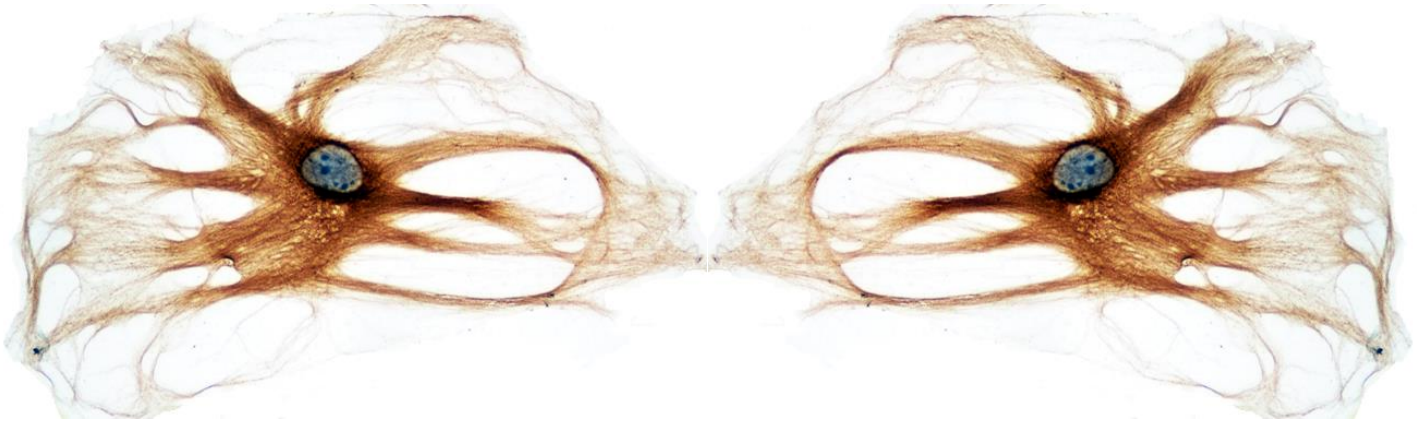
Universidad de Alcalá

Programa de Doctorado en Señalización Celular

ESTUDIO EXPERIMENTAL Y TRASLACIONAL DE LOS EFECTOS RENALES DEL FACTOR AMBIENTAL BISFENOL-A. PODOCITOPATÍA E HIPERTENSIÓN



Inmunocitoquímica de podocito humano marcado con podocina. Figura propia.

Tesis doctoral presentada por

RAFAEL MORENO GÓMEZ-TOLEDANO

2022

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ESTUDIO EXPERIMENTAL Y TRASLACIONAL DE LOS EFECTOS RENALES DEL FACTOR AMBIENTAL BISFENOL-A. PODOCITOPATÍA E HIPERTENSIÓN

Tesis doctoral presentada por

RAFAEL MORENO GÓMEZ-TOLEDANO

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Alcalá de Henares, 10 de marzo de 2022

Al eterno olvidado,
pero siempre presente.
Gracias por cuidarnos
inmerecidamente.

Nos sentimos gigantes
resolviendo el puzle de Dios;
no podríamos ni intentarlo
sin la ayuda del ratón.

Muchas han sido las personas que me han influido en mayor o menor medida, y no sólo a nivel profesional, sino a múltiples niveles. Mi forma de entender el mundo ha cambiado gracias a ellos, mi forma de expresarme, mi forma de relacionarme y, en definitiva, mi forma de ser. He trabajado con grandes científicos que me han regalado aspiraciones, he convivido con humanistas que me han ayudado a entender mi campo de estudio desde otras perspectivas, e incluso he compartido espacio con virtuosos que me han abierto la mente al lenguaje musical. Aunque si alguien merece atención especial, una persona cuya influencia me haya motivado desde mi infancia a comprender las causas de las enfermedades, y cuya presencia en mi vida ha desencadenado que hoy esté aquí escribiendo estas líneas, es mi tía Isa. El cromosoma 21 que le sobraba alimentó mi curiosidad y forjó la base de mi carrera científica.

Por supuesto y como no podía ser de otra manera, quiero comenzar agradeciendo al Dr. Ricardo J. Bosch la gran oportunidad que me ha brindado de desarrollarme personal y profesionalmente en el laboratorio. Fruto de la casualidad de un reencuentro fortuito, retomé la senda del investigador gracias a la confianza depositada en mí aquel día. Gracias, Ricardo, por tus enseñanzas, por tus consejos y por prepararme para el futuro. No sólo he aprendido el trabajo de laboratorio, sino que me has ayudado a formarme en la redacción de textos, proyectos, e incluso me has dado la libertad suficiente como para desarrollar mis ideas (y reconocerlas, posicionándome como “corresponding autor”). La forma de hacer ciencia, con un fuerte componente interdisciplinar, con total transparencia y plena colaboración entre personas y equipos, me parece la mejor enseñanza que me has podido trasmitir, y sin lugar a dudas desarrollaré en mis futuros proyectos.

También quiero agradecer a mis dos directoras su labor, sus enseñanzas y las oportunidades que me han brindado. Citaré por orden cronológico de incorporación a la tesis, pues no quiero restar inmerecidamente importancia. A la Dra. M. Isabel Arenas, Maribel, gracias por tu tiempo, tus enseñanzas y consejos, por el buen ambiente de trabajo, y por todos esos momentos en los que hemos sacado adelante los manuscritos gracias a que hacemos un gran equipo. A la Dra. Marta Saura, gracias por dejarme entrar en tu laboratorio y formar parte de él, donde siempre me he sentido a gusto y rodeado de gente maravillosa. Gracias por permitirme ser cotutor de prácticas y desarrollar un trabajo académico, y por las charlas de café, que me han mantenido cuerdo en las temporadas en que he trabajado solo en el laboratorio.

Muchas son las personas que me han influido, e intentaré recordar con mayor o menor acierto, haciendo lo que, en palabras de mis amigos humanistas, sería una suerte de *genealogía del paradigma*. Comenzaré por mis inicios, cuando aún no había terminado la carrera de Biología, en los que tuve la ayuda de la Dra. Nuria Olea, quien me acompañó en mis primeros pasos en el laboratorio. Gracias, Nuria, por darme numerosos consejos y trucos que a día de hoy sigo utilizando. Estas primeras experiencias son críticas a la hora de forjar el carácter del estudiante, y sin lugar a dudas agradezco la mía, pues creo que me ha influido positivamente en numerosos aspectos de mi actual trabajo de laboratorio. La otra persona de mis inicios a la que estoy agradecido es a la Dra. Carmen Muñoz. Carmen, gracias por enseñarme (y años después ayudarme a recordar) el trabajo con el animal de laboratorio. Siempre me ha gustado el trato que le das a los ratones,

quienes se merecen todo nuestro respeto y atención. Por último, gracias Michele por tu forma de ser y la alegría que creabas en el laboratorio.

En aquella época tuve una interesante experiencia investigadora gracias al Dr. Emilio Sola, fundador del CEDCS[1], y al Dr. Pablo M. Testa y a Pablo R. Ayuso, quienes me invitaron a formar parte del GIMEC[2]. Gracias a ambos y al resto del equipo, Cristian, Esteban, Griselda, Fátima, Andrés y Eugenio, por abrirme la mente a nuevas formas de entender la ciencia, y por hacer tangible la posibilidad de desmontar axiomas y crear nuevos paradigmas. Gracias a vuestra influencia he (re)descubierto la filosofía, la base de mi trabajo, habitualmente olvidada o relegada a un segundo plano, y hemos podido publicar un interesante manuscrito [1] y acudir a varios congresos. También estuve medio año en al laboratorio de análisis clínicos del Hospital San José, donde el Dr. José Luis Valle me ayudó a desarrollar el enfoque clínico de mi disciplina y el trato con los pacientes.

Varios años después me reincorporé a la Universidad de Alcalá. Durante ese período intermedio, el *entreacto*, pude dirigir la academia de Macarena, a la que agradezco enormemente la confianza que tuvo en mí, y después me convertí en comercial. Edu, me enseñaste que "en todas las casas cuecen habas", y que hasta en un trabajo tan duro como aquel, puedes forjar fuertes lazos con las personas, lo que hizo incluso divertida nuestra labor. Finalmente, el tiempo que viví en Asturias lo atesoro con nostalgia y alegría, momento en que me replanteé mi camino en la vida, dándome cuenta de que aquel que se considera científico lo es para el resto de su vida.

Mi vuelta a la investigación básica fue mucho más fácil gracias a la Dra. Saura y a su equipo. Irene, Paula y Rafa me ayudasteis a recordar todo lo necesario para volver a cacharrear en el laboratorio, dándome todo lo que necesitaba, y además vivimos muchos momentos divertidos tanto dentro como fuera de la universidad. Posteriormente se fueron incorporaron al laboratorio Sandra, Alberto y María, personas a las que aprecio mucho y con quienes he compartido cientos de ideas, resultados y cafés, así como alguna que otra cervecita. Hemos debatido largo y tendido sobre la forma de hacer ciencia y hemos jugado mucho al pádel; tanto que Alberto y yo llegamos a creer que jugábamos bien. Conozco pocas personas tan buenas, con tan poca vergüenza (en el buen sentido) y con tantas aptitudes para la ciencia como Alberto, y espero que se me haya pegado un poco de ello.

En el laboratorio de al lado tuve la suerte de tratar con los doctores Rafael Ramírez y Matilde Alique, quienes siempre han sido muy amables conmigo, me han ayudado e incluso me han dejado utilizar su equipo, y es que me cambió la vida poder usar su máquina de revelado. Antes de ello iba a la planta de isótopos radiactivos a revelar a la antigua usanza. Todos los días llamaba a Guillermo y Juan para que fueran encendiendo la máquina y siempre lo hacían de buen grado. También tengo que destacar a Isa y Cris de la Unidad de Cultivos Celulares, Angélica, secretaria del departamento, y Rosa, quien

[1] Centro Europeo para la Difusión de las Ciencias Sociales
[2] Grupo de Investigación multidisciplinar en Estudios Culturales

me ha ayudado en innumerables ocasiones con la burocracia, cuya dificultad crece exponencialmente con el tiempo.

Paralelamente también trabajé con el Dr. Eduardo Arilla, científico brillante y extraordinaria persona. Eduardo, me has enseñado que los científicos de vocación siguen maravillándose y aprendiendo de los nuevos descubrimientos con la misma intensidad que al principio de su carrera (o incluso más), y espero que también me ocurra a mí. Durante un año compartí laboratorio y buenos ratos con Clara, y me demostró que en las condiciones adecuadas las segundas oportunidades pueden ser muy provechosas. Y con otros compañeros como Héctor, Coral, Ágata y Lorena también he compartido espacio, ideas, dudas y divagaciones, lo cual siempre ha sido muy enriquecedor.

También estoy muy agradecido por haber podido aprender de las doctoras Adriana Izquierdo, que me abrió las puertas de su laboratorio en la Universidad Rey Juan Carlos, Pilar López Aparicio, que me ayudó a cuantificar bisfenol, y Esperanza Vélez, que me dio las herramientas que necesitaba para aprender meta-análisis. Respecto a esta clase de estudios también debo dar las gracias al Dr. Ariyán Mottaghi, con quien reflexionaba sobre estadística y metodologías durante mi estancia de auxiliar en Vitaldent.

Y por supuesto, las fuentes de financiación públicas, a las que debo mi beca de colaboración y mi contrato asociado al proyecto del Consorcio para el Estudio del Fracaso Renal Agudo (CIFRA), coordinado por el Dr. Lisardo Boscá.

Los consejos y recomendaciones de la Dra. María Sarabia me ayudaron a ganar la V edición del concurso “Tesis en 3 minutos”, pero también se lo debo a mi familia (la de sangre y la de elección) que escucharon mis charlas y me animaron en todo momento.

Mucho debo a mi familia, a los que están y a los que ya se fueron. Especial atención merece mi madre, pues sin quererlo me introdujo en la lucha contra los disruptores endocrinos, con aquella frase que siempre me decía cuando era pequeño: “hijo, no calientes el tupper en el microondas”.

Para finalizar, agradecer a todas aquellas personas que se han prestado voluntarias para la realización de los análisis recopilados en esta tesis, y por supuesto nefrólogos como los doctores Gabriel de Arriba o Laura Bucalo, quienes nos han ayudado con ello. Además, me parece imprescindible concluir con el agradecimiento a todos los animales cuyo sacrificio ha permitido profundizar en nuestras investigaciones, desarrollando evidencias mucho más completas, complejas y coherentes.

Y a mis dos compañeros de viaje, Nicole y Lord Puchito, gracias por hacerme disfrutar del camino como si cada día llegara a mi destino.

1. Publicaciones que conforman formalmente la tesis doctoral, cumpliendo los requerimientos del Programa de Doctorado en Señalización Celular para las tesis por compendio de publicaciones.

- Capítulo 1: **Moreno-Gómez-Toledano, R.**, Arenas, M. I., Muñoz-Moreno, C., Olea-Herrero, N., Reventun, P., Izquierdo-Lahuerta, A., Antón-Cornejo, A., González-Santander, M., Zaragoza, C., Saura, M. & Bosch, R. J. (2022). Comparison of the renal effects of bisphenol A in mice with and without experimental diabetes. Role of sexual dimorphism. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1868*(1), 166296.
- Capítulo 2: **Moreno-Gómez-Toledano, R.**, Arenas, M. I., González-Martínez, C., Olea-Herrero, N., Reventún, P., Di Nunzio, M., Sánchez-Esteban, S., Arilla-Ferreiro, E., Saura, M. & Bosch, R. J. (2020). Bisphenol A impaired cell adhesion by altering the expression of adhesion and cytoskeleton proteins on human podocytes. *Scientific reports, 10*(1), 1-15.
- Capítulo 3: **Moreno-Gómez-Toledano, R.**, Arenas, M. I., Vélez-Vélez, E., Coll, E., Quiroga, B., Bover, J., & Bosch, R. J. (2021). Bisphenol A Exposure and Kidney Diseases: Systematic Review, Meta-Analysis, and NHANES 03–16 Study*. Biomolecules, 11*(7), 1046.

2. Publicaciones relevantes para el contexto de la tesis

- Prólogo: Olea-Herrero, N., Arenas, M. I., Muñóz-Moreno, C., **Moreno-Gómez-Toledano, R.**, González-Santander, M., Arribas, I., & Bosch, R. J. (2014). Bisphenol A induces podocytopathy with proteinuria in mice. *Journal of cellular physiology, 229*(12), 2057-2066.
- Anexo III: Saura, M., Marquez, S., Reventun, P., Olea-Herrero, N., Arenas, M. I., **Moreno-Gómez-Toledano, R.**, Gómez-Parrizas, M., Muñoz-Moreno, C., González-Santander, M., Zaragoza, C. & Bosch, R. J. (2014). Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS*. The FASEB Journal, 28*(11), 4719-4728.
- Anexo IV: Reventun, P., Sanchez-Esteban, S., Cook, A., Cuadrado, I., Roza, C., **Moreno-Gomez-Toledano, R.**, Muñoz, C., Zaragoza, C., Bosch, R.J. & Saura, M. (2020). Bisphenol A induces coronary endothelial cell necroptosis by activating RIP3/CamKII dependent pathway. *Scientific reports, 10*(1), 1-17.
- Anexo V: **Moreno-Gómez-Toledano, R.**, Sánchez-Esteban, S., Cook, A., Mínguez-Moratinos, M., Ramírez-Carracedo, R., Reventún, P., Delgado-Marín, M., Bosch, R.J. & Saura, M. (2021). Bisphenol A Induces Accelerated Cell Aging in Murine Endothelium. *Biomolecules, 11*(10), 1429.
- Anexo VI: **Moreno-Gómez-Toledano, R.**, Arenas, M. I., Vélez-Vélez, E., Saura, M., & Bosch, R. J. (2021). New Evidence of Renal and Cardiovascular Alterations Promoted by Bisphenol A. *Biomolecules, 11*(11), 1649.
- Anexo VII: **Moreno-Gómez-Toledano, R.**, Arenas, M. I., Vélez-Vélez, E., & Bosch, R. J. (2022). New evidence for a role of Bisphenol A in cell integrity. Implications in the human population. *Biocell, 46*(2), 305.
- **Moreno-Gómez-Toledano, R.**, Arenas, M. I., Sánchez-Esteban, S., Cook, A., Saura, M., & Bosch, R. J. (2021). Critical analysis of human exposure to bisphenol A and its novel implications on renal, cardiovascular and hypertensive diseases. Heshmati HM (ed). *Hot Topics in Endocrinology and Metabolism* (pp. 1–20). IntechOpen.

