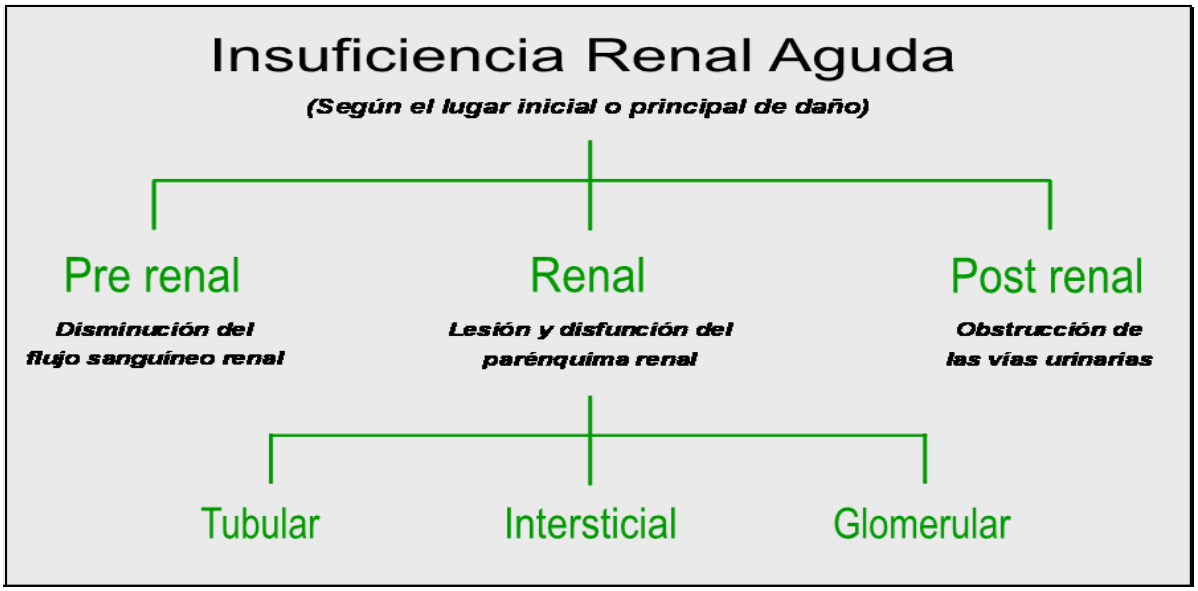

Figura 5. *Principales categorías de insuficiencia renal aguda.*

Como se indica en las secciones siguientes, una causa importante de FRA, relacionada directamente con el objeto de esta tesis doctoral, es la nefrotoxicidad aguda, o daño renal agudo producido por fármacos y toxinas. La nefrotoxicidad constituye un problema de salud y socioeconómico muy serio en todo el mundo. Aproximadamente el 25% de los 100 fármacos más utilizados en las unidades de cuidados intensivos son potencialmente nefrotóxicos (Taber y Mueller, 2006). Además, se estima que la nefrotoxicidad es la causa del 10-20% de los casos de FRA (Brivet y cols., 1996).

4.2 Fisiopatología general del FRA.

Esta enfermedad se clasifica en pre-renal, renal o post-renal, de acuerdo con el mecanismo que la desencadena (figura 5). Las causas pre-renales y renales, representan el mayor porcentaje de casos, aunque no es infrecuente observar que la etiología sea multifactorial y que un mismo agente nocivo produzca simultáneamente efectos pre-renales y renales (Esteller y Cordero, 1998; Singri N, 2003).

4.3. FRA pre-renal.

Constituye la forma de FRA más prevalente (55-60% de los casos). Se debe a una perfusión sanguínea renal deficiente que resulta de una disminución súbita y aguda de la presión arterial (shock) o de una interrupción del flujo de sangre hasta los riñones debido a un traumatismo o enfermedad grave. Esta situación lleva a una caída en la filtración glomerular. Cuando la presión arterial cae, ocurre una dilatación gradual de la arteriola aferente (autorregulación del flujo sanguíneo renal) mediada por la generación de óxido nítrico y una constricción eferente concomitante mediada por la angiotensina II, que tienden a mantener una presión hidrostática capilar constante. Sin embargo, hay un punto en que la hipoperfusión es tal, que estos cambios compensatorios se hacen insuficientes y comienza a caer rápidamente la presión hidrostática capilar, con la consiguiente caída de la filtración glomerular y el aumento de reabsorción tubular de agua, electrolitos y otros integrantes de la orina primaria, lo que produce oliguria. Además, como consecuencia de la hipoperfusión se produce una isquemia renal que da lugar a una necrosis tubular aguda cortical, que es la primera causa de insuficiencia renal aguda, y que, dependiendo del grado de isquemia, puede afectar también a otras estructuras. La lesión isquémica de la célula epitelial tubular, incluye turgencia celular, pérdida del borde en cepillo, pérdida de la polaridad por una redistribución de las proteínas de membrana (por ejemplo la bomba Na^+K^+ ATPasa, que aumenta la liberación distal de sodio, lo que activa la retroalmentación tubuloglomerular contribuyendo a la vasoconstricción), además de necrosis y apoptosis. Como consecuencia de esto, se agotan todas las reservas de ATP, se acumula calcio, y se activan enzimas que alteran y dañan la estructura de la célula y que inducen apoptosis. Las células dañadas se desprenden y obstruyen la luz del túbulo, aumentando la presión intratubular con la consiguiente disminución del filtrado glomerular. Además se producen lesiones endoteliales que aumentan la liberación de endotelina (vasocontrictor), lo que suma la disminución de la producción de NO y PGI_2 (Rivero Sánchez y cols., 2000; Esteller y Cordero, 1998).

Entre las principales causas de hipoperfusión renal destacan las siguientes:

- La hipovolemia (por hemorragias, deshidratación, uso de diuréticos, etc.).

- La insuficiencia cardiaca.

- El uso de ciertos medicamentos como los antiinflamatorios no esteroideos (AINEs) que disminuyen la producción de las prostaglandinas que normalmente dilatan la arteriola aferente y aumentan la presión hidrostática capilar, o los inhibidores de la enzima convertidora de angiotensina (IECAs), que disminuyen la producción de angiotensina II, que contrae la arteriola eferente y aumenta la presión

hidrostática glomerular (Singri, 2003).

4.4. FRA renal o intrínseco.

Se trata de una enfermedad del parénquima renal debida a inflamación, toxinas, medicamentos, infecciones o disminución del riego sanguíneo. El FRA renal o intrínseco puede deberse a alteraciones de los glomérulos, de los túbulos y del intersticio renales.

4.4.1. Afecciones glomerulares.

El glomérulo es el primer sitio de la nefrona que se pone en contacto con los agentes químicos, y hay varias nefrotoxinas que alteran la permeabilidad glomerular para las proteínas. La acción directa de determinados fármacos y tóxicos sobre las células que forman la barrera de filtración glomerular alteración de las propiedades fisico-químicas (p.e. eléctricas) de la barrera, o contracción o relajación de sus estructuras, que determinan alteraciones en la selectividad del filtrado y en la tasa de filtración glomerular, respectivamente. Concretamente una hipofiltración puede deberse a lesión glomerular que se manifiesta como una disminución del K_f que puede estar provocado por alteraciones en la permeabilidad hidráulica de la barrera de filtración, o bien por una vasoconstricción y proliferación de las células mesangiales intraglomerulares. Son ejemplos de este tipo de daño, la ciclosporina, la anfotericina B y la gentamicina

La lesión glomerular inducida por sustancias químicas también puede estar mediada por factores endógenos extrarrenales, como ocurre en las reacciones de hipersensibilidad tipo III. Los complejos inmunes circulantes pueden depositarse en los glomérulos. En la glomerulonefritis membranosa suelen observarse neutrófilos y macrófagos dentro de los glomérulos, y la liberación de citocinas y de radicales libres de oxígeno (ROS) puede contribuir a causar la lesión glomerular. Los metales pesados, los hidrocarburos, la penicilina y el captopril pueden producir también esta clase de lesión. Por último, ciertas infecciones y enfermedades inmunológicas (p.e. autoinmunes) producen una inflamación del glomérulo (glomerulonefritis) que altera la filtración (Rivero Sánchez, 2000; Esteller y Cordero, 1998).

4.4.2. Afecciones tubulares.

Las afecciones tubulares es la causa más frecuente de FRA en adultos, que junto a las causas pre-renales constituyen el 75% de todos los casos. Son tres los desencadenantes más importantes de enfermedad tubular aguda:

1. La obstrucción intratubular. Esto puede ocurrir por la precipitación de ácido úrico (como efecto secundario de la quimioterapia), de proteínas (mieloma) o de pigmentos (en casos de hemólisis masiva), por precipitación del xenobiótico o bien por un depósito del propio epitelio lesionado (Sierra y cols., 2000).

2. Lesión tubular directa, provocada por una necrosis celular producida por el agente nefrotóxico, que aumenta la permeabilidad de los túbulos de modo que el filtrado pasa de nuevo a la circulación (Molitoris, 1991). Es decir, se produce una disminución en la magnitud practica de la filtración glomerular.

3. La isquemia. Normalmente los túbulos renales están irrigados por los capilares peritubulares (ramas de las arteriolas eferentes), recibiendo el O_2 necesario para el transporte activo de sustancias en el proceso de reabsorción, especialmente el de sodio. En la necrosis tubular por isquemia hay falta de oxigenación de las células tubulares, lo que lleva a necrosis tubular y a que las células muertas se desprendan hacia el túbulo; esto lleva a la caída de la filtración glomerular tanto por la obstrucción del túbulo (restos celulares) como por la vasoconstricción de la arteriola aferente desencadenada por la retrosalimentación túbulo-glomerular, mediado por la mácula densa que detecta la gran concentración de sodio que no puede reabsorberse por el daño de las células tubulares y la falta de oxígeno (Lamiere y

Vanholder, 2004; Rivero, 2000; Valdivielso y cols., 2001).

4. La alteración de la función tubular causada por acción directa de fármacos o sustancias tóxicas sobre dianas moleculares tubulares (Sierra y cols., 2000).

Lesión del túbulo proximal.

Los túbulos son las estructuras más susceptibles al daño renal por efectuar principalmente la reabsorción isoosmótica y la secreción. Es el lugar donde con más frecuencia actúan los tóxicos, y esto se debe en parte a la acumulación de éstos en esta parte de la nefrona. El transporte tubular de aniones o cationes orgánicos, sustancias de bajo peso molecular, péptidos y metales pesados se hace exclusivamente en este tramo. El poder nefrotóxico de los xenobióticos depende de la capacidad intrínseca de cada sustancia para reaccionar con las dianas subcelulares o moleculares. Las células del túbulo proximal parecen ser más sensibles a las lesiones isquémicas que las del túbulo distal (Rivero y cols., 2000).

Lesiones del Asa de Henle y de los túbulos distal y colector.

Las alteraciones funcionales de estos tramos de la nefrona se manifiestan principalmente por disminución de la capacidad de concentración, por defectos de acidificación o por ambas cosas. La anfotericina B, el cisplatino y el metoxiflurano producen poliuria resistente a la hormona antidiurética (ADH), sugiriendo que el defecto de concentración tiene lugar en la porción gruesa de la rama ascendente

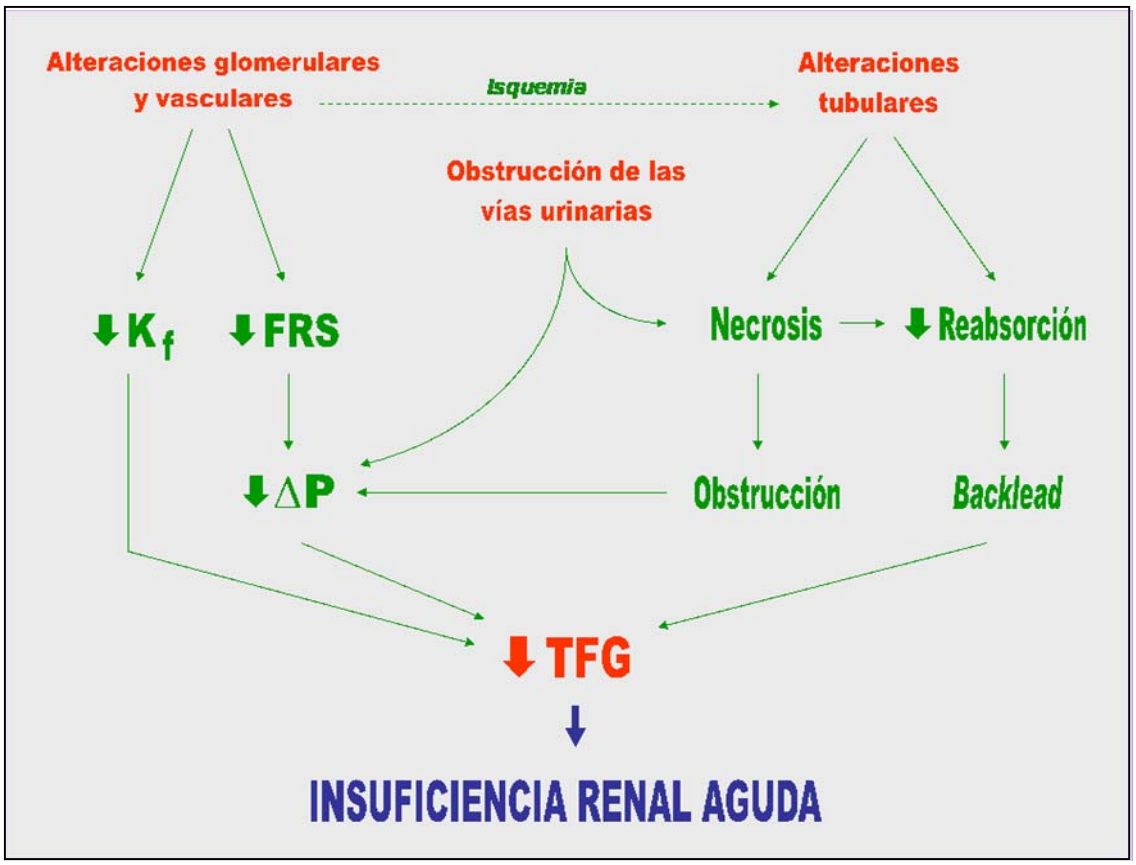

Figura 6. *Mecanismos frecuentes que conducen a la disminución del filtrado glomerular durante el daño renal agudo. Abreviaturas: K_f: Coeficiente de ultrafiltración; FSR: Flujo sanguíneo renal; ΔP: Presión neta de ultrafiltración; TFG: Tasa de filtración glomerular (Rivas y cols., 1995).*

del asa de Henle o bien en el conducto colector (Rivero y cols., 2000).

Lesión papilar.

La toxicidad papilar suele ser consecuencia de tratamientos de larga duración con fármacos tales como los analgésicos y los antiinflamatorios no esteroideos (AINEs). Las concentraciones elevadas de un posible tóxico y la inhibición de las prostaglandinas vasodilatadoras comprometen el flujo sanguíneo de la médula y las papilas renales y provocan isquemia tisular (Rivero y cols, 2000).

4.4.3. Afecciones del intersticio.

Normalmente se caracterizan por una inflamación del intersticio (nefritis intersticial aguda) que determina una obstrucción del lumen capilar. Esto ocurre en casos de alergia a medicamentos, entre otras causas. Tras la administración de algunos xenobióticos se han registrado trastornos renales con oliguria, proteinuria, hematuria y elevación de la tensión arterial que puede llegar al cuadro de insuficiencia renal aguda. La biopsia de estos casos ha mostrado infiltración del intersticio por linfocitos y plasmocitos, mientras el hemograma suele presentar una eosinofilia indicativa de afectación alérgica (Casarett y Doull, 2005).

4.5. FRA post-renal.

Esta forma de FRA es consecuencia de un obstrucción súbita del flujo de orina debido a aumento de tamaño de la próstata, cálculos, tumores o traumatismos de la vejiga y las vías urinaria. El FRA post-renal es el responsable al menos del 5% de los casos. Dado que un solo riñón posee la capacidad de depuración suficiente para excretar los productos de desecho, para que se produzca una FRA de causa obstructiva es necesario que exista una obstrucción en la uretra, en ambos uréteres o una obstrucción unilateral en un paciente con un solo riñón.

El mecanismo principal que conduce a FRA es la hipertensión retrógrada; esto significa que por la obstrucción aumenta la presión en las vías urinarias y ésta es transmitida hacia las zonas más proximales, hacia los túbulos renales y glomérulo; se produce un aumento de la presión hidrostática en el espacio de Bowman, disminuyendo el gradiente de presión de filtración, por lo que disminuye la FG.

4.6. Agentes nefrotóxicos y susceptibilidad renal.

Multitud de fármacos y contaminantes medioambientales (metales, insecticidas, etc.) pueden alterar rápidamente la función renal, modificando directamente los procesos renales básicos, dañando las estructuras renales, o mediante ambas acciones. La tabla 1 recopila las características fundamentales del efecto nefrotóxico de muchos de estos agentes, la mayoría de los cuales han sido presentados en las secciones anteriores.

A pesar de que los riñones constituyen sólo el 0.5% de la masa corporal total, las características anatómicas y fisiológicas les proporcionan una especial susceptibilidad frente a los efectos tóxicos de muchos de estos xenobióticos, ya que son los órganos que reciben mayor irrigación por gramo de tejido (alrededor del 20 al 25% del gasto cardíaco en reposo) y son la principal vía de eliminación de fármacos y de sus metabolitos.

Los procesos que intervienen en la concentración de la orina sirven también para concentrar los tóxicos en el interior del túbulo. Por lo tanto, puede ocurrir que un agente químico cuya concentración no llega a ser tóxica en el plasma alcance concentraciones tóxicas en el riñón. Finalmente, el transporte, el depósito y acúmulo, y el metabolismo de los xenobióticos en las células renales contribuyen a la predisposición del riñón para sufrir lesiones tóxicas (Klaassen y Watkins, 2005).

Los lugares concretos en los que se produce el daño van a depender de las características del agente tóxico, especialmente de sus propiedades

EFECTO PATOLÓGICO	MECANISMO PRIMARIO	NEFROTÓXICOS
Hipoperfusión/ Hipofiltración	Vasoconstricción renal. Daño glomerular.	Anfotericina B Aminoglucósidos Ciclosporina AINEs Agentes de radiocontraste.
Necrosis tubular directa.	Daño tubular directo	Aminoglucósidos Anfotericina B Acetaminofeno Cisplatino Metales pesados ß-lactámicos
Obstrucción Nefritis Tubulo intersticial	Obstrucción intratubular. Inmunológica, inflamatoria.	Agentes de radiocontraste ß-lactámicos AINEs Sulfamidas

Tabla 1. Efectos renales de los principales nefrotóxicos.

fisicoquímicas, que en definitiva determinan su interacción con sistemas de transporte, receptores, enzimas y estructuras celulares y tutulares específicas, y por lo tanto su distribución en los distintos compartimentos del organismo y sus efectos tóxicos.

5. EVALUACIÓN DEL DAÑO RENAL.

Existen diversos procedimientos para evaluar el daño renal, que van desde sencillos análisis cualitativos y ensayos bioquímicos hasta estudios anatomo-patológicos más complejos. A continuación se describen los más comúnmente utilizados.

5.1. Determinación del volumen y osmolalidad urinarios.

Un aumento del volumen urinario (poliuria), junto a un descenso de la osmolalidad inducida por un agente químico, puede ser un signo de deterioro de la capacidad de concentración de la orina. Así mismo, una disminución del volumen urinario (oliguria) es un signo indicativo de algún problema renal importante. Las alteraciones en la osmolaridad de la orina también son indicativas del estado de hidratación del paciente, que tiene gran importancia en la prevención y el tratamiento.

5.2. Medida de la filtración glomerular.

La filtración glomerular se puede medir directamente calculando el aclaramiento de la inulina (sustancia exógena) o la creatinina (producto endógeno derivado de la musculatura esquelética), pues ambas sustancias se filtran fácilmente y no se reabsorben ni secretan en gran medida. Las concentraciones del nitrógeno ureico en sangre (BUN), de la creatinina sérica y de la urea son marcadores indirectos de la filtración glomerular. Así, un aumento de su concentración en sangre sugiere una disminución de la filtración glomerular.

Actualmente se recomienda, con limitaciones, el uso de determinadas ecuaciones para valorar la filtración glomerular y, en general, la función excretora renal. Estas ecuaciones toman como base los valores de la concentración plasmática de creatinina, pero los corrigen con ciertos datos antropométricos de los pacientes, como la edad, el sexo y el peso. Las ecuaciones más utilizadas son las denominadas Ecuación de la Modificación de la Dieta en la Enfermedad Renal (MDRD, por sus siglas en inglés) y la Ecuación de Cockcroft-Gault en los adultos, y la Schwartz en los niños (Herget-Rosenthal y cols., 2007).

5.3. Análisis del sedimento urinario.

El análisis del sedimento urinario también proporciona una cierta información pues la presencia de células epiteliales indica una lesión tóxica, mientras que la presencia de células de la serie blancas es indicativa de una posible infección.

5.4. Estudio histopatológico del riñón.

El análisis histopatológico del riñón después de la administración de un agente químico es muy útil para identificar la localización, la naturaleza y la intensidad de la lesión nefrotóxica. Sin embargo su uso está muy restringido por la dificultad que entraña la obtención de muestras tisulares. La mera observación de una preparación de tejido renal, debidamente procesada y teñida (normalmente con hematoxilina y eosina) proporciona una idea de las estructuras más afectadas. Mediante estudios histoquímicos e inmunocitoquímicos puede detectarse la presencia o ausencia de antígenos marcadores de daño menos evidente, de forma muy localizada en cada estructura y tipo celular.

5.5. Estudio de la composición de la orina

La lesión renal puede ser detectada también mediante la evaluación de una serie de compuestos presentes en la orina, como proteínas, glucosa y electrolitos. Una elevada concentración de glucosa en orina (glucosuria), en tanto que su concentración plasmática es normal, puede estar relacionada con defectos de reabsorción de los azúcares en el túbulo proximal causados por un tóxico.

La excreción urinaria de proteínas de elevado peso molecular, como la álbumina, sugiere la existencia de lesiones glomerulares, mientras que la excreción de proteínas de bajo peso molecular, como la β-2-microglobulina debe hacer sospechar una lesión del túbulo proximal. Además son marcadores de proteinuria tubular: alfa-1-microglobulina, proteína unida a retinol (RBP), cistatina C, amilasa, etc., así como las proteínas *villin* (del citoesqueleto tubular e intestinal) y Tamm-Horsfall (THP) (del asa de Henle). La eliminación por la orina de enzimas que ocupan el borde en cepillo de las células tubulares, como la fosfatasa alcalina (FAL) y la gamma-glutamil-transferasa (GGT), se debe a lesiones del borde en cepillo, mientras que la excreción de otras enzimas, como la lactatodeshidrogenasa (LDH), glutatión transferasa (GST) y la alanina-aminopeptidasa (AAP), puede reflejar una lesión celular más generalizada. La lesión de la papila renal por agentes papilotóxicos libera N-acetil-beta-D-glucosaminidasa (NAG), hidrolasa lisosómica que posee varias isoenzimas

(presente también tras la lesión del túbulo proximal), acompañadas por un aumento del volumen de orina con osmolalidad baja.

5.6. Una nueva generación de marcadores sensibles y tempranos de daño renal agudo.

En la práctica clínica, el fallo renal agudo se diagnostica cuando la disfunción renal produce síntomas que se pueden medir. Estos típicamente se basan en la determinación de los niveles de creatinina y urea. Lo más habitual es que sus concentraciones en suero aumenten a medida que la TFG disminuye. Sin embargo, cuando ya se observa aumento de los niveles séricos de urea y creatinina, se ha perdido mas del 50 % de la función renal y el fallo renal agudo resulta difícil de tratar. Así, las tendencias actuales en el diagnóstico buscan detectar eventos fisiopatológicos incipientes producidos en etapas tempranas, cuando el daño está menos extendido (Vaidya y cols., 2008).

Los procesos fisiopatológicos característicos de la insuficiencia renal aguda comprenden tanto mecanismos relacionados con el daño como con la respuesta reparadora del organismo. Durante el daño a las estructuras renales, sus componentes o derivados metabólicos, compuestos de degradación o restos de ellos podrían verterse a la orina donde podrían ser detectados y utilizados como marcadores de lesión. El contacto directo de los epitelios renales con la orina facilita la aparición en ésta de moléculas y fracciones celulares procedentes de los procesos fisiopatológicos de estos tejidos. Los posibles marcadores pueden tener su origen, entre otros, en la síntesis, activación o inhibición de mediadores de los procesos bioquímicos y de constituyentes estructurales celulares relacionados con procesos como la apoptosis y la regeneración tisular. Así mismo, podrían encontrarse en la orina indicios de la destrucción de los tejidos (células, matriz extracelular, membranas basales, etc.), bien sean moléculas enteras, fracciones de éstas, organelas o restos de ellas y de fracciones celulares o tisulares.

La orina humana es un fluido inmediatamente asequible que contiene marcadores biológicos útiles para el diagnóstico precoz y monitorización de

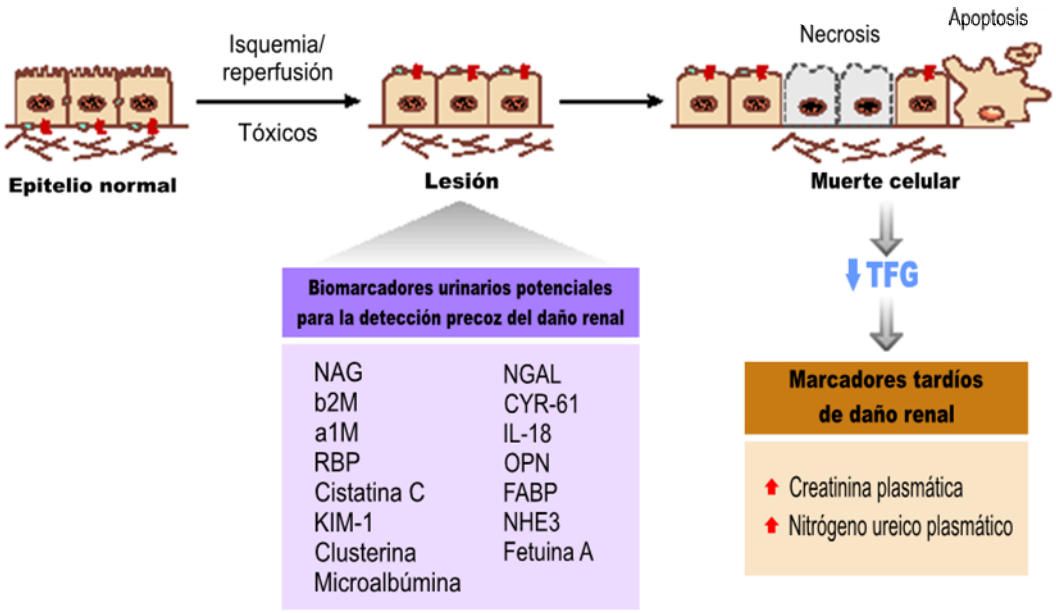

Figura 7. *Biomarcadores para el diagnóstico de la lesión renal (adaptada de Vaidya y cols., 2008)*

muchas enfermedades en la practica clínica diaria.

La detección en la orina de ciertas enzimas celulares procedentes de la lesión de células renales, es actualmente el procedimiento más refinado para la detección temprana del FRA que cursa con daño tubular. Estas enzimas incluyen la NAG, pero también otras como LDH, AAP o GGT. La mayor parte de estas enzimas tienen un valor moderado como marcadores urinarios tempranos y sensibles del fallo renal agudo, debido principalmente a problemas de estabilidad e inhibición por otros componentes de la orina (Vaidya y cols., 2008). La determinación de la actividad de la NAG en la orina es una de las técnicas más finas para la detección de daño leve, aunque su uso no está consolidado como técnica diagnóstica habitual (Price, 1982, 1992, 2002, Vaidya y cols., 2008)

En la última década se ha identificado, validado y desarrollado una nueva generación de marcadores sensibles y tempranos, capaces de detectar el daño renal agudo en sus fases iniciales, mucho antes de que se agote la reserva funcional renal y, por tanto, se manifieste la disfunción. Éstos incluyen, entre otros, la medida en la orina de la molécula de lesión renal 1 (KIM-1, por sus siglas en inglés *kidney injury molecule 1*), la lipocalina asociada a la gelatinasa neutrófila (NGAL, por sus siglas en inglés *neutrophil gelatinase associated lipocalin*), inhibidor 1 del activador del plasminógeno (PAI-1, por sus siglas en inglés *plasminogen activator inhibitor 1*), cistatina C, interleuquina 18, proteína unida al retinol (RBP, por sus siglas en ingles, *retinol binding protein*) y otros. Es probable que estos marcadores sean útiles para medir el tiempo inicial del daño y la evaluación de la IRA, así como para distinguir entre los diversos tipos y etiologías de IRA (Bonventre, 2007; Nguyen y Devarajan, 2007; Vaidya y cols., 2008 ; Waikar y Bonventre., 2008).

El biomarcador ideal de IRA sería aquel de alta sensibilidad y especificidad, fácil de cuantificar, reproducible, barato, específico para el riñón, que aparezca precozmente en el curso del fracaso renal.

KIM-1, es una glicoproteína de membrana de tipo I, que se ha desarrollado como un marcador precoz más sensible que la NAG. De hecho, el ectodominio de KIM-1 aparece antes que la NAG en la progresión del daño renal agudo, como resultado de una gran variedad de daños (Ichimura, 2004; Waikar y cols., 2008), incluyendo el tratamiento con la gentamicina (Zhou y cols., 2008), tanto en animales de experimentación como en humanos (Van Timmeren y cols., 2007). Un análisis de 31 estudios publicados sobre la capacidad de diagnóstico de varios marcadores de última generación en la orina humana, ha puesto de manifiesto que KIM-1 es un buen marcador para diferenciar el daño renal agudo (especialmente asociado a la necrosis tubular aguda) y otros tipos de daño renal, como la enfermedad renal crónica (Coca y cols., 2008).