3. Participación en otras publicaciones

- Ortega, A., Olea-Herrero, N., Arenas, M. I., Vélez-Vélez, E., **Moreno-Gómez-Toledano, R.**, Muñoz-Moreno, C., Lázaro, A., Esbrit, P., Tejedor, A. & Bosch, R. J. (2019). Urinary excretion of parathyroid hormone-related protein correlates with renal function in control rats and rats with cisplatin nephrotoxicity. *American Journal of Physiology-Renal Physiology, 317*(4), F874-F880.

El crecimiento tecnológico de las últimas décadas ha generado nuevos retos en investigación, puesto que el creciente consumo de plásticos ha expuesto a la población a nuevas moléculas con la capacidad de afectar al sistema hormonal, como el bisfenol-A (BFA). Recientemente se ha correlacionado positivamente el BFA urinario con un mayor riesgo de desarrollar diabetes y enfermedades cardiovasculares, lo que ha supuesto el punto de partida de la presente tesis doctoral, que ha pretendido explorar los posibles efectos del BFA sobre el sistema renal.

Comenzando con un modelo de estudio de podocitos de ratón, se observó que el BFA a baja concentración es capaz de inducir hipertrofia y apoptosis del podocito, alterando la expresión de las proteínas funcionales podocina y nefrina, así como de las proteínas reguladoras del ciclo celular TGF-β y $p27^{kip1}$. De manera análoga, la administración de BFA intraperitoneal en ratones indujo podocitopenia, proteinuria e hiperfiltración, así como la sobrexpresión renal de TGF-β, $p27^{kip1}$ y colágeno tipo IV. Estos datos representan la primera evidencia científica que señala que la proteinuria asociada al BFA no se limita a cambios funcionales renales, sino que implica fundamentalmente trastornos estructurales (podocitopatía).

En base a la podocitopatía, hiperfiltración y proteinuria observada en el modelo animal, alteraciones características de la nefropatía diabética (ND) precoz, se realizó un estudio comparativo de los efectos renales del BFA en ratones de ambos sexos con y sin diabetes experimental. Los animales que recibieron BFA en el agua de bebida desarrollaron cambios renales funcionales, estructurales e hipertensión, de forma similar a los animales diabéticos. Los animales diabéticos que recibieron BFA (D+BPA) presentaron un significativo aumento de la proteinuria y del número de glomérulos colapsados, en comparación con los demás grupos. Además, el empeoramiento observado en los machos D+BPA, que murieron precozmente, pudo estar relacionado con desequilibrios hidroelectrolíticos. En conjunto, nuestros datos señalan que el BFA promueve alteraciones similares a las lesiones características de la ND precoz.

El segundo modelo celular, realizado en podocitos humanos, demostró que el BFA a baja concentración es capaz de alterar la adhesión celular. Los estudios transcriptómicos y proteómicos, el Western blot y la inmunocitoquímica demostraron cambios significativos en la expresión de una treintena de proteínas estructurales y de adhesión. Además, se observó que este efecto estaba significativamente relacionado con el estrés oxidativo y con la modulación de receptores estrogénicos. En conjunto, los datos señalan que el BFA es capaz de promover un nuevo tipo de podocitopatía caracterizada por la alteración de la capacidad de adhesión celular, favoreciendo su pérdida por la orina y, por tanto, promoviendo daño renal.

Por último, se realizó un meta-análisis unificando todas las publicaciones que exploran el paradigma BFA-riñón, se analizó la mayor cohorte mundial de BFA urinario (NHANES) y se desarrolló un estudio cuantitativo del BFA urinario en la población enferma. De este modo, los resultados obtenidos, en conjunto con los modelos experimentales, han permitido obtener pruebas directas que señalan al BFA como un factor ambiental implicado en el desarrollo y/o progresión de enfermedad renal. Estos nuevos datos, asimismo, señalan la necesidad de futuros estudios traslacionales que profundicen en la relación de causalidad, con especial atención en el paciente diabético.

ABREVIATURA	SIGNIFICADO
17β-E_2	17β-estradiol
4-HNE	4-Hydroxynonenal /4-hidroxinonenal
ACR	Albumin-to-creatinine ratio / Ratio de albúmina corregido por creatinina
AM	Arithmetic mean / Media aritmética
AR	Androgenic receptor / Receptor androgénico
ASTM	American Society for Testing and Materials / Sociedad Americana para Pruebas y Materiales
BADGE	Bisphenol A diglycidyl ether / Diglicidil-éter de bisfenol-A
BFA / BPA	Bisfenol-A / Bisphenol A
BFAl	Bisfenol-A libre (sin metabolizar)
BFAt	Bisfenol-A total
BMD	Benchmark dose / Dosis de Referencia Experimental
BSA	Bovine serum albumin / Albúmina de suero bovino
BUN	Blood urea nitrogen / Nitrógeno ureico en sangre
CABP-9k	Calbindin-D9K / Calbindina-D9K
CAM	Comunidad Autónoma de Madrid
CaMKII	Calcium-calmodulin kinase II / Calcio calmodulina quinasa II
CHOP	DNA damage-inducible transcript 3 / C / EBP homologous protein
CI	Confidence interval / Intervalo de confianza
CKD	Chronic kidney disease / Enfermedad renal crónica
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CLARITY-BPA	Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity
DAB	3,3'-Diaminobenzidine / 3,3'-Diaminobencidina
DAF-DA	Diaminofluorescein diacetate / Diacetato de diaminofluoresceína
DES	Diethylstilbestrol / Dietilestilbestrol
DHE	Dihydroethidium / Dihidroetidio
DMSO	Dimethyl sulfoxide / Dimetil sulfóxido
DM	Diabetes mellitus
DN	Diabetic nephropathy / Nefropatía diabética
dsDNA	Double stranded DNA / ADN de doble cadena
EDI	Estimated daily intake / Consumo diario estimado
EE_2	Ethinylestradiol / Etinilestradiol
EED	Exposición estimada diaria
EFSA	European Food Safety Authority / Autoridad Europea de Seguridad Alimentaria
eGFR	Estimated glomerular filtration rate / Tasa de filtración glomerular estimada
EPA	U.S. Environmental Protection Agency / Agencia de Protección Ambiental de Estados Unidos
ER	Estrogen receptor / Receptor estrogénico

ERC	Enfermedad renal crónica
ERR	Estrogen-related receptor / Receptor relacionado con estrógenos
FEDER	Fondo Europeo de Desarrollo Regional
G-BPA	BPA-glucuronide / BFA glucurónido
GBM	Glomerular basal membrane / Membrana basal glomerular
GFR	Glomerular filtration rate / Tasa o Índice de filtrado glomerular
GM	Geometric mean / Media geométrica
GSEA	Gene Set Enrichment Analysis
HPLC	High performance liquid chromatography / Cromatografía líquida de alta presión o alta eficiencia
HRP	Horseradish peroxidase / Peroxidasa de rábano
ICI	ICI 182,780 / Fulvestrant
IDT	Ingesta Diaria Tolerable
ILK	Integrin-linked protein kinase / Quinasa unida a Integrinas
IP	Intraperitoneal
IR	Interquartile rank / Rango intercuartil
KIM-1	Kidney Injury Molecule-1 / Molécula de lesión renal-1
KT	Kilotoneladas
LADME	Liberación, Absorción, Distribución, Metabolismo, Excreción
LC-MS	Liquid chromatography-tandem mass spectrometry / Cromatografía líquida-espectrometría de masas en tándem
MAX	Valor máximo
MCP-1	Monocyte chemoattractant protein-1 / Proteína quimiotáctica de monocitos-1
MDA	Malondialdehyde / Malondialdehído
MDRD-4	Modification of Diet in Renal Disease / Modificación de la dieta en la enfermedad renal
MTT	Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol
NAC	N-acetylcysteine / N-acetil cisteína
NCHS	US National Center for Health Statistics
NET	Neutrophil extracellular traps / Trampas extracelulares de neutrófilos
ND	Nefropatía diabética
NGAL	Neutrophil Gelatinase-Associated Lipocalin / Lipocalina asociada a la gelatinasa de neutrófilos
NHANES	National Health and Nutrition Examination Survey
NL	Nefritis lúpica
NMDRC	Non-monotonic dose–response curves / Curvas dosis-respuesta no monotónicas
NO	Nitric oxide / Óxido nítrico
NOEL / NOAEL	No observable (adverse) effect level / Nivel al que no se observan efectos (adversos)
OC	Organic cations / Cationes orgánicos
OL-HDF	Online haemodiafiltration / Hemodiafiltración en línea

OR	Odds ratio
P95	95th percentile / Percentil 95
PBS	Phosphate-buffered saline / Tampón fosfato salino
PCNA	Proliferating cell nuclear antigen / Antígeno nuclear de proliferación celular
PE-HD	Polyethylene-high density / Polietileno de alta densidad
PE-LD	Polyethylene-low density / Polietileno de baja densidad
PEC	Parietal epithelial cells / Células epiteliales parietales
PET	Polyethylene terephthalate / Tereftalato de polietileno
PP	Polypropylene / Polipropileno
PS	Polystyrene / Poliestireno
PVC	Polyvinyl chloride / Cloruro de polivinilo
ROS	Reactive oxygen species / Especies reactivas de oxígeno
SD	Standard deviation / Desviación estándard
SDS	Sodium dodecyl sulfate / Dodecilsulfato sódico
SE	Standard error / Error estándar
SEM	Standard error mean / Error estándar de la media
SO	Superoxide / Superóxido
STZ	Streptozotocin / Estreptozotocina
TβIIR	Receptor de tipo II de TGF-β
TBS	Tris buffer saline / Tampón Tris salino
TDI	Tolerable Daily Intake / Consumo Diario Tolerable
TGF-β	Transforming growth factor β / Factor de Crecimiento Transformante β
TNF-α	Tumour Necrosis Factor alpha / Factor de necrosis tumoral alfa
TRPM2	Transient receptor potential cation channel, subfamily M, member 2 / Canal de catión potencial de receptor transitorio, subfamilia M, miembro 2
TRVP5	Miembro 5 de la subfamilia V del canal catiónico potencial receptor transitorio
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TX	Tamoxifen / Tamoxifeno
UAE	Urinary albumin excretion / Excreción urinaria de albúmina
UE	Unión Europea
UGT	Uridine diphosphate glucuronosyltransferase / Uridina difosfato glucuronosiltransferasa
VCAM-1	Vascular cell adhesion protein 1 / Proteína 1 de adhesion cellular vascular
VEGFA	Vascular Endothelial Growth Factor A / Factor de crecimiento del endotelio vascular A
WT-1	Tumor Wilms-1 / Proteína del tumor de Wilms-1

La experimentación es el método menos arrogante para obtener conocimiento. El experimentador hace, humildemente, una pregunta a la naturaleza [2]

Isaac Asimov (1920 – 1992)

El primer hombre de ciencia fue aquel hombre que contempló el interior de una cosa por el puro placer de conocer [2]

Samuel Taylor Coleridge (1772-1834)

I. INTRODUCCIÓN

1. Bisfenol-A

1.1. Introducción: Contexto histórico y actual del bisfenol-A

A lo largo del último siglo se ha producido un desarrollo tecnológico inigualable a ningún otro momento de la historia de la humanidad. Este hecho, si bien resulta excepcional, no queda exento de una problemática subyacente; y es que la sociedad moderna ha generado una dependencia hacia la industria de los polímeros plásticos. Su producción, uso, eliminación y reciclado conllevan la liberación de diversas partículas que hasta ahora nunca se habían encontrado ni en el medio natural, ni circulando por el torrente sanguíneo de la población. Desde una perspectiva cuantitativa, se han llegado a determinar más de 200 compuestos que no deberían tener cabida en el interior de ningún ser vivo [3] llamados compuestos xenobióticos[3] [4]**.** Y precisamente por su continua exposición y heterogénea distribución, cada vez más autores encuentran indicios de la posible relación entre los xenobióticos ambientales y el desarrollo de diversas alteraciones fisiológicas o patologías [5–9].

Dentro de la variabilidad de compuestos que podemos encontrar a nivel ambiental, uno de los más destacados en los últimos años es el bisfenol-A (BFA o BPA, por sus siglas en inglés) (figura 1). El BFA es un compuesto de tipo fenólico que se encuentra ampliamente distribuido debido a sus múltiples usos como monómero, aditivo y plastificante en la producción de polímeros plásticos [10]. Este compuesto puede encontrarse en una infinidad de elementos cotidianos, como pueden ser los envases y recipientes alimentarios, las tuberías de PVC, juguetes, prótesis y obturaciones dentales, aparatos electrónicos, material médico-quirúrgico e incluso en prendas de ropa [11–16]. Su importancia, además de encontrarse influida por su gran versatilidad y productividad, reside en su capacidad de modular la acción de los receptores estrogénicos; y es que el BFA es conocido por ser uno de los principales

[3] Los compuestos xenobióticos se definen como "[compuestos] químicos orgánicos que no son producidos mediante biosíntesis" o "[compuestos] químicos que no son componentes naturales del organismo expuesto a él". Traducido de [4, 5].

disruptores endocrinos que se encuentran actualmente bajo el punto de mira de la comunidad científica.

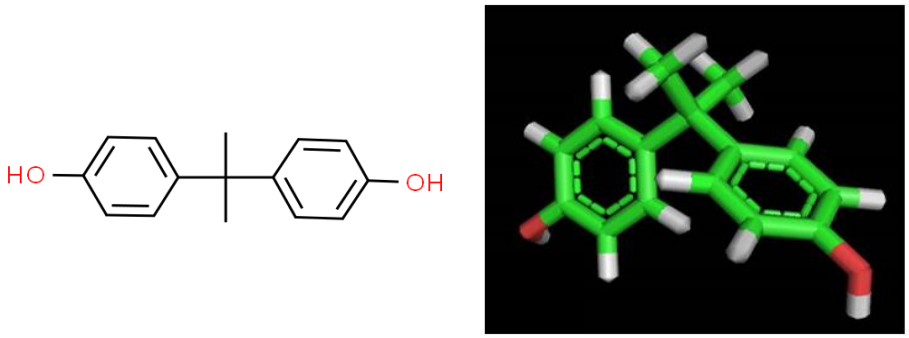

Figura 1.- Estructura química del bisfenol-A. Imagen propia realizada con el programa PyMOL (derecha) y obtenida a través de la Agencia Europea de Sustancias y Mezclas Químicas [17] *(izquierda).*

El concepto de disruptor endocrino es relativamente reciente, y pese a que desde principios del siglo XX ya se conociera la existencia de compuestos sintéticos con la capacidad de emular acciones hormonales, no fue hasta los años 90 cuando se acuñó el término. En ese momento surgió una mayor toma de conciencia sobre la posible relación entre la exposición a contaminantes ambientales y el mayor riesgo de desarrollo de patologías. La definición de disruptor endocrino fue modificándose a lo largo de los años por organismos internacionales como la *Agencia de Protección Ambiental de Estados Unidos* [18], la *Organización Mundial de la Salud* [19], la *Unión Europea* [20] o la *Sociedad Endocrina* [18]. Una de las definiciones más completas del término se encuentra en un libro recientemente publicado por Nicolás Olea [21], que manifiesta: "Los disruptores endocrinos son sustancias químicas capaces de alterar la síntesis, liberación, transporte, metabolismo, enlace, acción o eliminación de las hormonas naturales en el organismo, es decir, con la habilidad de alterar el equilibrio hormonal y la regulación del desarrollo embrionario y, por tanto, con potencial de provocar efectos adversos sobre la salud de un organismo o de su descendencia.".

Resulta de gran interés, y suscita gran controversia la dicotomía que puede observarse en la problemática del BFA; y es que pese a que es un compuesto cuyas propiedades estrogénicas fueron determinadas hace más de 80 años por Dodds y Lawson [22], su demanda y producción se ha incrementado en las últimas décadas.

Aunque el BFA era un prometedor candidato para el tratamiento de trastornos ginecológicos, siguieron probando diferentes compuestos químicos hasta que encontraron una sustancia con una actividad estrogénica mucho mayor, el dietilestilbestrol, cuya comercialización finalmente se realizó en los años 40 [23]. Es interesante destacar que se utilizó para prevenir y tratar complicaciones del embarazo hasta el año 1971, cuando se relacionó con el desarrollo de adenocarcinoma vaginal en las hijas de las mujeres tratadas con este fármaco [24].

El BFA nunca llegó a emplearse como fármaco *per se*, pero años después encontró su utilidad en la síntesis de polímeros plásticos. Pasaron cerca de 50 años desde que el químico ruso Alexander P. Dianin lo sintetizara en 1891 [25–28] hasta que el BFA comenzara a utilizarse en la manufacturación industrial de resinas epoxi, y gracias a la versatilidad de usos que poseen este tipo de resinas, el BFA rápidamente alcanzó una gran importancia en la industria americana. A mediados de los años 70 se consideraba que el BFA formaba parte, directa o indirectamente, de todas las grandes industrias de EEUU [23].

De forma paralela, las contribuciones de Schnell en 1956 demostraron que el BFA también podía utilizarse como monómero en la síntesis de policarbonatos [29, 30]. Esta clase de plásticos, debido a su única combinación de propiedades físicas, ha tenido una gran repercusión en la industria mundial, al igual que las resinas epoxi. En la actualidad, siguen empleándose en numerosas aplicaciones, como en la industria automovilística o la tecnología LED [30]. Además, el BFA se utiliza en la actualidad como aditivo en otros tipos de plásticos [31], lo que ha provocado que se encuentre presente en la mayoría de objetos cotidianos. Por ello, los estudios de mercado prevén un aumento tanto de la producción y demanda de BFA [32, 33] como de su valor de mercado [34] (figura 2). Este patrón de crecimiento también se ha descrito en el mercado de los policarbonatos [32, 35] o del fenol [36]. La consecuencia directa de todo ello es el impacto a la calidad del aire, con una liberación estimada de BFA de más de 100 toneladas anuales a la atmósfera [37].

Debido la heterogénea distribución del BFA, resulta preocupante la magnitud y el impacto potenciales que pueden llegar a tener sobre la población. En los últimos años

se ha desarrollado un sistema para el reciclado de plásticos que los clasifica en 7 categorías. En la mayoría de objetos plásticos de uso cotidiano se puede distinguir un triángulo con un número del 1 al 7 en su interior. Este código fue creado por la *Sociedad de la Industria del Plástico* [38] y mejorado recientemente en colaboración con la *Sociedad Americana para Pruebas y Materiales* (ASTM[4]), desarrollando el actual estándar de consenso voluntario ASTM D7611 [39], que incluye las siguientes categorías: 1) Tereftalato de polietileno (PET[4]); 2) Polietileno de alta densidad (PE-HD[4]); 3) Cloruro de polivinilo (PVC[4]); 4) Polietileno de baja densidad (PE-LD[4]); 5) Polipropileno (PP[4]); 6) Poliestireno (PS[4]); 7) Otros (incluyen materiales realizados con más de una resina de las categorías 1-6) (figura 3) [40].

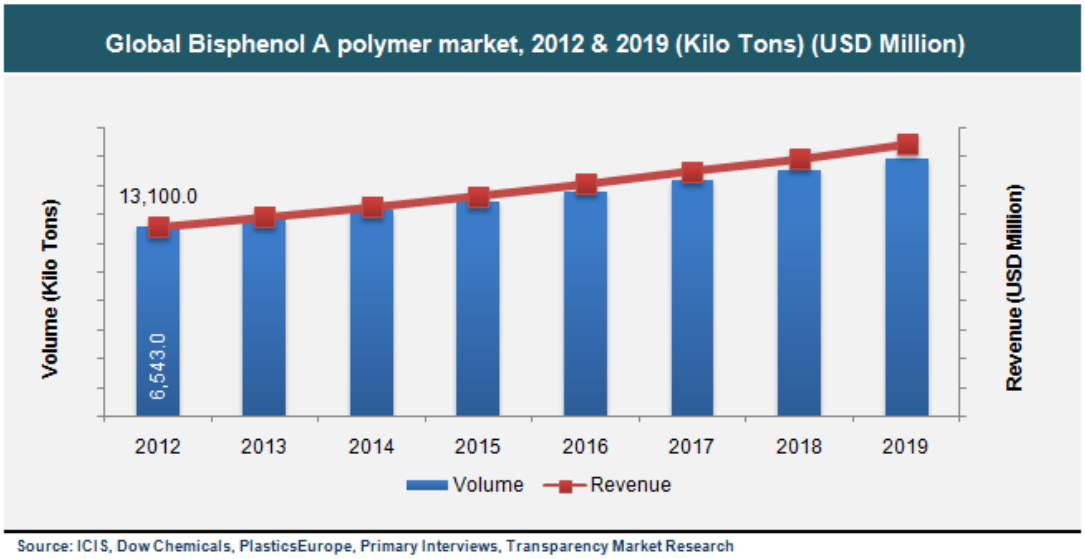

Figura 2.- Evolución del mercado de BFA en el mundo (producción y valor de mercado). Adaptado de https://www.transparencymarketresearch.com/pressrelease/bisphenol-a-market.htm.

Es habitual encontrar en la literatura académica textos que describen al BFA como el principal monómero utilizado en la producción de policarbonato, plastificante en la síntesis de resinas epoxi y aditivo en la manufactura de PVC (códigos 3 y 7) [11, 41]. A pesar de que puede utilizarse como aditivo en numerosos tipos de plásticos, habitualmente no se contempla la posibilidad de que podría encontrarse en otros materiales. De hecho, existen numerosos espacios en internet donde se afirma que ciertos polímeros son perjudiciales y otros no tanto. Estos espacios se retroalimentan

[4] Por sus siglas en inglés.

con la información de medios de comunicación que reafirman los mismos axiomas. De este modo, es común encontrar frases que inciden en la seguridad de los plásticos posicionados en las categorías 1, 2, 4 y 5, siendo los de las categorías 3, 6 y 7 los peligrosos para la salud [42–44]. Sin embargo, existen evidencias en la literatura académica de que el BFA se encuentra presente en todos los elementos del sistema de clasificación de plásticos [31, 45–49]**,** como puede observarse en la figura 3. Por todo lo anteriormente expuesto, resulta crucial entender la magnitud del grado de exposición humano y las potenciales consecuencias que ello supone.

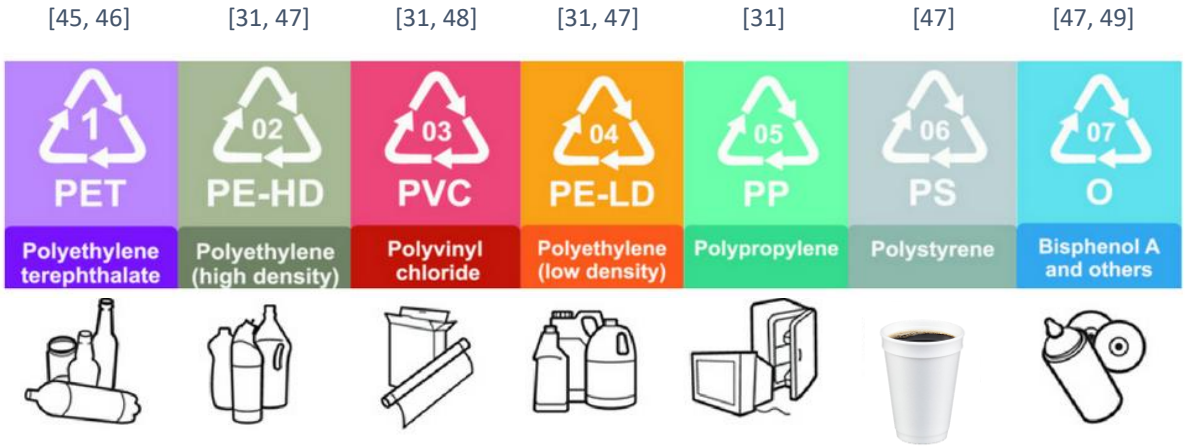

Figura 3.- Categorías del sistema de reciclado de plásticos actual y publicaciones académicas que identifican la presencia de BFA en cada una de ellas [31, 45–49]*. Las publicaciones relativas a la categoría 7 identificaron la presencia de BFA en policarbonatos* [47] *y poliamidas* [49]*. Adaptado de plasticoceans.org.*

1.2. Bisfenol-A en la población humana: Farmacocinética y exposición

Tradicionalmente se ha considerado al BFA como un compuesto de entrada oral, puesto que numerosos tipos de envases alimentarios se fabricaban con policarbonatos, y además las resinas epoxi son las responsables de que no se altere el sabor de los alimentos y bebidas enlatados [50]. Por ello, los modelos de estudio farmacocinéticos humanos han empleado generalmente esta vía de administración. Esta clase de modelos suelen utilizar un reducido tamaño muestral (n≈10), al que administran una forma modificada de BFA, permitiendo su fácil identificación y evitando de este modo la

sobreestimación con el BFA propio de cada individuo[5][51–53]. No obstante, existen otras fuentes de entrada al organismo, algunas tan importantes como la vía oral. Para profundizar en la cuestión, se procederá a abordar la farmacocinética del BFA mediante el modelo **LADME**[6]**.** En primer lugar, la **liberación** y entrada al organismo del compuesto se puede producir por diferentes vías:

1. A través de la vía oral, mediante líquidos y alimentos contaminados con BFA [54, 55] o, en el caso de los niños, a través del contacto directo con sus juguetes [56].
2. A través de la piel también podría existir entrada del BFA al organismo, fundamentalmente usando como vehículo los tickets de compra termosensibles y los cosméticos (y puesto que el agua contiene BFA, cualquier acción de bañarse o ducharse implica la exposición dérmica a este compuesto) [41, 56–58].
3. A través del aire y el polvo ambiental podría producirse la entrada inhalatoria del compuesto [41, 56, 57, 59].
4. La transmisión materno-fetal es de gran relevancia para el correcto desarrollo embrionario. Se ha detectado BFA tanto en sangre de cordón umbilical como en la placenta, líquido amniótico e incluso en el propio hígado fetal [37, 60].
5. La vía intravenosa es otra posibilidad de gran relevancia en el ámbito clínico, pues numerosos materiales médico-quirúrgicos contienen BFA en su composición [61–74].
6. Por último, también es posible que la entrada al organismo se produzca mediante la vía sublingual [56, 58, 75] (de gran relevancia en la odontología) o incluso la vía ocular [76].

Si bien se consideraba la vía oral como la mayoritaria, existen evidencias que sugieren que la vía dérmica podría tener el mismo grado de relevancia en algunos grupos poblacionales [56, 77]. Finalmente, cabe destacar la nueva posibilidad que suscitan los

[5] La forma deuterada del bisfenol-A (d(16)-bisfenol A), permite su detección específica por HPLC, obviando de ese modo el posible BFA residual y/o ambiental. El peso molecular del BPA es 228,29 g/mol, mientras que el de su forma deuterada es de 244,38 g/mol.

[6] Los modelos LADME estudian la farmacocinética de los compuestos en 5 fases: Liberación, Absorción, Distribución, Metabolismo y Excreción.

estudios relativos a los microplásticos, pues se han identificado partículas plásticas de 50 a 500 µm en heces humanas [78]. Si dichos elementos se degradaran en el interior del organismo, liberando compuestos como el BFA, supondría otra forma de entrada cuya concentración dependería de la capacidad del organismo para descomponerlos.

Continuando con la **absorción**, se estima que entre el 85 y el 100% del BFA ingerido es capaz de absorberse a través del intestino. La facilidad de absorción se ha atribuido a sus propiedades físicas, ya que el BFA es considerado como una sustancia lipofílica pero con cierta solubilidad acuosa, lo que le confiere la potencialidad de atravesar cualquier barrera fisiológica, como la transplacentaria o la hematoencefálica, y distribuirse por cualquier fluido y tejido biológico [37, 51, 55]. En el caso de la vía dérmica, la concentración estimada de BFA capaz de absorberse e introducirse en el organismo se considera en un porcentaje mucho menor, en torno al 10 % [79, 80]. Por su parte, la vía de entrada sublingual parece que podría llegar a ser tan eficiente o incluso más que la entrada intestinal, puesto que se ha llegado a observar, tras la administración de BFA por esta vía, niveles internos superiores a los observados tras la administración oral [75].

En tercer lugar, con respecto a la **distribución**, gracias a sus propiedades físicas el BFA tiene la capacidad, por un lado, de desplazarse a través de fluidos biológicos sin necesidad de un transportador, aunque puede existir una fracción unida a proteínas [76], y por otro lado, atravesar membranas lipídicas con facilidad. Gracias a ello, se ha detectado la presencia de BFA en sangre, orina, en la leche materna, líquido amniótico [37, 81, 82], numerosos órganos [83–85] e incluso se ha descrito que puede acumularse en el tejido adiposo [86, 87]. Tradicionalmente se consideraba que existían importantes diferencias en la distribución del BFA entre roedores y primates [51, 52, 55, 81, 88], pero estudios más recientes afirman que no hay diferencias significativas entre los modelos de mamíferos estudiados [89].

El **metabolismo** del BFA también resulta similar en todas las especies de mamíferos estudiadas. En todos ellos coinciden las reacciones de Fase II, que son mecanismos bioquímicos capaces de modificar la estructura de compuestos exógenos con el fin de facilitar su excreción [90]. De este modo, se ha comprobado que tanto en

el intestino como en el hígado, el BFA es metabolizado hacia formas glucuronizadas o sulfatadas, siendo la ruta mayoritaria la que lo conjuga con ácido glucurónico a través de la enzima uridina difosfato glucuronosil transferasa [90–92].

También se ha sugerido que una parte del BFA que llega al intestino podría ser degradado a p-cresol por la microbiota intestinal, del mismo modo que sucede con las proteínas alimentarias, participando de este modo en la generación de toxinas urémicas (figura 4) [93]. Otra posible ruta metabólica estudiada ha sido la hidroxilación a catecol seguido de una transformación a o-quinona. Esta ruta, al igual que la anterior, puede generar toxicidad asociada a estrés oxidativo. Se ha comprobado que pequeñas cantidades de o-quinonas son suficientes para generar elevadas cantidades de especies reactivas de oxígeno (ROS, por sus siglas en inglés) [94]. Finalmente, para terminar con el aspecto metabólico, cabe destacar que el metabolismo del BFA se encuentra fuertemente influido por el genotipo [95], el estado de salud (puede verse afectado en patologías como la enfermedad hepática, diabetes u obesidad [96]) o la vía de entrada al organismo [80, 88, 97–99].

En último lugar, con respecto a la **excreción**, se han llegado a observar importantes diferencias entre las especies estudiadas. Mientras que en primates humanos y no humanos se ha considerado que la totalidad del BFA responde a la excreción urinaria, en el caso de roedores se ha observado que una parte de la excreción se realiza a través de las heces. Tradicionalmente se ha atribuido este fenómeno a una mayor presencia de circulación enterohepática [51, 52, 55, 81, 88]**,** pero actualmente existen evidencias que desmienten dicha afirmación [89].

En humanos, se consideraba que el 100% del BFA se excretaba por vía urinaria en su forma glucuronizada [51], pero actualmente se ha demostrado que se excreta en sus formas glucuronizada (mayoritaria), sulfatada y en su forma libre (sin metabolizar) [100–102]. En cualquier caso, gracias a su eliminación urinaria es relativamente fácil calcular la exposición diaria, tal y como hemos desarrollado en un capítulo de libro recientemente publicado [64]. En este trabajo, gracias al análisis de la concentración urinaria de BFA de las publicaciones académicas, se pudo estimar la exposición poblacional al compuesto, tanto en la población general como en grupos de especial

riesgo, como en el entorno hospitalario. Sin embargo, el grado de exposición a BFA estimado en la mayoría de grupos suele encontrarse por debajo de los límites o umbrales de seguridad de este compuesto. En Europa, la *Autoridad Europea de Seguridad Alimentaria* (EFSA, por sus siglas en inglés) ha definido la *Ingesta Diaria Tolerable* (IDT)[7] en 4 µg/kg peso/día [56, 103]. Es interesante destacar que el IDT se calculó a partir de modelos animales que identificaron la concentración de BFA más baja a la que no se producían efectos sobre el peso del riñón (lo denominaron NOEL[8] renal) en 50 mg/kg [104].