NGAL, es una lipocalina que se sintetiza rápidamente en el epitelio dañado, se detecta en la orina y plasma tras el inicio del daño renal (Devarajan., 2008) independientemente del mecanismo de daño (Nickolas y cols., 2008), y de forma sustancial es detectado antes que otros marcadores (Devarajan, 2008). Hasta el momento, diferentes estudios de cohortes de tamaño medio en pacientes, no sólo han corroborado la utilidad de NGAL como una herramienta de diagnóstico y pronóstico, sino también como uno de los mejores marcadores predictivos de daño renal agudo (Nickolas y cols., 2008).

La cistatina C es una proteína producida por las células nucleadas y se cree que es uno de los inhibidores más importantes de la proteasa cisterina. Su bajo peso molecular y alto punto isoeléctrico le permite ser filtrada libremente y reabsorbida en el túbulo renal. Además, su producción es estable por lo que es un buen indicador para evaluar la tasa de filtración glomerular. Algunos estudios demuestran que es un marcador más sensible que la creatinina sérica y el aclaramiento de creatinina en diversas

enfermedades renales como por ejemplo la nefropatía IgA, y, por tanto, puede ser utilizada para predecir el pronóstico de una manera más temprana en estos pacientes. (Shimizu-Tokiwa y cols., 2002, Arias y cols., 2005). Se han encontrado valores elevados de cistantina C en orina en los individuos con disfunción tubular (Conti M y cols., 2006, Uchida y Gotoh, 2002).

La vimentina es una proteína del citoesqueleto de las células poco diferenciadas, y por ello es un marcador de células regenerativas y desdiferenciadas, y un marcador de regeneración tisular (Gröne y cols., 1987). Aumenta en los riñones tras el daño agudo y crónico, como consecuencia de diferentes etiologías (tóxica, isquémica, etc.) (Villanueva y cols., 2006; Yang y cols., 2007). Incluso parece que esta molécula es importante para la reparación renal tras el daño, ya que los ratones deficientes en ella recuperan peor la función renal post isquémica (Runembert y cols., 2004).

IL-18 es un mediador de la inflamación y lesiones del tejido isquémico en muchos órganos. Recientemente se ha demostrado que la IL-18 es un mediador del FRA en ratones. La IL-18 se detectó en los túbulos proximales (Melnikov y cols., 200. Las concentraciones de IL-18 en orina humana predicen la lesión proximal tubular tras isquemia o trasplante renal (Melnikov y cols., 2001; Parikh y cols., 2004). La rápida disminución en la orina tras el trasplante predice una más rápida normalización en la sangre los niveles de creatinina (Parikh y cols., 2004). Por lo tanto, la medición del nivel de IL-18 urinaria puede ser una herramienta valiosa en el diagnostico diferencial de la disfunción renal aguda, sobre todo después del trasplante.

Algunos de estos marcadores disponen ya de sistemas automáticos de medida (NGAL) o de química seca (KIM-1) que permiten utilizarlos en la escala del análisis poblacional, aunque todavía deben consolidarse en la práctica clínica habitual para la detección precoz del daño renal agudo.

6. PERSPECTIVAS EN EL DIAGNOSTICO DEL FRA.

A pesar de los avances que pueden proporcionar los nuevos marcadores precoces de daño renal agudo, todavía quedan facetas por explorar que permitirán mejorar sustancialmente el diagnóstico de esta enfermedad y que, como se comenta más adelante, pueden abrir nuevas vías para la medicina preventiva y la medicina personalizada. Entre otras nuevas facetas diagnósticas queremos destacar dos que constituyen el objeto de esta tesis doctoral.

6.1 Diagnóstico de la predisposición adquirida al FRA.

Los tratamientos con fármacos potencialmente nefrotóxicos constituyen factores de riesgo de FRA para la administración conjunta de otros fármacos potencialmente nefrotóxicos. En general, esta circunstancia se ha considerado tradicionalmente como la suma de dos elementos que desencadenan un FRA. Sin embargo, los estudios que se muestran en este trabajo demuestran que el tratamiento con un fármaco (en nuestro caso la gentamicina) potencialmente nefrotóxico, en un régimen terapéutico que no produce ningún síntoma de daño renal, predispone al desarrollo de FRA. Esta predisposición se pone de manifiesto cuando los animales previamente tratados con la gentamicina se someten a un segundo agente potencialmente nefrotóxico, en un régimen que en un animal no predispuesto resulta totalmente inocuo. Así, más que de sumación de efectos, debemos hablar de una sinergia entre los dos agentes, que tiene consecuencias farmacológicas, clínicas y socioeconómicas de gran importancia. Por ejemplo, un 0.6-2.3% de los pacientes sometidos a una radiografía de contraste, sin historia previa de enfermedad renal, desarrollan algún grado de FRA (Mehran y cols., 2006). Algunos de estos pacientes podrían cursar silenciosamente con un incremento del riesgo al FRA debido a un tratamiento previo con un nefrotoxico como la gentamicina, el cisplatino o por la exposición a un agente ambiental

(como el cadmio o el uranio) sin ninguna evidencia clínica, ni síntomas de lesiones renales, donde la TFG y los niveles urinarios de marcadores sensibles al daño renal (por ejemplo, KIM-1, NGAL) se mantienen en los valores normales. Así, estos pacientes tendrían un riesgo teórico incrementado, pero difícil de evaluar individualmente. Por este motivo, la identificación de marcadores o sistemas de diagnóstico que sean capaces de diagnosticar la predisposición al FRA adquirida mediante tratamientos farmacológicos o agentes potencialmente nefrotóxicos podrían ser de gran utilidad para identificar los pacientes de riesgo y estratificar su condición de una manera personalizada, antes de someterlos a nuevas intervenciones, procesos o tratamientos que puedan desencadenar el daño. Los datos presentados en este trabajo constituyen una primera prueba de concepto en esta nueva faceta diagnóstica. Para ello hemos utilizado un modelo de predisposición concreto, con el antibiótico aminoglucósido gentamicina. Sin embargo, nuestro grupo de investigación tiene sólidas evidencias (pendientes de publicación) de que este no es un caso aislado. Por el contrario, otros fármacos (como el cisplatino) y otros tóxicos (como algunos metales pesados) también causan este tipo de predisposición, para los que se han encontrado marcadores con posibilidades de uso como método de diagnóstico. La investigación para la identificación y desarrollo de marcadores de predisposición debe, según nuestra opinión, extenderse a otras causas de daño renal de diferente naturaleza, e incluso a la predisposición de otros efectos tóxicos de los fármacos, como la hepato, cardio o neurotoxicidad. Esto dará lugar a un nuevo concepto "teranóstico" en el que nuestra capacidad diagnóstica se anticipe a los primeros signos o síntomas de las enfermedades y prevenga su aparición mediante la identificación de indivíduos con alto riesgo adquirido.

6.2 Diagnóstico etiológico diferencial del FRA.

Otra posibilidad para mejorar el diagnostico del daño renal agudo es el diagnóstico etiológico diferencial, es decir, la identificación de la causa. Por ejemplo, sería de gran utilidad poder diferenciar el daño renal causado por un fármaco o agentes determinados a la ejercida por otros (Cataldi y cols., 2002). Actualmente esto es casi imposible ya que casi todas las formas de daño renal agudo dan lugar a los mismos marcadores, especialmente dentro del grupo de causas de un mismo tipo de FRA, como por ejemplo, la necrosis tubular aguda. Esto sería de gran utilidad en aquellas situaciones clínicas en las que en un mismo paciente convergen a la vez diferentes fármacos y procedimientos potencialmente nefrotóxicos. En ese contexto, en aquellos pacientes en los que aparecen síntomas precoces de daño renal, sería muy útil poder conocer cuál de todas las causas potenciales de daño renal es la responsable principal del daño. De esta manera se podría actuar específicamente sobre esa y respetar las demás. Un ejemplo específico de estas situaciones es el caso de los pacientes polimedicados. En ellos, cuando aparecen síntomas de daño renal, es imposible determinar con la tecnología existente cuál de esos fármacos es el desencadenante del efecto tóxico. La identificación de marcadores específicos de cada fármaco y de cada causa de FRA permitirá realizar un tratamiento más racional, individualizado y específico de éstas situaciones clínicas cotidianas. El trabajo de esta tesis doctoral también supone una prueba de concepto sobre el diagnóstico etiológico diferencial, ya que identifica marcadores urinarios para su uso potencial en la diferenciación de la nefrotoxicidad de la gentamicina de la del cisplatino y abre nuevas posibilidades de mejora del diagnóstico del FRA.

7. NEFROTOXICIDAD DE LA GENTAMICINA.

La gentamicina es un antibiótico aminoglucósido ampliamente utilizado en la práctica clínica para el tratamiento de infecciones por microorganismos gram negativos y de la endocarditis bacteriana. La nefrotoxicidad es su principal limitación terapéutica, que afecta a de un 10-25% de

los pacientes tratados con este fármaco, y puede dar lugar a un FRA. A pesar de eso, los aminoglucósidos continúan siendo, la única alternativa terapéutica efectiva contra los gérmenes sensibles a otros antibióticos, e incluso los fármacos de elección en muchas circunstancias, por su eficacia y bajo coste. La nefrotoxicidad de la gentamicina se describe en dos de nuestros artículos de revisión, que se incluyen en las páginas siguientes.

7.1 Integración de los mecanismos fisiopatológicos de la nefrotoxicidad de la gentamicina:

ARTICULO I: New insights into the mechanism of aminoglycoside nephrotoxicity. an integrative point of view.

7.2. Mecanismos de la citotoxicidad de la gentamicina:

ARTICULO II: An integrative overview on the mechanisms underlying the renal tubular cytotoxicity of gentamicin.

ARTÍCULO I

NEW INSIGHTS INTO THE MECHANISM OF AMINOGLYCOSIDE NEPHROTOXICITY. AN INTEGRATIVE POINT OF VIEW

Jose M. Lopez-Novoa, Yaremi Quiros, Laura Vicente, Ana I. Morales, Francisco J Lopez-Hernandez.

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New insights into the mechanism of aminoglycoside nephrotoxicity. An integrative point of view

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Nephrotoxicity is one of the most important side effects and therapeutical limitations of aminoglycoside antibiotics, especially gentamicin. Despite rigorous patient monitoring, nephrotoxicity appears in 10–25% of therapeutic courses. Traditionally, aminoglycoside nephrotoxicity has been considered to result mainly from tubular damage. Both lethal and sub-lethal alterations in tubular cells handicap reabsorption and, in severe cases, may lead to a significant tubular obstruction. However, a reduced glomerular filtration is necessary to explain the symptoms of the disease. Reduced filtration is not solely the result of tubular obstruction and tubular malfunction, resulting in tubuloglomerular feedback activation; renal vasoconstriction and mesangial contraction are also crucial to fully explain aminoglycoside nephrotoxicity. This review critically presents an integrative view on the interactions of tubular, glomerular, and vascular effects of gentamicin, in the context of the most recent information available. Moreover, it discusses on therapeutic perspectives for prevention of aminoglycoside nephrotoxicity derived from the pathophysiological knowledge.

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KEYWORDS: gentamicin; aminoglycoside antibiotics; nephrotoxicity; pathophysiology; prevention

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INTRODUCTION: AMINOGLYCOSIDE ANTIBIOTICS AND NEPHROTOXICITY

Aminoglycoside antibiotics (AG) are widely used in the treatment of a variety of infections (for example, ocular, pulmonary, and intestinal infections) produced by Gram-negative bacteria and bacterial endocarditis.[1] Their cationic structure, which depends on the number of amino groups and on their distribution within the molecule, seems to have an important role in their toxicity, mostly affecting renal (nephrotoxicity;[2]) and hearing (ototoxicity) tissues in which they accumulate. In spite of their undesirable toxic effects, AGs still constitute the only effective therapeutic alternative against germs insensitive to other antibiotics. This is primarily because of their chemical stability, fast bactericidal effect, synergy with betalactamic antibiotics, little resistance, and low cost.[3] In spite of being one of the most nephrotoxic AG, gentamicin is still frequently used as a first- and second-choice drug in a vast variety of clinical situations. Moreover, this aminoglycoside has been widely used as a model to study the nephrotoxicity of this family of drugs, both in experimental animals and human beings.[4–6] Most of the available data on the mechanisms responsible for AG nephrotoxicity has been obtained from gentamicin, especially at the preclinical level, in animal models or cell culture studies.

Although there are some reviews about the mechanisms explaining the toxic effects of gentamicin in the tubular epithelium, renal vasculature, and glomeruli, they lack an integrative view that brings together glomerular and tubular effects and their possible interplays. Thus, the purpose of this article is to review the effects of gentamicin in several kidney compartments with an integrative approach in order to further explain its nephrotoxicity.

NEPHROTOXICITY OF GENTAMICIN

Incidence and risk factors

The incidence of aminoglycoside nephrotoxicity has progressively increased since its introduction, until reaching 10–25% of the treatments, despite the accurate control and follow-up exercised on patients.[5–9] Clinical studies lead to the conclusion that the incidence of renal damage varies depending on the target population,[10–13] which indicates that some

individuals seem to be more sensitive than others. Table 1 shows the most important risk factors for the nephrotoxicity of gentamicin and, in general, of AGs.[14–17]

Clinical manifestations

The typical clinical manifestation of aminoglycoside toxicity is nonoliguric or even polyuric renal excretion dysfunction,[10,18–20] accompanied by an increase in plasma creatinine, urea and other metabolic products of the organism, proteinuria, enzymuria, aminoaciduria, glycosuria, and electrolyte alterations (hypercalciuria, hypermagnesuria, hypocalcemia, and hypomagnesemia).[21,22]

Table 1 | Risk factors of aminoglycoside antibiotics related to patient and treatment characteristics, and to the concomitant administration of other drugs

Patient	Treatment	Other drugs
Older age	Longer treatment	NSAIDs
Reduced renal function	Higher dosage	Diuretics
Pregnancy	Split dosage	Amphotericin
Dehydration	—	Cisplatin
Renal mass reduction	—	Cyclosporin
Hypothyroidism	—	Iodide contrast media
Hepatic dysfunction	—	Vancomycin
Metabolic acidosis	—	Cephalosporin
Sodium depletion	—	—

Abbreviation: NSAID, nonsteroidal anti-inflammatory drugs.

TUBULAR EFFECTS

The tubular toxicity of gentamicin presents two aspects: (i) the death of tubular epithelial cells, mainly within the proximal segment, with a very important inflammatory component associated and (ii) the nonlethal, functional alteration of key cellular components involved in water and solute transport.

Mechanisms of tubular cell death

A central aspect of aminoglycoside nephrotoxicity is their tubular cytotoxicity. Treatment of experimental animals with gentamicin results in apoptosis[23–25] as well as necrosis[26] of tubular epithelial cells. In culture, gentamicin also causes both apoptosis[27] and necrosis of these cells.[28] The phenotype of death might depend on the concentration of the drug, as with other cytotoxic compounds such as cisplatin and H_2O_2.[29,30] It might also depend on the concurrence of other triggering or predisposing factors, such as the degree of ischemia, on specific points of the renal parenchyma. Apoptosis is an ATP-requiring process. When the cell's ATP reserve drops, the death mode loses the typical characteristics of apoptosis and acquires those of necrosis.[31] Hypoxia inhibits respiration, ATP production, and sensitizes cells to Fas ligand[32] and induces cell death.[33,34] However, the most commonly observed phenotype *in vitro* is apoptosis, probably because it is necessary to expose cultured cells to high concentrations of the drug (>1 to 2 mg/ml) to observe a modest cytotoxic effect.[28,35,36] Figure 1 graphically depicts the

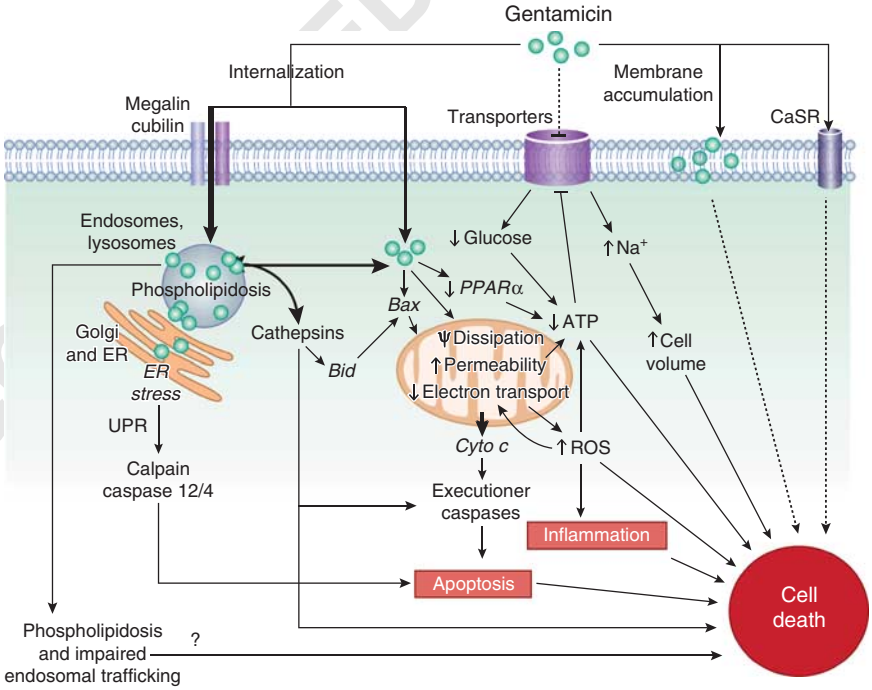

Figure 1 | Mechanisms and cell signaling pathways underlying the cytotoxic effect of gentamicin. ATP, adenosine triphosphate; CaSR, extracellular calcium-sensing receptor; Cyto *c*, cytochrome *c*; ER, endoplasmic reticulum; PPARα, peroxisome proliferator-activated receptor-α; ROS, reactive oxygen species; UPR, unfolded protein response.

mechanisms of cytotoxicity detailed in the following paragraphs.

Gentamicin cytotoxicity occurs in those cell types in which the drug accumulates. In the kidneys, these cells constitute the epithelial cells in the cortex, mainly in the proximal tubule of experimental animals[37] and humans,[16] and also in the distal and collecting ducts.[38] A higher accumulation of gentamicin in these cells is consistent with the expression of a transporter of proteins and cations, namely, the giant endocytic complex formed by megalin and cubilin, which is restricted to the proximal tubule. This complex is known to transport gentamicin and, in general, AGs, by endocytosis.[39] These drugs then traffic through the endosomal compartment and accumulate mostly in lysosomes, the Golgi, and endoplasmic reticulum.[40–41] Gentamicin binds to membrane phospholipids, alters their turnover and metabolism, and, as a consequence, causes a condition known as phospholipidosis that has been observed in humans[7] and experimental animals treated with the drug.[42,43] Lysosomal phospholipidosis results from (i) the reduction in the available negative charge necessary for the correct function of phospholipases[44] and (ii) inhibition of A1, A2, and C1 phospholipases.[4,45,46] Phospholipidosis correlates tightly with the level of toxicity of aminoglycosides.[43,47,48] Moreover, agents protecting from phospholipidosis, such as polyaspartic acid, also prevent aminoglycoside nephrotoxicity.[49–51] However, the effect of polyaspartic acid has been ascribed to its capacity to bind gentamicin and thus to prevent its union to phospholipids.[52] Binding to phospholipids is also a requirement for gentamicin endocytosis,[53,54] indicating that further investigation is necessary to ascertain the exact role of phospholipidosis in tubular cell death.

When the concentration of aminoglycoside in endosomal structures exceeds an undetermined threshold, their membrane is disrupted and their content, along with the drug, is poured into the cytosol.[55,56] Cytosolic gentamicin then acts on mitochondria directly and indirectly,[57,58] and thus activates the intrinsic pathway of apoptosis, interrupts the respiratory chain, impairs ATP production,[58,59] and produces oxidative stress by increasing superoxide anions and hydroxyl radicals,[60,61] which further contributes to cell death. The indirect mitochondrial effect is mediated by increasing Bax levels[62] through the inhibition of its proteosomal degradation.[35] In addition, the lysosomal content bears highly active proteases named cathepsins, which are capable of producing cell death.[63] Cathepsin-mediated cell death occurs through apoptosis by directly cleaving active executioner caspases and indirectly unleashing the intrinsic pathway through the proteolytic activation of Bid.[64,65] In high amounts, cathepsins also cause a massive proteolysis that, especially under low ATP conditions, leads to a rapid, necrotic-like mode of cell death.[66]

In the endoplasmic reticulum, gentamicin inhibits protein synthesis,[67,68] impairs translational accuracy,[69] and might interfere with the correct posttranslational protein folding.[62] This generates endoplasmic reticulum stress and activates the unfolded protein response that, on continuous stimulation, activates apoptosis through calpains and caspase 12.[70–72] Finally, activation of the extracellular calcium-sensing receptor (CaSR) with gentamicin and other aminoglycosides has also been shown to induce a mild degree of apoptosis in CaSR-expressing tubule cells and not in those lacking it. However, CaSR is also expressed in gentamicin-resistant cells including bone, brain, colon, parathyroid gland, smooth muscle, endothelial cells, and so on. Clearly, more information is necessary to clarify the exact role and the relative weight of CaSR stimulation in tubule cell death induced by aminoglycosides.

Sub-lethal alterations in tubular reabsorption

In experiments carried out with cultured cells or membrane vesicles from tubular cells, it has been shown that gentamicin, independently of cell injury, inhibits a variety of cell membrane transporters of both the brush-border and the basolateral membrane (reviewed in Mingeot-Leclercq and Tulkens[20]) including (i) Na-Pi cotransporter[73] and Na-H exchange;[74] (ii) carrier-mediated dipeptide transport;[75] (iii) electrogenic Na transport;[76] and (iv) Na-K adenosine triphosphatase.[77,78] Transport inhibition affects tubular reabsorption, but it may also compromise cell viability (Figure 1). For example, Na-K adenosine triphosphatase is a key component of cell volume homeostasis, and deregulated swelling may lead to necrosis or apoptosis.[79,80] As early as 30 min after gentamicin renal perfusion[21] or 3 h after gentamicin administration to rats,[81] deficient reuptake of calcium and magnesium is observed, leading to hypercalciuria, hypermagnesiuresis, and hypomagnesemia, before alterations in renal handling of Na^+ and K^+, and before detectable signs of renal damage and toxicity are evident. Gentamicin is transported by and also competes with proteins, organic cations, and other molecules for the megalin–cubilin endocytic complex in the proximal tubule, and thus impairs their reabsorption.[82–85]

Tubular effects cannot solely explain the reduced glomerular filtration rate

The spilling of tissue and cellular residues to the tubular lumen partially or totally obstructs the tubules.[86,87] Tubular obstruction reduces, or even voids, the excretory function of the affected nephrons. In addition, it increases the hydrostatic pressure inside the tubule and in the Bowmans' capsule, which reduces filtration pressure gradient and, therefore, the glomerular filtration rate (GFR). Moreover, the increase of intratubular pressure increases the leak of the ultrafiltrate toward the interstitial space (backleak) and peritubular capillaries, and, thus, decreases excretion of the filtrate products.[86] Accordingly, tubular obstruction may account for a part of the reduced filtration caused by gentamicin. However, in mild cases and early stages of severe cases, that is, in the absence of significant tubular obstruction, a relevant accumulation of creatinine and uremic products can be detected in the blood, which is usually the evidence that alerts

on the underlying renal damage, and indicates that, by that time, GFR is already reduced. In the absence of significant nephron obstruction, an increase in plasma creatinine (and other products) can only be explained by a reduced GFR.

Tubular damage leads to a dysfunctional reabsorption process that produces an excessive delivery of water and electrolytes to the distal part of the nephron, which in turn triggers the tubuloglomerular feedback (TGF) mechanism. TGF is brought about by an angiotensin-II and adenosine-mediated afferent and efferent arteriole effects, and the subsequent decrease in GFR.[88,89] TGF is activated as a protective mechanism to avoid massive loss of water and electrolytes.[90] The TGF mechanism is known to adapt in a period of time ranging from 1 to 24 h.[91,92] Therefore, its role in the reduction of glomerular filtration should, theoretically, disappear after this interval. However, GFR continues to decrease as long as gentamicin treatment is maintained. As described in the following sections, oxidative stress, inflammation, and the release of vasoconstrictors induce mesangial and vascular contraction (see below). These may explain why GFR remains low even in the absence of an active TGF and of significant tubular obstruction. In addition, it can also be hypothesized that gentamicin might inhibit or modulate TGF-adaptive mechanisms.

GLOMERULAR EFFECTS

The glomerulus is the first part of the nephron to come into contact with chemical agents. Gentamicin has glomerular effects that alter filtration (Figure 2). (i) Gentamicin produces mesangial contraction (reviewed in Martínez-Salgado *et al.*[93]) andresults in K_f (ultrafiltration coefficient) and GFR reduction;[94,95] (ii) gentamicin also stimulates mesangial proliferation paralleled by an increase in apoptosis of these cells, which basically compensate each other;[93,96] (iii) despite the fact that gentamicin does not generate significant morphological changes in the glomerulus, in high-dose treatments, a slight increase in size, alteration of their round shape and density, and a diffuse swelling of the filtration barrier associated with neutrophil infiltration have been detected,[97] although their pathophysiological significance is uncertain; and (iv) loss of glomerular filtration barrier selectivity, due to the neutralization of its negative charges,[98] contributes to proteinuria, especially under circumstances in which tubular reabsorption is impaired such as in tubular necrosis.

Early studies demonstrated that gentamicin reduces the number and pore size of glomerular endothelial fenestrae,[99–101] correlating with a decrease in the sieving coefficient of low-molecular-weight proteins such as lysozyme,[100] and supporting a reduction in GFR. These effects seem to be the consequence of mesangial contraction. Gentamicin activates contraction of cultured mesangial cells and isolated glomeruli,[102,103] and thus reduces K_f. Several factors induced by gentamicin increase intracellular calcium concentration and cause mesangial cell contraction (reviewed in Martínez-Salgado *et al.*[93]; Figure 2). They include (i) platelet-activating factor (PAF) secretion and autocrine action;[102] (ii) activation of the renal renin–angiotensin system; (iii) production and action of vasoconstrictors such as endothelin-1 and

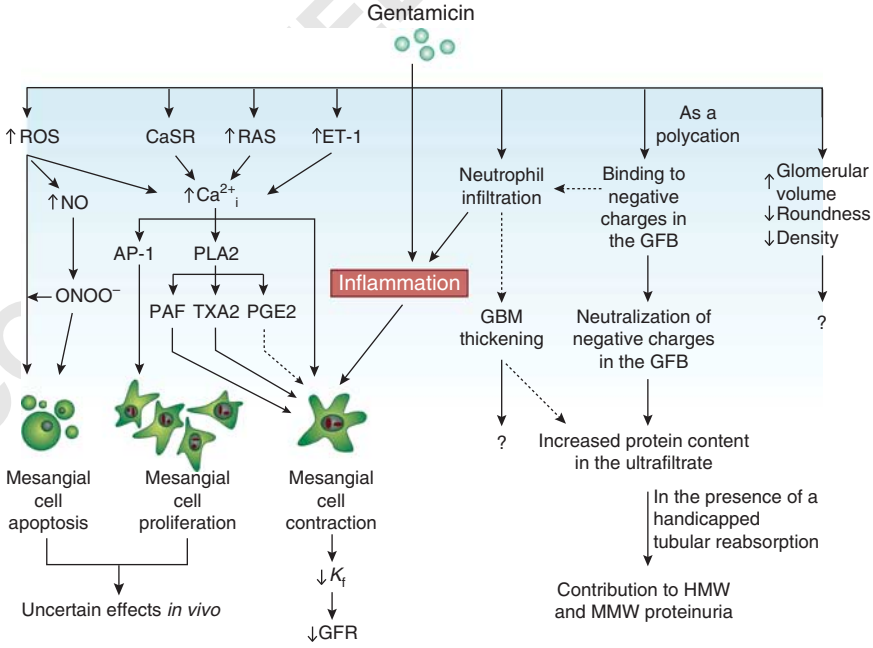

Figure 2 | Glomerular effects of gentamicin. AP-1, activator protein 1; CaSR, extracellular calcium-sensing receptor; ET-1, endothelin-1; GBM, glomerular basement membrane; GFB, glomerular filtration barrier; GFR, glomerular filtration rate; HMW, high molecular weight; K_f, ultrafiltration coefficient; MMW, medium molecular weight; NO, nitric oxide; PAF, platelet activating factor; PGE2, prostaglandin E2; PLA2, phospholipase A2; RAS, renin–angiotensin system; ROS, reactive oxygen species; TXA2, thromboxane A2.

thromboxane A2 arising from endothelial dysfunction or imbalance;[104] (iv) CaSR stimulation; and (v) increase in reactive oxygen species (ROS) production and oxidative stress.[105]

Activation of phospholipase A2 has also been associated with the synthesis of some of the above mediators and with the effect of gentamicin on mesangial cells.[103] Phospholipase A2 catalyzes the formation of arachidonic acid, a soluble phospholipid. Arachidonic acid generates, through cyclooxygenase, the synthesis of thromboxane A2 and leads to mesangial contraction. PAF is also synthesized from the soluble phospholipids that result from phospholipase A2 activity. PAF is recognized as an important mediator of mesangial contraction, which decreases K_f and GFR.[106–108] In fact, PAF antagonists partially inhibit gentamicin-induced reduction in GFR,[95,109,110] and mesangial contraction in isolated glomeruli and cultured mesangial cells.[92,102,110]

In rats treated with gentamicin, both proliferation and apoptosis take place at the same time in the mesangial compartment. Both effects apparently compensate one another, because no net variation in the number of mesangial cells has been reported.[93,111] Mesangial proliferation is mediated by calcium-dependent AP-1 activation.[96] Mesangial cell apoptosis is mediated by increased ROS[96,111] and probably by nitric oxide (NO) overproduction.[93] Gentamicin stimulates inducible nitric oxide synthase (iNOS) expression and NO production in isolated glomeruli and mesangial cells.[112–114] Excessive NO production due to expression of iNOS, especially under oxidative stress circumstances, interacts with superoxide anion to forms peroxynitrite, which causes nitrosative stress and cytotoxic effects.[115] The role of mutually counterbalancing mesangial apoptosis and proliferation is not clear. Probably, one is the homeostatic consequence of the other, in order to maintain tissue integrity. Gentamicin might cause a mild degree of apoptosis in mesangial cells followed by a repairing proliferation. Alternatively, gentamicin might promote the proliferation of mesangial cells (through the increment in Ca_i^{2+}) that, in the absence of tissue damage, would lead to apoptosis.[93] However, both increased proliferation and apoptosis have been detected in cultured mesangial cells treated with gentamicin,[111] which obscures both of these interpretations. As argued in Martínez-Salgado *et al.*,[93] *in vivo* the primary effect would be apoptosis, with subsequent homeostatic proliferation.