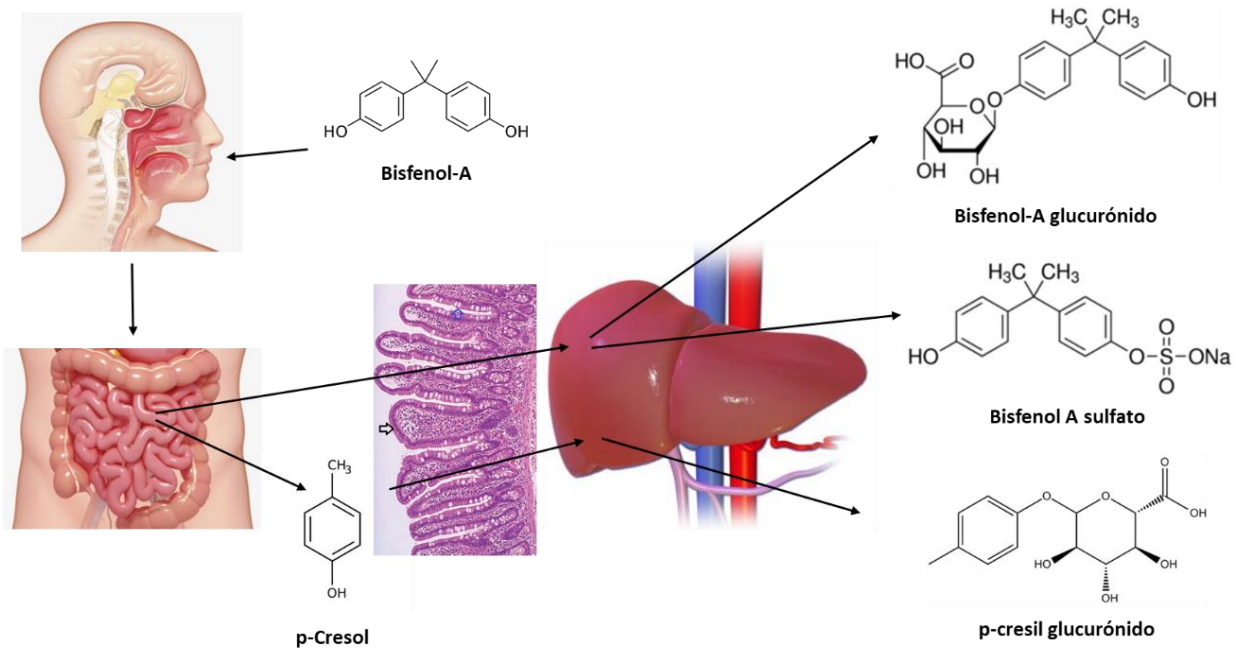

Figura 4.- Representación esquemática de las vías metabólicas del BFA. Incluye la posible degradación por la microbiota intestinal a p-cresol, una toxina urémica, y su posterior glucuronización hepática. Imagen propia.

Por último, y a pesar de la existencia de los IDT propuestos por instituciones europeas y americanas, es importante recordar que el BFA es un compuesto xenobiótico que hasta hace medio siglo no circulaba por el torrente sanguíneo de las personas. Hoy en día, la mayoría de estudios que cuantifican BFA en fluidos biológicos suelen encontrarlo en prácticamente toda la población de estudio. Por poner algunos ejemplos,

[7] Las instituciones anglohablantes utilizan el concepto *Tolerable Daily Intake* (TDI) cuya traducción sería *Consumo Diario Tolerable*. Sin embargo, en los documentos oficiales españoles como el *Boletín Oficial del Estado* se denomina *Ingesta Diaria Tolerable* (IDT) [422].

[8] No observable effect level / no observed adverse effect level. El término, si bien se refiere al mismo concepto, varía en función de las publicaciones consultadas [56, 104, 423].

se ha detectado BFA en la orina del 95 % de la población analizada en EEUU [105], del 97 % en España [106], de entre el 90 y 100 % en China [107, 108] o del 100 % en Canadá [109]. Y precisamente en los estudios humanos, cuyas concentraciones de BFA urinario suelen encontrarse por debajo del IDT, se han encontrado numerosas evidencias estadísticas que correlacionan la exposición a este compuesto con el riesgo a desarrollar diferentes patologías. Estos trabajos, en conjunto con las numerosas evidencias descritas en investigación básica, han situado al BFA en el punto de mira de la comunidad científica, motivo por el cual se ha comenzado a limitar su uso en biberones y botellas infantiles [110, 111], así como en los tickets de compra termosensibles [112].

1.3. Efectos del bisfenol-A sobre la salud

El BFA es un compuesto cuya naturaleza lipofílica le permite atravesar con facilidad barreras fisiológicas y membranas celulares, motivo por el cual se ha detectado en numerosos órganos y sistemas, desde el sistema reproductor, digestivo o excretor hasta incluso en el sistema nervioso central [83–85]. Tradicionalmente considerado como un modulador estrogénico, numerosos estudios se han realizado sobre el sistema reproductor, determinando que el BFA puede actuar como un agonista de los receptores estrogénicos (ER[9]) y como antagonista de los receptores androgénicos (AR[9]) [113, 114]. Sin embargo, su heterogénea presencia en el organismo y sus propiedades físicas lo convierten en un candidato con la potencialidad de relacionarse con numerosas patologías. En las últimas dos décadas se han publicado numerosos estudios que lo relacionan con trastornos reproductivos [115–119], cardiovasculares [9, 120–122], renales [82, 123, 124], hepáticos [125, 126], respiratorios [127–129], tiroideos [130–132], cognitivos y conductuales [133–136], con la obesidad [137, 138], diabetes [9, 139, 140], cáncer [141–143] e incluso con alteraciones del desarrollo embrionario [136, 144, 145].

Desde el punto de vista nefro-vascular, el trabajo de Lang y cols. [9] sentó las bases de nuestra hipótesis de trabajo, pues descubrió una relación positiva y estadísticamente significativa entre el BFA urinario y las enfermedades

[9] Por sus siglas en inglés.

cardiovasculares, la diabetes y la desregulación de las enzimas hepáticas en la cohorte americana NHANES (National Health and Nutrition Examination Survey). Puesto que la enfermedad renal se encuentra íntimamente relacionada con la enfermedad cardiovascular y la diabetes [146–148], el estudio del BFA en el contexto renal era una propuesta necesaria y coherente.

Hasta ese momento no se habían publicado trabajos que exploraran el potencial efecto del BFA sobre el sistema renal. Las **primeras evidencias** publicadas observaron, en diferentes **líneas celulares renales**, que el BFA a elevadas concentraciones (μM) podía afectar a la viabilidad celular [149, 150], generar estrés oxidativo [151], activar ERs o inhibir ARs [114] y modular el calcio celular [150], alterando los canales de calcio [152, 153]. Respecto a los **modelos animales**, Tyl y cols. definieron el NOEL renal [104] en 50 mg/kg en ratones adultos. A la misma dosis en adultos, y dosis inferiores en neonatos, Kabuto y cols. [154, 155] determinaron que el BFA podía generar estrés oxidativo a nivel renal. En modelos animales que utilizaron dosis superiores al NOEL, describieron alargamiento del riñón e hidronefrosis en ratas juveniles [156] y reducción en la expresión del ARNm de citoquinas pro-inflamatorias (IL-2, IL-12, IFN-γ y TNF-α) en la descendencia de animales tratados con BFA [157]. También se observó que podía afectar a la capacidad de absorción de calcio, reduciendo su concentración sérica [158, 159]. Por último, los modelos farmacocinéticos demostraron que el riñón es uno de los órganos con mayor concentración de BFA [83–85]. Los **estudios humanos** habían determinado dos interesantes evidencias: por un lado, desde el año 2001 se ha demostrado la presencia de BFA en el suero de pacientes con enfermedad renal crónica terminal sometidos a hemodiálisis, ya que el propio hemodializador libera BFA en un rango de concentración dependiente del material con que esté fabricado [70–74]. Por otro lado, los estudios epidemiológicos observaron que el BFA urinario se correlacionaba positivamente con la albuminuria de bajo grado[10] en una cohorte de niños [124] y otra de adultos [123]. También se observó una relación positiva, aunque

[10] El concepto se refiere a todos los individuos que, pese a tener unos valores de albuminuria inferiores al rango patológico (menores de 30 mg/g creatinina), se encuentran en el cuartil 4; es decir, los que presentan las mayores concentraciones de albuminuria dentro del rango normal.

inconsistente, entre el BFA urinario y la función renal[11] [160], y de manera muy preliminar, entre el BFA en sangre y la función renal[12] [73].

En la actualidad, se han publicado interesantes avances en el paradigma BFA-riñón. Comenzando con los **modelos celulares**, por un lado, se ha observado que el BFA a bajas dosis puede afectar a la viabilidad celular (exposición prolongada) [161] y promover alteraciones en la expresión y la metilación de genes asociados con cáncer [162]. Por otro lado, las dosis elevadas se han relacionado con estrés oxidativo [163–165], mecanismos pro-inflamatorios [164], respuesta fibrótica [166], mecanismos de autofagia [166], respuesta autoinmune [167] y alteración de los canales de calcio [165]. Es interesante destacar la última publicación, de Çiğ y cols. [165], puesto que la co-administración de resveratrol, un conocido antioxidante, redujo el daño oxidativo inducido por BFA. En los últimos años se han publicado numerosos trabajos sobre el uso de antioxidantes para prevenir el daño renal inducido por BFA en **modelos animales**. De este modo, se ha observado que el co-tratamiento con N-acetil-cisteína [168–171], quercetina [172–174], luteolina [175], ácido gálico [176], oleuropeína [177], hidroxicortisol [177], cúrcuma [178], curcumina [179], taurina [179], astaxantina [180], vitamina D [181] o con extracto de *Asparagus officinalis* [182], de *Genista tinctoria* [183] o de *Pistacia integerrima* [184] podría mejorar o prevenir el daño renal inducido por BFA. Evidentemente, dichos trabajos estudiaban al BFA dentro del contexto del estrés oxidativo y/o la inflamación. Otros trabajos de interés han explorado el posible efecto del BFA sobre el desarrollo embrionario renal [136, 144, 145] o el agravamiento de la enfermedad autoinmune renal [167, 185] y la respuesta inflamatoria [166]. También destacan los trabajos relacionados con la mezcla de compuestos, pues en el mundo real no sólo estamos expuestos a un único contaminante ambiental. De este modo, se ha observado que la mezcla de tetrabromobisfenol A y triclosan [186], o la mezcla de ambos junto con parabenos y pftalatos [187] puede aumentar la concentración de BFA en los riñones.

[11] En el trabajo de You y cols. [160] observaron un valor medio de BFA urinario menor en los individuos con menor función renal determinada con la fórmula MDRD, pero no tras utilizar la CKD-EPI.
[12] Murakami y cols. [73] observaron una relación negativa entre BFA en sangre y función renal (utilizando la fórmula MDRD) en 22 pacientes con enfermedad renal crónica.

Por último, es importante destacar el estudio **CLARITY-BPA**, realizado durante dos años en ratas Sprague-Dawley, a los que se administró mediante cánula oral y de manera continuada o intermitente dosis de 2,5, 25, 250, 2500 y 25000 µg/kg de BFA o dosis de 0,05 y 0,5 µg/kg de EE_2 [188]. Los autores manifestaron que las dosis inferiores a 25000 µg/kg de BFA no mostraron un claro patrón de respuestas consistente, el cual sí se llegó a apreciar tras el tratamiento con EE_2. Sólo consideraron que la dosis más elevada de BFA mostró un patrón consistente a nivel de tracto reproductor femenino y pituitaria masculina. Este trabajo se encuentra rodeado de controversia, pues existen evidencias en la literatura que sugieren que el BFA puede actuar de manera similar a una hormona, con patrones de respuesta que no corresponden con las clásicas rectas de dosis-respuesta, generando repuestas no monotónicas cóncavas o convexas [37, 189–191]. De este modo, los autores que han reevaluado los resultados del estudio desde el prisma de los efectos no monotónicos afirman que el BFA es capaz de afectar a múltiples niveles a concentraciones por debajo de las consideradas seguras [192–194].

En la población humana, nuevos estudios han profundizado en la relación entre el BFA y el tipo de hemodializador, la técnica de diálisis o los materiales implicados en el proceso [16, 66, 68, 195–199]. Es interesante destacar que el rango de concentraciones de BFA urinario o sérico en estos pacientes se ha observado en rangos cercanos a la población general [72–74, 196, 199], ligeramente superiores [195, 197] o muy superiores [66, 68]. En este último caso, se llegaron a determinar valores medios de 52.73 ± 60.6 y 163.03 ± 155.84 ng/ml en pacientes sometidos a diálisis convencional. Por su parte, los estudios epidemiológicos han encontrado nuevas relaciones entre la concentración de BFA en orina o sangre y la predisposición a desarrollar enfermedad renal. Como se detalla y analiza en el meta-análisis del capítulo 3, en los últimos 10 años se han publicado 12 artículos originales que estudian en diferentes cohortes la posible relación causal entre el BFA y la enfermedad renal. De ellas, sólo dos son estudios longitudinales, ambos realizados por Hu y cols. [200, 201], en los que observaron que los individuos hipertensos o diabéticos con elevados niveles de BFA en sangre tienen un riesgo muy superior a desarrollar enfermedad renal crónica. Los otros 10 corresponden con estudios transversales, casi todos realizados en orina [123, 124, 160, 195, 199, 202–206].

Para concluir el subcapítulo, la tabla 1 recoge las principales cifras utilizadas en la tesis para estudiar al BFA en la población humana y definir los modelos de investigación básica.

Grupo	***[BFA]***	***Unidades***	***Aplicabilidad***
NOEL renal [104]	50	mg/kg peso/día	Modelos animales y exposición humana
IDT (EFSA) [51, 80]	4	µg/kg peso/día	
IDT (FDA) [207]	50	µg/kg peso/día	
EED Pobl. general [64]	30	ng/kg peso/día	
EED Exp. ocupacional [64, 208]	5,12	µg/kg peso/día	
Pobl. general [64]	≈10	nM	Modelos celulares
Pobl. hospitalaria [64]	≈100	nM	
Exp. ocupacional [64, 208]	≈1000	nM	
Diálisis convencional [64, 68]	231 - 714	nM	
OL-HDF [64, 68]	38 – 102	nM	

Tabla 1. Principales cifras utilizadas en el estudio del BFA. Los valores indicados en los grupos poblacionales corresponden con valores medios. Abreviaturas: NOEL, nivel al que no se observaron efectos (adversos); IDT, Ingesta Diaria Tolerable; EFSA, Autoridad Europea de Seguridad Alimentaria; FDA, Administración de Medicamentos y Alimentos Estadounidense; EED, exposición estimada diaria; Pobl., población; Exp., exposición, OL-HDF, hemodiafiltración en línea.

2. Fisiología renal

2.1. Estructura y función de los riñones

Los riñones son órganos retroperitoneales situados a ambos lados de la columna vertebral, encontrándose el derecho ligeramente por debajo del izquierdo. Cada riñón posee una fina cápsula fibrosa, dos polos (superior e inferior) y, en su parte media, se encuentra localizado el hilio [209]**.** Si se visualizan transversalmente, se pueden diferenciar claramente dos regiones principales: la zona más externa, llamada corteza, presenta una coloración café-rojizo y aspecto granular debido a la abundancia de capilares. La región más profunda, denominada médula, se caracteriza por un aspecto rayado debido a la presencia de numerosos túbulos microscópicos y vasos sanguíneos. Es en esta región donde se pueden distinguir macroscópicamente las pirámides renales, las cuales se encuentran a su vez separadas por las columnas renales (figura 5) [210].

Profundizando en el interior del riñón, a nivel microscópico, se distinguen aproximadamente un millón de estructuras denominadas nefronas, que componen la unidad funcional del riñón [211–213]. Gracias a ellas, el riñón [210, 211]:

1. Regula la composición del medio interno (líquido extracelular)
2. Regula la presión arterial
3. Regula el equilibrio ácido-base
4. Posee función excretora
5. Posee función endocrina (juega un papel relevante en la síntesis de vitamina D, en la mineralización ósea y en el desarrollo eritrocitario [214])

La nefrona, a su vez, está constituida por dos elementos básicos, el glomérulo y el túbulo renal. El glomérulo (u ovillo glomerular), con casi 200 µm de diámetro, es una estructura globular formada por la invaginación de capilares en disposición hacia el extremo inicial y dilatado de la porción tubular de la nefrona (cápsula glomerular o de Bowman) [210–213]. La cápsula de Bowman y su glomérulo acompañante se sitúan en la corteza renal, y ambos constituyen el corpúsculo renal. Cabe destacar una particularidad exclusiva de esta región fisiológica, y es la disposición de los vasos

sanguíneos. Una arteriola aferente libera sangre en el glomérulo y lo abandona a través de la arteriola eferente, la cual libera la sangre en otra red capilar que circunda alrededor de los túbulos de la nefrona (capilares peritubulares) [210–213]. Existe un área, o espacio, comprendido entre la cápsula de Bowman y el ovillo glomerular, llamado espacio de Bowman. En él se forma el filtrado glomerular, y a partir de ahí penetra en la luz del primer segmento del túbulo proximal [212].

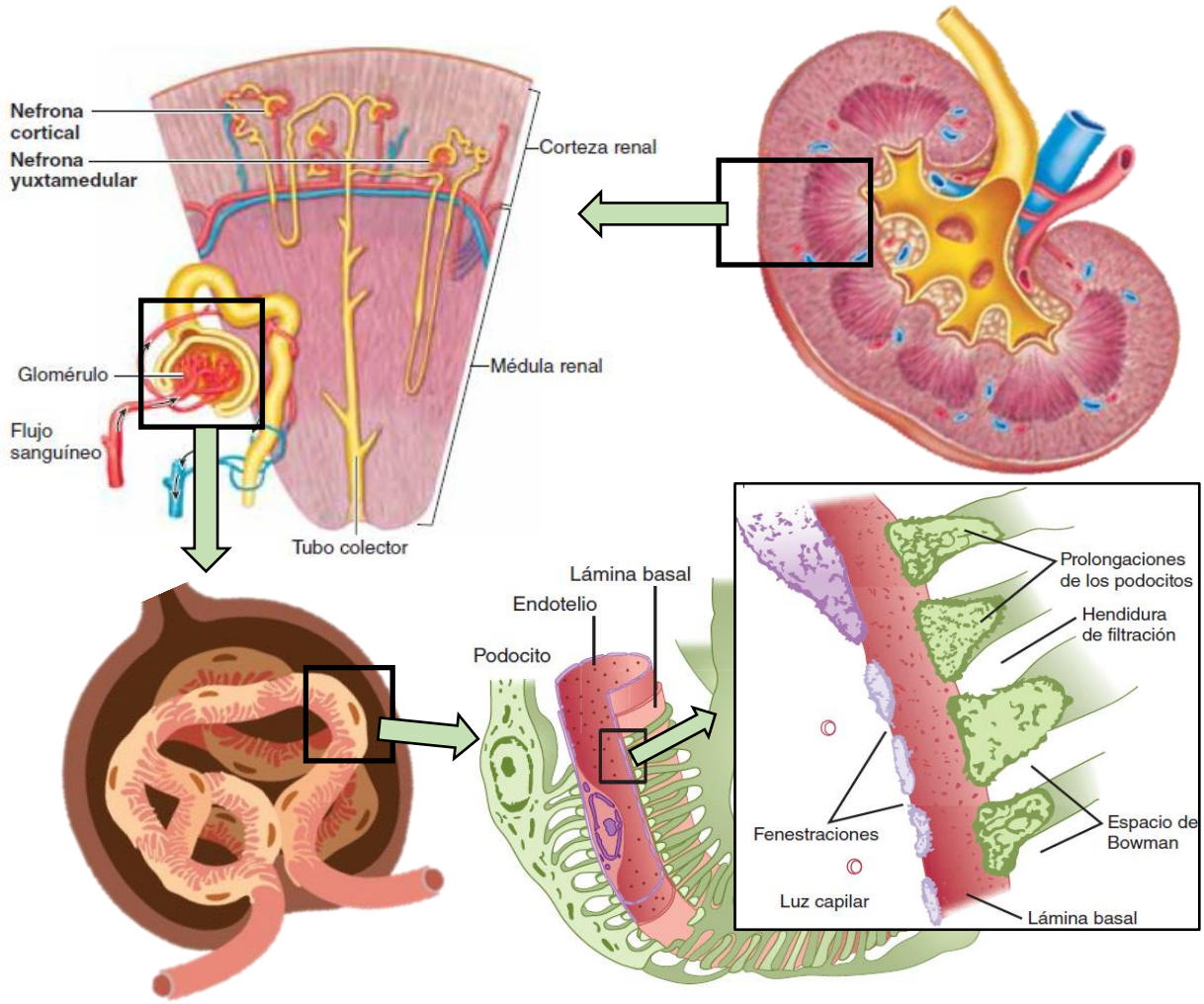

Figura 5.- Representación visual de las diferentes estructuras del riñón. Profundizando en el corte transversal del riñón se distinguen claramente dos regiones bien diferenciadas, la corteza (más externa) y la médula (interna). Profundizando aún más aparecen las nefronas, unidades básicas funcionales, cuyos corpúsculos se encuentran en la zona cortical, y sus túbulos en diferentes zonas de la región medular. Detalle de las estructuras internas del corpúsculo renal y la barrera de filtración glomerular. Las células endoteliales destacan por la presencia de fenestraciones, y las prolongaciones citoplasmáticas de los podocitos conforman y delimitan la hendidura de filtración glomerular. Adaptado de [210, 213] *y biorender.com.*

2.2. Corpúsculo renal: Elementos encargados de la filtración glomerular

La filtración glomerular depende de la superficie disponible de filtrado, así como de la permeabilidad de la barrera de filtración. Dicha barrera incluye: la capa de células endoteliales que revisten los vasos sanguíneos glomerulares, una membrana basal glomerular (GBM, por sus siglas en inglés [214]) y una capa de células epiteliales especializadas, conocidas como podocitos [211] (figura 5).

El endotelio capilar está perforado por numerosos orificios, denominados ventanas, por lo que se le considera un **endotelio fenestrado**. Como consecuencia de dicho fenómeno, los capilares glomerulares son de 100 a 400 veces más permeables al agua y los solutos disueltos en plasma que los capilares del músculo esquelético. No obstante, aunque la fenestración es relativamente grande, las proteínas celulares endoteliales están dotadas de numerosas cargas negativas fijas que dificultan el paso de las proteínas plasmáticas [210, 215].

Tras superar la primera barrera de filtración potencial, la siguiente barrera la constituye la **membrana basal glomerular**, formada fundamentalmente por colágeno tipo IV, cuya finalidad es la de proporcionar elasticidad y resistencia a la presión hidrostática. Por su parte, la membrana también contiene laminina, fibronectina y proteoglucanos con grupos heparán sulfato cargados negativamente, formando de este modo una barrera electrostática para las proteínas filtradas [211].

Por último, la tercera barrera la constituyen las células epiteliales especializadas o **podocitos**, cuyas prolongaciones o extensiones citoplasmáticas, conocidas como pedicelos (o pies), forman interdigitaciones con los de las células colindantes formando una enmarañada red. En ella existen estrechas hendiduras, sutiles espacios, entre los pedicelos adyacentes[13], por donde las moléculas del filtrado acceden al espacio de Bowman [210].

[13] A este tipo de espacios remanentes entre las interconexiones de los pedicelos podocitarios se les denomina “glomerular slit diaphragm”[424], y podemos encontrarlo traducido como “hendidura de filtración” [213] o, de manera más literal, “diafragma de hendidura” [425] o “diafragma hendido” [212].

De este modo, todos los solutos plasmáticos pasan con facilidad a través de las tres barreras de filtración potenciales e ingresan al interior de la cápsula de Bowman (figuras 5 y 6). No obstante, en el caso de las proteínas plasmáticas, existe un importante bloqueo selectivo, influido, fundamentalmente, por el tamaño, forma y carga eléctica [210, 211]. Como ejemplo, moléculas como el agua (18 Da), sodio (23 Da) o la glucosa (180 Da) poseen una capacidad de filtración de 1, es decir, la sustancia se filtra con una rapidez del 100 % del agua filtrada. Sin embargo, la albúmina (69.000 Da o 69 kDa) tiene una capacidad de filtración de tan sólo 0,005 [215]. Este ejemplo es de gran relevancia en el contexto de la enfermedad renal, pues existen numerosos estudios que afirman que la microalbuminuria es un marcador precoz de enfermedad renal (y cardiovascular) [216–220].

En el contexto global del corpúsculo renal no sólo deben tenerse en consideración los dos tipos celulares involucrados de forma directa en la barrera de filtración glomerular, pues existen otros dos tipos celulares que, si bien no se encuentran en el filtro *per se*, de su correcto funcionamiento dependerá el buen desarrollo de la actividad glomerular. Estas son las células mesangiales y las células epiteliales parietales.

Las células mesangiales son las encargadas de sintetizar la matriz extracelular, así como de generar un soporte estructural para los capilares glomerulares. Cuando se realiza el filtrado sanguíneo, numerosas macromoléculas quedan retenidas en la lámina basal glomerular. Estas células, actúan, en consecuencia, como macrófagos en las regiones intraglomerulares, fagocitando el material acumulado, evitando, de este modo, que se obstruya [221]. Algunas de estas células pueden encontrarse fuera del corpúsculo renal, formando parte del aparato yuxtaglomerular, que participa en la regulación de la presión arterial y balance hidroelectrolítico a través del sistema renina-angiotensina [222, 223].

Las células epiteliales parietales (PEC, por sus siglas en inglés) se encuentran configurando una monocapa en la capsula de Bowman, y proceden del mismo progenitor mesenquimal que los podocitos. Sin embargo, y pese al origen común, estas células son capaces de proliferar de manera normal (los podocitos tienen una capacidad muy limitada de regeneración) [224]. Es interesante destacar la existencia de estudios

que sugieren la posibilidad de que algunas células parietales pudieran diferenciarse en podocitos [225–228].

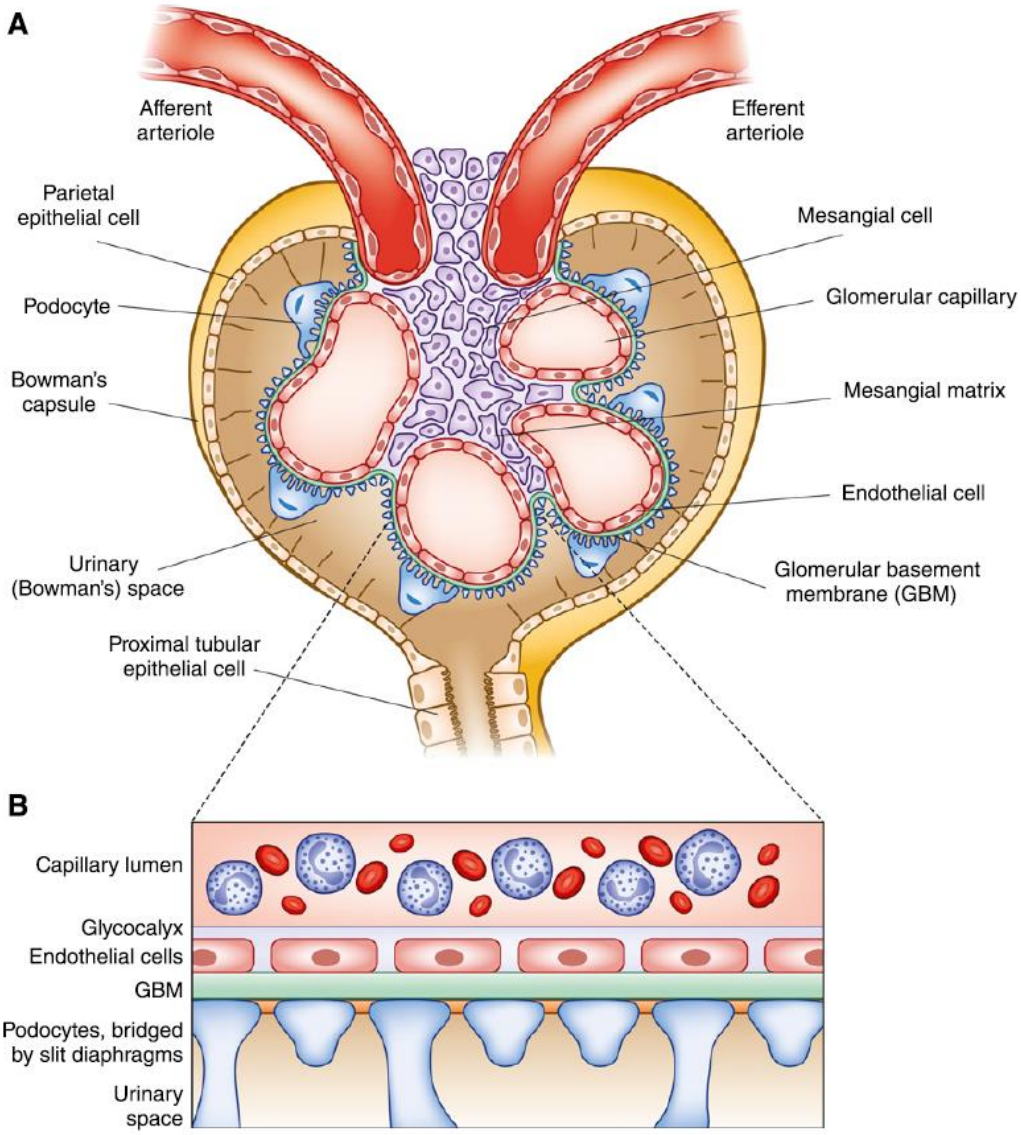

Figura 6.- Representación esquemática de las estructuras básicas del corpúsculo renal (A) y de la barrera de filtración glomerular (B). Adaptado de [224].

Continuando con la fisiología de la filtración glomerular, existen fenómenos, fuerzas físicas, capaces de favorecer o de disminuir la filtración de la sangre. Las fuerzas que favorecen el proceso son la presión hidrostática de la sangre dentro del capilar y la presión oncótica del filtrado en el espacio de Bowman (la presión oncótica suele ser insignificante por la ausencia de proteínas de medio y alto peso molecular). En contrapartida, las fuerzas que se oponen a la filtración, son la presión oncótica

plasmática dentro del capilar y la presión hidrostática en el espacio de Bowman. La relación existente entre dichas fuerzas se denomina presión de filtración neta (P_f)[14] [212]. Así pues, la cantidad total de filtrado depende de las diferencias de presiones y del coeficiente de filtrado, el cual, a su vez, depende del área capilar total disponible para la filtración, y de la permeabilidad del mismo [229]. De este modo, el riñón es capaz de filtrar alrededor de 180 litros de plasma diario (con un contenido de 10 – 16 kg de proteína) con una superficie de filtración de menos de 1 metro cuadrado [230].

Evidentemente, los tres elementos que constituyen la barrera de filtración glomerular se encuentran sometidos a las fuerzas físicas anteriormente mencionadas. Sin embargo, en la literatura académica se considera a la capa de podocitos como el elemento crítico de entre las diferentes estructuras que conforman dicha barrera [231], y es que el podocito es la célula diana en la patogénesis del síndrome nefrótico [232]. Del mismo modo, y debido a que el podocito es la célula fundamental sobre la que se centra la presente tesis, se procederá a analizar en profundidad su relevancia e impacto en la fisiopatología renal, así como las proteínas implicadas en los consiguientes fenómenos relacionados con su alteración estructural y /o pérdida de adhesión.

2.3. El podocito

El podocito es considerado como una célula altamente especializada, con una morfología única, y que, como puede apreciarse en la figura 5, se encuentra localizado en la parte externa de la membrana basal glomerular [232]. Su formación se produce dentro del glomérulo en desarrollo, a partir de un origen mesenquimal, mientras va adquiriendo, a lo largo de su maduración, algunas características de una célula epitelial [233]. Existen autores que manifiestan la particularidad de que los podocitos, aunque son conocidos como células epiteliales viscerales, poseen algunas características más propias de células mesenquimales.

Su elevado grado de especialización se encuentra atribuido a su morfología única, que consta de tres zonas o compartimentos diferentes: el cuerpo celular, los

[14] $P_f = P_{cg} - (\pi_b + P_t)$. P_f es la presión de filtrado neta. P_{cg} corresponde con la presión hidrostática capilar. π_b es la presión oncótica capilar y P_t la presión hidrostática del ultrafiltrado [212].

procesos podales primarios, que emergen del cuerpo celular, y los procesos podales secundarios[15], que se ramifican a partir de los primarios, y son los responsables de interdigitarse con los procesos podales secundarios de otros podocitos y formar, de este modo, la hendidura de filtración (figura 7) [212, 213]. Cabe destacar que existen diferencias notables en la estructura del citoesqueleto de cada tipo de proceso podal. Así, la composición del citoesqueleto de los procesos podales primarios está constituido, fundamentalmente, por microtúbulos y filamentos intermedios de vimentina, mientras que el citoesqueleto de los procesos secundarios se encuentra limitado a filamentos de actina [214, 234]. La ultraestructura del citoesqueleto podocitario, además de mantener estable la hendidura de filtración, permite estabilizar, sostener y mantener a una determinada presión el conjunto completo de la barrera de filtración glomerular, puesto que, debido a las fuerzas que interactúan con ella, necesitan un soporte adecuado a las circunstancias [235]. Los procesos podales, para ello, se unen a la membrana basal mediante un complejo entramado proteico [236].