VASCULAR EFFECTS

Gentamicin induces a reduction in renal blood flow (RBF),[116,117] which is the consequence of an increased resistance of the renal vascular bed rather than that of a lower perfusion pressure.[118] A lower RBF causes GFR to fall[119] (see Figure 3), and sensitizes tubule cells to cell death by reduction of oxygen and ATP availability (as explained above). RBF reduction arises initially (i) from the activation of TGF by the handicapped tubular reabsorption, in order to prevent massive fluid and electrolyte loss and (ii) progressively, superseding TGF adaptation, by production of vasoconstrictors within the renal vascular tree and mesangial compartment; and by direct effects of gentamicin on vascular cells (Figure 3).

The production of several vasocontrictors is increased on gentamicin treatment, including endothelin-1,[104] PAF, and arachidonic acid metabolites, mainly prostaglandins and thromboxane A2,[103,120,121] arising from endothelial and mesangial cells,[93] as explained in the previous section. They act in a paracrine manner on vascular myocytes and cause vasoconstriction. In addition to stimulating the production

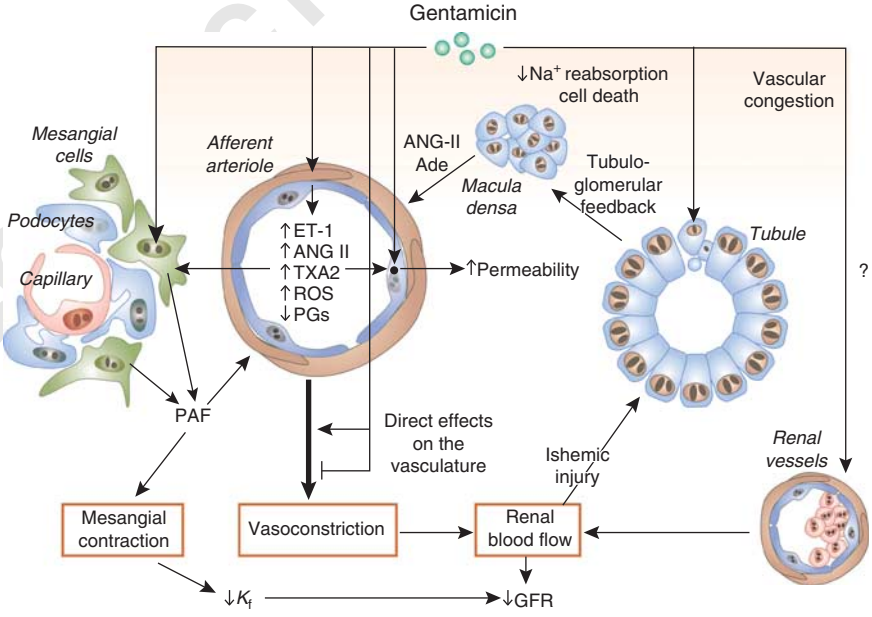

Figure 3 | Vascular effects of gentamicin. Ade, adenosine; ANG-II, angiotensin-II; ET-1, endothelin-1; GFR, glomerular filtration rate; K_f, ultrafiltration coefficient; PAF, platelet-activating factor; PGs, prostaglandins; ROS, reactive oxygen species; TXA2, thromboxane A2.

of vasoconstrictors, gentamicin also blocks the synthesis of vasodilator prostaglandins.[120] Endothelial NO synthase-derived NO, at low levels, mediates physiological vasodilatation, whereas excessive NO production due to the over-expression of iNOS (see above, section 'Vascular effects') can cause cytotoxic effects in surrounding cells. NO interacts with superoxide anion to form peroxynitrite, which induces protein and cell damage and uncouples endothelial NO synthase to become a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress.[122]

Gentamicin also impairs vascular smooth muscle-relaxing capacity through an unraveled mechanism, theoretically contributing to vasoconstriction and RBF reduction, to an undetermined extent.[123] However, gentamicin has also been shown to relax isolated, precontracted arteries,[124,125] through the inhibition of PLC, protein kinase C, and calcium movements.[124,125] This relaxing effect is exerted directly on smooth muscle cells and occurs despite gentamicin inhibiting the release of endothelium-derived relaxing factor, secondary to inhibition of PLC.[126]

Finally, leukocyte margination, leading to vascular plugging, congestion, and infarction, is induced by gentamicin in retinal vessels after 48–72 h of treatment.[127] It can be speculated that vascular plugging contributing to ischemia might also occur in the kidneys, especially under a strong proinflammatory environment, although this has to be specifically corroborated.

INTEGRATIVE PATHOPHYSIOLOGY OF GENTAMICIN NEPHROTOXICITY

Classically, the nephrotoxicity of gentamicin has been considered as a tubulopathy in which tubular damage and tubular dysfunction are the main cause of renal insufficiency. This may explain some clinical observations, such as proteinuria, enzymuria, and electrolytic alterations. However, as explained in the section 'Tubular effects cannot solely explain the reduced glomerular filtration rate', in the absence of tubular obstruction, tubular damage itself cannot account for a reduced GFR without the concourse of extratubular determinants. GFR reduction needs to be justified in order to fully explain the alterations in renal excretory function, leading to the accumulation of metabolic products in the blood, azotemia, uremia, and the whole renal syndrome produced by gentamicin.

Tubular and glomerular mechanisms differentially contribute to the reduced GFR

Tubular dysfunction leads to the loss of fluid and electrolytes that swiftly fire the TGF response, which reduces RBF and GFR to the appropriate level. Because, under physiological circumstances ~99% of water and electrolytes in the ultrafiltrate are reabsorbed along the tubule, a drastic reduction in GFR must be accomplished to compensate for a small reduction in tubular reabsorption, thus preventing the life-threatening loss of water and electrolytes. That is why even a mild injury to the tubular epithelium may bring about a pathological reduction in GFR and renal failure. However, TGF adapts within hours and its control over GFR is lost even in the presence of an increasing tubular incompetence. Yet, clinical and experimental observations demonstrate that, despite TGF adaptation, GFR grows lower as gentamicin-induced damage progresses, as described in previous sections.

Figure 4 shows the mechanisms leading to a reduced GFR. It can be observed that tubular malfunction leading to a defective reabsorption is the only mechanism that causes no GFR reduction directly, although it decreases GFR indirectly by activating the TGF mechanism, at least transitorily. Tubular obstruction increases progressively with tubular damage, as does its contribution to the reduced GFR. As such, it only partially explains the whole reduction in GFR, especially in the initial phase of acute kidney injury, which is the most relevant clinical situation. In these circumstances (Figure 5), a number of factors may hold GFR low in the absence of TGF-mediated control. Contracting factors produced by mesangial, vascular, and tubular cells, including ROS, PAF, angiotensin-II, and endothelin-1 act in an autocrine and paracrine manner to induce contraction of glomerular vessels and mesangial cells, which reduce RBF and K_f, respectively, and lower GFR. A question for the future is if a part of the reduction in GFR caused by gentamicin would still occur, should tubular alterations be completely and specifically prevented, or, whether most glomerular and vascular effects are, at least partially, independent of tubular damage. As explained above, gentamicin-induced mesangial activation and contraction have been documented in cultured, isolated mesangial cells,[93] indicating that no tubular-derived stimulation is necessary for these effects. In addition, reduced GFR and RBF may contribute to aggravating gentamicin-induced tubular damage,[128] probably because they limit oxygen and nutrient availability to tubular cells and facilitate oxidative stress, as it has been demonstrated in the ischemic renal failure.

Central role of oxidative stress and inflammation: a loop of damage amplification and a connection between tubular and glomerular mechanisms

Oxidative stress has been suggested to have a key role in gentamicin nephrotoxicity.[129–131] This is mainly based on a myriad of studies conducted in experimental models demonstrating that cotreatment with a variety of antioxidants protects from gentamicin-induced renal damage,[61,117,132,133] although clinical data is not so conclusive.[134] Gentamicin directly increases the production of mitochondrial ROS,[58] which (i) are able of damaging many cellular molecules including proteins, lipids, and nucleic acids, thus impairing cell function and leading to cell death; (ii) contribute to mesangial and vascular contraction (as described in sections 'Glomerular effects' and 'Vascular effects'); and (iii) participate in inflammation.

The nephrotoxicity of gentamicin has been shown to involve an inflammatory response in experimental animals[135,136] and humans,[137] with cell infiltration, activation

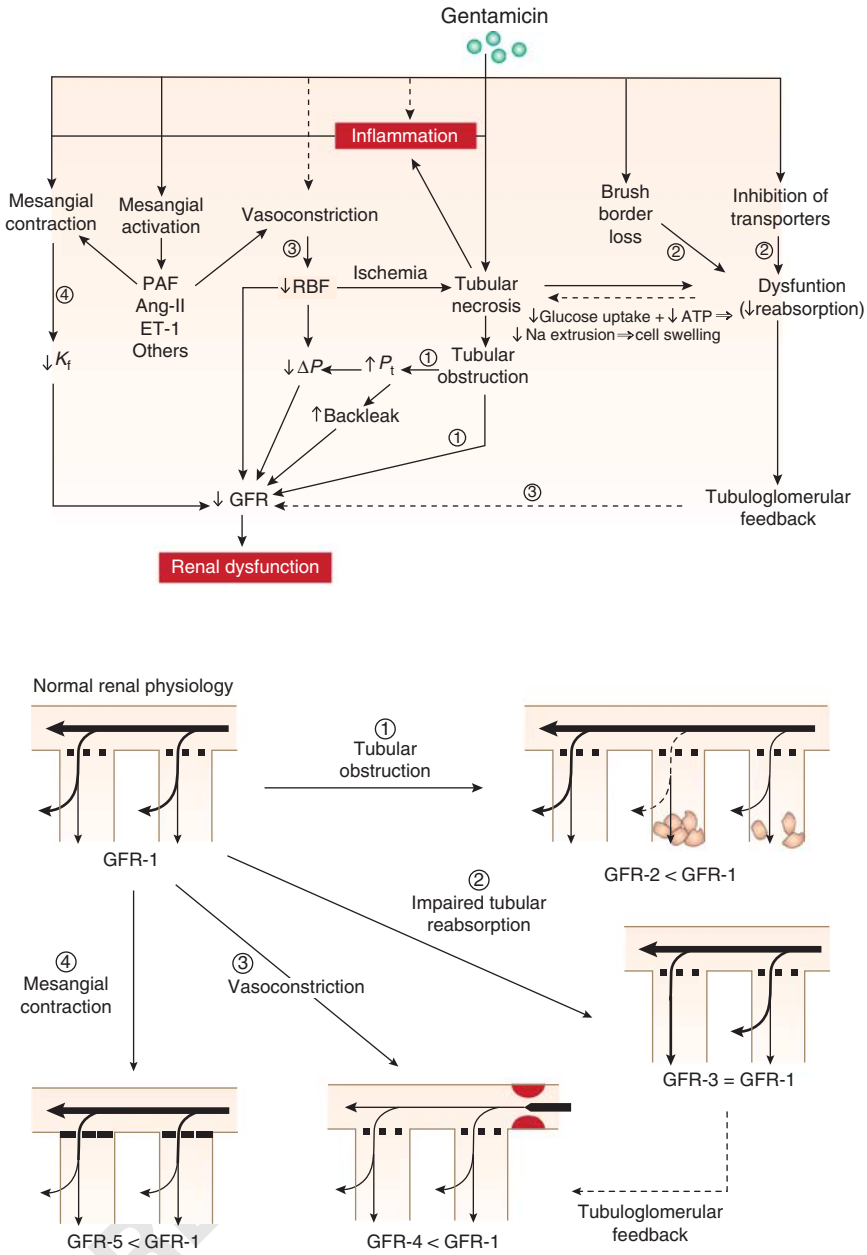

Figure 4 | Integrative view of the mechanisms leading to gentamicin nephrotoxicity. It can be appreciated that, in the absence of a significant tubular obstruction, vascular and mesangial mechanisms are necessary to explain the reduction in glomerular filtration (GFR) and renal excretion, once the tubuloglomerular feedback adapts. ATP, adenosine triphosphate; ANG-II, angiotensin-II; ET-1, endothelin-1; GFR, glomerular filtration rate; K_f, ultrafiltration coefficient; ΔP, net ultrafiltration pressure; PAF, platelet-activating factor; P_t, intratubular pressure; RBF, renal blood flow.

of resident cells, increased cytokine production,[138,139] and capillary hyperpermeability.[140] The inflammatory response, initially unleashed as a defense and repair mechanism, when globally considered seems to contribute to renal damage progression. In fact, strategies that protect from gentamicin-induced renal damage usually inhibit the inflammatory response.[135,141] In this sense, ROS are known to participate in the inception and signaling of inflammation,[142] which might explain why antioxidants are very effective at softening the renal damage inflicted by gentamicin[117,143,144] (Figure 6) and, in general, by other tubular necrosis-inducing nephrotoxins.[134,145] ROS such as superoxide anion[146] and hydrogen peroxide[147] activate nuclear factor κB, which has a key role in the inception of the inflammatory process. Indeed, nuclear factor κB inhibitors protect the kidney against gentamicin-induced damage.[148] Nuclear factor κB induces the expression of proinflammatory cytokines[149] and iNOS.[150] As described above, iNOS-derived NO can react with superoxide anion and produce peroxinitrite, a highly reactive radical that contributes to cell damage and reduced vascular relaxation.

It can be speculated that the effect of antioxidants might be related to a combined action at different levels, including the following: (i) softening of gentamicin's direct cytotoxicity (as explained above); (ii) inhibiting vasoconstriction and mesangial contraction; and (iii) an antiinflammatory action. However, there is little information on the ability of antioxidants to modulate the direct cytotoxic effect of gentamicin on cultured tubule cells. To our knowledge, only Juan *et al.*[151] have reported a protective effect in this sense. In their article, tetramethylpyrazine reduces ROS accumulation and apoptotic events in rat renal NRK-52E cells. However, the effect of tetramethylpyrazine on cell viability is not reported. Because there are many apoptotic and necrotic pathways leading to cell death as a consequence of gentamicin action, and because their redundancy and hierarchical organization are not well understood, the magnitude of the direct cytoprotection afforded by ROS inhibition is unknown.

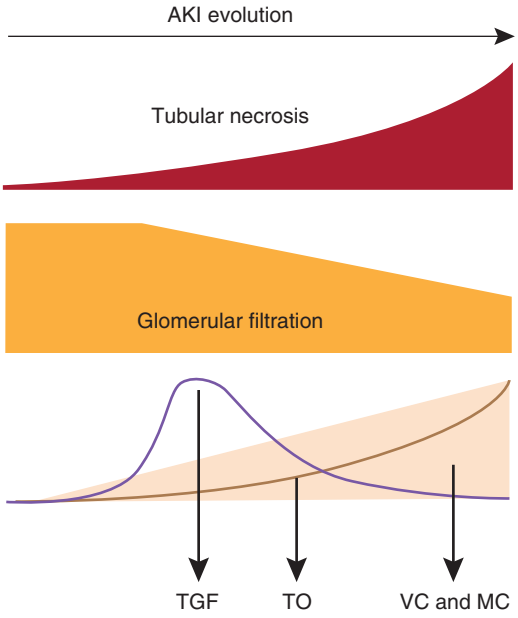

Figure 5 | Comparative temporal evolution of the acute kidney injury, tubular necrosis, glomerular filtration, tubuloglomerular feedback, and vascular and mesangial contraction on treatment with gentamicin. Initially, tubuloglomerular feedback (TGF) controls glomerular filtration rate. As TGF adapts, increasing tubular obstruction (TO), and vascular and mesangial contraction (VC and MC) take over and make GFR progressively lower.

In any case, it is reasonable to think that the inflammatory response acts as an amplifying mechanism of damage (Figure 6). Initially, cell destruction through necrosis would lead to the onset of an inflammatory response. Tissue debris and cell content shed into the extracellular space trigger inflammation,[152] whereas an exaggerated inflammation would contribute to further damage that, in turn, would exacerbate the inflammatory response.[153] Inflammation also activates glomerular cells, such as mesangial cells, podocytes and epithelial cells, endothelial cells, and resident and infiltrated leukocytes. These, in turn, produce cytokines and growth factors that contribute to the pathophysiological process with different effects (Figure 6), including amplification of tubular damage.[154] As such, inflammation and oxidative stress provide a connection between tubular necrosis and glomerular and vascular activation and contraction, which ultimately further contribute to tubular damage, mainly through a reduction in RBF.

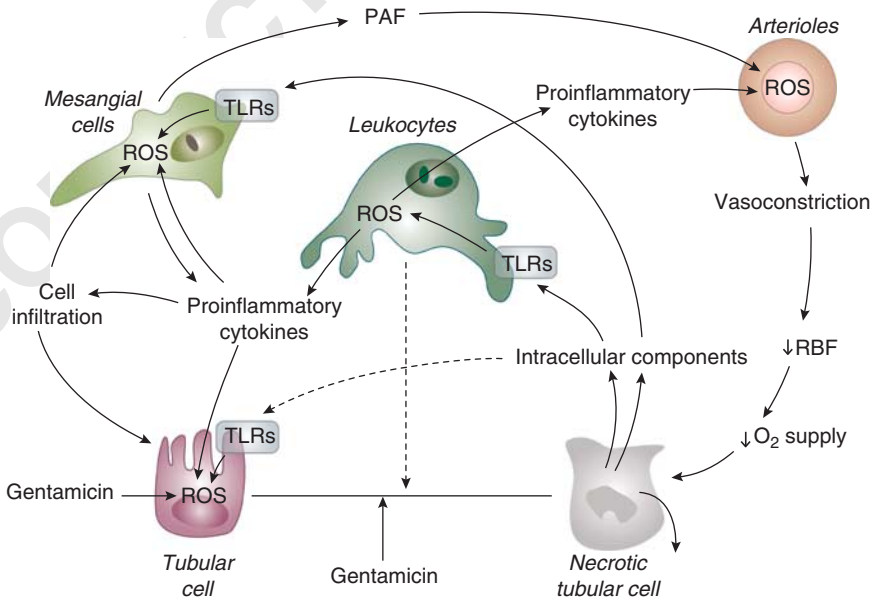

Figure 6 | Role of inflammation in the amplification of tubular, glomerular, and vascular effects of gentamicin. PAF, platelet-activating factor; RBF, renal blood flow; ROS, reactive oxygen species; TLRs, toll-like receptors.

CLINICAL IMPLICATIONS FOR THE PREVENTION OF NEPHROTOXICITY

Prevention of nephrotoxicity is an unmet therapeutic objective that will improve the pharmacotoxicological profile and the clinical utility of many drugs significantly, including AGs. In many cases, nephrotoxicity is the most important limitation to the dosage or intensity of the therapeutic regimen, and may lead to serious health complications and even death in determined cases. Nephrotoxicity is a concern in all clinical settings, but takes special relevance among critically ill patients. Indeed, it is estimated that ~25% of the 100 most used drugs in intensive care units are potentially nephrotoxic,[155] and that nephrotoxicity is responsible for 10–20% of acute renal failure cases.[156] Besides a correct monitoring, maintenance of patient's hydration, and application of dialysis when necessary, there are no therapeutic tools available to prevent or palliate drug nephrotoxicity. There are much less tailored preventive strategies for individual nephrotoxic drugs, based on specific mechanisms of action. Nonetheless, this is another challenge for the future. In addition to the identification of less toxic compounds, several new strategies for the prevention of aminoglycoside nephrotoxicity are currently under different degrees of development, mostly at the preclinical level.

Inhibition of tubular accumulation

A proposed strategy focuses on finding drugs that prevent the accumulation of aminoglycosides by interfering with transport mechanisms. An obvious target is the megalin-related endocytic machinery responsible for AG transport and accumulation in tubular and auditory cells. Inhibition of aminoglycoside transport can be approached by administering (i) competitors for the receptor that displace aminoglycosides from binding to it or (ii) specific inhibitors of this endocytic pathway. Certain protein, fragments thereof and basic peptide ligands of megalin reduce the accumulation of gentamicin in cultured tubular cells and renal tubuli *in vivo* by inhibiting drug binding to the brush border.[85,157,158] Statins have been shown to reduce gentamicin accumulation in tubule cells and renal damage through a mechanism involving geranyl isoprenoids.[159] Megalin-mediated endocytosis involves other proteins with binding, adaptor, and unknown functions, such as cubilin, disabled-2, nonmuscle myosin heavy chain IIA and β-actin, which seem to participate in endocytic trafficking.[160] These proteins, and others resulting from a deeper knowledge of the endocytic mechanisms, are potential targets for pharmacological prevention of aminoglycoside accumulation. Indeed, genetic disruption of myosin VI[161] or treatment with the myosin inhibitor blebbistatin[160] reduces the uptake of proteins transported by the megalin complex. Myosin VI knockout mice show albuminuria with no alterations in urine output or electrolyte excretion. These initial results show a potential avenue for further exploration. Yet, the clinical consequences (for example, proteinuria) of interfering with megalin-mediated endocytosis as a mechanism of nephroprotection need to be determined in the short- and long term. In this line, myosin VI knockout mice show tubular dilation and fibrosis, consistent with persistent proteinuria.[161]

Cotreatment with renoprotective drugs

Another strategy relies on nephroprotective drugs for cotreatment along with aminoglycosides. At the preclinical level, many molecules have been shown to exert protective effects on drug nephrotoxicity and, specifically on aminoglycoside nephrotoxicity. By far, most of the studies have tested the ability of antioxidants to alleviate aminoglycoside nephrotoxicity. With one exception studied in patients,[162] all of them have been conducted in experimental animals. Preclinical studies offer unambiguous information on the beneficial effects of antioxidants. However, these results need to be further explored in the clinical setting, as promising, although inconsistent, results have been obtained on the protection exerted by antioxidants on the nephrotoxicity of other drugs.[134] In most studies in which inflammation has been evaluated, it is concluded that they might exert their effects through a cytoprotective and antiinflammatory action.

Improvement of RBF may also attenuate aminoglycoside nephrotoxicity, even independently from tubular damage.[162] An increased RBF by preglomerular or general vasodilatation can enhance GFR and attenuate the tubular damage caused or amplified by the reduced flow. In this sense, promising results have been obtained in animals with PAF inhibitors, although they have not progressed further into human investigation. This could be an attractive strategy to pursue. In general, vasodilators also relax mesangial cells and augment K_f. As such, the increase in GFR is not only the result of hemodynamic improvement but also of K_f modulation. Thromboxane A2 inhibitors have been used in one study with protective results.[120] Calcium antagonists have also been used with contradictory results at the preclinical level. We have found only two studies conducted in humans. They document protection afforded by calcium channel blockers verapamil and nifedipine on gentamicin nephrotoxicity.[162,163] The effect of calcium antagonists may depend on the relative level of contraction of preglomerular and postglomerular vessels and mesangial cells, and on the weight of vasoconstriction and mesangial contraction in the overall effect of a determined experimental or clinical therapeutic regimen with aminoglycosides. This, in turn, may also depend on the dose and length of treatment, drug accumulation, and so on. A note of caution should also be introduced here, because the clinical consequences of augmenting GFR without a parallel amelioration of tubular damage may result in massive proteinuria, and water and electrolytic loss, which need to be addressed.

Other strategies

Another potentially nephroprotective effect that should be pursued is the blockade of the immune response. In fact, genetic knockdown of toll-like receptor-4 has been shown to

alleviate the renal lesion induced by cisplatin[164] and ischemia reperfusion[165] in mice, in which inflammation has a central pathological role. Indeed, ROS are involved in toll-like receptor-mediated inflammation.[166] Perhaps, cocktails containing several drugs aimed at providing protection against tubular damage and inflammation, and improvement of renal hemodynamics should be evaluated at the preclinical and clinical levels.

CONCLUSIONS AND PERSPECTIVES

An integration of tubular, glomerular, and vascular effects of aminoglycosides based on the evidence discussed in this paper is consistent with an important component of tubular injury. In severe degrees of acute kidney injury induced by gentamicin, tubular obstruction may account, at least partly, for the reduced GFR. However, in mild cases and early stages of severe cases, that is, in the absence of significant tubular obstruction, GFR reduction can only be explained by extratubular mechanisms, namely, mesangial and vascular contraction. These later result from (i) the TGF mechanism, with the temporal restriction explained in section 'Tubular effects cannot solely explain the reduced glomerular filtration rate'; (ii) direct mesangial and vascular contraction; and (iii) indirect mesangial and vascular contraction produced by inflammation and paracrine mediators. Inflammation is known to result from tissue damage, specially arising from cell necrosis. Still, it remains to be elucidated (i) whether all the inflammatory responses are the consequences of tubular damage or whether they are also partly activated or amplified by tubular necrosis-independent mechanisms; and (ii) what is the contribution of direct extratubular effects of gentamicin to the overall syndrome, which are completely independent of tubular damage, and of mechanisms derived from tubular damage that alter glomerular and vascular function.

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Finally, it should be stressed that known and new nephroprotective strategies should also be tested for their potential effects on the bactericidal effect of aminoglycosides. This issue has not been addressed in renal studies. For example, oxidative stress has been proposed to contribute to aminoglycoside bactericidal effect.[167] Then, treatment with antioxidants with the objective of reducing their nephrotoxicity may also impair their antibiotic activity. Thus, combined models of nephrotoxicity/nephroprotection and sepsis should be developed.

DISCLOSURE

JML-N and FJL-H are minority shareholders of Bio-inRen, SL, a biotech company holding licence on the patent on the use of GM2AP as a marker for the diagnosis of kidney injury.

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ARTÍCULO II

AN INTEGRATIVE OVERVIEW ON THE MECHANISMS UNDERLYING THE RENAL TUBULAR CYTOTOXICITY OF GENTAMICIN.

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An Integrative Overview on the Mechanisms Underlying the Renal Tubular Cytotoxicity of Gentamicin

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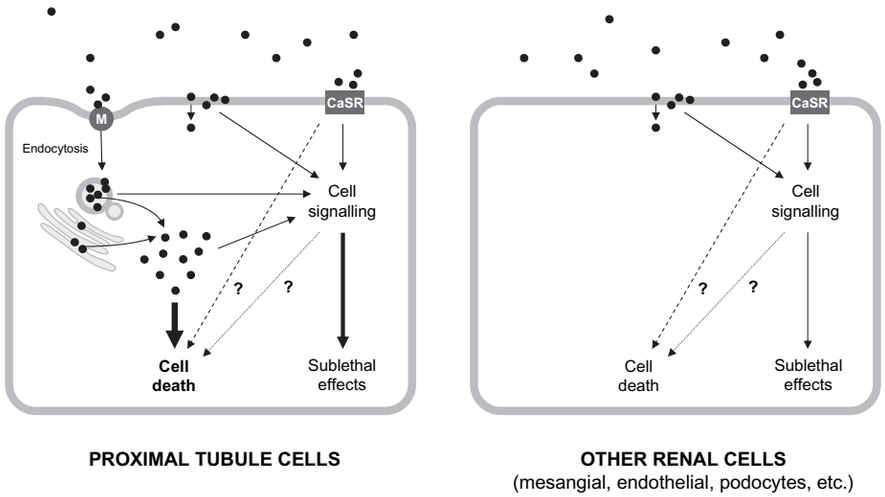
Gentamicin is an aminoglycoside antibiotic widely used against infections by Gram-negative microorganisms. Nephrotoxicity is the main limitation to its therapeutic efficacy. Gentamicin nephrotoxicity occurs in 10–20% of therapeutic regimes. A central aspect of gentamicin nephrotoxicity is its tubular effect, which may range from a mere loss of the brush border in epithelial cells to an overt tubular necrosis. Tubular cytotoxicity is the consequence of many interconnected actions, triggered by drug accumulation in epithelial tubular cells. Accumulation results from the presence of the endocytic receptor complex formed by megalin and cubulin, which transports proteins and organic cations inside the cells. Gentamicin then accesses and accumulates in the endosomal compartment, the Golgi and endoplasmic reticulum (ER), causes ER stress, and unleashes the unfolded protein response. An excessive concentration of the drug over an undetermined threshold destabilizes intracellular membranes and the drug redistributes through the cytosol. It then acts on mitochondria to unleash the intrinsic pathway of apoptosis. In addition, lysosomal cathepsins lose confinement and, depending on their new cytosolic concentration, they contribute to the activation of apoptosis or produce a massive proteolysis. However, other effects of gentamicin have also been linked to cell death, such as phospholipidosis, oxidative stress, extracellular calcium–sensing receptor stimulation, and energetic catastrophe. Besides, indirect effects of gentamicin, such as reduced renal blood flow and inflammation, may also contribute or amplify its cytotoxicity. The purpose of this review was to critically integrate all these effects and discuss their relative contribution to tubular cell death.