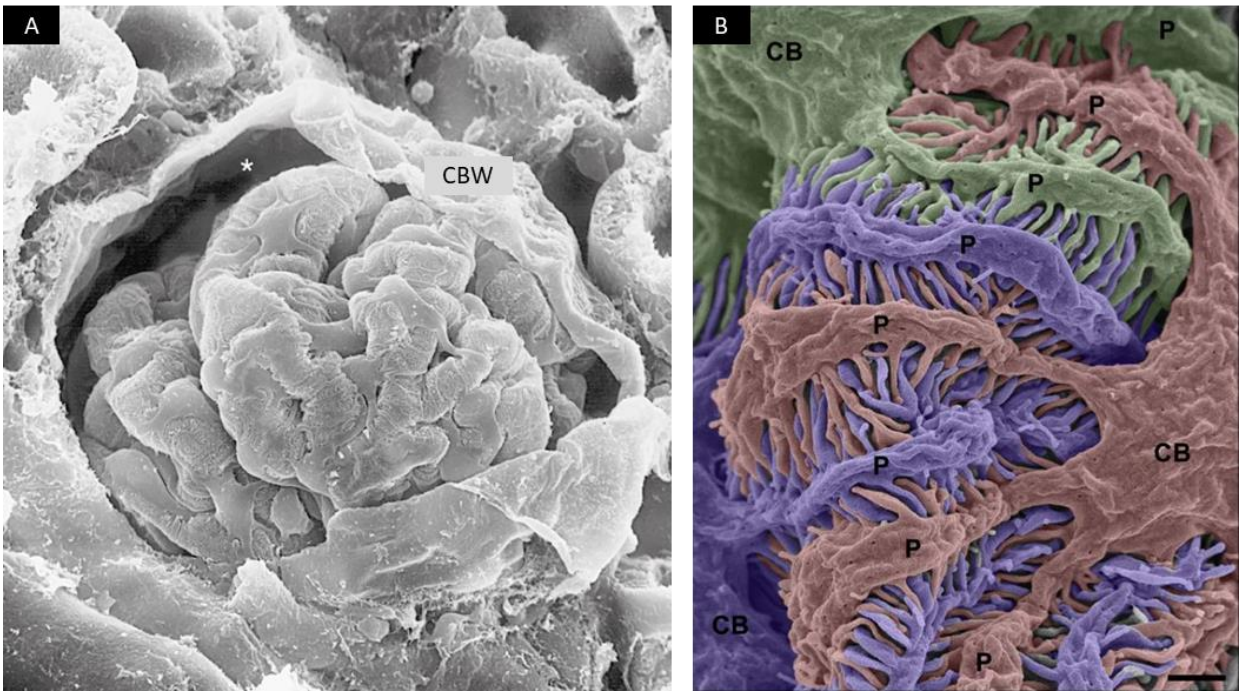

Figura 7.- Imagen de microscopía electrónica de barrido del corpúsculo renal (A) y ampliación con falso color de la hendidura de filtración glomerular (B). Significado de abreviaturas: CBW, Cápsula de Bowman; CB, cuerpo celular (cell body); P, procesos principales. El asterisco identifica el espacio de Bowman. Adaptado de [212, 237].

[15] Otros autores emplean la terminología procesos principales (major processes), para referirse a los primarios, y procesos del pie (foot processes) para referirse a los secundarios [234].

De este modo, se ha descrito que, en la hendidura de filtración glomerular, existe una red de proteínas que conectan al citoesqueleto de actina los procesos podales de los podocitos adyacentes [238]. Una de las principales proteínas implicadas en este proceso de adhesión intercelular es la nefrina, puesto que se ha demostrado que mutaciones en el gen que la codifica, NPHS1, se encuentra asociado con el síndrome nefrótico congénito, una enfermedad que manifiesta proteinuria severa o síndrome nefrótico [239]. Como puede apreciarse en la figura 8, otra de las principales proteínas implicadas en este proceso es la podocina. La podocina es una proteína de membrana con un dominio transmembrana, cuya alteración se encuentra directamente relacionada con la reorganización del citoesqueleto de actina en los procesos podales secundarios [240]**.** Estas dos proteínas, junto con las proteínas Neph1 y Neph2, forman el complejo Nefrina-Neph-Podocina, el cual coordina la dinámica del citoesqueleto [241].

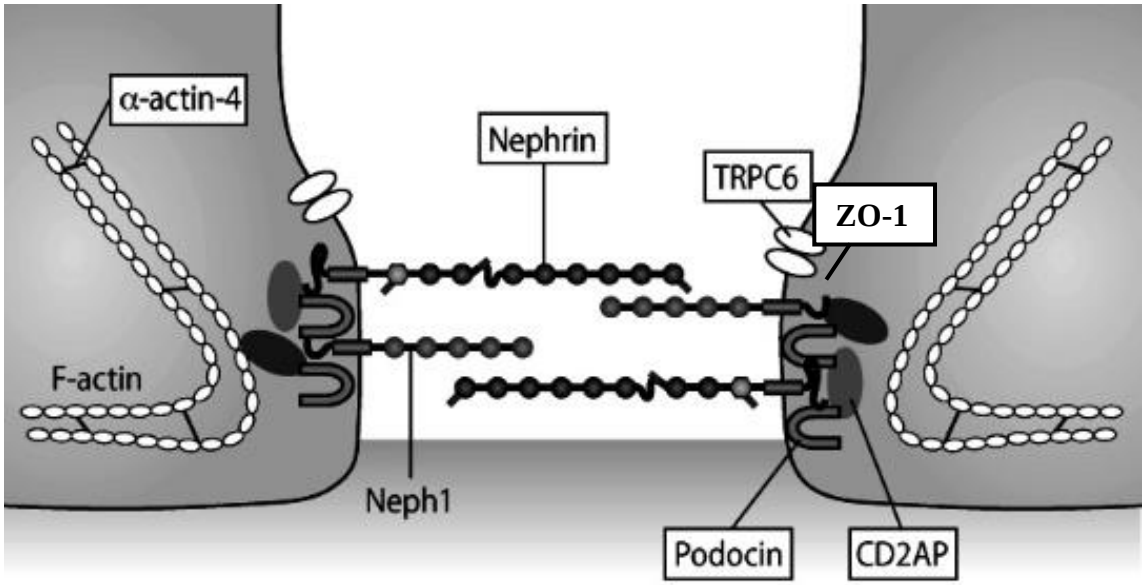

Figura 8.- Principales proteínas involucradas en los procesos de unión intercelular en la hendidura en filtración glomerular. Adaptado de [239].

Por su parte, los procesos de adhesión a la membrana basal están fuertemente condicionados por la presencia de proteínas como la integrina $\alpha_3\beta_1$ [242]. A su vez, existen proteínas capaces de interrelacionar ambos mecanismos de unión como la α-actinina-4. Esta proteína, en coordinación con la proteína ILK, son capaces de organizar, en una única estructura proteica, la integrina unida a la membrana basal, con la nefrina

implicada en los procesos de unión célula-célula, y todo ello a su vez al citoesqueleto de actina (figura 9) [243].

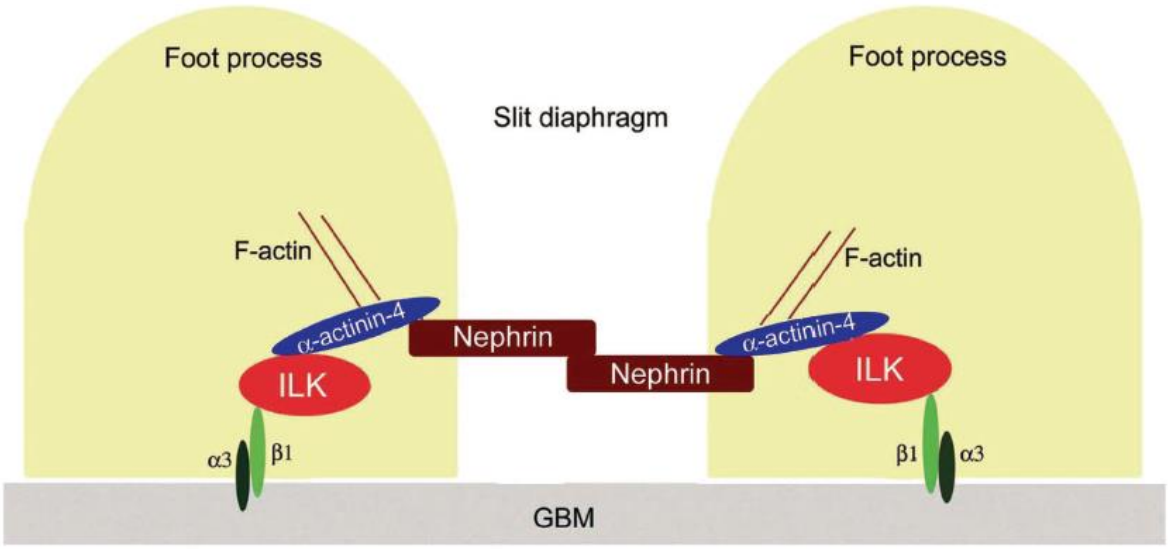

Figura 9.- Proteínas involucradas en la interconexión de las uniones intercelulares de la hendidura de filtración glomerular y de las uniones con la membrana basal al citoesqueleto de actina. Abreviaturas: GBM, membrana basal glomerular. Adaptado de [243].

Los mecanismos de adhesión, en el contexto de la enfermedad renal, son tan importantes como los mecanismos de muerte celular; y es que debido a la nula capacidad de replicación del podocito, así como la reducida tasa de renovación celular a partir de células progenitoras, un aumento en la pérdida de podocitos (por cualquier causa) supone la progresiva reducción de la capacidad de filtración glomerular, es decir, de la función renal. Se ha observado que, ante la pérdida podocitaria, se pueden estimular mecanismos de hipertrofia celular que compensen la pérdida de volumen ocupado en el glomérulo [244]. Sin embargo, cuando la pérdida llega alrededor del 40 %, los podocitos remanentes son incapaces de mantener el mecanismo compensatorio, lo que desemboca en la esclerosis del glomérulo acompañado de la degeneración tubular [231]. También se ha observado que, ante elevadas concentraciones de glucosa, se pueden activar los mecanismos de hipertrofia podocitaria a través de la vía de señalización mediada por las proteínas TGF-β y $p27^{Kip1}$ [245–249] (figura 10). $p27^{Kip1}$ interactúa con los complejos CDK-ciclina e inhibe su actividad, lo que conlleva una detención de la célula en fase G_1 y un aumento del tamaño celular, probablemente por

la acumulación de proteínas de matriz extracelular [245–247, 249–252]. Por otro lado, el aumento de la expresión de TGF-β también puede inducir mecanismos de apoptosis del podocito, lo que conduce hacia la progresión de la glomerulosclerosis [253, 254].

El podocito también se encarga de la síntesis de proteínas de la matriz extracelular, como laminina β_2 y colágeno tipo IV [$(\alpha_1 IV)_2\alpha_2 IV$], requeridas para el correcto desarrollo de la membrana basal glomerular [235]. Estas células son, además, productoras del factor de crecimiento del endotelio vascular A, proteína imprescindible para el desarrollo del glomérulo, así como el mantenimiento de la correcta funcionalidad de la barrera de filtración glomerular [224, 247, 255]. En definitiva, el podocito juega un papel crítico en los cambios estructurales y funcionales tempranos que suceden en la enfermedad renal crónica.

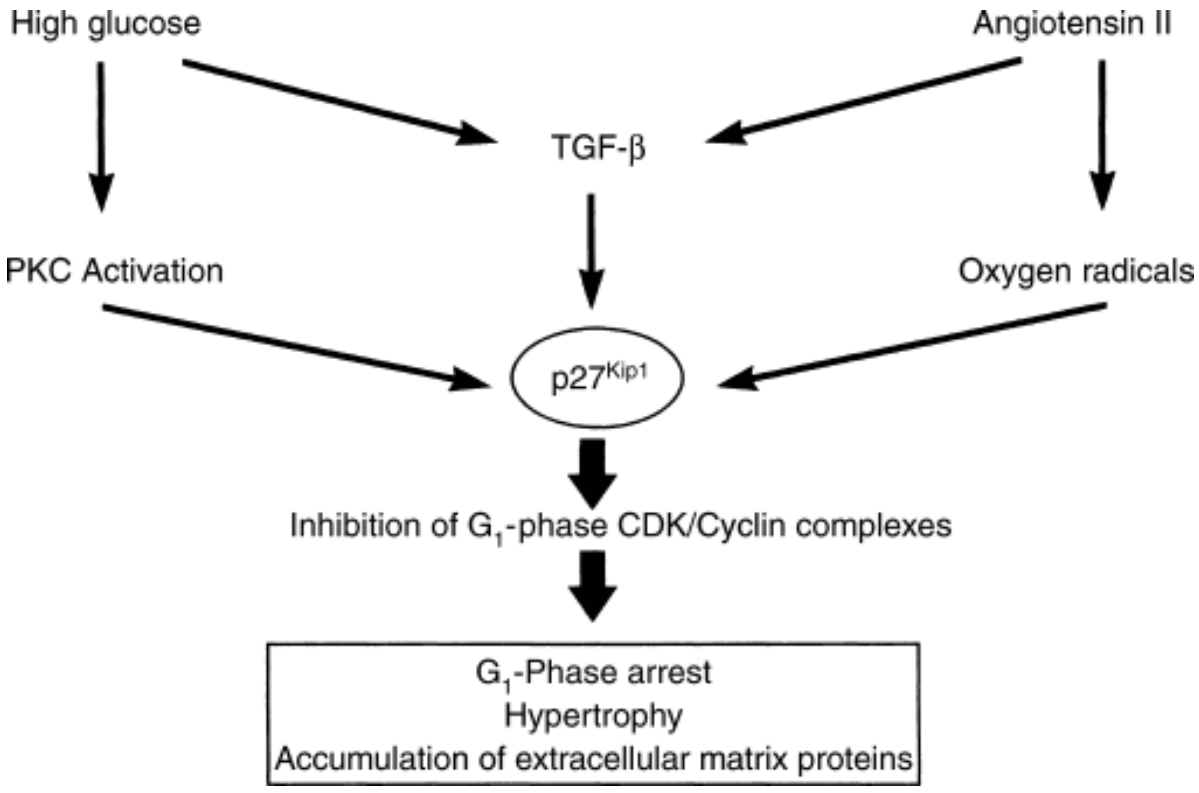

Figura 10.- Papel de las proteínas TGF-β y $p27^{Kip1}$ en los mecanismos hipertróficos de los podocitos. Adaptado de [251].

2.4. Riñón en la diabetes mellitus

La diabetes mellitus se define como un grupo de alteraciones fisiológicas caracterizadas por un estado de hiperglucemia como resultado de presentar resistencia a la insulina, una inadecuada secreción de la misma o por una excesiva secreción de glucagón [256]

Según la *American Diabetes Association*, la diabetes puede clasificarse en cuatro categorías [257]**:**

1. Diabetes tipo 1: Destrucción de las células beta del páncreas, usualmente ligado a una deficiencia absoluta de insulina.
2. Diabetes tipo 2: Pérdida progresiva de la insulina secretada por las células beta, normalmente subyacente a una situación de resistencia a la insulina.
3. Diabetes mellitus gestacional: Diabetes diagnosticada en el segundo o tercer trimestre de embarazo, sin enfermedad diabética previa.
4. Tipos específicos de diabetes debidos a otras causas: síndrome de diabetes monogénica, enfermedades del páncreas exocrino y diabetes inducida por fármacos/químicos.

El riñón diabético es extremadamente variable, pudiendo comprender desde un tamaño reducido y aspecto contraído hasta alargado e hinchado. Dicho aumento del tamaño renal se ha asociado frecuentemente con fenómenos de hiperplasia e hipertrofia. Puesto que la enfermedad renal diabética o nefropatía diabética (ND) es una enfermedad multifactorial, es coherente que exista dicha variabilidad renal; y es que se ha descrito que dichos cambios pueden atribuirse al tipo y duración de la diabetes, así como a la presencia o ausencia de hipertensión y/o infecciones [258].

La ND se define como la alteración de la función renal en pacientes diabéticos que no incluya otras causas de enfermedad renal crónica [259]. Teniendo en cuenta los cambios funcionales y estructurales que se producen en el riñón diabético, la progresión de la ND se ha organizado en cinco fases o estadios, que tradicionalmente se han definido de la siguiente manera [260]:

1. Hipertrofia renal: Caracterizada por un aumento del tamaño del riñón junto con un aumento de la filtración glomerular (hiperfiltración), sin variaciones en la excreción urinaria de albúmina. Siempre que exista un adecuado control de la glucemia, las alteraciones descritas pueden revertirse a la situación basal.

2. Lesión renal sin aparición de signos clínicos: Se produce un engrosamiento de la membrana basal glomerular a lo largo de los 2-3 años siguientes al desarrollo de la nefropatía. La excreción urinaria de albúmina permanece dentro de los límites de la normalidad, con la excepción de algunos pacientes tras la realización de ejercicio [261].

3. Nefropatía diabética incipiente: Este estadio se manifiesta por la aparición de microalbuminuria[16]. A su vez se produce un incremento en la síntesis de colágeno tipo IV de la membrana basal glomerular, favoreciendo la glomeruloesclerosis, así como la fibrosis tubulointersticial [262].

4. Nefropatía diabética establecida: Caracterizada por la presencia de proteinuria, hipertensión y reducción del filtrado glomerular por debajo de 30 ml/minuto. Es común la presencia de lesiones vasculares junto con lesiones glomerulares [263].

5. Insuficiencia renal crónica: El estadio final se caracteriza por la reducción del filtrado glomerular por debajo de 15 ml/minuto. La patología irá progresando hasta alcanzar un deterioro completo de la función renal, requiriendo tratamiento de diálisis y/o trasplante renal.

No obstante, es importante matizar en que la clasificación de los diferentes estadios en la progresión de la ND ha ido acompañada de discrepancias ante la imposibilidad de su uso en ciertas subpoblaciones de pacientes, por lo que el *Comité Conjunto de Nefropatía Diabética* (*Joint Committee on Diabetic Nephropathy*) recientemente ha propuesto una clasificación centrada en la determinación de

[16] La microalbuminuria se define como la excreción urinaria de albúmina en el rango de 30 a 300 mg/24 horas en humanos [426].

albúmina urinaria / creatinina urinaria y la tasa o índice de filtrado glomerular[17] (GFR, por sus siglas en inglés) de la siguiente manera [264]:

1. Estadio 1 (pre-nefropatía): Normoalbuminuria (<30 mg albúmina/g creatinina); GFR ≥ 30 ml/min/1.73 m^2.
2. Estadio 2 (nefropatía incipiente): Microalbuminuria (30-299); GFR ≥ 30.
3. Estadio 3 (nefropatía manifiesta): Macroalbuminuria (≥ 300); GFR ≥ 30.
4. Estadio 4 (fallo renal): Cualquier condición de albuminuria; GFR ≤ 30. Todos los pacientes con GFR menor de 30 ml/min/1.73 m^2 se clasifican en la categoría de fallo renal sin tener en cuenta los valores de proteinuria/albuminuria.
5. Estadio 5 (tratamiento de diálisis): Cualquier estado en la terapia de diálisis continua.

Continuando con el estudio de la progresión de la enfermedad a nivel histológico, en primer lugar, se produce la aparición de hipertrofia celular renal, aumento de la matriz extracelular y expansión mesangial. En estadios más avanzados de la enfermedad (ND establecida), se manifiesta la presencia de glomerulosclerosis nodular intercapilar (descrita ya en 1938 por Kimmestiel-Wilson [263]), conocida por ser la lesión más característica de la ND. También es común observar una glomeruloesclerosis difusa intercapilar generalizada, dispuesta a lo largo de todo el glomérulo. A su vez, también pueden encontrarse lesiones exudativas en la cápsula de Bowman, llamadas *gotas capsulares*, que corresponden con una masa eosinofílica de tamaño variable. La lesión exudativa eosinofílica también puede suceder en el glomérulo *per se*, denominándose lesión en gorro de fibrina (figura 11) [258, 265].

A la hora de abordar el estudio de la ND en modelos animales, existen diferentes métodos de acercamiento, como el uso de químicos o manipulación génica. En el desarrollo de la presente tesis doctoral, se procedió a utilizar estreptozotocina (STZ)

[17] En el diagnóstico de patologías como la ERC se suele utilizar la tasa de filtración glomerular estimada o eGFR, la cual se realiza a partir de la creatinina plasmática y la aplicación de factores de corrección en función de la edad, etnia y sexo [264, 427]. No obstante, el uso de la GFR se considera más preciso puesto que se determina a partir de las concentraciones plasmáticas y urinarias de creatinina, teniendo en cuenta además el volumen de orina en 24 horas.

para recrear un estado fisiológico lo más parecido posible a la diabetes tipo I, lo que promueve un daño renal en pocas semanas [266–268].

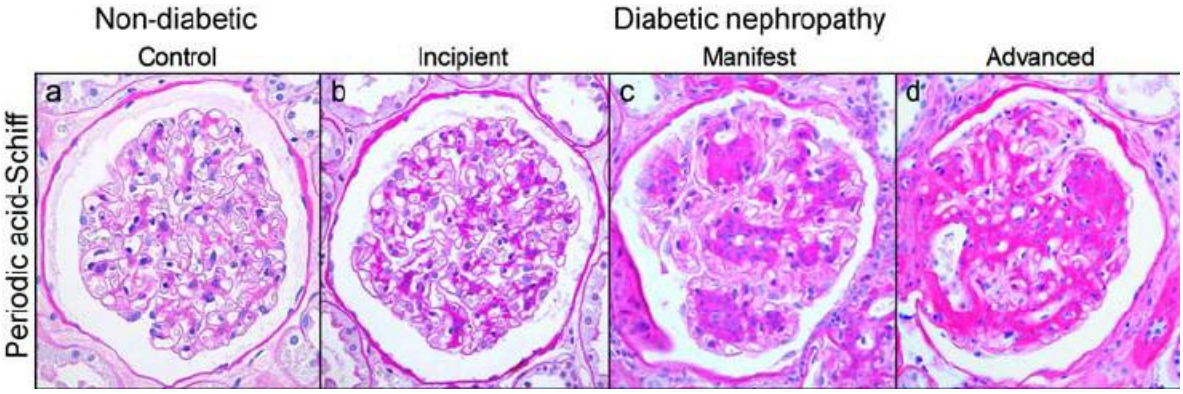

Figura 11.- Cambios estructurales manifestados en el glomérulo renal del paciente con nefropatía diabética a lo largo del transcurso de la enfermedad. Nótese el creciente aumento y expansión de la matriz mesangial, así como la progresiva aparición de esclerosis difusa y nodular, con la reducción en el número de células. Adaptado de [265].

2.4.1. Modelos animales de inducción de diabetes por STZ

La STZ es un antibiótico extraído de la bacteria S*treptomyces acromogens*. Dicho compuesto tiene la capacidad de actuar como un tóxico selectivo de los islotes de células β pancreáticos [266] y, a su vez, también puede ejercer cierta toxicidad inespecífica, especialmente administrada a elevadas concentraciones. Por ello, la tendencia actual se centra, en lugar de en la administración de una única dosis de 150 – 200 mg/kg, en múltiples administraciones de dosis más bajas (40 - 60 mg/kg) reduciendo, de este modo, la toxicidad inespecífica y la mortalidad consiguiente [267].

El efecto conseguido tras su administración, resulta en un estado muy similar a la diabetes tipo I [266–268]; y es que, como se ha descrito en la publicación de Reddy y cols. [268], tras la administración consecutiva de STZ a bajas dosis, la generación de la diabetes se establece tras un periodo de latencia de 5 a 7 días[18]. A dicho fenómeno precede una fase de aumento en la infiltración de células del sistema inmune en los

[18] Al contrario de lo que sucede tras la administración de una dosis elevada, donde la necrosis de las células beta se produce en 72 horas, con el desarrollo de la consecuente diabetes [268].

islotes pancreáticos, de forma análoga a los procesos descritos en la diabetes tipo I humana.

Por otro lado, si bien existen diversos modelos de estudio de diabetes, la administración de STZ en ratones CD1 (el modelo usado en la presente tesis), se considera, tal y como se expresa en la publicación de Kitada y cols. [267], un modelo útil para el estudio de la nefropatía diabética humana. Sin embargo, existen limitaciones asociadas a esta clase de modelos experimentales, ya que no reproduce completamente la enfermedad humana. Los animales tratados con STZ fundamentalmente desarrollan lesiones incipientes de la ND, manifestando un reducido número de lesiones funcionales y estructurales características de los estadios avanzados de la enfermedad [269, 270].

La mera formulación de un problema es muchas veces más importante que su solución. Plantear nuevas cuestiones, nuevas posibilidades, considerar problemas viejos desde un ángulo nuevo, todo ello requiere imaginación creadora y marca los progresos reales de la ciencia [2]

Albert Einstein (1879 – 1955)

Una hipótesis puede ser definida como una simple conjetura. Una hipótesis científica es una conjetura inteligente [2]

Isaac Asimov (1920 – 1992)

II. HIPÓTESIS Y OBJETIVOS

La **hipótesis general** del proyecto manifiesta que la exposición al contaminante ambiental BFA puede afectar al desarrollo y/o progresión de la enfermedad renal. Esta idea se basa en los estudios epidemiológicos que han descrito una relación significativa entre el BFA urinario y el riesgo de desarrollar diabetes y enfermedades cardiovasculares [9]. Para comprobarlo:

1. Se procederá con el estudio inicial del podocito de ratón en cultivo, puesto que nuestras primeras aproximaciones descubrieron que es la célula más sensible al BFA, una célula que participa activamente en el desarrollo de glomeruloesclerosis, nefropatía diabética o enfermedad renal crónica [232].
2. Se evaluará el papel del BFA como inductor de enfermedad renal en un modelo animal murino. Para ello, se realizará la administración por la vía intraperitoneal con el fin de identificar posibles daños estructurales en la membrana de filtración, puesto que las publicaciones académicas que estudian albuminuria y BFA urinario atribuyen su posible efecto a una alteración funcional [123, 124].

Los resultados de los primeros modelos de estudio plantearon una **segunda hipótesis general**, y es que la exposición a BFA puede generar un daño renal análogo a la nefropatía diabética, empeorando la función renal en el paciente diabético. Para investigarlo:

3. Se realizará un estudio en animales a los que se ha inducido diabetes tipo I mediante estreptozotocina. El tratamiento de BFA se realizará a través de la vía oral (agua administrada en biberones de vidrio) debido a que la vía intraperitoneal podría presentar diferente farmacocinética [55, 81, 88].
4. Se desarrollará un enfoque traslacional comenzando con el estudio del podocito humano en cultivo. Para ello, se plantearán estudios funcionales, analizando los posibles cambios fisiopatológicos inducidos por BFA en esta clase de células.
5. Por último, se realizará un análisis estadístico combinado (meta-análisis) de los estudios humanos que exploran el paradigma BFA - enfermedad renal, completando el estudio traslacional con el análisis de la mayor cohorte mundial de BFA urinario (NHANES) y el desarrollo de un estudio cuantitativo del BFA urinario en la población enferma.

III. PRÓLOGO

BISPHENOL A INDUCES PODOCYTOPATHY WITH PROTEINURIA IN MICE.

JOURNAL OF CELLULAR PHYSIOLOGY (3.839, Q1 PHYSIOLOGY).

DOI:10.1002/JCP.24665

Journal of Cellular Physiology

Bisphenol-A Induces Podocytopathy With Proteinuria in Mice

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Bisphenol-A, a chemical used in the production of the plastic lining of food and beverage containers, can be found in significant levels in human fluids. Recently, bisphenol-A has been associated with low-grade albuminuria in adults as well as in children. Since glomerular epithelial cells (podocytes) are commonly affected in proteinuric conditions, herein we explored the effects of bisphenol-A on podocytes in vitro and in vivo. On cultured podocytes we first observed that bisphenol-A—at low or high concentrations—(10 nM and 100 nM, respectively) was able to induce hypertrophy, diminish viability, and promote apoptosis. We also found an increase in the protein expression of TGF-β1 and its receptor, the cyclin-dependent kinase inhibitor p27Kip1, as well as collagen-IV, while observing a diminished expression of the slit diaphragm proteins nephrin and podocin. Furthermore, mice intraperitoneally injected with bisphenol-A (50 mg/Kg for 5 weeks) displayed an increase in urinary albumin excretion and endogenous creatinine clearance. Renal histology showed mesangial expansion. At ultrastructural level, podocytes displayed an enlargement of both cytoplasm and foot processes as well as the presence of condensed chromatin, suggesting apoptosis. Furthermore, immunohistochemistry for WT-1 (specific podocyte marker) and the TUNEL technique showed podocytopenia as well as the presence of apoptosis, respectively. In conclusion, our data demonstrate that Bisphenol-A exposure promotes a podocytopathy with proteinuria, glomerular hyperfiltration and podocytopenia. Further studies are needed to clarify the potential role of bisphenol-A in the pathogenesis as well as in the progression of renal diseases.

J. Cell. Physiol. 229: 2057–2066, 2014. *Journal of Cellular Physiology* Published by Wiley Periodicals, Inc.

Podocytopathy is a complex of cellular lesions involving glomerular visceral epithelial cells, also known as podocytes. Several proteinuric glomerular diseases result from podocyte abnormalities such as genetic, infectious, immune, and toxic aminoglucosides including diabetes mellitus, with diabetic nephropathy (DN) being the most common cause of end-stage renal disease in developed countries (Ritz et al., 1999; Shankland, 2006; Barisoni et al., 2007).

In many human glomerular diseases the degree of proteinuria correlates with a decrease in podocyte number and a reduction in nephrin and podocyn proteins in the slit diaphragm (D'Agati, 2008). Podocyte depletion is known to occur as a result of necrosis, apoptosis, and detachment or decompensated podocyte hypertrophy (Wiggins et al., 2005; Shankland, 2006). The cytokine TGF-β1, besides its well-known involvement in the mechanism of glomerulosclerosis, is also known to trigger podocyte hypertrophy by activating a cell cycle regulatory protein, the cyclin-dependent kinase inhibitor p27Kip1. Moreover, TGF-β1 is also known to be a proapoptotic factor in most cells including podocytes (Griffin and Shankland, 2008; Jefferson et al., 2008; Ziyadeh and Wolf, 2008; Diez-Sampedro et al., 2011).

In recent years, humans have suffered considerable exposure to Bisphenol-A (BPA)—an enviromental estrogen (xenoestrogen)—which is widely used in the production of polycarbonate plastic and epoxy resins for lining food and beverage containers (Kurosawa et al., 2002). It is known that BPA, particularly after oral exposure, is conjugated by the liver, thus losing its estrogenic activity, and excreted mainly through the intestine. Both non-conjugated (bioactive) and conjugated BPA (inactive) are excreted in the urine (Teeguarden et al., 2011; Gonzalez-Parra et al., 2013).

The authors have declared that no conflict of interest exists.

Contract grant sponsor: Ministerio de Ciencia e Innovación;
Contract grant number: SAF2009-12009-C02-01.
Contract grant sponsor: Instituto de Salud Carlos III;
Contract grant number: PI12/02825.
Contract grant sponsor: The Eugenio Rodríguez Pascual Foundation.

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Manuscript Received: 3 July 2013
Manuscript Accepted: 6 May 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 8 May 2014.
DOI: 10.1002/jcp.24665

The US Centers for Disease Control and Prevention found BPA present in the urine of 95% of US adults (Calafat et al., 2005). Animal studies have suggested that exposure to BPA might lead to abnormal metabolism of glucose and lipids, and exacerbate the development of cardiovascular diseases (Lang et al., 2008). The National Health and Nutrition Examination Survey (NHANES) (2003–2004 and 2005–2006) found that higher urinary BPA concentrations were associated with various diseases including cardiovascular diseases and type 2 diabetes. Moreover, a high BPA concentration up to 66.91 ng/mL (or 293 nM) has been reported in human blood (Sriphrapradang et al., 2013). Recently, exposure to BPA has been associated with low-grade albuminuria in adults as well as in children (Li et al., 2012; Trasande et al., 2013). In any case, the controversy exists by reason of a recent report claiming that the benefits of BPA far outweigh the risks (Tyl et al., 2008). Since no studies have examined the potential role of BPA in the pathogenesis of renal diseases, in this study we assessed the effects of BPA on glomerular podocytes in vitro as well as in vivo.

Materials and Methods

Cell cultures

Conditionally immortalized mouse podocytes were cultured as previously reported (Izquierdo et al., 2006; Romero et al., 2010). In brief, podocytes were cultured on type I collagen (Sigma) and grown in RPMI 1640 medium with 5% FBS and antibiotics, supplemented with 10 U/ml recombinant interferon-γ to enhance T antigen expression, at 33 °C (permissive conditions). To induce differentiation, podocytes were maintained on type I collagen at 37 °C without interferon-γ (nonpermissive conditions). Differentiation of podocytes was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by immunocytochemistry.

To analyze podocyte hypertrophy and viability as well as apoptosis, we used a BPA concentration previously described by several investigators as low or high BPA concentrations—10 nM and 100 nM—, respectively (Wetherill et al., 2007; Benachour and Aris, 2009; Qin et al., 2012). Both of these concentrations were lower than the concentration recently found by Sriphrapradang et al. (2013) in human blood. Protein expression was analyzed by Western blot on podocytes cultured in the presence of the above mentioned BPA concentrations. In all cases BPA was dissolved in DMSO and then added to the culture medium, reaching a DMSO final concentration of less than 0.05%. Control cells were incubated in culture medium with the addition of the same concentration of DMSO.