Key Words: **gentamicin; aminoglycoside antibiotics; cytotoxicity; apoptosis; necrosis.**

Nephrotoxicity is one of the main side effects of the aminoglycoside antibiotics, especially of gentamicin, and also one of its main therapeutic limitations. Gentamicin accumulates in the renal cortex (see below) and induces renal morphological changes and an overall syndrome very similar in humans and experimental animals (Luft *et al*., 1977). However, the precise characterization of the pathophysiological and molecular mechanisms underlying gentamicin's nephrotoxicity at the organism, tissue, cell, and molecular levels has been mostly obtained in animal and cellular experimental models. Gentamicin nephrotoxicity is typically characterized by tubular damage arising from tubular epithelial cell cytotoxicity. Treatment of experimental animals with gentamicin produces apoptosis (Li *et al*., 2009a) as well as necrosis (Edwards *et al*., 2007) of tubular epithelial cells *in vivo* and also in cultured cells (Pessoa *et al*., 2009). For other toxins, such as chemotherapeutic agents (Edinger and Thompson, 2004) and H_2O_2 (Saito *et al*., 2006), a relationship also exists between toxin concentration and death phenotype. Low concentrations cause apoptosis, whereas high ones cause necrosis. The death phenotype strongly depends on the cell energy status and ATP reserve. Apoptosis requires ATP, at least for the initial steps. At such, other circumstances different from drug concentration may modulate the death mode. For example, a severely diminished renal blood flow (RBF) may lower oxygen availability in some areas of the kidneys and limit respiration and ATP pool. In these circumstances, cell death may lose the typical characteristics of apoptosis and acquires those of necrosis (Chiarugi, 2005). Still, the most commonly observed phenotype *in vitro* is apoptosis, an observation that is in agreement with the fact that high concentrations of the drug (>1–2 mg/ml) are necessary to induce a modest cytotoxic effect in cultured cells (Pessoa *et al*., 2009; Servais *et al*., 2006).

ACCUMULATION OF GENTAMICIN IN TUBULAR CELLS

In the kidneys, aminoglycosides distinctively accumulate in epithelial cells of the proximal tubule (PTECs). This has been verified both in humans and in experimental animals (Luft *et al*., 1977). However, the mechanism of accumulation has



[AQ24] **FIG. 1.** Mechanisms of uptake and subcellular redistribution of gentamicin in tubular and other renal cells. M, Megalin.

been mostly studied in animals. This specific accumulation is because of the existence in these cells of a membrane endocytic complex involving the proteins megalin and cubilin (Cui *et al.*, 1996; Moestrup *et al.*, 1995; Nagai *et al.*, 2002, 2006), which has also been described as an endocytic receptor in human [AQ7] proximal tubules (Lee *et al.*, 2009). This complex transports cations present in the ultrafiltrate, such as a vast variety of proteins and certain xenobiotics, as for example aminoglycoside antibiotics (Schmitz *et al.*, 2002; Fig. 1). Accumulation of aminoglycosides inside the PTECs alters the function of several organelles and processes that are crucial for cell viability. Moreover, gentamicin activates the extracellular calcium–sensing receptor (CaSR), a membrane receptor sensitive to the amount of extracellular calcium, which has also been associated with tubular cell death.

It has been demonstrated in animal models and cultured cells that, quantitatively, most gentamicin enters tubular cells via endocytosis mediated by the megalin/cubilin complex. This process requires the electrostatic binding of gentamicin to the negative charges of membrane phospholipids (Frommer *et al.*, 1983; Lipsky *et al.*, 1980). Gentamicin then passes via pinocytosis to the endosomal compartment. The drug mostly accumulates in the lysosomes, travels retrograde through the secretory pathway to the Golgi and endoplasmic reticulum [AQ8] (ER; Sandoval and Molitoris, 2004; Silverblatt, 1982; Silverblatt and Kuehn, 1979) and alters vesicular traffic (Giurgea-Marion *et al.*, 1986; Jones and Wessling-Resnick, 1998). In the lysosomes, gentamicin produces membrane destabilization, lysosomal aggregation (De Broe *et al.*, 1984), alteration of lipid metabolism, and phospholipidosis, which have been associated with cell death (see below). It also generates multilamelar structures known as myeloid bodies (Edwards *et al.*, 1976; Houghton *et al.*, 1978; Silverblatt, 1982), whose pathophysiological role is uncertain.

ER STRESS AND UNFOLDED PROTEIN RESPONSE

Accumulation of gentamicin in the ER may originate ER stress (Fig. 2). ER stress activates the unfolded protein response (UPR) and cell cycle arrest (Zhang *et al.*, 2006). Under circumstances of UPR overload, the cell undergoes apoptosis (Fribley *et al.*, 2009), which is mediated by the classical route of calpains and caspase 12 (maybe caspase 4 in humans) activated by the release of Ca from the ER; UPR-activated apoptosis also involves Jun kinase and C/EBP homologous protein transcription factor (Kim *et al.*, 2008; Lai *et al.*, 2007; Peyrou and Cribb, 2007; Peyrou *et al.*, 2007). In this line, a calpain inhibitor reduces the cytotoxicity of gentamicin in cultured auditory hair cells (Shimizu *et al.*, 2003). Once activated, these enzymes promote the proteolytic activation of executor caspases and unleash the mitochondrial pathway of apoptosis (Kerbiriou *et al.*, 2009; Peyrou *et al.*, 2007). In fact, gentamicin joins calreticulin and inhibits its necessary chaperon activity for a correct posttranslational protein folding (Horibe *et al.*, 2004). It is well known that the bactericidal effect of gentamicin is related to its capacity to bind the small subunit of the ribosome and skew protein translation (Recht *et al.*, 1999). However, it is not yet well characterized whether gentamicin exerts similar effects in mammalian cells, which could be the cause or participate in cell death. Recht *et al.* (1999) reported that the minimum [AQ9] inhibitory concentration of gentamicin for the eukaryotic 16S rRNA was 0.23mM, 128 times higher than that for the prokaryotic rRNA. Despite this, different reports have suggested [AQ10] that aminoglycosides alter ribosomal accuracy (Buchanan *et al.*, 1987) and inhibit protein synthesis (Bennett *et al.*, 1988; Monteil [AQ11] *et al.*, 1993; Sundin *et al.*, 2001). Protein synthesis is reduced by 50% before gross cellular morphological alterations appear (Sundin *et al.*, 2001). The implications of these effects need to be further clarified.

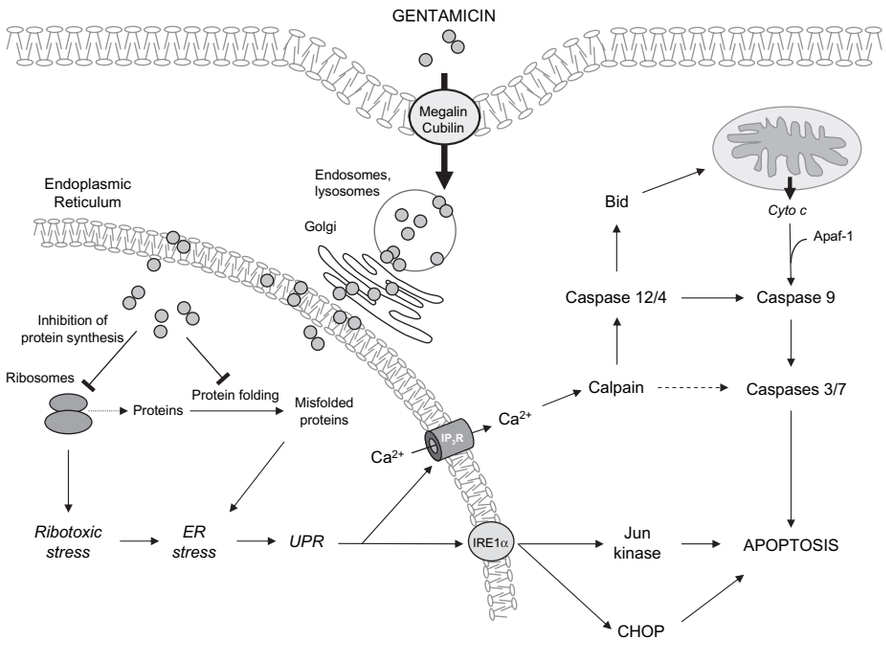


FIG. 2. Schematic representation of the ER stress and UPR caused by gentamicin. Cyto c, Cytochrome c.

CYTOSOLIC REDISTRIBUTION AND MITOCHONDRIAL TARGETING

Recent studies with cultured cells have shown that a critical aspect of gentamicin's tubular cytotoxicity is its cytosolic concentration and not, as previously thought, its accumulation in lysosomes (Servais *et al.*, 2006, 2008; Fig. 3). In comparison, a small amount of gentamicin directly enters the cytosol and nucleus independently from the endocytosis mediated by the megalin/cubilin complex (Myrdal *et al.*, 2005). Very recently, it has been demonstrated that gentamicin also enters cultured tubule cells through an unspecific cation channel, namely the transient receptor potential vanilloid type 4 (Karasawa *et al.*, 2008) channel. However, this channel is expressed in epithelial cells of the distal tubule but not in the proximal tubule (Karasawa *et al.*, 2008). Besides, the relative contribution of this entry mechanism is probably small.

The most important effect occurs when the concentration of gentamicin inside the lysosomes, the Golgi, and ER exceeds a threshold and destabilizes their membrane (Ngaha and Ogunleye, 1983; Regec *et al.*, 1989; Fig. 3). The accumulated gentamicin is released into the cytosol from where it acts on mitochondria and activates the mitochondrial pathway of apoptosis, produces oxidative stress, and reduces the ATP reserve (Morales *et al.*, 2010; Simmons *et al.*, 1980). On the other hand, the rupture of lysosomes causes the release of proteases into the cytosol, such as L, B, D, and other cathepsins, which intervene in the induction of cell death (Schnellmann and Williams, 1998). Cathepsins catalyze the proteolytic activation of executor caspases 3 and 7 and activate the mitochondrial pathway of apoptosis through the activation of Bid (Chwieralski *et al.*, 2006; Yin, 2006). In the absence of ATP, cathepsins in the cytosol produce a massive proteolysis that leads to necrotic cell death (Golstein and Kroemer, 2007).

In cell cultures, cytosolic gentamicin acts on mitochondria and triggers the translocation of cytochrome c and other proapoptotic proteins, such as apoptosis-inducing factor (AIF). In the cytosol, cytochrome c activates caspase 9 and, finally, the executor caspases 3 and 7, which result in cellular death by apoptosis (Servais *et al.*, 2008). The effect of gentamicin on mitochondria is produced in a direct and also in an indirect fashion. The mechanism of the direct action is unknown. However, it has been demonstrated that incubation of isolated mitochondria with gentamicin induces the release of proapoptotic proteins from the intermembrane space (Mather and Rottenberg, 2001), a requisite for the activation of the intrinsic pathway of apoptosis. The indirect action is mediated by Bax, and it is inhibited by overexpression of Bcl-2. In this sense, gentamicin binds the proteasome (Horibe *et al.*, 2004), which might affect the degradation of Bax and increase its cellular levels (Servais *et al.*, 2006).

CELL ENERGY STATUS IMPAIRMENT

Studies carried out in rats and mice demonstrate that peroxisome proliferator–activated receptor alpha (PPAR-α) activation (1) maintains ATP production by sustaining fatty

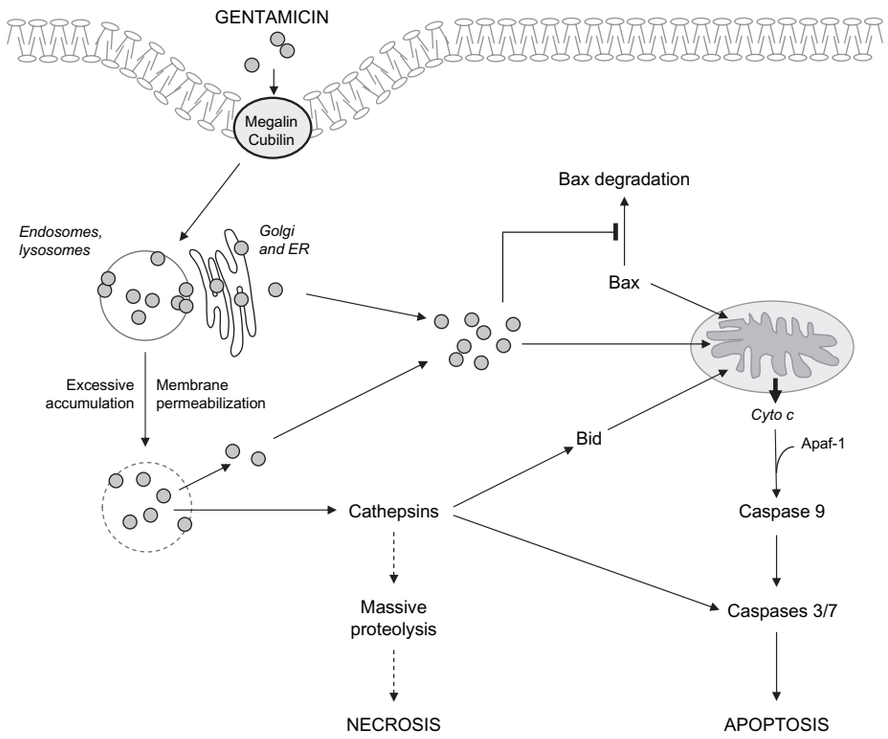


FIG. 3. Cytosolic redistribution of gentamicin and mechanisms leading to cell death through necrosis and the apoptotic intrinsic pathway.

acid oxidation; (2) prevents the increase in reactive oxygen species (ROS) and oxidative stress; and (3) reduces apoptosis of tubule cells, both *in vitro* and *in vivo*, during the acute kidney injury induced by ischemia and a variety of drugs, including cisplatin (Li *et al*., 2004, 2009b), doxorubicin (Lin *et al*., 2007), and gentamicin (Hsu *et al*., 2008). Indeed, these drugs reduce the level of PPAR-α in tubular cells through ubiquitination-dependent degradation, which has been shown to be crucial for their tubular toxicity (Lopez-Hernandez and Lopez-Novoa, 2009). The inhibition of cell membrane transporters might also contribute to an undetermined extent to the cytotoxicity of gentamicin. Indeed, both glucose intake inhibition and reduced Na+ efflux can theoretically lead to decreased cellular ATP levels and cell swelling. Glucose transport in proximal tubule cells depends on the sodium gradient generated by adenosin triphosphatases (ATPases). Deficient sodium extrusion caused by gentamicin may (1) indirectly reduce intracellular glucose availability and contribute to ATP pool reduction and (2) lead to sodium and, consequently, water accumulation, cell swelling, and necrotic death. Na–K ATPase is a key component of cell volume homeostasis, and deregulated swelling may lead to necrosis (DiBona and Powell, 1980; Lieberthal and Levine, 1996). In experiments carried out with cultured cells or membrane vesicles from tubular cells, it has been shown that gentamicin inhibits a variety of cell membrane transporters (reviewed in Mingeot-Leclercq and Tulkens, 1999) of both the brush border and the basolateral membrane, such as the Na–Pi cotransporter and Na–H exchanger (Levi and Cronin, 1990), brush-border dipeptide transporters (Skopicki *et al*., 1996), electrogenic Na transport (Todd *et al*., 1992), and the Na–K ATPase (Fukuda *et al*., 1991; Lipsky *et al*., 1980). Figure 4 schematically represents the cellular events activated by gentamicin that lead to ATP exhaustion.

MEMBRANE DESTABILIZATION AND PHOSPHOLIPIDOSIS

Another mechanism potentially involved in its cytotoxicity is the accumulation of gentamicin in cell membranes. Because of its polycationic properties, gentamicin binds to phospholipids. This has been shown to cause cell membrane structure alterations (Forge *et al*., 1989) and a condition known as phospholipidosis, which has been observed in humans (De Broe *et al*., 1984) and experimental animals treated with the drug (Giuliano *et al*., 1984; Nonclercq *et al*., 1992). Phospholipidosis is derived from (1) the disruption of phosphatidylinositol signalling pathways (Ramsammy *et al*., 1988), (2) the reduction of phospholipid turnover (Ramsammy *et al*., 1989a) and phospholipid accumulation in cell membranes (Kacew, 1987; Laurent *et al*., 1982), (3) the reduction in the available negative charge necessary for the correct function

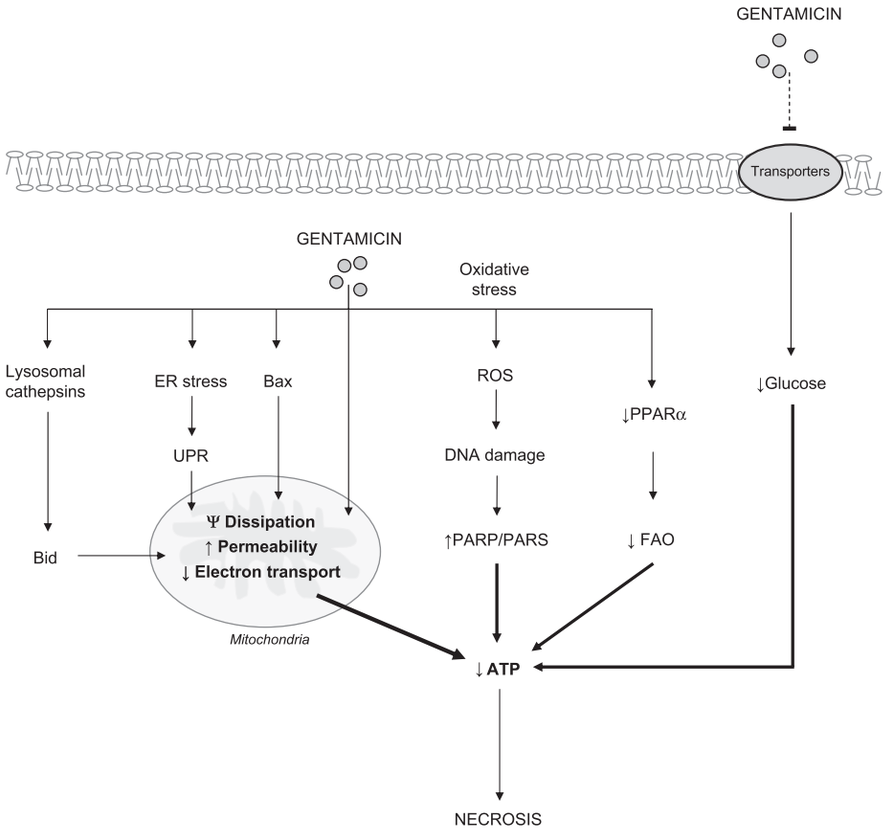


FIG. 4. Mechanisms contributing to the energetic catastrophe caused by gentamicin. FAO, fatty acid oxidation; PARP, poly (ADP-ribose) polymerase; PARS, poly (ADP-ribose) synthase. Ψ, mitochondrial transmembrane potential.

of phospholipases (Mingeot-Leclercq *et al.*, 1995), and (4) the inhibition of calcium-dependent phosphodiesterases by competing with and displacing calcium from the enzyme (van Rooijen and Agranoff, 1985). Binding to plasmalemmal phospholipids and plasma membrane accumulation occurs in other cell types exposed to the drug, in which intracellular accumulation and cell death are comparatively much less significant or absent. This indicates that these effects initiated in the cell membrane might not contribute largely to tubule cell death.

However, because aminoglycosides accumulate in lysosomes, lysosomal phospholipidosis has been more closely linked to cell death. In fact, lysosomal phospholipidosis correlates tightly with the level of toxicity of aminoglycosides (Kaloyanides, 1992; Nonclercq *et al.*, 1992; Tulkens, 1989). Precisely, lysosomal phospholipidosis has been proposed to be the result of (1) the reduction in the available negative charge, which is necessary for the proper function of lysosomal phospholipases (Mingeot-Leclercq *et al.*, 1995) and (2) the direct inhibition of A1, A2, and C1 phospholipases (Abdel-Gayoum *et al.*, 1993; Laurent *et al.*, 1982; Ramsammy *et al.*, 1989a). Support for a role of phospholipidosis in cell death comes from experiments in which rats were treated with polyaspartic acid (PAA), which has been shown to mitigate (Ramsammy *et al.*, 1989b) or to completely prevent the nephrotoxicity of gentamicin (Swan *et al.*, 1991). The effect of PAA has been ascribed to its capacity to bind gentamicin and thus to prevent its union to phospholipids (Ramsammy *et al.*, 1990). However, binding to phospholipids is also a requirement for gentamicin endocytosis (as described above), which blurs conclusions. As such, it is not known to what extent (if to any) lysosomal or endosomal phospholipidosis contribute to cell death or to other sublethal alterations. [**AQ15**]

A glimpse of light on this issue was provided by the study of Kishore *et al.* (1990). These authors used three different polyanionic peptides, namely poly-L-Asp with poly-L-Glu and poly-D-Glu to inhibit the nephrotoxicity and lysosomal phospholipidosis caused by gentamicin in rats. These peptides showed similar capacity to bind gentamicin, and thus to displace it from phospholipids in wide range of pH, including acidic pH. However, they showed a significantly different degree of hydrolysis in the presence of lysosomal extracts. Interestingly, their capacity to prevent gentamicin-induced phospholipidosis and gentamicin's nephrotoxicity was inversely proportional to their hydrolysis rate, supporting the hypothesis that their site of action was inside the lysosomes and not at the level of other renal membranes. Clearly, further research is necessary to shed light on this matter.

CaSR STIMULATION

CaSR, a member of the family C of cell membrane G-protein–coupled receptors (Trivedi *et al*., 2008), has also been implicated in gentamicin-induced tubule cell death (Fig. 1). *In vitro* experiments using HEK-293 cells have shown that gentamicin induces the death of cells expressing CaSR but not of those lacking it (Ward *et al*., 2005). Moreover, pharmacological antagonism of CaSR prevents the cell death induced by gentamicin in CaSR-expressing cells (Gibbons *et al*., 2008). However, a number of issues invites to caution when interpreting these results. First, there has been some controversy about the origin and phenotype of HEK-293 cells. Second, the extent of cell death induced by gentamicin in CaSR-expressing cells is low. Finally, *in vivo* evidence is missing because there are no useful tools to manipulate the CaSR. Moreover, an important pathophysiological role of gentamicin-induced CaSR–mediated tubule cell death odds with the evidence showing that the critical event is its cytosolic concentration, as explained above. In addition, the CaSR has been found in many other cell types outside the kidneys, where gentamicin has no evident cytotoxicity, including bone, brain, colon, parathyroid gland, smooth muscle, endothelial cells, etc. Clearly, more information is necessary to clarify the exact role of CaSR in tubule cell death.

OXIDATIVE STRESS

Treatment with gentamicin produces oxidative stress in tubular cells, both *in vivo* (in rats; Karataş *et al*., 2004) and in cultured tubular cells (Juan *et al*., 2007). This oxidative stress is mediated by hydroxyl radicals from hydrogen peroxide and by superoxide anions (Basnakian *et al*., 2002; Nakajima *et al*., 1994) from mitochondrial origin (Yang *et al*., 1995). Gentamicin directly increases the production of mitochondrial ROS from the respiratory chain (Morales *et al*., 2010). Reduced glutathione (GSH; Ali *et al*., 1992; Sandhya and Varalakshmi, 1997) and superoxide dismutase (SOD; Nakajima *et al*., 1994; Kadkhodaee *et al*., 2007) levels have been found to be low in the kidneys upon treatment with gentamicin.

Oxidative stress plays an important role in the nephrotoxicity of gentamicin (Koyner *et al*., 2008). Cotreatment of rats with a variety of antioxidants significantly reduces renal dysfunction and tissue damage (Ali, 2003; Cuzzocrea *et al*., 2002; Koyner *et al*., 2008; Martínez-Salgado *et al*., 2002; Morales *et al*., 2002). However, a note of caution was introduced by the study of Stratta *et al*. (1994), who demonstrated that GSH administration had no effect on the nephrotoxicity of gentamicin, despite reducing lipid peroxidation and increasing renal GSH content. Interestingly, this absence of effect was observed with a dosage of gentamicin that apparently resulted in an excess of the drug. With a lower dosage, GSH implementation softened renal damage, which curiously correlated with a lower accumulation of gentamicin in the kidneys. It is thus possible that (1) the critical level of highly cytotoxic oxidative stress induced by gentamicin depends on the dose or accumulation of gentamicin, and consequently, that the weight of oxidative stress in the nephrotoxicty of gentamicin depends on the dose of the drug and (2)the mechanism of damage is, at least partially, derived from the prevention of its renal accumulation. These aspects need further investigation.

ROS, mainly superoxide anions and hydroxyl radicals, cause cellular damage and death through diverse mechanisms, including the following (Cuzzocrea *et al*., 2004; Morgan *et al*., 2007; Ott *et al*., 2007; Ryter *et al*., 2007): (1) inhibition of the electron transport chain and suppression of cellular respiration and ATP production; (2) stimulation of the release of cytochrome c, AIF, etc. from the mitochondrial intermembrane space; (3) DNA damage, which triggers an increase in poly ADP ribose synthase activity, a decrease in the cell's ATP reserve, and cell cycle arrest; (4) lipid peroxidation, destabilization of the cellular membrane, activation of death receptors (Fas, etc.) by alteration of lipid rafts, and generation of proapoptotic lipid metabolites, such as 4-hydroxynonenal and ceramide; (5) stress on different organelles and cellular structures, such as the ER (Yokouchi *et al*., 2008; Santos *et al*., 2009) and (6) inhibition of transmembrane sodium flow, by oxidative inhibition of the Na+/K+ ATPase pump and of sodium channels, which originates cellular swelling, loss of membrane integrity, and necrosis.

However, there is little information on the ability of antioxidants to modulate the direct cytotoxic effect of gentamicin on cultured tubule cells. To our knowledge, only Juan *et al*. (2007) have reported a protective effect in this sense. In their article, tetramethylpyrazine (TTP) reduces ROS accumulation and apoptotic events in rat renal NRK-52E cells. However, the effect of TTP on cell viability is not reported. Because there are many apoptotic and necrotic pathways leading to cell death as a consequence of gentamicin action, and because their redundancy and hierarchical organization are not well understood, the magnitude of the direct cytoprotection afforded by ROS inhibition is unknown. The question that remains to be solved is, is the increment in ROS production the consequence of the mitochondrial injury directly and indirectly exerted by gentamicin? Or are ROS increased by gentamicin previously to or independently from its mitochondrial proapoptotic effects, which in turn the trigger apoptosis? Speculatively, oxidative stress can be viewed at least as an amplification factor.

INTEGRATIVE OVERVIEW OF TUBULAR CELL DEATH

From the information presented above, it can be concluded that gentamicin needs to accumulate inside the cells to a significant level in order to induce cell death. CaSR stimulation (from the outside) has also been shown to induce some degree of cell death in tubule cells and might participate

in mesangial and tubular cell death (Martínez-Salgado *et al.*, 2007). However, this is proportionally small compared with the cytotoxicity caused by intracellular accumulation, and might show cell type dependency, because many other CaSR-expressing cells do not die when exposed to gentamicin.

Inside the cells, a critical factor appears to be its cytosolic concentration rather than its accumulation in endosomal structures. Cytosolic gentamicin directly and indirectly attacks mitochondria, inhibits respiration and ATP production, and produces oxidative stress (Morales *et al.*, 2010), all of which activate the intrinsic pathway of apoptosis. These data indicate that cytosolic gentamicin has the ability to trigger apoptosis. However, they do not discard contributions from other damaged structures or signalling pathways. In fact, the cytosolic redistribution of gentamicin probably coincides with the leakage from the ER, permeabilization of lysosomes, and the release of lysosomal proteases (i.e., cathepsins) into the cytosol, which may add a redundant mediation toward cell death. Gentamicin also induces stress of other cellular structures, such as the ER, including protein synthesis inhibition, which, depending on the intensity, can affect cell viability. Unresolved and persistent stress also unleashes apoptosis from the damaged structures. Because the route to cytosolic accumulation goes through accumulation in intracellular membrane structures, including ER, it is difficult to imagine how gentamicin can accumulate in the cytosol without inducing some degree of ER stress. As such, we propose that besides mitochondrial damage, gentamicin also activates other pathways of cell death resulting from stress to other structures and organelles, which add an unknown level of redundancy. Probably, the predominance of some over the others, as well as the phenotype of cell death (highly dependent on energy status), might be a matter of concentration of the drug to which the cell is exposed. It can be hypothesized that low concentrations of the drug would traffic through the endocytic pathway and leak through the ER into the cytosol to a sufficient amount to active mitochondrial apoptosis, without inducing a significant injury to the ER and without causing lysosomal breakage or energetic catastrophe. High concentrations would cause further leakage through the ER, significant ER stress and protein synthesis inhibition, lysosomal rupture, and redundant apoptotic stimulation. In extreme cases of drug accumulation, massive and rapid cathepsin-driven proteolysis and ATP exhaustion may abort the execution of apoptosis and cause necrotic-like cell death. Also, as a result of accumulation in endosomal vesicles and lysosomes, phospholipidosis may also contribute to an undetermined extent to tubular cell death. A challenge for the coming future is to elucidate the relative contribution of all these mechanisms of cytotoxicity to the different cell death phenotypes, under a range of drug concentrations. This will unravel the key targets for the pharmacological prevention of the tubular cytotoxicity of aminoglycosides, which cannot be achieved with the present level of knowledge.

INDIRECT DETERMINANTS OF CYTOTOXICITY

In general, cultured tubular cells exhibit a significant resistance to cell death by exposure to gentamicin. Only very high concentrations of the drug (>1–3mM), over long periods of time (>1–4 days), cause a mild degree of cell death (<20%), only in determined cell lines (El Mouedden *et al.*, 2000; Pessoa *et al.*, 2009; Servais *et al.*, 2006; Wu *et al.*, 2009). As such, other factors independent from a direct cytotoxic action of gentamicin might exist, which would amplify deadly stimulation, and which are present *in vivo* and absent in cultured cells. One hypothesis (Fig. 5) is that inflammation and ischemia may be two of those amplification factors. Alternatively, it might be speculated that tubule cells in culture lose their capacity to efficiently accumulate gentamicin (Servais *et al.*, 2006). This topic is of special interest because therapeutic targets to prevent gentamicin-induced tubular cell death should be sought out of target cells if additional factors are essential for an extensive tubular necrosis.

Gentamicin causes a reduction in RBF in experimental animals (Hishida *et al.*, 1994; Morales *et al.*, 2002), which has been associated to tubular damage (Moran *et al.*, 1992). Although the mechanisms linking reduced RBF to tubular cell death are not well understood, it is hypothesized that limitation in O_2 and glucose supply lead to a diminished ATP production, all of which causes or sensitizes to cell death (Jeong *et al.*, 2003; Sato *et al.*, 2010; Seppet *et al.*, 2009). In fact, hypoxia activates inducible nitric oxide synthase (iNOS) expression, which leads to cell death by inducing oxidative stress, inhibiting ATP synthesis, and activating the mitochondrial pathway of apoptosis (Kiang and Tsen, 2006). Platelet-activating factor (PAF) and thromboxane A2 (TXA2) are two mediators of gentamicin-induced renal vasoconstriction (López-Novoa, 1999; Martínez-Salgado *et al.*, 2007). PAF and TXA2 inhibitors improve RBF and lessen tubular damage (Dos Santos *et al.*, 1991; Papanikolaou *et al.*, 1992; Rodriguez-Barbero *et al.*, 1992). Gentamicin is also known to impair vascular smooth muscle relaxing capacity, contributing to the reduced RBF (Seçilmiş *et al.*, 2005). Coadministration of L-arginine normalizes vascular relaxation and softens tubular injury (Seçilmiş *et al.*, 2005). However, results from Hishida *et al.* (1994) contradict this notion. These authors found that cotreatment with desoxycorticosterone acetate or SOD normalized gentamicin-induced RBF decline but did not reduce the severity of tubular necrosis. Moreover, cotreatment with dimethylthiourea, a hydroxyl radical scavenger, attenuated tubular necrosis but did not ameliorate the reduction in RBF. These data break the link between tubular necrosis and reduced RBF but, interestingly, indicate that intervention on different ROS species may have preferential vascular or tubular effects in gentamicin's nephrotoxicity. Clearly, more investigation is necessary.

The nephrotoxicity of gentamicin has been shown to involve an inflammatory response in experimental animals (Bledsoe *et al.*, 2006; Kalayarasan *et al.*, 2009; Kourilsky *et al.*, 1982).

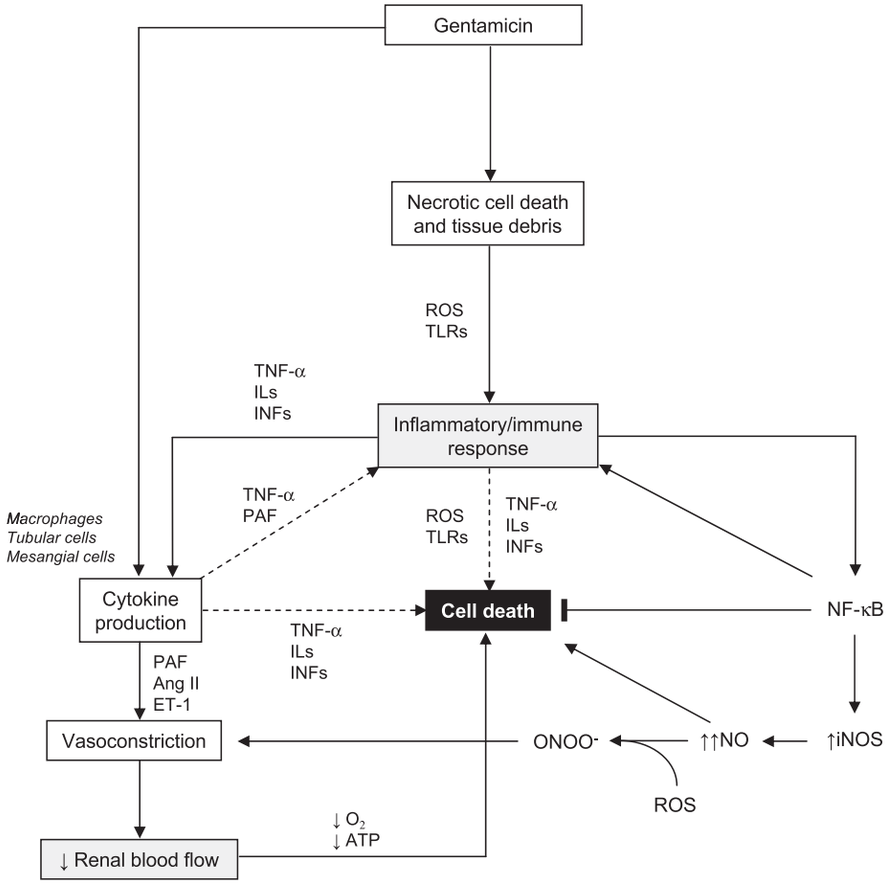


FIG. 5. Indirect mediators of gentamicin's cytotoxicity: inflammation and reduced RBF. Ang II, angiotensin II; ET-1, endothelin-1; Ils, interleukins; INFs, interferons; TLRs, toll-like receptors; TNF-α, tumor necrosis factor alpha.

An exaggerated or pathologically skewed inflammatory response seems to be involved in tubular injury and contribute to renal damage progression (Karkar, 2008). In fact, strategies that protect from gentamicin-induced renal damage usually inhibit the inflammatory response (Bledsoe *et al*., 2006; Sue *et al*., 2009) An increased or unbalanced ROS production and oxidative stress mediate the inflammatory response unleashed by gentamicin (Kadkhodaee *et al*., 2005; Maldonado *et al*., 2003; Morales *et al*., 2002; Fig. 5). Superoxide anion (Schreck *et al*., 1991) and hydrogen peroxide (Meyer *et al*., 1993; Lu *et al*., 2010) activate nuclear factor κB (NFκB), a key mediator of several inflammatory pathways. Indeed, NFκB inhibitors protect the kidney against gentamicin-induced damage (Tugcu *et al*., 2006). NFκB induces the expression of proinflammatory cytokines (Sánchez-López *et al*., 2009) and iNOS (Xie *et al*., 1994). Endothelial NOS-derived NO, at low levels, mediates physiological vasodilatation, whereas excessive NO production because of the overexpression of iNOS can cause cytotoxic effects in surrounding cells. Excessive iNOS-derived NO can react with superoxide anion and produce peroxinitrite, a highly reactive radical that contributes to cell damage (Pedraza-Chaverrí *et al*., 2004) and reduced vascular relaxation (Förstermann, 2010; Fig. 5). Inflammatory cytokines, such as tumor necrosis factor alpha can activate tubular apoptosis, especially in the pathological environment (Justo *et al*., 2006).

PERSPECTIVES

Many cellular effects of gentamicin have the capacity to cause cell death or contribute significantly to it, including activation of the mitochondrial pathway of apoptosis, ER stress, and onset of an UPR and phospholipidosis. Others have an uncertain capacity to lead directly to cell death, such as oxidative stress and ATP-depleting mechanisms. However, besides the relative contribution of these pathways considered individually, the hierarchic relation among them is still unknown. For example, can gentamicin pass through the endosomal vesicles (endosomes, lysosomes, Golgi, etc.) toward the cytosol without producing ER stress leading to cell death? If mitochondrial effects were inhibited, would other

mechanisms lead the way to cell death? To what extent are some of these mechanisms redundant? Does the participation of each individual mechanism vary depending on the level of stimulation (i.e., gentamicin dosage)? After reviewing the existing information on gentamicin tubular cytotoxicity, it must be concluded that these questions remain incompletely answered. These are important aspects of future research, which will yield critical information on the key mechanisms that should be targeted for the pharmacological prevention of gentamicin's undesired renal side effects. Selective inhibition of specific mediators of individual mechanisms will led further light on these issues.

A potential limitation to progression in this line is the uncertainty on the reliability of the available tubular cell lines and primary cultures at reproducing the effects of gentamicin in tubular cells *in vivo*. The relative resistance of cultured cells to gentamicin cytotoxicity might be the result of an experimental artifact or it might reflect the real nature of tubular cells in their tissue environment. If this is the case, indirect mechanisms of cytotoxicity, as those addressed in the previous section (i.e., reduced RBF, inflammation, and the immune response), will need to be invoked to fully explain tubular necrosis and may gain a central role in therapeutics. [AQ22]

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OBJETIVOS

Con objeto de mejorar el diagnóstico del daño renal agudo, nos propusimos identificar marcadores potencialmente útiles en la práctica clínica para nuevas facetas diagnósticas dirigidas a detectar de forma anticipada el riesgo adquirido de padecer esta enfermedad como consecuencia de tratamientos farmacológicos aparentemente inocuos, pero que predisponen a la acción nefrotóxica de otros fármacos o tóxicos ambientales. Para ello planteamos los siguientes objetivos concretos:

OBJETIVO 1. Estudiar si el tratamiento subnefrotóxico con el antibiótico aminoglucósido gentamicina predispone o sensibiliza a los animales de experimentación al desarrollo de un fracaso renal agudo.

OBJETIVO 2. De ser así, identificar marcadores urinarios que puedan servir para identificar esta condición.

En relación con el desarrollo de herramientas diagnósticas que permitan diferenciar la nefrotoxicidad de unos fármacos de la de otros, con utilidad potencial en la medicina personalizada de pacientes polimedicados, y a modo de prueba de concepto, plateamos el siguiente objetivo concreto:

OBJETIVO 3. Identificar marcadores urinarios que sean capaces de distinguir el daño renal agudo producido por el anitibiótico gentamicina del producido por el antineoplásico cisplatino.

El sentimiento más importante que el hombre puede experimentar es su propio respeto al misterio: éste es la fuente del arte y la ciencia.

Quien no puede contemplar el mundo con asombro, es que tiene los ojos cerrados.

A. Einstein

RESULTADOS, METODOLOGÍA Y DISCUSIÓN

GENERALIDADES

Los resultados de esta tesis doctoral se exponen en dos artículos y sus correspondientes patentes. El primero de ellos, titulado **"SUB-NEPHROTOXIC DOSES OF GENTAMICIN PREDISPOSE ANIMALS TO DEVELOPING ACUTE KIDNEY INJURY AND TO EXCRETE GANGLIOSIDE M2 ACTIVATOR PROTEIN"** en el mismo se describe el estudio y la identificación de marcadores urinarios (proteína activadora del ganglósido M2) de predisposición al daño renal agudo por la administración de dosis subnefrotoxicas gentamicina y la administración de un segundo sudnefrotoxico.

EL Segundo articulo se titula: "**URINARY REG IIIB AND GELSOLIN: DIFFERENTIAL MARKERS OF GENTAMICIN-INDUCED ACUTE KIDNEY INJURY".** En este artículo se describe la identificación de biomarcadores urinarios para diagnóstico etiológico del FRA por la nefrotoxicidad de la gentamicina, además su capacidad de distinguir, el daño renal agudo producido por la gentamicina del producido por el cisplatino.

ARTÍCULO I

SUB-NEPHROTOXIC DOSES OF GENTAMICIN PREDISPOSE ANIMALS TO DEVELOPING ACUTE KIDNEY INJURY AND TO EXCRETE GANGLIOSIDE M2 ACTIVATOR PROTEIN

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Sub-nephrotoxic doses of gentamicin predispose animals to developing acute kidney injury and to excrete ganglioside M2 activator protein

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We studied whether nephrotoxic drug administration sensitizes to acute renal failure (ARF) by administering a sub-nephrotoxic dose of gentamicin. This pre-treatment sensitized animals with no sign of renal injury to develop ARF when exposed to a second potential nephrotoxic drug, also given at sub-nephrotoxic doses that would be otherwise harmless to non-sensitized animals. We identified urinary ganglioside M2 activator protein (GM2AP) as a biomarker of an enhanced sensitivity to suffer ARF following sub-nephrotoxic treatment with gentamicin. Sub-nephrotoxic gentamicin did not alter renal *GM2AP* gene expression or protein levels, determined by reverse transcriptase-PCR, western blot, and immunostaining, nor was its serum level modified. The origin of increased GM2AP in the urine is thought to be a defective tubular handling of this protein as a consequence of gentamicin action. Hence, markers of acquired sensitivity may improve the prevention of ARF by enhancing our capacity to monitor for this condition, in a preemptive manner.

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KEYWORDS: gentamicin; GM2AP; nephrotoxicity; sensitization to acute kidney injury; urinary markers

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Acute renal failure (ARF) is an extremely serious condition in which the renal excretory function abruptly falls within a few hours or days after an insult to the kidneys.[1,2] ARF still leads to death in 50% of the cases, a number that grows to 80% if multiorgan damage occurs.[3–6] The most common causes of ARF are renal ischemia, obstruction of the urinary ways, and drug nephrotoxicity.[7] A key determinant for a successful clinical handling of ARF is an early diagnosis, which significantly improves therapeutic intervention and outcome.[8,9] Traditionally, ARF has been diagnosed through measurable symptoms of renal dysfunction, such as the increase in serum creatinine and blood urea nitrogen (BUN) concentrations, or changes in the fractional excretion of sodium.[9] However, owing to compensatory adaptation, renal dysfunction only appears after an extensive loss of functional nephrons occurs.[10] Consequently, a new generation of biomarkers (mostly urine biomarkers) is under development, associated with early pathophysiological events underlying the incipient acute kidney injury (AKI), before it turns into an overt ARF. They, most significantly, include kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin, and others.[9]

Gentamicin is an aminoglycoside antibiotic widely used against Gram-negative infections. The most important side effect of this drug is its nephrotoxicity,[11,12] which occurs in ~10–25% of therapeutic courses, despite correct dosage and hydration status monitoring.[13–15] Gentamicin nephrotoxicity may range from a mere transient renal dysfunction to a severe ARF. Gentamicin produces tubular lesions, affecting mostly the proximal segment, with no gross modification of glomerular structures. Tubular lesions vary from a mild sloughing of the brush border to a generalized tubular necrosis.[11,16,17] Besides, gentamicin has been shown to exert functional glomerular[12,18–22] and vascular effects[23–25] that, depending on the dose, contribute to a larger or lesser extent of renal dysfunction.[26]

However, it is not yet well characterized to what extent a subtoxic treatment with gentamicin sensitizes individuals to ARF, such as the ARF induced by subsequent sub-nephrotoxic

exposure to another potentially nephrotoxic agent. A typical clinical situation exists, where a patient treated with gentamicin showing no signs of renal disease is thereupon given another potentially nephrotoxic agent, such as another drug, or a diagnostic contrast medium, also within a theoretically subtoxic regime. These scenarios pose relevant clinical situations of special importance for its hidden nature, which should be addressed from the diagnostic and therapeutic perspectives. In this study, we demonstrate that a sub-nephrotoxic regime of gentamicin primes the rats to develop an ARF induced by a subsequent or concomitant exposure to sub-nephrotoxic doses of a second nephrotoxicant. We also show that the urinary level of ganglioside M2 activator protein (GM2AP) may be used to identify this condition. Urinary levels of GM2AP could also serve for the early diagnosis of gentamicin-induced ARF. Detection of the increased risk enables a preemptive handling of drug toxicity by anticipating situations that can result in injury, before the slightest alterations that are usually observed arise.

RESULTS

Characterization of a sub-nephrotoxic regime of gentamicin

A sub-nephrotoxic regime of gentamicin was identified to test whether it would induce sensitisation to ARF in the absence of a direct deleterious effect on the kidneys. After pilot studies, a regime of six daily consecutive doses of 50 mg/kg/day gentamicin (G-50 group) was observed to exert no obvious renal injury symptoms. It was further characterized to ensure the absence of nephrotoxicity. Survival during the whole treatment was identical to that of control rats (100%), whereas in rats treated with a nephrotoxic regime of gentamicin (150 mg/kg/day; G-150 group), which developed a clear ARF, survival decreased to 50%. Similarly, body weight increased by a 3–4% in control and G-50 animals, whereas it was reduced by 4–5% in G-150 rats. As shown in Figure 1, we were unable to find a single marker of renal damage or dysfunction in G-50 rats, when compared with controls. On the contrary, G-150 rats underwent a typical and overt ARF characterized by an increase in plasma creatinine concentration (Cr_{pl}) and BUN, proteinuria, increased fractional excretion of sodium, and the presence of urinary (i.e., increased *N*-acetyl-glucosaminidase (NAG) excretion) and renal tissue (KIM-1, plasminogen activator inhibitor 1, and vimentin) markers of tubular lesion (Figure 1a–f). A gross morphological examination of renal slices showed that renal parenchyma in G-50 was indistinguishable from that of control rats, whereas a clear tubular necrosis and obstruction was evident in G-150 rats (Figure 1g). These results indicate that the G-50 regime exerts no apparent deleterious action on the kidneys, as evaluated by the finest diagnostic methods available.

Sub-nephrotoxic gentamicin sensitizes to ARF

Under these sub-nephrotoxic circumstances, we tested whether the G-50 regime sensitizes rats to ARF, e.g., by reducing the nephrotoxicity threshold of another potential nephrotoxicant. We first used uranyl nitrate (UN), which we titrated for dose-nephrotoxic effect in pilot studies. A single dose of 0.5 mg/kg UN was found to lack nephrotoxic effects, which was confirmed in further experiments (Figure 2). However, when this dose of UN was administered to rats previously treated with G-50, a clear ARF ensued, which was not observed in control rats or in that treated with UN or G-50 alone. This ARF was characterized (Figure 2a–d) by an increase in Cr_{pl}, BUN and NAG excretion, proteinuria, and a decrease in creatinine clearance. This sensitization appears along with the first sub-nephrotoxic dose of gentamicin and lasts at least 1 week after gentamicin withdrawal. This is evidenced by the increase in serum creatinine in rats that were given the single dose of UN at the onset of the gentamicin regime, as well as in rats in which UN is administered 1 week after cessation of the gentamicin treatment (data not shown). Interestingly, the sensitization produced by gentamicin is also effective on other potentially nephrotoxic drugs, such as the antineoplastic cisplatin or the iodinated contrast medium iohexol. Figure 2e–f shows how rats, previously exposed to G-50 for 6 days, suffered a renal damage when subsequently exposed to iohexol (in 24 h) and cisplatin (in 2 days), both used at sub-nephrotoxic doses (titrated in previous pilot studies). This is evidenced by increased serum creatinine and BUN, and elevated NAG excretion.

GM2AP is identified in the urine as a marker of sensitized animals

Next, we performed a differential proteomic analysis comparing the urine of control rats and G-50 rats at the end of the treatment, before the administration of the second nephrotoxicant. The objective of this study was to identify whether proteins increased or decreased in the urine of G-50 (sensitized to AKI) compared with control (non-sensitized to AKI) rats, which might prospectively serve as biomarkers of gentamicin-induced sensitization to AKI. As shown in Figure 3a, both urinary proteomes were almost identical. However, a protein was clearly increased in the urinary proteome of G-50, which was unambiguously identified using liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as GM2AP. A polyclonal antibody raised in rabbits against an epitope found in rat and human GM2AP further confirmed the increased levels of GM2AP in the urine of G-50 using western blot analysis (Figure 3b). Furthermore, the urine of eight patients treated with gentamicin for at least 2 days was analyzed using western blot for the level of GM2AP, and compared with that of eight sex and age matching untreated individuals. A total of seven of eight gentamicin-treated patients, whereas only two of eight untreated controls, showed increased levels of GM2AP in the urine, also determined using western blot analysis (Figure 3c).

Early diagnosis capacity of GM2AP in gentamicin-induced AKI

We also decided to study the early diagnostic capacity of urinary GM2AP on an animal model of gentamicin-induced

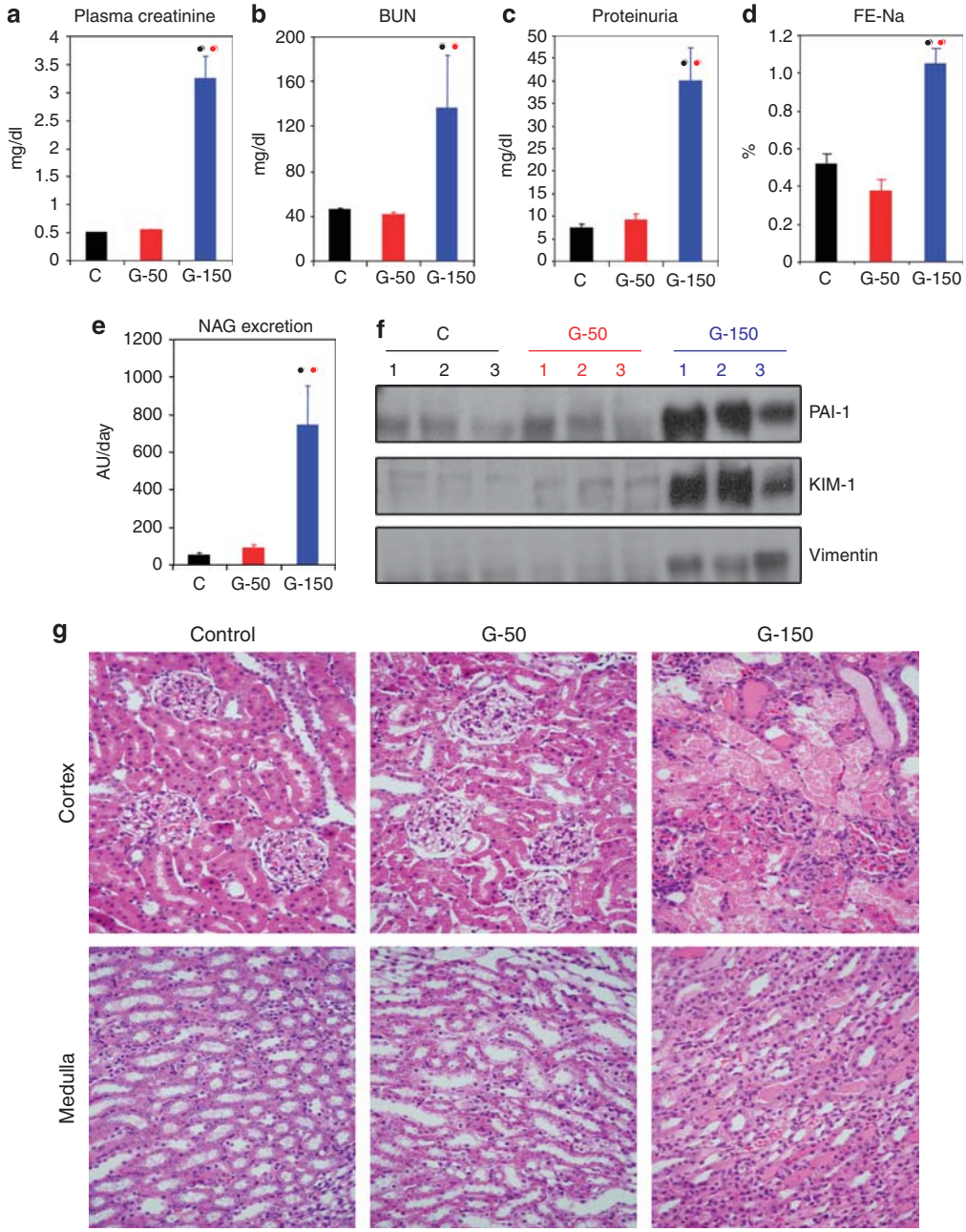

Figure 1 | Absence of renal effects after sub-nephrotoxic gentamicin. (**a**) Plasma creatinine concentration, (**b**) blood urea nitrogen concentration (BUN), (**c**) proteinuria, (**d**) fractional excretion of sodium (FE-Na), and (**e**) *N*-acetyl-glucosaminidase (NAG) excretion after 6 days of treatment with saline (C), 50 mg/kg/day gentamicin (G-50), or 150 mg/kg/day gentamicin (G-150); $n > 12$. (**f**) Representative images of western blot analysis of plasminogen activator inhibitor 1 (PAI-1), kidney injury molecule 1 (KIM-1), and vimentin levels in renal tissue homogenates from three randomly selected animals from C, G-50, and G-150 groups. (**g**) Representative images (400 ×) of the cortex and medulla of hematoxilin–eosin-stained renal sections from C, G-50, and G-150 rats ($n = 6$). ●, $P < 0.05$ with respect to C; •, $P < 0.05$ with respect to G-50.

ARF. A time course experiment revealed that, in this model, GM2AP appears in the urine from the first day of treatment with overtly nephrotoxic doses of gentamicin, largely preceding not only classical markers such as serum creatinine, BUN, NAG excretion, or proteinuria, but also the new, earlier, and more sensitive urinary markers of AKI, KIM-1, and plasminogen activator inhibitor 1 (Figure 4a). Furthermore, the level of GM2AP in the urine progressively increased with time, which makes it potentially suitable for monitoring AKI evolution induced by gentamicin in a much more specific manner than other novel markers. GM2AP also appears in the urine of overtly nephrotoxic rats as a consequence of cisplatin or UN administration at toxic doses (Figure 4b). In the case of cisplatin, GM2AP appears in the urine in parallel or after KIM-1 (Figure 4c). These results indicate that urinary GM2AP, in the absence of damage

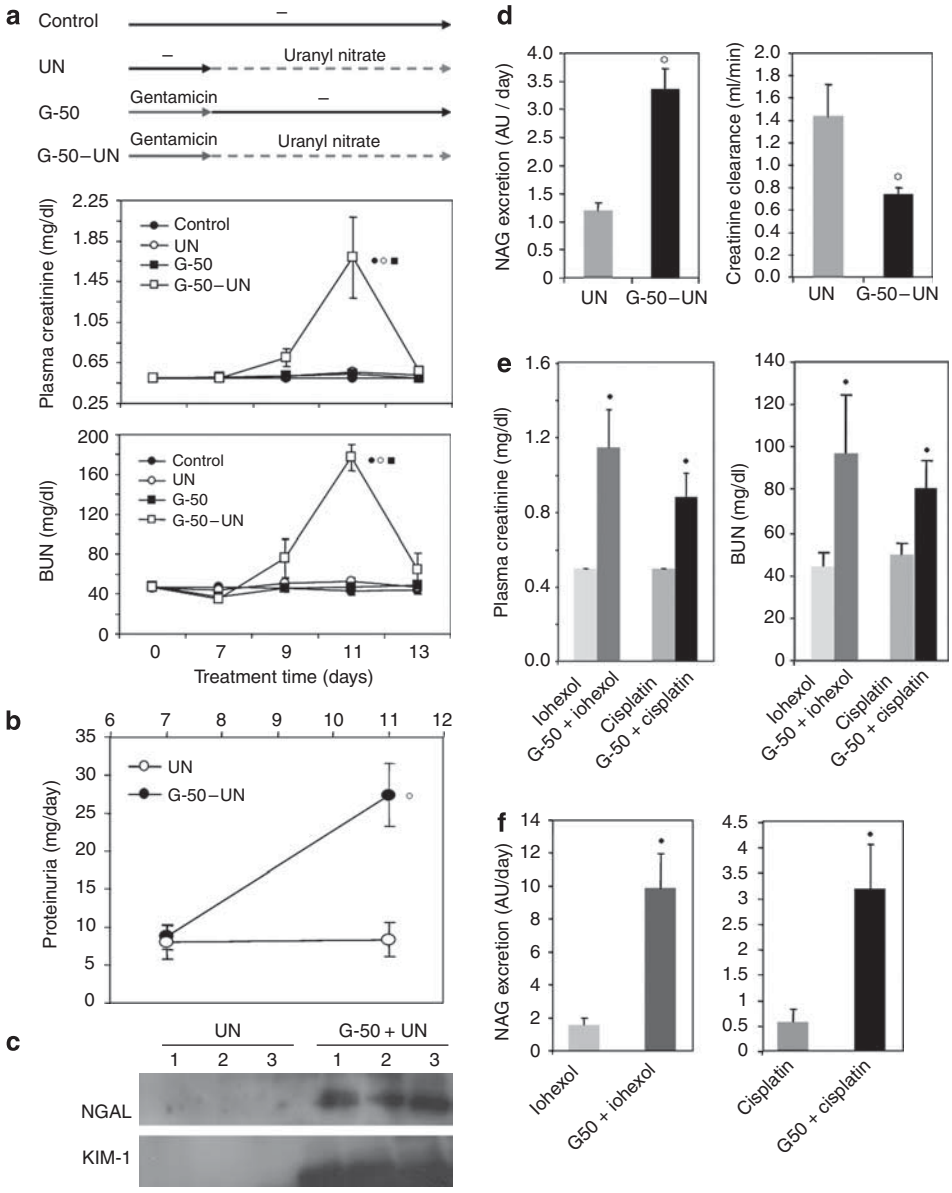

Figure 2 | Sub-nephrotoxic gentamicin sensitizes to acute renal failure. (**a**) Time-course evolution of plasma creatinine and BUN concentration, (**b**) proteinuria, (**c**) urinary neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1) levels, (**d**) *N*-acetyl-glucosaminidase (NAG) excretion, and creatinine clearance of rats treated as indicated in the upper, left panel of **a**; $n = 6$. ●, $P < 0.05$ with respect to C; ○, $P < 0.05$ with respect to uranyl nitrate (UN); and ■, $P < 0.05$ with respect to G. (**e**) Plasma creatinine and BUN concentration and (**f**) NAG excretion of rats treated as indicated in the left panel, in which uranyl nitrate was substituted by iohexol or cisplatin; $n = 4$; ●, $P < 0.05$ with respect to iohexol or cisplatin.

markers, likely reflects the increased risk of ARF induced by gentamicin, because further exposure to the drug produces an overt ARF.

Urinary GM2AP results from an altered renal handling of the filtered blood-borne protein

To unravel the origin of the urinary GM2AP, we studied the effect of gentamicin on the presence and production of GM2AP in renal tissue, the urine, and the blood. The immunohistochemical analysis of GM2AP distribution in renal tissue shows (Figure 5a and b) that this protein is mainly located in the renal cortex, with great selectivity, within the proximal tubules. The latter is evidenced by a perfect co-staining of GM2AP with the proximal tubule-restricted protein megalin, and the absence of co-staining with the distal tubule-borne protein calbindin. Sub-nephrotoxic gentamicin (G-50) did not modify the histological distribution or apparent expression level of GM2AP (Figure 5a and b). Western blot analysis of total GM2AP protein level in renal tissue homogenates (Figure 5c), as well as *GM2AP* gene expression (by reverse transcriptase-PCR; Figure 5d), confirmed the lack of effect of gentamicin on the renal expression of the protein. GM2AP was detected in the serum and its serum levels were not altered by G-50.

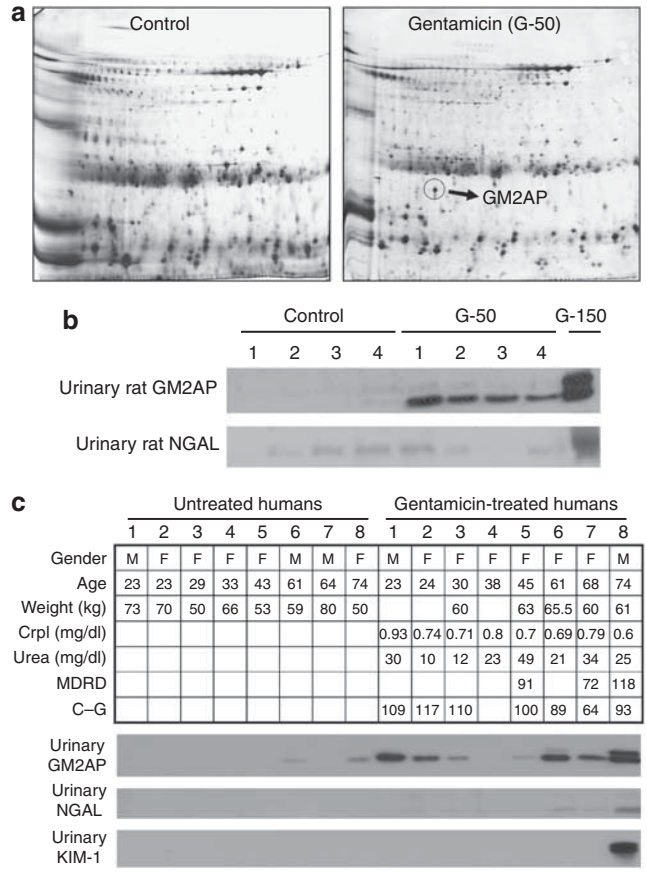

Figure 3 | Increased urinary level of ganglioside M2 activator protein (GM2AP) in gentamicin-sensitized rats and gentamicin-treated humans. (**a**) Representative bidimensional electrophoresis (2D) gels of urine from rats treated with saline (control) or 50 mg/kg/day gentamicin (G-50) during 6 days ($n = 4$). (**b**) Representative images of western blot analysis of GM2AP and neutrophil gelatinase-associated lipocalin (NGAL) in the urine of four randomly selected rats from control and G-50 groups, and an acute kidney injury-positive control (urine from a rat treated with 150 mg/kg/day gentamicin, otherwise as in G-50). (**c**) Representative images of western blot analysis of GM2AP, NGAL, and kidney injury molecule 1 (KIM-1) in the urine of eight patients treated with gentamicin and eight sex- and age-matched untreated individuals. Their gender, age, weight, plasma creatinine concentration (Cr_{pl}), blood urea concentration, and glomerular filtration rate estimated by the Modification of Diet in Renal Disease (MDRD) study equation or the Cockroft–Gault (C–G) equation are provided (when known).

However, G-150 treatment significantly increased serum GM2AP (Figure 5e).

In acute experiments with anesthetized rats, a bolus administration of a high dose of gentamicin induced a rapid increase in the urinary excretion of GM2AP (Figure 5g). The increased excretion declined after 3 h, probably correlating with the bioavailability of gentamicin. Interestingly, when, under similar circumstances, kidneys were perfused *in situ* with Krebs solution (instead of blood) by means of an extracorporeal circuit, gentamicin did not produce an increase in urinary GM2AP (Figure 5h). All together, these results indicate that the increase in urinary GM2AP produced by gentamicin is an acute effect which is tightly dependent on the presence of gentamicin and, most importantly, that the urinary GM2AP comes from the blood and not from renal tissues. These results can be explained by an altered renal handling of GM2AP (e.g., reduced reuptake) as the mechanism responsible for its increase in the urine. In fact, GM2AP appears in the urine shortly after treatment with maleate (Figure 5f), indicating that it is transported by the megalin complex (see discussion). This is further supported by the colocalization of GM2AP and megalin in proximal tubule cells within subcellular structures, probably being endocytosis vesicles, as revealed by confocal microscopy (Figure 5b; lower panel).

DISCUSSION

Our experiments show that gentamicin-induced sensitization to ARF, a condition hitherto largely underestimated, is distinctly differentiated from early and mild renal injury. It has a potentially high clinical relevance because it poses an unnoticed risk of ARF. The recognition of acquired sensitization to ARF as an existing and relevant pathological state makes obvious the necessity to identify tools to create a level of diagnosis for its detection and appropriate clinical handling. Our results also show that the increased urinary level of GM2AP is associated with the sensitization to AKI induced by sub-nephrotoxic gentamicin. They further show that an increased level of GM2AP also appears in the urine of rats undergoing an overt ARF and, in the case of gentamicin-induced AKI, urinary GM2AP appears earlier than other sensitive markers such as KIM-1 or plasminogen activator inhibitor 1. However, in the case of other nephrotoxicants, such as cisplatin, urinary GM2AP is elevated in parallel or even after the appearance early damage marker KIM-1. This fact has special importance because GM2AP might be exploited also for an etiological and selective diagnosis of AKI within the very early stages of the disease. The sub-nephrotoxic and the early nephrotoxic situations related to gentamicin treatments show the common characteristic of lacking the markers of tissue damage, while showing increased urinary levels of GM2AP. As such, monitoring the progressive increment of the urinary level of GM2AP from the onset of a gentamicin regime will provide means of detecting the increasing risk of an AKI burst as a consequence of further gentamicin administration or treatment with another potential nephrotoxicant.

In the case of the sensitization to AKI, GM2AP urinary excretion would serve as a diagnostic tool to discern which patients have acquired an increased risk as a consequence of a gentamicin regime, when contemplating the need of subjecting them to additional potentially nephrotoxic circumstances like the administration of other drugs. A typical and relevant case, from the clinical and socioeconomic point of views, is posed by the fact that 0.6–2.3% of not-at-risk patients undergoing a contrast radiography, with no previous history of renal disease, develop some degree of AKI.[27] We propose that a part of this patient group might be silently coursing

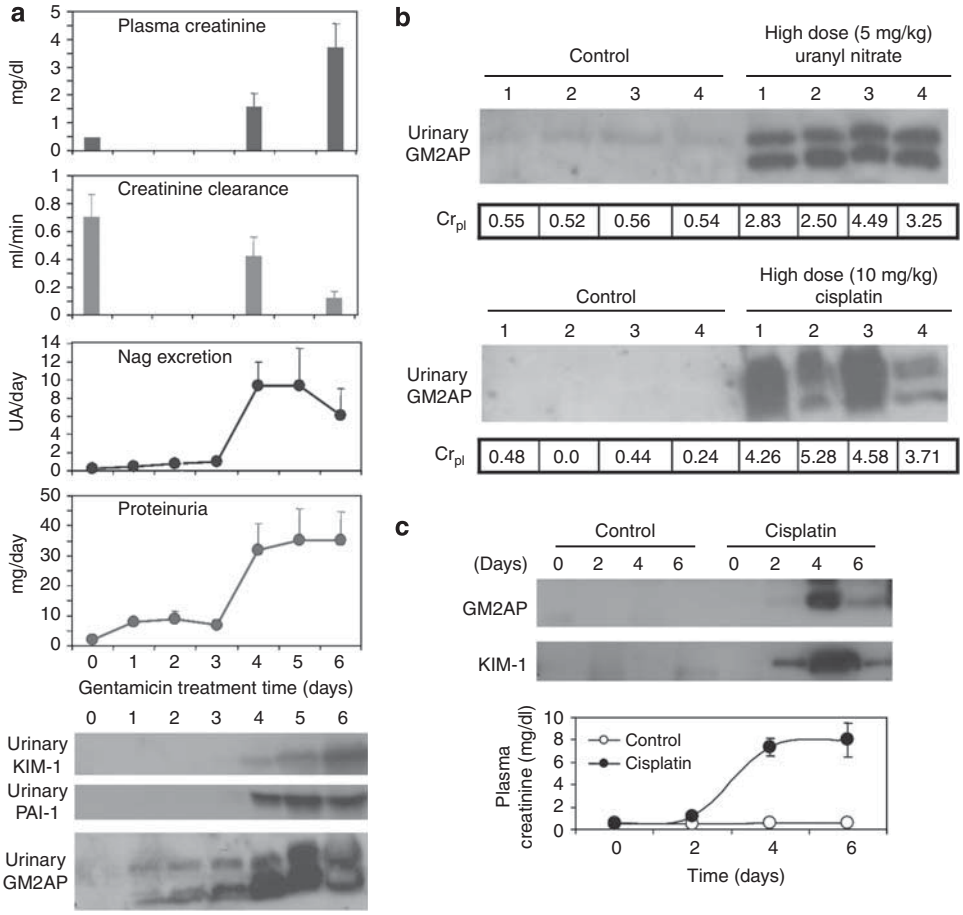

Figure 4 | Ganglioside M2 activator protein (GM2AP) in the urine of nephrotoxic rats. (**a**) Time-course evolution of plasma creatinine concentration (Cr_{pl}), creatinine clearance, urinary excretion of *N*-acetyl-glucosaminidase (NAG), proteinuria ($n = 6$), and representative ($n = 3$) images of western blot of urinary kidney injury molecule 1 (KIM-1), plasminogen activator inhibitor 1 (PAI-1), and GM2AP excretion of rats treated during 6 days with 150 mg/kg/day gentamicin. (**b**) Cr_{pl} and representative images of western blot analysis of urinary GM2AP of rats treated with saline (control) or a nephrotoxic dose of uranyl nitrate (5 mg/kg) or cisplatin (10 mg/kg). (**c**) Time-course evolution of Cr_{pl} ($n = 4$) and representative images of western blot analysis of urinary GM2AP and KIM-1 of rats treated with saline (control) or cisplatin (10 mg/kg).

with an increased risk to AKI owing to a previous treatment or exposure to an environmental agent that has induced no clinical symptoms of renal lesions, such as a treatment with gentamicin. The urinary level of GM2AP could be used as a marker to detect this risk. Very interestingly, urinary GM2AP is found to be increased (to a variable degree) in most patients treated with gentamicin for at least 3 days, and whose estimated glomerular filtration rate and urinary levels of sensitive renal damage markers (e.g., neutrophil gelatinase-associated lipocalin, KIM-1) remain normal during analysis. Identification of markers of risk should be extended over other nephrotoxic drugs and insults, including renal ischemia and obstruction of the urinary ways.

GM2AP is an 18–24 kDa substrate cofactor for the lysosomal β-hexosaminidase A implicated in GM2 ganglioside metabolism.[28] It has also been proposed as an intra and intercellular glycosphingolipid transporter.[29–31] GM2AP is mainly driven to the lysosomes through typical trans-Golgi mannose-6-phosphate receptor-dependent[32] and -independent[33] pathways. About one third of the synthesized protein is secreted.[32] Because, even at low doses, gentamicin accumulates in the lysosomes, endosomes, and Golgi;[34–37] modulates lipid metabolism, signalling, and function;[14,38–41] and interferes with endosomal traffic,[42,43] it was tempting to think that the mechanism of gentamicin-induced increase in urinary GM2AP has to do with alterations in the cellular handling of this protein resulting in its higher secretion, production, or both. Our experiments indicate that, as a result of gentamicin treatment, the renal expression of GM2AP is not modified, and that the origin of the increased urinary level of GM2AP is not the renal tissue, but a blood origin. Owing to its low molecular weight, GM2AP is expected to cross the glomerular filtration barrier freely.[44–46] Our data point at an alteration in its tubular handling as the cause of its urinary appearance.

In the case of the sub-nephrotoxic regime of gentamicin (G-50), the increased urinary level of GM2AP is a highly specific event, being one of the very few proteins showing higher excretion than in control animals. Contrarily, in the urine of animals treated with nephrotoxic regimes of

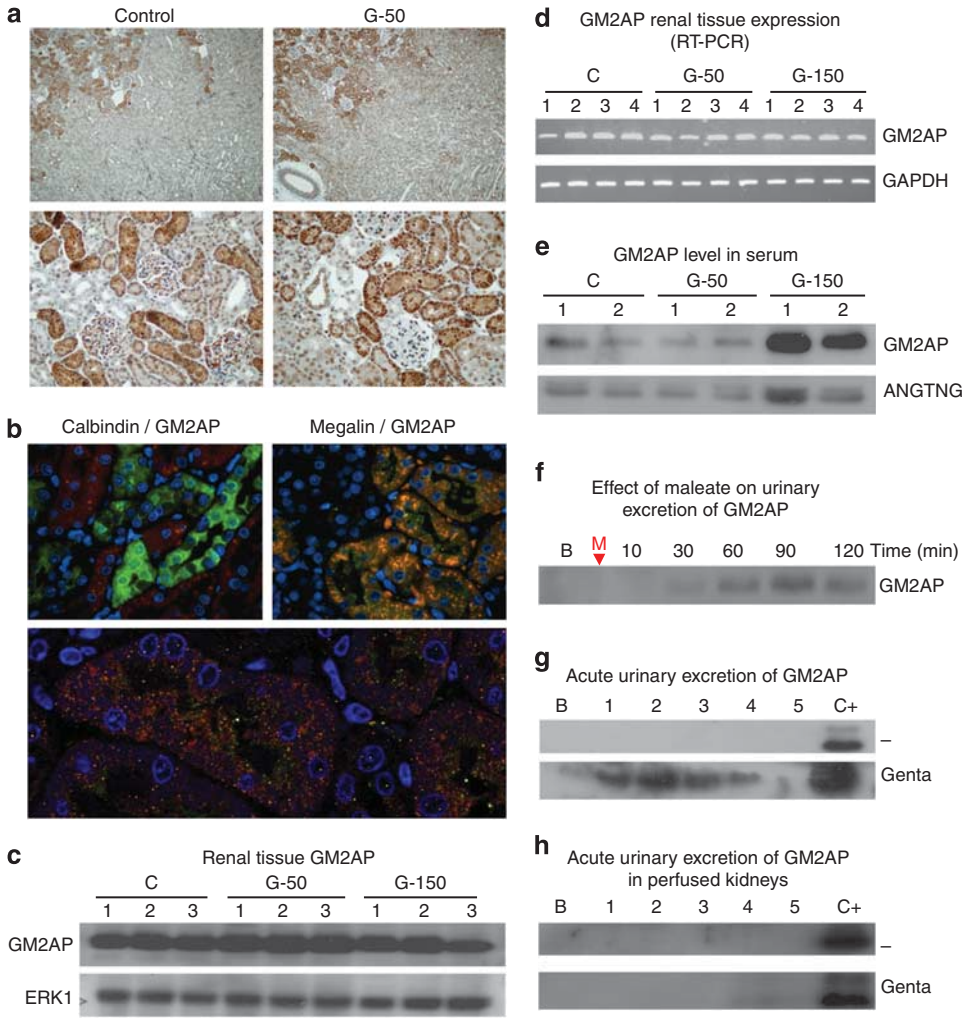

Figure 5 | An altered tubular handling of ganglioside M2 activator protein (GM2AP) causes its increase in the urine. (**a**) Representative images ($n = 3$) of renal tissue sections from rats treated with saline (C) or 50 mg/kg/day gentamicin (G-50) during 6 days, stained with anti-GM2AP. Upper panels (100 ×) show the cortex and medulla. Lower panels (400 ×) show an amplified area of the renal cortex. (**b**) In the upper panels, representative ($n = 3$) immunofluorescence co-staining images of GM2AP and calbindin (left), and GM2AP and megalin (right). In the lower panels, detailed confocal microscopy image of megalin and GM2AP co-staining. Red represents GM2AP, green represents calbindin or megalin, and orange shows co-staining. (**c**) Western blot analysis of GM2AP and extracellular signal-regulated kinase 1 (Erk-1) level in three randomly selected renal tissue homogenates from rats treated with saline (C), 50 mg/kg/day gentamicin (G-50), or 150 mg/kg/day gentamicin (G-150) during 6 days. (**d**) Reverse transcriptase-PCR (RT-PCR) amplification of the renal mRNA of GM2AP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from four randomly selected C, G-50, and G-150 rats. (**e**) Representative images of western blot analysis of GM2AP and angiotensinogen (ANGTNG) level in two randomly selected serum samples from C, G-50, and G-150 rats. (**f**) Representative images ($n = 5$) of western blot analysis of the time-course level of GM2AP in the urine of rats treated intravenously with a bolus of sodium maleate (400 mg/kg). (**g**) Representative images ($n = 4$) of western blot analysis of the time-course level of GM2AP in the urine of rats treated intravenously with a bolus of saline (C +) or 100 mg/kg gentamicin (Genta). (**h**) Representative images ($n = 4$) of western blot analysis of the time-course level of GM2AP in the urine of rats with their left kidney perfused with isosmotic solution (C +) or isosmotic solution containing 1.5 mg/ml gentamicin during 5 h. B, basal.

gentamicin, cisplatin, and UN, the increased GM2AP urinary levels might be the result of a less specific and defective renal handling of proteins leading to an overt and unselective proteinuria. Gentamicin is transported into the proximal tubule epithelial cells by the apical protein–endocytic transport complex megalin/cubilin.[47] The complex megalin/cubilin has been shown to mediate the reabsorption of a wide variety of proteins.[48] Gentamicin competes with filtered proteins for this transporter and augments their urinary excretion,[21,49–52] even in an acute manner.[51]

As such, it is reasonable to hypothesize that a plausible origin of the increased urinary excretion of GM2AP in sub-nephrotoxic circumstances is a submaximal competition of gentamicin for the GM2AP tubular transporter (likely the megalin/cubilin complex). Indeed, our results suggest that GM2AP is reabsorbed in the proximal tubule by the megalin complex. First, megalin and GM2AP colocalize in spotty subcellular structures in proximal tubule cells, which are likely the endosomal vesicles. Second, acute treatment with maleate produces a rapid appearance of GM2AP in the urine.

Maleate treatment has been shown to induce the shedding of megalin into the urine and to impair megalin-mediated reabsorption.[53] In fact, both maleate[54] and gentamicin[55,56] have been used experimentally to inhibit megalin-dependent endocytosis. It is further hypothesized that under mild competition circumstances, only a few proteins showing the least affinity for the system would be displaced, GM2AP being one of the first. This is supported by the uneven effect of gentamicin on the increased excretion of different proteins, the excretion of ones being increased by orders of magnitude that of others.[51] Increasing competition (i.e., by higher gentamicin concentrations) would bring about the displacement of more proteins that, together with a dysfunctional necrotic tubular epithelium, would produce a full range proteinuria like that seen during overtly nephrotoxic circumstances with gentamicin and other drugs.

In conclusion, our study opens new possibilities for the improvement of AKI diagnosis by providing a potential marker of the enhanced risk of AKI induced by gentamicin, a condition that has hitherto been mostly uncharacterized, quite underestimated, and impossible to diagnose, but which might have a high clinical and socioeconomic repercussion. The capacity to detect and prospectively quantify AKI risk through the determination of biochemical parameters may help to overturn drug-induced sensitization to AKI. However, the most important result of this work is the realization, through this proof-of-concept, of new possibilities for a more rational and personalized utilization of drugs in the clinical practice, by anticipating the increased susceptibility and risk of damage, injury, or alteration posed by drugs even in the absence of detectable signs of toxicity.

METHODS

Reagents

Unless otherwise indicated, products were purchased from Sigma. Gentamicin sulphate was kindly provided by Schering-Plough. For the preparation of an anti-GM2AP serum, female New Zealand White rabbits were injected with a synthetic immunogen corresponding to the rat and human GM2AP partial peptide sequence SWDNCDEGKDPAVI. After three immunizations, the serum was purified through a HiTrap TM Protein G HP column (GE Healthcare Bio-Sciences AB, Madrid, Spain) and kept at −20 °C for further use.

Animal models and experimental protocols

In-house bred, female Wistar rats weighing 190–230 g were divided into the following experimental groups: (i) control: rats treated intraperitoneally during 7–13 days with saline (0.9% NaCl), once daily, (ii) G-50: rats treated with 50 mg/kg/day gentamicin during 6 days, (iii) G-50-NU: rats treated with 50 mg/kg/day gentamicin during 6 days and on the seventh day, treated with a single intraperitoneal dose of 0.5 mg/kg UN or cisplatin (10 mg/kg) or iohexol (2.1 g I/kg, GE Healthcare), (iv) NU: rats treated intraperitoneally with saline (0.9% NaCl) once daily during 6 days and on the seventh day, treated with a single intraperitoneal dose of 0.5 mg/kg UN or cisplatin (10 mg/kg) or iohexol (2.1 gI/kg), and (v) G-150: rats treated with 150 mg/kg/day gentamicin during 6 days. Rats were individually allocated in metabolic cages under controlled conditions. Urine and plasma were obtained and stored at −80 °C. Only for western blot studies, some plasma was depleted of albumin through Murine Albumin Depletion Columns (Qiagen, Madrid, Spain) at the moment of use. At the end of treatments, kidneys were perfused with heparinized saline and immediately dissected. Half of the kidney was fixed in paraformaldehyde for histological studies. The remaining renal mass was frozen at −80 °C for ulterior western blot and gene expression studies.

Characterization of renal function

Plasma and urine creatinine, (Cr_p and Cr_u, respectively) and BUN concentrations were determined using an automated analyzer (Reflotron, Roche Diagnostics, Barcelona, Spain). This method has a lower detection limit of 0.5 mg/dl for creatinine. Creatinine clearance (Cl_{Cr}) = $Cr_u \times$ 24-hour urine output $\times Cr_p^{-1}$. Urine protein concentration was measured by the Bradford method,[57] and urine NAG activity by a commercial enzymatic test (Roche Diagnostics). Sodium (Na) concentration was measured by indirect potentiometry in a Modular Analytics System (Roche Diagnostics). Fractional excretion of sodium (FE-Na) was calculated as (100 × urinary Na × Cr_p)/(plasma Na × Cr_u).

Histological studies

Paraformaldehyde-fixed tissue samples were immersed in paraffin, cut into 5 µm-thick slices and stained with (i) hematoxylin and eosin, (ii) anti-GM2AP, (iii) anti-megalin, (Santa Cruz, Santa Cruz, CA, USA) and (iv) anti-calbindin (Santa Cruz); followed by horseradish peroxidase-, fluorescein isothiocyanate- or cyanine 3-conjugated secondary antibodies.

Western blotting

Protein extracts were obtained from renal tissue homogenates in lysis buffer (25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mmol/l NaCl, 1% Igepal CA-630, 10 mmol/l $MgCl_2$, 1 mmol/l EDTA, 2% v/v glycerol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 5 mmol/l phenylmethylsulfonyl fluoride, 5 µM NaF, 1 mmol/l Na_3VO_4, pH = 7.5). A total of 50 µg of tissue lysates, 21 µl of albumin-deprived plasma, or a volume of urine from each animal corresponding to the same excretion fraction were subject to polyacrylamide gel electrophoresis, transferred to an Inmobilon-P membrane (Millipore, Madrid, Spain) and hybridized with antibodies against KIM-1 (R&D Systems, Minneapolis, MN, USA and Santa Cruz), neutrophil gelatinase-associated lipocalin (MBL, Woburn, MA, USA), bone morphogenetic protein 7 (Santa Cruz Biotechnology), plasminogen activator inhibitor 1 (BD Biosciences, Madrid, Spain), vimentin (Dako, Glostrup, Denmark), GM2AP.

Urinary differential proteomic studies

Urine proteins were precipitated and isoelectrically focused (500–8000 V) through 18-cm long immobilized pH gradient (IPG) strips, pH 4–7 (GE Healthcare). Then, proteins in IPG strips were separated by polyacrylamide gel electrophoresis, fixed, and silver stained. The spots of interest were in-gel digested with porcine trypsin (Promega, Madrid, Spain). Peptides were extracted and injected in a liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometer QSTAR XL (Applied Biosystems, Carlsbad, CA, USA) with an 1100 micro high-performance liquid chromatography (Agilent, Madrid, Spain). MS/MS spectra were obtained. Mass spectrometry was confirmed

by matrix-assisted laser desorption/ionization time-of-flight. Protein identification was performed with the MASCOT software (http://www.matrixscience.com) against non-redundant protein sequence databases (Swiss Prot and National Center for Biotechnology Information).

Gene expression analysis

reverse transcriptase-PCR amplification of GM2AP and glyceraldehyde 3-phosphate dehydrogenase was performed with the following primers: for rat GM2AP, 5′-ATCAAAAGCCTCACGCTCCA-3′ and 5′-TATT TGCCCTTGAGAGAGGC-3′; for rat glyceraldehyde 3-phosphate dehydrogenase, 5′-GTGGTCATGAGCCCTTCCA-3′ and 5′-AACTC CCTCAAGATTGTCAGCAA-3′. PCR conditions were: $1 \times (95\,^{\circ}C \times 4\,min)$; $30 \times (95\,^{\circ}C \times 1\,min + T_m \times 1\,min)$; $1 \times (72\,^{\circ}C \times 10\,min)$; where T_m was 55 °C for GM2AP and 55.9 °C for glyceraldehyde 3-phosphate dehydrogenase.

Excretion of GM2AP

In a set of experiments, rats were anesthetized and after a single injection of gentamicin (100 mg/kg), sodium maleate (400 mg/kg) or saline through the jugular vein, urine fractions were collected from the bladder during the last 15 min of each subsequent hour, during 5 h. In another set of experiments, rats were anesthetized and an extracorporeal circuit for kidney perfusion was set up, as described[58] with some modifications. Briefly, the renal artery, vein, and ureter of the right kidney were ligated. The renal artery and vein of the left kidney and the urinary bladder were canulated. Oxygenated and warm (37 °C) Krebs-dextran (40 g/l of dextran (molecular weight 64K–76K) in Krebs solution (118.3 mmol/l NaCl, 4.7 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 0.026 mmol/l EDTA, 11.1 glucose, pH = 7.4)) or Krebs-dextran containing 1.5 mg/ml gentamicin was perfused through the renal artery at 3 ml/min, and was discarded through the renal vein. Urine fractions were also collected as before. All urine samples were kept at −80 °C until assayed by western blot for the presence of GM2AP.

Human samples

The urine and blood from eight unselected patients treated for at least 3 days with gentamicin, and the urine from eight sex- and age-matched untreated donors was obtained from volunteers from the Hospital Universitario de Salamanca (Spain). Glomerular filtration rate was estimated by the Modification of Diet in Renal Disease (MDRD) study or the Cockroft–Gault (C–G) equations.

Statistical analysis

Data are expressed as the average ± s.e.m. of the indicated number of experiments. Statistical analysis was performed by the Scheffe's test or the Kruskal–Wallis test, as appropriate. $P < 0.05$ was considered statistically significant.

DISCLOSURE

JML-N and FJL-H are minority shareholders of Bio-inRen, S.L., a biotech company holding license of the patent on the use of GM2AP as a marker for the diagnosis of kidney injury.

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PATENTE I

PATENTE I

URINARY GM2 ACTIVATOR PROTEIN AS A MARKER OF ACUTE RENAL FAILURE OR THE RISK OF DEVELOPING ACUTE RENAL FAILURE

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Title	URINARY GM2 ACTIVATOR PROTEIN AS A MARKER OF ACUTE RENAL FAILURE OR THE RISK OF DEVELOPING ACUTE RENAL FAILURE

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V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	
VI-1	**Priority Claim**	NONE
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)

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VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description (excluding sequence listing part)	29	✓
IX-3	Claims	4	✓
IX-4	Abstract	1	✓
IX-5	Drawings	11	✓
IX-7a	Sub-total number of sheets	50	
IX-6a	Sequence listing part of description	2	✓
IX-7	TOTAL	52	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	-	✓
IX-16(A)	Sequence listing submitted for the purposes of international search under Rule 13ter	-	✓
IX-18	PCT-SAFE physical media	-	-
IX-20	**Figure of the drawings which should accompany the abstract**		
IX-21	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**	(PKCS7 Digital Signature)	
X-1-1	Name	UNIVERSIDAD DE SALAMANCA	
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X-1-3	Capacity	(Applicant)	

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10-1	**Date of actual receipt of the purported international application**	
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10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
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10-6	**Transmittal of search copy delayed until search fee is paid**	

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ARTÍCULO I I

URINARY REG IIIB AND GELSOLIN: DIFFERENTIAL MARKERS OF GENTAMICIN-INDUCED ACUTE KIDNEY INJURY

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Urinary reg IIIb and gelsolin: differential markers of gentamicin-induced acute kidney injury

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A key aspect for the clinical handling of acute kidney injury (AKI) is an early diagnosis, for which a new generation of urine markers is currently under development, including kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL) and others. A further diagnostic refinement is necessary for the identification of the specific cause of ARF. This gains especial importance under those circumstances where several potentially nephrotoxic insults converge, such as in multidrug therapeutic courses. In this study we have identified regeneratin islet-derived protein III beta (reg IIIb) and gelsolin as potentially diferential urinary markers of gentamicin's nephrotoxicity. Indeed, both reg IIIb and gelsolin urinary levels differentiate the nephrotoxicity caused by gentamicin from that caused by cisplatin. Reg IIIb is overexpressed in the kidneys of gentamicin-treated rats and poured into the urine, whereas gelsolin proceeds from the glomerular ultrafiltrate. Our results pose a proof-of-concept for the aetiological diagnosis of AKI through the biochemical analysis of the urine, with potential application for an enhanced drug theranostic and a more personalized medicine of polimedicated and critically ill patients at multifactorial risk of AKI.

Key words: gentamicin, urinary markers, acute kidney injury, reg IIIb, gelsolin.

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Introduction

Gentamicin is an aminoglycoside antibiotic widely used worldwide against Gram negative infections. Its therapeutic efficacy and use are severely handicapped by its toxicity, which mainly occurs at the renal and auditory levels (1). Gentamicin-induced nephrotoxicity appears in 10-25% of therapeutic courses (2-4). It is characterized mostly by tubular damage (5,6), but glomerular (1,7-11) and vascular (12-14) alterations might also appear in a dose-dependent manner (15). Tubular damage affects mainly the proximal compartment (5), and it results in a reduced glomerular filtration rate (GFR) through (i) an impairment of the reabsortion capacity that activates the tubuloglomerular feedback to prevent massive fluid loss (16); and (ii) an increased intratubular pressure resulting from tubular obstruction by tissue debris (5). In the glomeruli, gentamicin produces contraction of mesangial cells,

which diminishes the ultrafiltration coefficient (K_f; 1,8,17,18). As a polycation, gentamicin also alters the electrical properties of the glomerular filtration barrier (GFB; 19,20). Finally, gentamicin reduces renal blood flow (RBF) by contracting both preglomerular arteries, and the afferent and efferent arterioles (12). As a consequence of a diminished RBF, GFR becomes deteriorated. A lower RBF also contributes to tissue necrosis, especially within the cortical area (21). Glomerular and vascular contraction is mediated by locally increased autacoids like arachidonic acid derivatives (i.e. thromboxane A2), platelet activating factor (22,23), and endothelin-1 (24-26). Vasoconstriction might also be related to deterioration of the relaxing capacity of the vascular smooth muscle (14).

Depending on other comorbid conditions, gentamicin-associated nephrotoxicity may reach to an acute renal failure (ARF), an extremely serious condition resulting from an abrupt loss of the kidneys' excretory function sufficient to prevent blood cleansing of waste products, and to impair water and electrolytic balance (27-29). ARF poses an enormous human and socio-economic burden derived from its high incidence and mortality rate. It is estimated that nearly 1% of hospital admissions are associated to ARF, and about 2-7% of hospitalized patients eventually develop ARF (30,31). Most importantly, mortality among ARF patients stays strikingly high at approximately 50% of the cases in spite of dialysis instauration (30-32). This rate grows up to an 80% when ARF courses with multi-organ damage (30,32,33). Most effective handling of ARF relies on the earliest possible detection, upon which hydration and treatment withdrawal or regime modifications are applied. Other occasional measurements include the pre-emptive administration of renoprotective drugs, such as the antioxidants N-acetylcisteine (34-37) or amifostine (38), and other molecules presently under development for the prevention of renal injury. Intervention on severe cases relies mostly on dyalisis.

In the clinical practice, ARF is diagnosed when renal dysfunction induces an increase in creatinine and urea levels in the blood. However, at this stage GFR is already decreased and ARF becomes difficult to handle. As such, present diagnostic tendencies aim at detecting incipient pathophysiological events occurring at early stages, when damage is less extensive (32,39-41). Measurement of urinary enzymes originating from renal tissue injury, including N-acetyl-β-D-glucosaminidase (NAG) and others (40), is presently the finest method for an early detection of ARF coursing with tubular damage. New, early urine markers are currently in an advanced degree of validation for the diagnosis and prognosis of ARF, including kidney injury molecule 1 (KIM-1), neutrophil gelatinase associated lipocalin (NGAL), and others (40,42).

The next step in the refinement of ARF diagnosis will be the capability to differentiate the renal damage inflicted by a determined drug or insult from that exerted by others (43). It could be based on specific collections of markers composing a molecular fingerprint for every potential nephrotoxicant. This new diagnostic capability will be very useful for a more rational, individualized and specific handling of clinical situations coursing with symptoms of renal damage, as for example, in the case of polymedicated patients receiving cotreatments containing two or more drugs being potentially nephrotoxic. This will allow us to correctly redirect the treatment by substituting only the harmful drug or by reshaping its therapeutic regime. In this article, we identify regenerating islet-derived protein 3 beta (reg IIIb) and gelsolin as new urinary markers to further profile and differentiate the AKI inflicted by gentamicin from that induced by cisplatin.

Results

Characterization of the renal lesion induced by gentamicin.

As expected, after 6 days of treatment, gentamicin caused a marked ARF with an associated

mortality of about a 50% (figure 1-B). Surviving animals coursed with a small but significant weight loss and polyuria. ARF was further characterized by a dramatic increase in serum creatinine and BUN concentration, indicating a reduction of GFR (figure 1-A). NAG (figure 1-A), KIM-1 and bone morphogenetic protein 7 (BMP7; figure 1-C) urinary excretion also increased, indicating tubular damage. Proteinuria was also evident in the urine of animals treated with gentamicin (figure 1-A). Hematoxiline-eosine stained renal sections (figure 1-D) reveal a clear tubular necrosis in gentamicin-treated rats. No

A

	Δ Weight (%)	Serum creatinine (mg/dL)	Cl_{Cr} (mL/min)	BUN (mg/dL)	Protein excretion (mg/day)	NAG excretion (AU/day)	Urinary flow (mL/day)
Control	105 ± 4	<0.5	0.93 ± 0.15	45.9 ± 1.9	7.3 ± 0.9	0.54 ± 0.11	10 ± 1
Gentamicin	95 ± 3 [a]	3.3 ± 0.4 [a]	0.17 ± 0.06 [a]	435.8 ± 47.9 [a]	40.0 ± 7.4 [a]	7.44 ± 2.12 [a]	18 ± 3 [a]

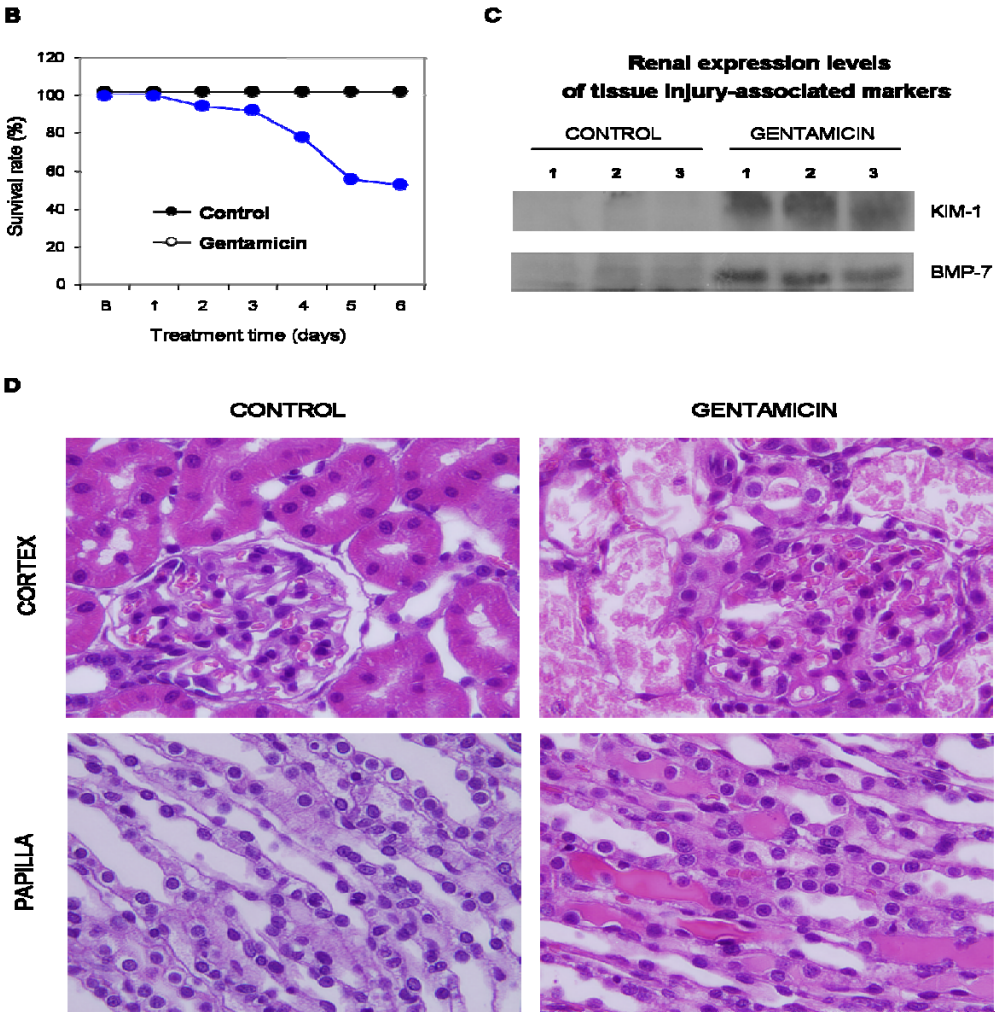
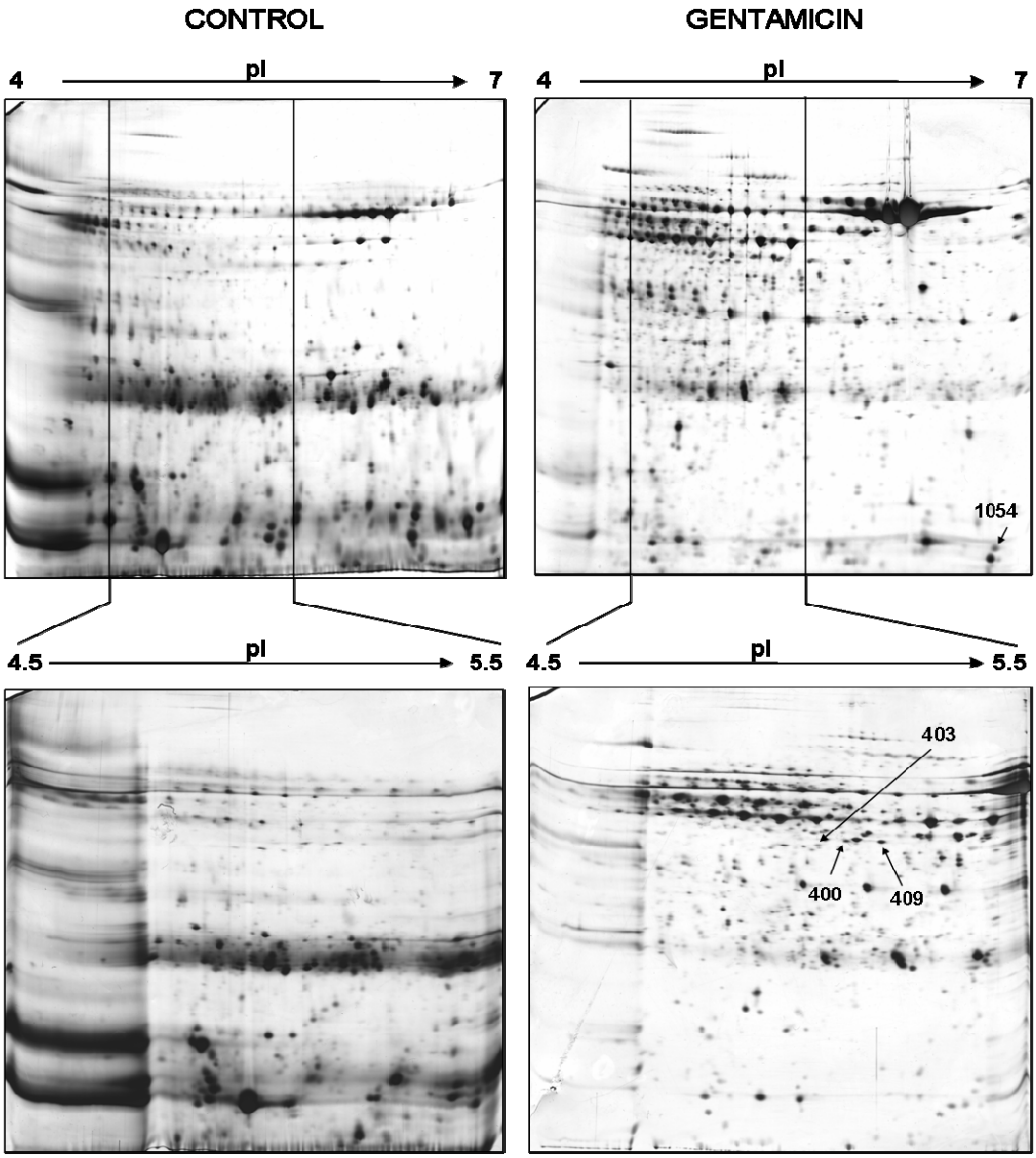


Figure 1. A. Body weight (shown as a percentage of the initial weight), plasma creatinine concentration, creatinine clearance (Cl_{Cr}), blood urea nitrogen (BUN), proteinuria, NAG excretion and urine flow parameters of rats from control and gentamicin groups after 6 days of treatment. **B.** Survival rate represented as percentage of surviving animals in each group after 6 days of treatment, **C.** Representative images of western blot analysis of KIM-1 and BMP-7 expression levels in kidney homogenates from 3 randomly selected, control and 3 gentamicin rats after 6 days of treatment. **D.** Representative images (1,000x magnification) of renal sections stained with hematoxilin and eosin from gentamicin and control rats. *AU: arbitrary units; B: basal; a, p≤0.05 with respect to the control group. n=12 at the beginning of the experiment in both groups.*

gross modification of the glomeruli is evident. At the papillary level, obstruction of collecting tubuli with hyaline material is widespread in gentamicin-treated animals.

Differential proteomic analysis of the urine

A representative image of 2D gels (pH range 4-7) of urines from control and gentamicin-treated rats is shown in the upper panels of figure 2. Many



Protein name	Accession No.	Protein MW (kDa) / pI	Spot No.	Score	No. of peptides	% Sequence coverage
Gelsolin	Q68FP1	86.1 / 5.75	400	100	3	3
			403	85	3	3
			409	116	3	3
Regenerating islet-derived protein 3 beta	P25031	20.0 / 7.56	1054	107	7	46

Figure 2. Urinary proteomics. 2D gel images of differentially expressed reg IIIb and gelsolin. Spots were subject to quantitative intensity analysis, labeled with numbers corresponding to those in table and identified by LC-ESI-Q-TOF mass spectrometry. Each gel shown in this figure is representative of 8 gels obtained with urine from 4 randomly selected animals in each group, each one analyzed in duplicate.

proteins concentrate in the rage of pH 4.5-5.5. For that reason, 2D separations in this pH range were also done with the same urines. A representative image of these latter is shown in the lower panels of figure 2. A great similarity was observed between samples from animals in the same group, and high reproducibility was obtained when repeating the 2D separation with the same sample, for quality assurance. However, the urine proteome of both groups is substantially different. Statistically significant, differentially present spots between control and gentamicin groups were recognized and numbered for chemical identification. Mass spectrometric analysis revealed the identity of three proteins increased in the urine of gentamicin-treated rats, which showed potential interest after discarding most of the other proteins, normally found in different proteinuric conditions. They were identified as regenerating islet-derived protein 3 beta (reg IIIb) and gelsolin (figure 2).

Reg IIIb and gelsolin are differentially excreted in the urine of rats treated with gentamicin

The increased urinary level of these proteins in the urine of gentamicin-treated rats was confirmed by Western blot analysis. Moreover, the urine from rats treated with a nephrotoxic regime of cisplatin was also analyzed. Figure 3-B shows data on serum creatinine concentration and BUN from 6 control rats, 6 rats treated with gentamicin and 6 rats treated with cisplatin. It demonstrates that treated animals developed an overt renal failure. Those urines were also analyzed for their content in reg IIIb and gelsolin. Figure 3-A clearly shows that the urinary level of reg IIIb is markedly increased only in gentamicin-treated animals, despite undergoing a similar degree of renal

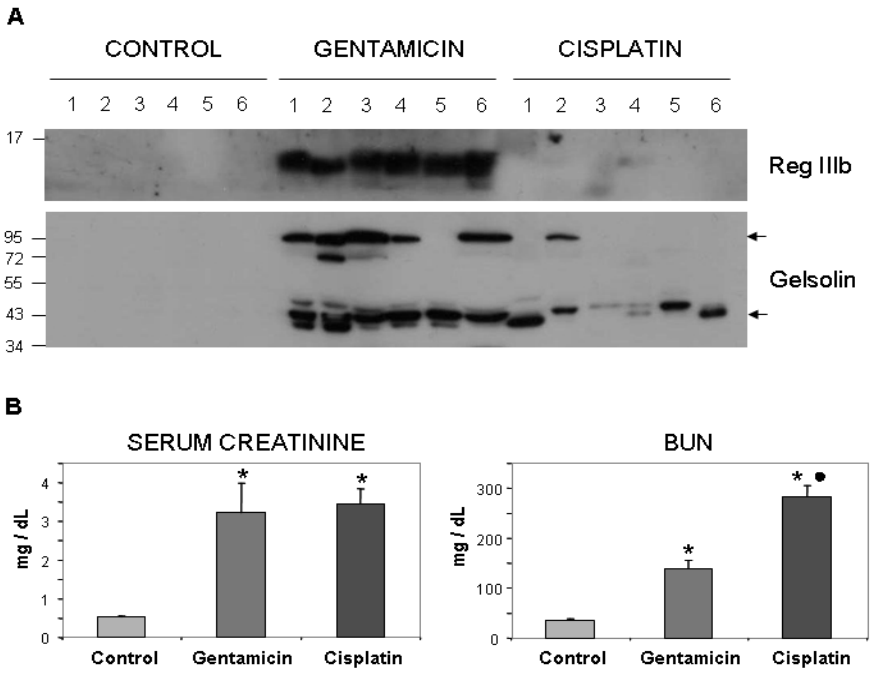


Figure 3. A. Western blot analysis of reg IIIb and gelsolin in the urine of 6 randomly selected rats treated with vehicle (control), gentamicin or cisplatin. Arrows indicate the full length and t-gelsolin fragment **B.** Serum creatinine and BUN concentration of rats as in A. **, $p < 0.05$ vs. control; ● $p < 0.05$ vs. gentamicin*

damage than cisplatin-treated rats. Western blot of gelsolin revealed two reactive bands. The higher one corresponds to the full length protein, whereas the lower one corresponds to a fragment thereof. The presence of gelsolin within the reactive bands was further re-confirmed by MS/MS mass spectrometry. Treatment with gentamicin induces the appearance in the urine of both the full length gelsolin and the ~ 43 kDa fragment. However, the full length band was absent in the urine from all of the rats treated with cisplatin, except for one of them. Extensive analysis of the urine from other cisplatin-treated rats shows no presence of the full length band (data not shown).

Time course evolution of reg IIIb and gelsolin urinary excretion

We further analyzed the time course evolution of the urinary excretion of these proteins in rats treated with gentamicin. Figure 4 shows the temporal profile of the renal damage inflicted by gentamicin. Significant damage only occurs after 4 days of treatment, as revealed by the evolution of serum creatinine, NAG excretion, proteinuria, and the

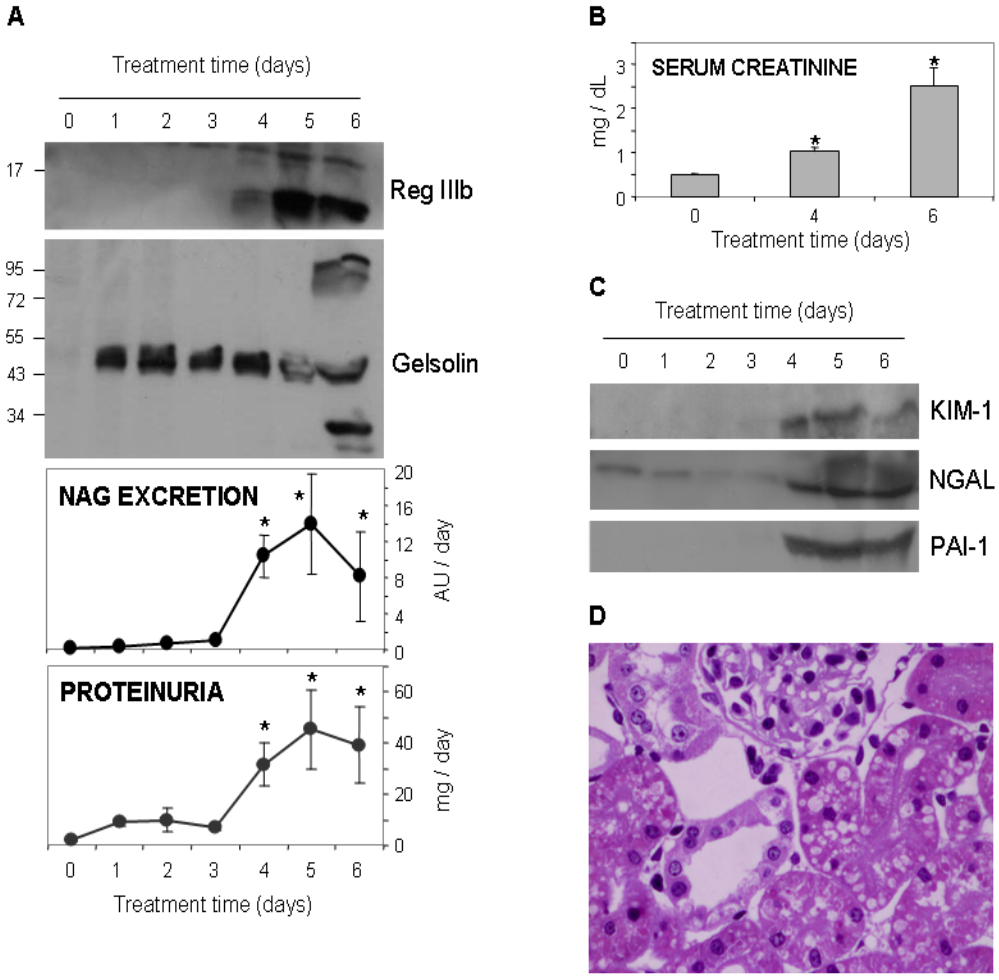


Figure 4. Time course evolution of urinary reg IIIb and gelsolin. **A.** Representative images of Western blot analysis of urinary reg IIIb and gelsolin, and NAG excretion and proteinuria of rats treated with gentamicin. B. Serum creatinine concentration. C. Western blot analysis of KIM-1, NGAL and PAI-1. D. Representative image of renal sections stained with hematoxilin and eosin from rats treated with gentamicin during 3 days. *n=3 in all experiments. *, $p < 0.05$ vs. time 0.*

urinary level of three sensitive markers of kidney injury, such as KIM-1, NGAL and plasminogen activator inhibitor 1 (PAI-1). Congruently with the accumulated knowledge (44), serum creatinine is the least sensitive of all the markers tested. Furthermore, histological analysis of renal sections after 3 days of treatment reveals no findings of tubular damage. At this time point, cytoplasmic vacuolation of tubule epithelial cells is evident, probably resulting from the elsewhere reported accumulation of gentamicin in the endosomal compartment (45,46), and of alteration of the endocytic pathway and the endosomal trafficking (47,48). In this scenario, Western blot analysis showed that reg IIIb appears in the urine along with most other sensitive markers of renal injury, starting on day 4. Interestingly, urinary gelsolin (the ~ 43 kDa fragment) appears as early as on day 1 and stays high through the treatment, long before all other sensitive markers do, including KIM-1, PAI-1, NGAL, and NAG.

Origin of urinary reg IIIb and gelsolin

Western blot analysis of albumin-depleted serum from control and gentamicin-treated rats indicated that reg IIIb is absent (to the detection limit of this technique), whereas gelsolin is normally found in the blood compartment. Even more, gentamicin slightly increases the serum level of this latter (figure 5-A). Gene expression analysis carried out on renal tissue by RT-PCR showed that these 2 proteins are normally expressed in the kidneys. Treatment of rats with gentamicin does not modify the renal expression pattern of gelsolin, but induces an increase in reg IIIb gene expression as early as on day 3 (figure 5-B), when no detectable kidney injury has occurred yet

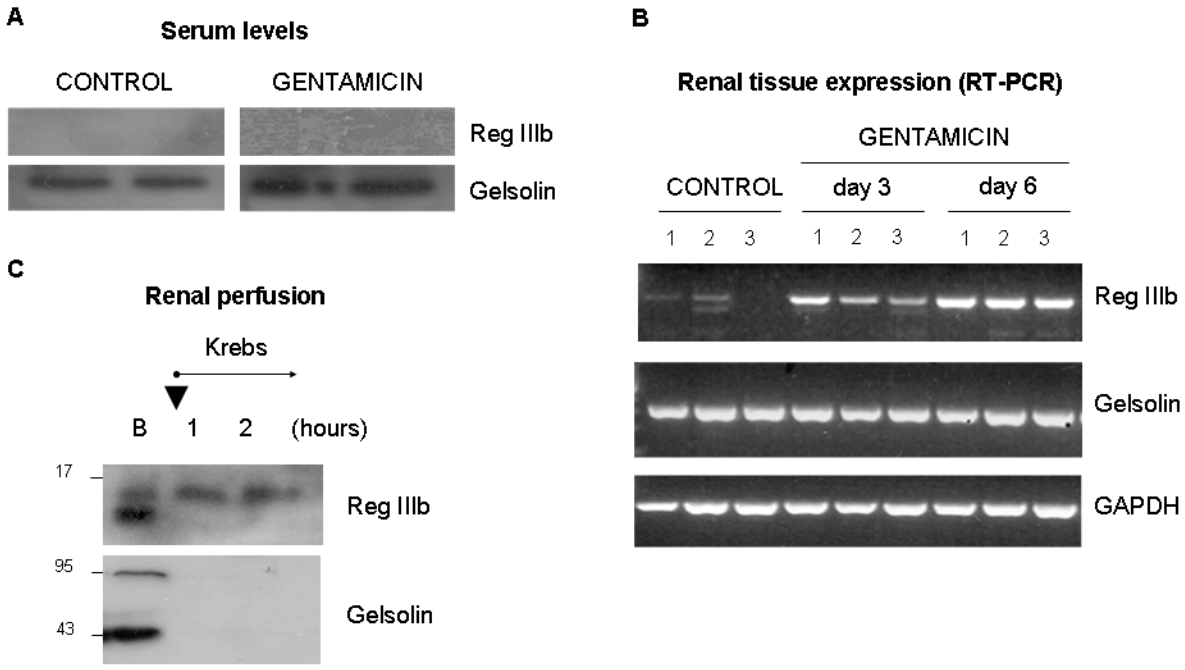


Figure 5. A. Representative images of Western blot analysis of serum level of reg IIIb and gelsolin from 2 randomly selected rats treated with vehicle (control) or gentamicin during 6 days. **B.** Renal tissue Reg IIIb, Gelsolin and GAPDH gene expression by RT-PCR from 3 randomly selected rats treated with vehicle (control) or gentamicin during 3 and 6 days. **C.** Renal perfusion experiments. Representative mages of Western blot analysis of the urinary level of reg IIIb and gelsolin from rats treated with gentamicin during 6 days, and then subject to renal perfusion with Krebs solution, immediately before the beginning of perfusion (B) and 1 and 2 hours during it; n=3.

(figure 4). On day 6, reg IIIb expression is highest.

In order to study whether the origin of these urinary proteins was the blood, which would shed them to the urine through the glomerular filtration barrier, we perfused the kidneys of rats treated during 6 days with gentamicin with Krebs solution (containing dextran to compensate for the oncotic pressure). We found that, immediately before substituting the renal blood flow with Krebs, we could still detect reg IIIb and gelsolin in the urine (figure 5-C). However, once the renal blood flow was substituted with Krebs flow, gelsolin disappeared from the urine; yet, the upper band of reg IIIb was still detected, whereas the lower one disappeared.

Discussion

Nephrotoxicity poses a considerable health and economic problem worldwide. It is an important reason of failure along the drug discovery process, which leads to discarding otherwise clinically interesting molecules (49,50). Most importantly, about 25% of the 100 most used drugs in intensive care units are potentially nephrotoxic (51). Overall, it is estimated that nephrotoxicity is responsible for 10-20% of the acute renal failure cases (52). A critical aspect for the optimal clinical handling of AKI is an early diagnosis (53,54). Important progress has been made in the last decade towards an increasingly earlier detection based on novel and more sensitive urinary markers (40). However, AKI diagnosis may still be improved in an individual-drug basis, for enhanced theranostics and a more individualized medicine. In this article we provide some evidence on new urinary markers with potentially to differentiate the nephrotoxicity of gentamicin from that caused by cisplatin, and to detect the renal effects of gentamicin earlier than with state-of-the-art AKI markers. They will help to better delineate the pharmacological profile of gentamicin and, in turn, to improve its clinical utility.

Both reg IIIb and full length gelsolin have potential for a differential or aetiological diagnosis of gentamicin's nephrotoxicity. They appear in the urine of rats with overt renal failure induced by gentamicin, but are not present in the urine of rats with a similar degree of renal damage inflicted by cisplatin. Reg IIIb is a 17 kDa member of the calcium dependent lectin (C-type lectin) superfamily (55) comprising several secretory protein products of four genes (Reg I, II, III and IV). Reg genes have been found in different mammal species including human, rat and mouse. Rat Reg genes map to the 4q33-q34 chromosomal region (56). In humans, all Reg genes except Reg IV, map to the 2p12 region (57). In general terms, Reg family proteins are involved in tissue regeneration in a number of physiological and pathological situations, most prominently including pancreatitis, but also hepatic injury, diabetes and cancer (55). Our experiments suggest that reg IIIb may be implicated in renal tissue injury and repair during gentamicin treatment and, importantly, that it might be used as a differential urinary marker. Because we could not detect this protein in the serum, we thought that urinary reg IIIb may be originated in the renal tissue. Indded, our data indicates that Reg IIIb expression is strongly induced by gentamicin in the kidneys, even preceding urine and serum markers and histological findings of nephrotoxicity (on day 3 of treatment; figure 5-B). The renal origin of reg IIIb upon treatment with gentamicin is further supported by our experiments on renal perfusion. When we acutely substituted the renal blood flow for perfused Krebs solution in rats previously treated with gentamicin, we still observed reg IIIb in the urine (figure 5-C). Urinary reg IIIb appears as a double band in Western blot analysis, corresponding to a double spot in 2D gels. However, when blood is substituted for Krebs in the renal circuit of gentamicin-treated rats, only the upper band is detected in the urine. We can only speculate that the lower band corresponds to a proteolytic fragment produced by serum proteases, or renal proteases activated by serum components.

Gelsolin is a highly conserved 82 kDa protein of the gelsolin superfamily. It is involved in

cytoskeleton organization and rearrangement in a number of normal cellular processes including motility, signalling and apoptosis (58); and pathophysiological conditions, such as inflammation, cancer and amyloidosis (59, 60). Gelsolin is expressed in many cell types and is also secreted and found normally in the blood of vertebrates (60,61). Gelsolin is a known substrate for effector caspase 3, which yields a 42 kDa proteolytic fragment (t-gelsolin; 62) involved in the execution (63) and regulation (58,62,64) of apoptosis. Our results indicate that urinary gelsolin may also be developed as a marker for the differential diagnosis of gentamicin's nephrotoxicity. In fact, the band corresponding to the full length protein in Western blot studies appears in the urine of gentamicin-treated rats, but it is mostly absent in cisplatin-treated rats. On the contrary, the ~ 43 kDa band in our gels, likely t-gelsolin, is common to both gentamicin and cisplatin groups. The results shown in figure 5 indicate that gelsolin gene expression is not modified in the kidneys of rats treated with gentamicin (with respect to controls). They further show that gelsolin disappears from the urine when renal blood flow is substituted for Krebs, suggesting that urinary gelsolin is probably filtered from the blood through the glomerular filtration barrier. This may also explain why the full length gelsolin is detected in the urine of rats treated with gentamicin and not in those treated with cisplatin, whereas t-gelsolin appears after both treatments. Gentamicin alters the GFB properties leading to increase filtration of specific proteins (19,20). The polycationic charge of gentamicin alters the electrostatic properties of the GFB, increasing the permeability of negatively charged proteins (such as gelsolin, pI = 5.75) and lowering the sieving coefficient of positively charged ones (65). No alterations of the GFB sieving properties have been reported for cisplatin. In this case, full length gelsolin would be excluded from passing through the GFB for size restriction (66,67). However, t-gelsolin would not be trapped in the blood in any case because its lower size allows it to filter more easily through the GFB. In a scenario of tubular necrosis, a larger amount of proteins scapes the handicapped tubular reabsorption capacity, which can be detected in the urine. As shown in figure 4, t-gelsolin appears in the urine of gentamicin-treated rats significantly earlier than traditional and new AKI markers, the latter including KIM-1, NGAL, NAG and PAI-1. This might also be exploited for an early monitoring of gentamicin's nephrotoxicity.

The present study provides two novel urinary biomarker candidates for the differential diagnosis of gentamicin's nephrotoxicity, which need to be further developed in the preclinical and clinical settings for a better theranostic usage and efficacy of this drug. Moreover, it poses a proof-of-principle for the potential application of the aetiological diagnosis of AKI to critical patients coursing with multiple conditions potentially affecting renal integrity, including polymedication. Aetiological diagnosis should be extended on many other potentially nephrotoxic drugs widely used in the clinical practice, and on pre-renal and post-renal causes of AKI. This will enable us to delineate patterns of markers that specifically discriminate the origin of undesirable renal effects in order to appropriately and selectively reshape the clinical handling and therapeutic regimes of patients at risk.

Materials and methods

Unless otherwise indicated, all reactives were purchased from Sigma. Gentamicin sulphate was kindly provided by Schering-Plough.

Animals and experimental protocol

Female Wistar rats weighing 200-250 g were allocated under controlled environmental conditions in individual metabolic cages, for 24-hour urine samples collection. Rats were randomly divided in two groups: (i) control group (C), receiving daily vehicle i.p. during 6 days; (ii) gentamicin group (G), receiving gentamicin i.p (150 mg/kg body weight/day) during 6 days; and (iii) cisplatin group (Cisp),

receiving one i.p. dose of cisplatin (7.5 mg/kg). At the end, kidneys were perfused by the aorta with saline (0.9% NaCl) and immediately dissected. One was frozen in liquid nitrogen and subsequently kept at -80 ºC for Western blot studies. The other one was fixed in buffered 3.7% p-formaldehyde for histological studies. Blood samples were also obtained in heparinized capillaries at different time points by a small incision in the tail tip. Blood was centrifuged and serum was kept at -80 ºC until use. Urine was cleared by centrifugation, and it was stored at -80 ºC until use.

Histological studies

Paraffin blocks were made and 5-µm tissue sections were stained with hematoxilin and eosin. Photographs were taken under an Olympus BX51 microscope connected to an Olympus DP70 colour, digital camera.

Biochemical measurements

Serum and urinary creatinine (Cr_s and Cr_u respectively) and blood urea nitrogen (BUN) concentration were measured by means of the automated analyzer Reflotron® (Roche Diagnostics; lower detection limit of 0.5 mg/dL). Creatinine clearance (Cl_{Cr}) = Cr_u x 24-h urine output x Cr_s^{-1}. Urine protein concentration was measured by the Bradford method (68). Urine NAG content was determined by a colorimetric method with a commercial kit (Roche Diagnostics) based on the conversion of 3-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminide into the purple 3-cresol-cresolsulfonphthaleinyl.

Western blot

Western blots were run with (i) urine samples (21 µl per sample), (ii) tissue extracts (100 µg total protein per sample) prepared by homogenizing the kidneys with a tissue mixer (Ultra-Turrax T8, IKA®-Werwe) at 4 ºC in homogenization buffer (140 mM NaCl, 20 mM Tris-HCl pH=7.5, 0.5 M ethylenediaminetetraacetic acid –EDTA-, 10% glycerol, 1% Igepal CA-630, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, 1 mM phenylmethylsulphonyl fluoride –PMSF-), or (iii) albumin-free blood serum. Albumin was removed from serum with a column-based, commercial kit based on the immunological retention of rat albumin (Qproteome Murine Albumin Depletion Kit, Quiagen). Samples were separated by electrophoresis in 10-15% acrylamide gels (Mini Protean II system, BioRad). Immediately, proteins were electrically transferred to an Immobilon-P membrane (Millipore). Membranes were probed with antibodies against KIM-1 (R&D Systems), bone morphogenetic protein 7 (BMP-7, Santa Cruz Biotechnology), NGAL (MBL), PAI-1 (BD Biosciences), reg IIIb (R&D Systems), and gelsolin (Santa Cruz Biotechnology).

Two-dimensional electrophoretic separation of proteins

Urine was concentrated and desalted through Amicon Ultra 5 K cut-off columns (Millipore). Protein concentration was determined by the Bradford method. For two-dimensional electrophoresis (2D), urine proteins (100 µg) were precipitated with the Clean-Up kit (GE Healthcare). Precipitated proteins were rehydrated in 7 M urea, 2 M thiourea, 4% (w/v) Chaps, 0.5% ampholytes (pH 4-7 or 4.5-5.5), 50 mM dithiothreitol (DTT) and bromophenol blue, and isoelectrically focused (500-8,000 V) through 18-cm long immobilized pH gradient (IPG) strips, pH 4-7 or 4.5-5.5 (GE Healthcare), using an IPGphor apparatus (GE Healthcare). After focusing, IPG strips were pre-equilibrated during 15 minutes in equilibration buffer [50 mM Tis-HCl pH=8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecylsulphate (SDS), 0.01% (w/v) bromophenol blue] containing 1% (w/v) DTT, and another 15 minutes in equilibration buffer containing 2.5% (w/v) iodoacetamide. Then, IPG strips were transferred to 18-cm long, 12% acrylamide gels and separated by electrophoresis with a SE 600 Ruby apparatus (GE Healthcare). Gels were fixed

overnight in 30% ethanol, 10% acetic acid and silver stained with a commercial kit (GE Healthcare).

For visualization and analysis, gels were scanned (Image Sanner, GE Healthcare), processed and statistically analyzed with the Image Master 2D Platinum 6.0 software (GE Healthcare). Spot discrimination was done with the next parameters: (i) smooth factor: 2; (ii) minimal area: 5 pixels; (iii) saliency: 100. Analysis was visually corrected for artefact elimination. For each individual spot, background was subtracted and individual intensity volume was normalized by total intensity volume (all-spot intensity). For comparison of the same spot among gels, a minimum of a two-fold intensity difference (with $p<0.01$ according to the Students t test) was established to consider a differential expression.

Protein identification by mass spectrometry

The spots of interest from 2D separations were cut off the gels, dehydrated in acetonitrile, vacuum-evaporated and resuspended in NH_4HCO_3. 2D-LC fractions were also vacuum-evaporated and residues ressuspended in NH_4HCO_3. Samples were then reduced with 10 mM DTT in 50 mM NH_4HCO_3 at 56 ºC, and alkylated with iodoacetamide in 50 mM NH_4HCO_3. Proteins were in-gel digested with porcine trypsin (Promega), and peptides were extracted with 0.5% (v/v) trifluoroacetic acid (TFA), vacuum-evaporated and redissolved in 0.1% (v/v) formic acid. Peptide-containing solutions were injected in a LC-ESI-QUAD-TOF mass spectrophotometer QSTAR XL (Applied Biosystems) with an 1100 micro HPLC (Agilent). A wide pore 150x0.32 mm (5 μm) Supelco column (Discovery BIO) was used. MS/MS spectra were obtained. Protein identification was performed with the MASCOT softwar (www.matrixscience.com) against non redundant protein sequence databases (Swiss Prot and NCBI). Mass tolerance was set at 50 ppm, MS/MS tolerance was 0.5 Da, and the taxonomic status was Rattus. Only significant hits, as identified by MASCOT probability analysis, were considered and at least one peptide match with ion score above 20 was set as the threshold of acceptance. For confirmation, some proteins where also identified with an Ultraflex I MALDI-TOF mass spectrophotometer (Bruker Daltonics).

Gene expression analysis

RT-PCR-amplification of reg IIIb, gelsolin and GAPDH was performed with the next primers: for rat reg IIIb, 5'-TTGTTTGATGCAGAACTGGC-3' and 5'-AGACGTAGGGCAACTTCACT-3; for rat gelsolin, 5'-CAAGGCTACTTCAAGTCTGG-3' and 5'-GCTACCCTCTTCAGACACAT-3; for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GTGGTCATGAGCCCTTCCA-3' and 5'-AACTCCCTCAAGATTGTCAGCAA-3'. PCR conditions were: 1 x (95 ºC x 4 min); 30 x (95 ºC x 1 min + T_m x 1 min); 1 x (72 ºC x 10 min); where T_m was 55.5 ºC for reg IIIb, 55.0 ºC for gelsolin, and 55.9 ºC for GAPDH.

Renal excretion studies

At the end of the treatment, rats treated with gentamicin during 6 days were anesthetized and an extracorporeal circuit for kidney perfusion was set up, as described elsewhere (69), with some modifications. Briefly, the renal artery, vein and ureter of the right kidney were ligated. The renal artery and vein of the left kidney and the urinary bladder were canulated. Oxygenated and warm (37 ºC) Krebs-dextran [40 g/L of dextran (molecular weight 64K-76K) in Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 0.026 mM EDTA, 11.1 glucose, pH=7.4)] was perfused through the renal artery at 3 mL/min, and was discarded through the renal vein. Urine fractions were collected from a catheter placed in the urinary bladder, starting before the perfusion with Krebs (when blood was still passing through the kidney), and during 2 hours after perfusion with Krebs started. All urine samples were kept at -80 ºC until assayed by Western blot for the presence of reg IIIb

and gelsolin.

Statistical analysis

Data are represented as the mean ± standard error of n experiments performed, as indicated in each case. Except for the study of proteomic results (as indicated above), statistical comparisons were assessed by the one-way ANOVA analysis.

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PATENTE II

PATENTE II

MÉTODO PARA LA DETECCIÓN DE DAÑO RENAL

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(7) TÍTULO DE LA INVENCIÓN:	METODO PARA LA DETECCION DE DAÑO RENAL
(8) PETICIÓN DE INFORME SOBRE EL ESTADO DE LA TÉCNICA:	
SI	[ ]
NO	[✓]
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN	
SI	[ ]
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(12) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS:	
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RESUMEN:	[✓] N.º de páginas: 1
FIGURA(S) A PUBLICAR CON EL RESUMEN:	[ ] N.º de figura(s):
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OTROS (Aparecerán detallados):	
(17) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE PAGO DE TASA PREVISTO EN EL ART. 162 DE LA LEY 11/1986 DE PATENTES, DECLARA: BAJO JURAMIENTO O PROMESA SER CIERTOS TODOS LOS DATOS QUE FIGURAN EN LA DOCUMENTACIÓN ADJUNTA:	[ ]
DOC COPIA DNI:	[ ] N.º de páginas:
DOC COPIA DECLARACIÓN DE CARENCIA DE MEDIOS:	[ ] N.º de páginas:
DOC COPIA CERTIFICACIÓN DE HABERES:	[ ] N.º de páginas:
DOC COPIA ÚLTIMA DECLARACIÓN DE LA RENTA:	[ ] N.º de páginas:
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FIRMA DEL SOLICITANTE O REPRESENTANTE:	ENTIDAD PONS PATENTES Y MARCAS INTERNACIONAL SL - CIF B84921709 - NOMBRE PONS ARIÑO ANGEL - NIF 50534279J
LUGAR DE FIRMA:	Madrid
FECHA DE FIRMA:	04 Agosto 2009

PATENTE III

PATENTE III

PCT GELSOLIN - REG IIIB
METHOD FOR THE DETECTION OF RENAL DAMAGE

Yaremi Quiros, Laura Ferreira, Sandra M. Sancho-Martínez, José M. González-Buitrago, José M. López-Novoa1 and Francisco J. López-Hernández.Garcia Sanchez, Omar.

European Patent Office (EPO) (RO/EP)

PCT1367.34

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PCT REQUEST

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0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT Online Filing Version 3.5.000.219 MT/FOP 20020701/0.20.5.9
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	European Patent Office (EPO) (RO/EP)
0-7	Applicant's or agent's file reference	PCT1367.34
I	Title of Invention	METHOD FOR THE DETECTION OF RENAL DAMAGE
II	Applicant	
II-1	This person is	Applicant only
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III-5	**Applicant and/or inventor**	
III-5-1	This person is	Applicant and inventor
III-5-2	Applicant for	US only
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III-5-6	State of nationality	ES
III-5-7	State of residence	ES

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III-6	**Applicant and/or inventor**	
III-6-1	This person is	Applicant and inventor
III-6-2	Applicant for	US only
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III-6-6	State of nationality	ES
III-6-7	State of residence	ES
III-7	**Applicant and/or inventor**	
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III-7-7	State of residence	ES
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	Agent
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IV-1-4	Facsimile No.	+34 91 308 61 03
IV-1-5	e-mail	patente@pons.es
IV-1-5(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address to send, if the Office or Authority so wishes, advance copies of notifications in respect of this international application.	Yes
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	

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VI-1	**Priority claim of earlier national application**		
VI-1-1	Filing date	04 August 2009 (04.08.2009)	
VI-1-2	Number	P200930559	
VI-1-3	Country	ES	
VI-2	**Incorporation by reference :** where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.		
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description (excluding sequence listing part)	55	✓
IX-3	Claims	7	✓
IX-4	Abstract	1	✓
IX-5	Drawings	8	✓
IX-7a	Sub-total number of sheets	76	
IX-6	Sequence listing part of the description	21	✓
IX-7	TOTAL	97	

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	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	-	✓
IX-16	Sequence listing submitted for the purposes of international search under Rule 13ter	-	✓
IX-18	PCT-SAFE physical media	-	-
IX-19	A statement confirming that "the information in Annex C/ST.25 text format submitted under Rule 13ter is identical to the sequence listing as contained in the international application"	-	✓
IX-20	**Figure of the drawings which should accompany the abstract**		
IX-21	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**	(PKCS7 Digital Signature)	
X-1-1	Name	UNIVERSIDAD DE SALAMANCA	
X-1-2	Name of signatory	ES, Pons Patentes y Marcas Internacional, S.L., A. Pons-Arino 7414	
X-1-3	Capacity	(Applicant)	

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10-1	**Date of actual receipt of the purported international application**	
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP
10-6	**Transmittal of search copy delayed until search fee is paid**	

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11-1	**Date of receipt of the record copy by the International Bureau**	

PATENTE IV

PATENTE IV

MÉTODO PARA LA DETECCIÓN DE DAÑO RENAL

Yaremi Quiros, Laura Ferreira, Sandra M. Sancho-Martıínez, José M. Gonzáez-Buitrago, José M. López-Novoa1 and Francisco J. López-Hernández.

European Patent Office, The Hague

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P201031407	
Fecha de recepción:	22 septiembre 2010, 17:14 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	ES1367.34bis div.	
Solicitante:	UNIVERSIDAD DE SALAMANCA	
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País:	ES	
Título:	MÉTODO PARA LA DETECCIÓN DE DAÑO RENAL	
Documentos enviados:	Descripcion.pdf (46 p.) Reivindicaciones-1.pdf (6 p.) Resumen-1.pdf (1 p.) Dibujos-1.pdf (5 p.) SEQLPDF.pdf (21 p.) SEQLTXT.txt	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=ENTIDAD PONS PATENTES Y MARCAS INTERNACIONAL SL - CIF B84921709 - NOMBRE PONS ARIÑO ANGEL - NIF 50534279J,OU=703015345,OU=fnmt clase 2 ca,O=FNMT,C=es	
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DE INDUSTRIA, TURISMO
Y COMERCIO

Oficina Española
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(1) MODALIDAD:	
PATENTE DE INVENCIÓN	[✓]
MODELO DE UTILIDAD	[ ]
(2) TIPO DE SOLICITUD:	
PRIMERA PRESENTACIÓN	[ ]
ADICIÓN A LA PATENTE EUROPEA	[ ]
ADICIÓN A LA PATENTE ESPAÑOLA	[ ]
SOLICITUD DIVISIONAL	[✓]
CAMBIO DE MODALIDAD	[ ]
TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA	[ ]
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(3) EXP. PRINCIPAL O DE ORIGEN:	
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N.º SOLICITUD:	200930559.0
FECHA SOLICITUD:	04 Agosto 2009
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5) DIRECCIÓN ELECTRÓNICA HABILITADA (DEH):	
(5-1) SOLICITANTE 1:	
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DNI/CIF/PASAPORTE:	Q3718001E
CNAE:	
PYME:	
DOMICILIO:	Patio de Escuelas, 1
LOCALIDAD:	Salamancca
PROVINCIA:	37 Salamanca
CÓDIGO POSTAL:	37008
PAÍS RESIDENCIA:	España
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CORREO ELECTRÓNICO:	
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MODO DE OBTENCIÓN DEL DERECHO:	
INVENCIÓN LABORAL:	[✓]
CONTRATO:	[ ]
SUCESIÓN:	[ ]
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DNI/PASAPORTE:	
(8) TÍTULO DE LA INVENCIÓN:	MÉTODO PARA LA DETECCIÓN DE DAÑO RENAL
(9) PETICIÓN DE INFORME SOBRE EL ESTADO DE LA TÉCNICA:	
SI	[ ]
NO	[✓]
(10) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESIÓN	
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NO	[✓]
(11) EFECTUADO DEPÓSITO DE MATERÍA BIOLÓGICA:	
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NO	[✓]
(12) DEPÓSITO:	
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(13) DECLARACIONES RELATIVAS A LA LISTA DE SECUENCIAS:	
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(14) EXPOSICIONES OFICIALES:	
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(15) DECLARACIONES DE PRIORIDAD:	
PAÍS DE ORIGEN:	
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(16) AGENTE/REPRESENTANTE:	
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CÓDIGO POSTAL:	28010
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CÓDIGO PAÍS:	ES

TELEFONO: FAX: CORREO ELECTRÓNICO: NÚMERO DE PODER:	
(17) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN: DESCRIPCIÓN: REIVINDICACIONES: DIBUJOS: RESUMEN: FIGURA(S) A PUBLICAR CON EL RESUMEN: ARCHIVO DE PRECONVERSION: DOCUMENTO DE REPRESENTACIÓN: LISTA DE SECUENCIAS PDF: ARCHIVO PARA LA BUSQUEDA DE LS: OTROS (Aparecerán detallados):	 [✓] N.º de páginas: 46 [✓] N.º de reivindicaciones: 36 [✓] N.º de dibujos: 4 [✓] N.º de páginas: 1 [ ] N.º de figura(s): [ ] [ ] N.º de páginas: [✓] N.º de páginas: 21 [✓]
(18) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE PAGO DE TASA PREVISTO EN EL ART. 162 DE LA LEY 11/1986 DE PATENTES, DECLARA: BAJO JURAMIENTO O PROMESA SER CIERTOS TODOS LOS DATOS QUE FIGURAN EN LA DOCUMENTACIÓN ADJUNTA: DOC COPIA DNI: DOC COPIA DECLARACIÓN DE CARENCIA DE MEDIOS: DOC COPIA CERTIFICACIÓN DE HABERES: DOC COPIA ÚLTIMA DECLARACIÓN DE LA RENTA: DOC COPIA LIBRO DE FAMILIA: DOC COPIA OTROS:	[ ] [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas: [ ] N.º de páginas:
(19) NOTAS:	
(20) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	 ENTIDAD PONS PATENTES Y MARCAS INTERNACIONAL SL - CIF B84921709 - NOMBRE PONS ARIÑO ANGEL - NIF 50534279J Madrid 22 Septiembre 2010

DISCUSIÓN GENERAL

El diagnóstico del daño renal agudo va a experimentar una mejora cualitativa en las próximas décadas con la introducción sistemática en la práctica clínica de la detección precoz facilitada por una nueva generación de marcadores muy tempranos. Sin embargo, los estudios de esta tesis doctoral, y sus correspondientes publicaciones, ponen de manifiesto que existen aspectos para seguir profundizando en el diagnóstico de esta enfermedad, para prevenirla, controlarla e identificarla cada vez mejor y de forma más precisa.

Así, nuestros estudios abren la puerta a dos nuevos conceptos médicos que, en nuestra opinión, tienen un gran potencial clínico. Por una parte hemos demostrado que un tratamiento farmacológico (con dosis bajas del antibiótico gentamicina) completamente inocuo para la estructura y la función excretora de los riñones, hace a los animales más sensibles a otros agentes nefrotóxicos, tanto farmacológicos como ambientales. Estos animales predispuestos tienen un riesgo adquirido mayor de sufrir esta enfermedad, no solamente por exposición a otros procedimientos médicos, sino a agentes presentes en los alimentos, en el agua o en el aire que pasan inadvertidos pues, con las exposiciones normales, no afectan a los animales no predispuestos. Esta condición tiene un correlato inmediato con la salud humana. Los pacientes tratados con este antibiótico pueden tener un mayor riesgo de sufrir un fracaso renal que las personas no tratadas con él.

La predisposición producida por la gentamicina no es un caso aislado. Aunque no es objeto de esta tesis doctoral, otros resultados de nuestro grupo de investigación demuestran que otros fármacos y agentes potencialmente tóxicos tienen los mismos efectos. Por este motivo, pensamos que estos estudios proporcionan pruebas de un nuevo concepto "teranóstico" que permitirá anticiparse al efecto tóxico, no solo nefrotóxico, de los fármacos, antes de que estos produzcan los más mínimos síntomas de toxicidad. Y esto será posible gracias a la monitorización paralela y posterior a los tratamientos farmacológicos, de marcadores conceptualmente nuevos que, potencialmente, informarán sobre el nivel de riesgo de padecer una determinada enfermedad de origen tóxico, para poder reajustar el manejo clínico de los pacientes de forma personalizada.

La nefrotoxicidad de los fármacos, y más aún la toxicidad en general, es un asunto de gran relevancia clínica y socioeconómica. Centrándonos en la nefrotoxicidad, se estima que aproximadamente 1 de cada 4 fármacos, de los 100 más usados en las unidades de cuidados intensivos, es nefrotóxico. Más aún, la nefrotoxicidad de los fármacos es responsable de entre un 10 y un 20% del total de casos de fracaso renal agudo. La nefrotoxicidad es, además, una causa importante de abandono de fármacos candidatos durante el proceso preclínico y clínico de desarrollo. Y, finalmente, la nefrotoxicidad o, mejor dicho, las actuaciones para evitar la nefrotoxicidad de los fármacos, normalmente reducen su eficacia terapéutica, pues limitan la dosis que puede ser administrada. Es el caso, por citar algunos, de la gentamicina y los antibióticos aminoglucósidos, los antiinflamatorios no esteroideos, los inmunodepresores de la familia de la ciclosporina, y muy paradigmáticamente, los anticancerosos platinados, como el cisplatino. El uso y la eficacia de terapéutica de todos ellos mejorarán sustancialmente cuando se disponga de marcadores teranósticos que permitan ir ajustando los tratamientos de acuerdo con las necesidades y con los riesgos, que varían significativamente de un paciente a otro.

Otro nuevo concepto surgido de nuestros

estudios es el del diagnóstico diferencial de la nefrotoxicidad. Hasta el momento, y con las herramientas diagnósticas más avanzadas, es imposible distinguir el daño renal que produce cada fármaco del que producen los demás. Así, en pacientes sometidos simultáneamente a varias circunstancias o agentes potencialmente dañinos para los riñones, resulta imposible saber la causa o causas de la lesión renal cuando aparecen los primeros síntomas. Un caso particular de estas situaciones es el de los enfermos polimedicados con más de un fármaco nefrotóxico. Otro caso es el de pacientes en estado crítico en los que, además de los medicamentos administrados, pueden concurrir en ellos otras causas de daño renal agudo, tales como la reducción del flujo sanguíneo renal total o local, por hipotensión extrema, oclusión de las arterias renales o la microangiopatía renal, o la obstrucción de las vías urinarias, fundamentalmente de los uréteres. En todos estos casos sería de gran utilidad poder conocer la causa de la lesión renal cuando ésta se presenta. De este modo sería posible reajustar el tratamiento y el manejo clínico de los pacientes de forma más específica y más personalizada. Nuestro resultados suponen el primer paso en el diagnóstico etiológico o diferencial de la nefrotoxicidad y, por lo tanto, proponen este nuevo concepto diagnóstico. Concretamente, nuestro estudio revela que es posible distinguir el daño renal causado por la gentamicina del producido por el cisplatino, mediante el análisis de la orina.

Nuestro objetivo inmediato es hacer parte del desarrollo clínico de los marcadores identificados. El reto para el futuro es extender estos dos nuevos conceptos a sectores más amplios de la Medicina, especialmente a los agentes causantes de daño re-

CONCLUSIONES

De los estudios expuestos en este trabajo de Tesis Doctoral podemos extraer las siguientes conclusiones:

1. El tratamiento subnefrotóxico con la gentamcina predispone a las ratas al fracaso renal agudo. Esta predisposición se pone de manifiesto por exposición a un segundo agente terapéutico o ambiental potencialmente nefrotóxico, incluso en condiciones que resultan inocuas para los animales no previamente predispuestos.

2. La predisposición al fracaso renal agudo que produce la gentamcina se correlaciona con una mayor excreción urinaria de la proteína activadora del ganglósido M2 (*GM2AP*).

3. La excreción urinaria de GM2AP también podría utilizarse para el diagnóstico muy precoz del daño renal agudo que produce la gentamicina, ya que esta proteína aparece elevada en la orina mucho antes que los marcadores más sensibles conocidos hasta el momento.

4. El incremento de la excreción urinaria de GM2AP parece deberse a la competición de la gentamicina y esta proteína por el complejo de endocitosis formado por la megalina y la cubilina, localizado en el túbulo proximal.

5. En pacientes tratados con la gentamicina también se detecta una mayor excreción urinaria de GM2AP, que podría servir potencialmente para estratificar el riesgo de sufrir un fracaso renal agudo derivado de la exposición a esta antibiótico.

6. Nuestros resultados proporcionan una prueba de concepto sobre la posibilidad de diferenciar la nefrotoxicidad de unos fármacos de la de otros mediante el análisis de la orina.

7. Concretamente, la gentamicina produce un incremento de la excreción urinaria de la gelsolina y de la proteína regenerativa derivada de los islotes 3 beta (reg III b) que no se observa en el caso del tratamiento con el cisplatino, para un mismo grado de daño renal producido.

8. El fragmento proteolítico de la gelsolina denominado t-gelsolina aparece en la orina de las ratas tratadas con un régimen nefrotóxico de gentamicina mucho antes que los marcadores más sensibles conocidos hasta el momento. Esto puede tener aplicación en el diagnóstico precoz de la nefrotoxicidad.

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