Measurement of podocyte hypertrophy

We analized the effect of low and high BPA concentration on podocyte hyperthropy by studying cell hypertrophy index, [H^3]-leucine incorporation, as well as by analyzing podocyte surface area. To study cell hypertrophy, index podocytes were trypsinized, washed with phosphate-buffered saline (PBS), and counted using a Neubauer hemocytometer. Equal numbers of cells were lysed in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1.0% Nonidet P-40, in PBS) and the total protein content was determined by the Bradford's method (Pierce, Rockford, IL), using BSA as standard. Total protein was expressed as percentage of hypertrophy index (micrograms of protein per 10^4 cells) as previously reported (Romero et al., 2010; Ortega et al., 2012). To determine [H^3]-leucine incorporation, podocytes were pulsed with 2 μCi/mL [H^3] leucine, washed with PBS, solubilized with 0.1% sodium dodecyl sulfate (SDS) and transferred into a tube containing 10% bovine serum albumin (BSA). Precipitated proteins (20% TCA) were centrifuged at 2000 g for 30 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in 0.5 N NaOH and counted in a scintillation counter (Romero et al., 2010). To evaluate the differences in the podocytes' size after BPA treatment, podocyte surface area was determined by using the stereologic software Motic Images Advanced 3.2 (Motic China Group Co, Ltd, Hong Kong, China). This program allows the selection of fields to be studied by random systematic sampling after the input of an appropriate sampling fraction. An average of 10 fields per well was scanned using the X20 objective.

Western blot analysis

After electrophoresis of total cell proteins, samples were immunoblotted as previously reported (Romero et al., 2010; Ortega et al., 2012). Membranes were then incubated overnight at 4 °C with the following rabbit polyclonal antibodies [dilution, -fold]: anti-p27Kip1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) [500], anti-TGF-β1 antibody (Santa Cruz Biotechnology) [300], and anti-TβIIR antibody (Santa Cruz Biotechnology) [500]. A mouse monoclonal anti-tubulin (Sigma–Aldrich, Saint Louis, MO), at a 1:1000-dilution, was used as an internal loading control. Blots were analyzed by densitometric scanning with Scion Image Program for Windows Release 4.0.2 (Scion Corp., Frederick, MD). Densitometric values were normalized against those of tubulin as previously reported (Romero et al., 2010; Ortega et al., 2012). Western blot studies in cultured cells were performed in at least three independent experiments and a representative figure is shown. In all cases values were normalized with respect to the corresponding control (100%) and the relative expression of each protein was expressed as percentage of changes with respect to those of the control as previously reported (Ortega et al., 2012).

MTT cell viability assay

After treatment, 100 μl of MTT (5 mg/ml) was added to each well in 1 ml of medium, and the plates were incubated for 1 h at 37 °C. Then, isopropanol was added to solubilize the cells. The absorbance was measured at a test wavelength of 570 nm with a reference wavelength of 690 nm.

Animal model

In all of the experiments below, CD1 mice (25–30 g) were used. All studies were performed in accordance with guidelines established by Institutional Animal Care and Use Committees at the University of Alcalá. Mice were housed in a temperature-controlled room (21 ± 2 °C) on a 14/10 h light/dark cycle under pathogen-free conditions and with free access to food and water (Romero et al., 2010). Even though the frecuency (or timing) of BPA exposure is unknown, it is improbable that it would occur in a significant level on a daily basis. Thus, to analyze the renal effects of BPA on the whole animal, mice (n = 17) were intraperitoneally injected with BPA (Sigma, Saint Louis, MO) at 50 mg/Kg—dissolved in oil—once a day from Monday through Friday for 5 weeks. Control mice (n = 6) were treated equally but injected only with oil. We selected this dose of BPA because it has been shown to affect several tissues, including the kidney (Kabuto et al., 2003). This experimental approach has also been reported by several investigators (Pottenger et al., 2000; Naciff et al., 2002; Zalko et al., 2003; Takeuchi et al., 2006; Richter et al., 2007).

Mice were placed in metabolic cages and 24-h urine was collected for creatinine and protein measurement as previously reported (Izquierdo et al., 2006). Blood was taken by cardiac puncture under ether anaesthesia, for creatinine and glucose measurements. One kidney of each animal was removed, weighed, frozen in liquid nitrogen, and stored at −80 °C for subsequent total protein extraction. The remaining kidney of each animal was weighed and fixed in 10% buffered formaldehyde for morphological and immunohistochemistry studies. The degree of renal hypertrophy was expressed as an index, the ratio of kidney weight to total body weight.

Blood BPA measurement

BPA was analyzed in plasma by liquid chromatography-tandem mass spectrometry (LC-MS) with a detection limit of 10 ng/ml. For LC-MS 20 μl of plasma was added with 20 μl acetonitrile, vortexed and centrifuged at 13000 rpm. Then 20 μl of methanol solution containing 5 ppm of the deuterated BPA was added, and after vortexing and centrifugation the clear supernatant was analyzed (Volkel et al., 2005).

Renal histology, immunohistochemistry and electron microscopy

Renal tissues were routinely processed, embedded in paraffin, and 5 μm sections were obtained. To evaluate the possible alterations present in the kidneys, sections were stained with PAS-Hematoxylin and/or PAS-Alcian blue. In order to evaluate the modifications in the mesangial area, 10 randomly selected glomeruli in the cortex per animal (total 100 glomeruli for each group) were examined under high magnification (X400). Glomerular tuft area was measured by manually tracing the glomerular tuft using an automatic image analyzer (Motic Images Advanced 3.2). Mesangial matrix area was defined as the PAS-positive area within the tuft area. The mesangial matrix index represented the ratio of mesangial matrix area divided by the tuft area. The results are expressed as means ± SEM (in micrometers squared).

For immunohistochemistry, samples were treated as previously reported (Ortega et al., 2012), using a rabbit anti-Wilms' tumor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1500. The number of podocytes WT-1 labeled per renal corpuscle was calculated in each section. Measurements were carried out using an Olympus microscope equipped with a X20 lens and using the stereologic software Motic Images Advanced 3.2. An average of 20 fields per section was scanned. The systematic field selection with a random start assures that the number of podocyte estimates were representative of all the kidneys.

For electron microscopy, small kidney fragments (1 mm^3) were fixed for 6 h at 4 °C in a mixture of 2.5% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde buffered with 0.1 M phosphate, at pH 7.4. After rinsing in PBS, samples were post-fixed in buffered 1% osmium tetroxide at 4 °C for 2 h and dehydrated through a graded acetone series and embedded in araldite according to conventional methods. Tissue blocks were sectioned and serial ultrathin sections of similar thickness producing gold interference color were collected on nickel grids. Staining was carried out with saturated solution of uranyl acetate in distilled water for 20 min, followed by Reynold's lead citrate for 10 min. Sections were observed in a Zeiss EM10 transmission electron microscope.

TUNEL assay

For the detection of apoptotic cells the DeadEndTM Fluorometric TUNEL System was applied (Promega, Madison, WI) to paraffin-embedded sections and cell culture according to the manufacturer's instructions. Then, the sections were incubated with an anti-FITC-HRP for 30 min, diluted 1:500, and the reaction product in nuclei was developed with DAB. In renal tissues, to confirm what cell type suffered an apoptotic process, we performed, after TUNEL assay, an immunostaining by using podocin (a specific marker of podocyte processes), diluted 1:400, and amplified with ABC-Complex-AP (Dako) and developed with Fast Red (Zymed).

Statistical analysis

All results are expressed as mean ± SD. $P < 0.05$ was considered statistically significant. The Kruskal–Wallis test was used to compare differences among samples in all cases, with the exception of samples in which there were renal histological changes, which were analyzed by the paired *t*-test.

Results

BPA induced hypertrophy on podocytes

We first investigated the potential role of BPA on podocyte hypertrophy, which is an early event in the mechanism of podocyte injury (Wiggins et al., 2005). To this end, we determined the hypertrophy index after the addition of BPA for 5 days. As shown in Figure 1A, BPA induced hypertrophy of podocytes. We then determined the incorporation of [H^3]-leucine, a measure of protein synthesis. Exposure of podocytes to BPA stimulated [H^3]-leucine incorporation (Fig. 1B). To further confirm podocyte hypertrophy, we evaluated podocyte size by studying cell surface area. We observed that BPA at both low and high concentrations significantly increased podocyte surface area (Fig. 1C). Although low BPA dosis

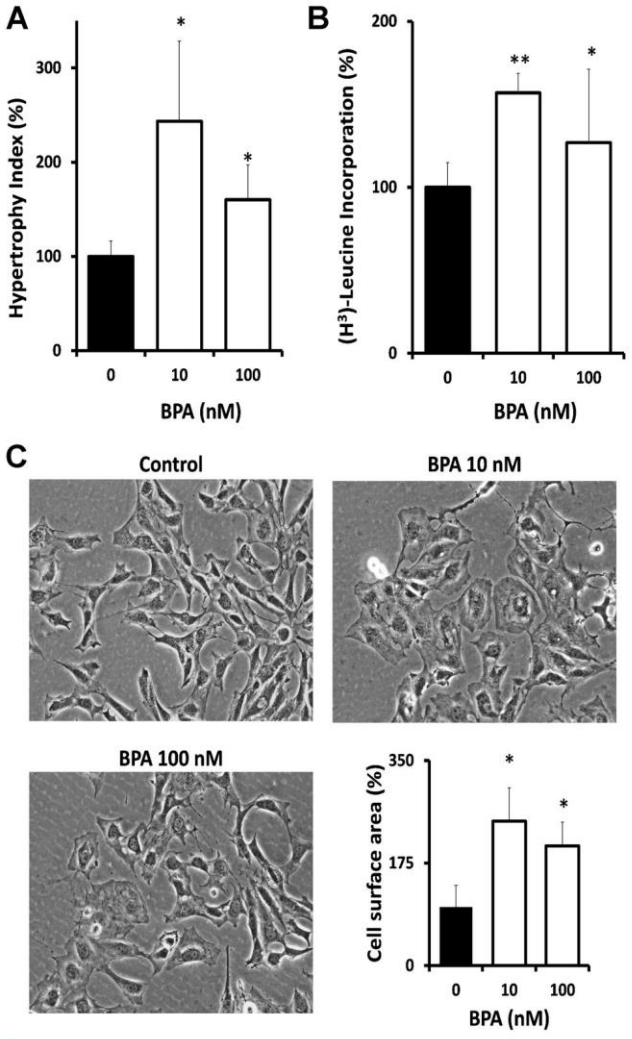

Fig. 1. BPA induces podocyte hypertrophy. (A) hypertrophy index, (B) [H^3]-leucine incorporation, (C) microphotographs of podocytes incubated with low and high BPA concentration as well as planar podocyte surface area analysis (X200). Data are the means ± SD of three different experiments, each performed in duplicate. *$P < 0.05$ and **$P < 0.01$ using ANOVA test for the comparison between control and BPA-treated cells.

tended to be more effective to increase podocyte protein synthesis, it did not reach statistical significance.

Effect of BPA on renal cell viability and apoptosis

We then investigated whether BPA affects podocyte viability, which is known to occur in several podocytopathies including decompensated podocyte hypertrophy (Wiggins et al., 2005). We found that viability significantly diminished after 9 days of BPA exposure at both low and high concentration (Fig. 2A).

We then explored whether apoptosis was involved in the mechanism by which BPA affects podocyte viability. We found that both low and high BPA concentrations were able to induce podocyte apoptosis after 9 days in culture (Fig. 2B). Although no statistically significant differences on both podocyte viability and apoptosis between low and high BPA doses were found, it is possible to speculate that longer BPA exposure may promote them.

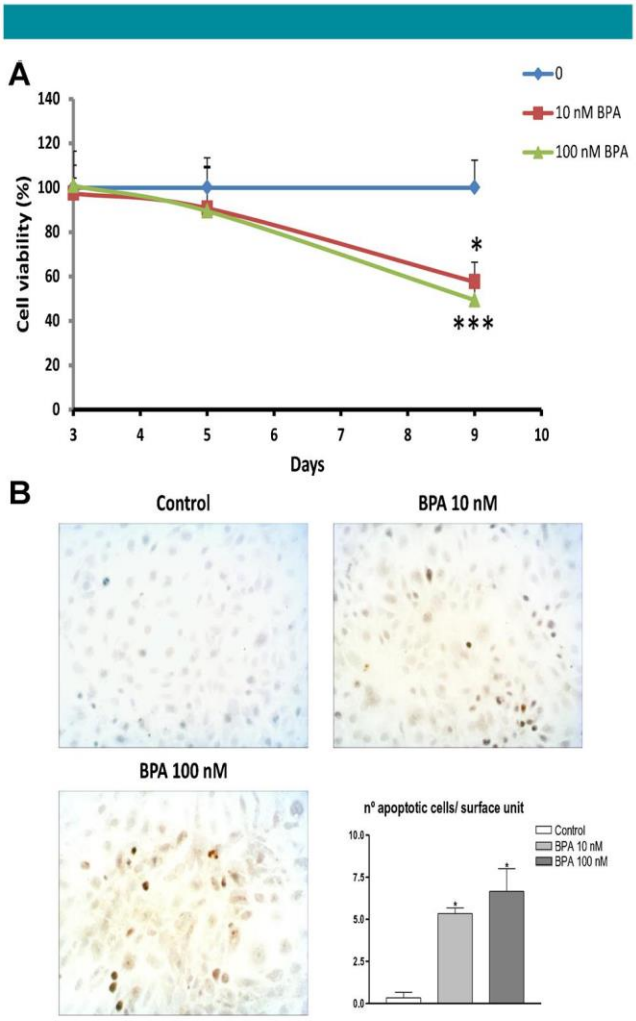

Fig. 2. Effect of BPA on podocyte viability. (A) Mouse podocytes were incubated with different doses of BPA during 3, 6, and 9 days, and podocyte viability was assayed by MTT. Data are the means ± SD of three different experiments, each performed in duplicate. *$P < 0.05$ and **$P < 0.01$ using ANOVA test for the comparison between control and BPA-treated cells. (B) Representative images of TUNEL assay in control, BPA 10 nM and BPA 100 nM treated cells (X300). The histogram shows statistically significant differences between control and treated cells. *$P < 0.05$ using ANOVA test for the comparison between control and BPA-treated cells.

BPA as a modulator of the expression of the TGF-β1 system as well as the cyclin-dependent kinase inhibitor p27Kip1

To analyze the mechanism involved in BPA-induced podocyte hypertrophy, we studied the expression of TGF-β1, which is a well established factor in the development of renal cell hypertrophy (Wolf and Ziyadeh, 1999; Romero et al., 2010). We found that BPA was able to induce an increase in the expression of TGF-β1 as well as its receptor—TβIIR—in cultured podocytes (Fig. 3A and 3B, respectively). We then studied the expression of the cell cyclin-dependent kinase inhibitor p27Kip1, a known downstream effector of TGF-β1 (Romero et al., 2010). As shown in Figure 3C, BPA was found to induce p27Kip1 significantly.

BPA stimulates collagen IV production in podocytes

Glomerulosclerosis, including an increase in collagen IV, is characteristic of several podocytopathies where TGF-β1 is known to play a key role in promoting collagen IV upregulation (Wolf and Ziyadeh, 1999; Romero et al., 2010). Thus, we studied the potential effect of BPA on collagen IV stimulation in cultured podocytes. As shown in Figure 3D, BPA is able to significantly increase collagen IV production on cultured podocytes.

BPA downregulates the slit diaphragm proteins nephrin and podocin

In order to gain insight into the cellular effects of BPA on podocytes, we analyzed the expression of the slit diaphragm proteins nephrin and podocin, which are known to play a key role in the mechanism of protein filtration (D'Agati, 2008; Coward et al., 2005). Downregulation of these proteins has been involved in the mechanism of proteinuria as well as in podocyte viability. As shown in Figure 4, BPA was able to downregulate the protein expression of both nephrin and podocin. This result suggests a mechanism by which BPA might induce both podocytophathy and proteinura.

Blood glucose and BPA quantification in experimental animals

Blood glucose and BPA concentration were analyzed in all experimental animals. There were no significant differences in fasting blood glucose levels between control (C) 73 ± 4 mg/dl vs 76 ± 4 mg/dl BPA-treated mice.

Non conjugated BPA concentration in the blood of control (C) animals was 29.04 ± 6 ng/ml vs BPA-injected animals 80.07 ± 31 ng/ml, $P < 0.05$; while plasma conjugated BPA was undetectable in control animals, it was only detected in two of eight BPA-injected mice (123,5 ± 7 ng/ml).

BPA induces proteinuria, renal hypertrophy and glomerular hyperfiltration

As expected from in vitro data showing early changes observed in several podocytopathies, BPA-injected mice (n = 17) displayed an increased urinary albumin excretion (controls (C) 2.31 ± 1.4 mg/24 h vs BPA 5.13 ± 1.5, $P < 0.05$); an increase in the hypertrophy index (C 0.60 ± 0.06 mg/g vs BPA 0.68 ± 0.04, $P < 0.05$); as well as an increase in the glomerular filtration rate (C 0.05 ml/min vs BPA 0.08, $P < 0.05$) as assessed by the endogenous creatinine clearance.

Renal morphological changes induced by BPA

Changes in glomerular morphology were studied using two different staining methods: PAS-hematoxilin and PAS-alcian

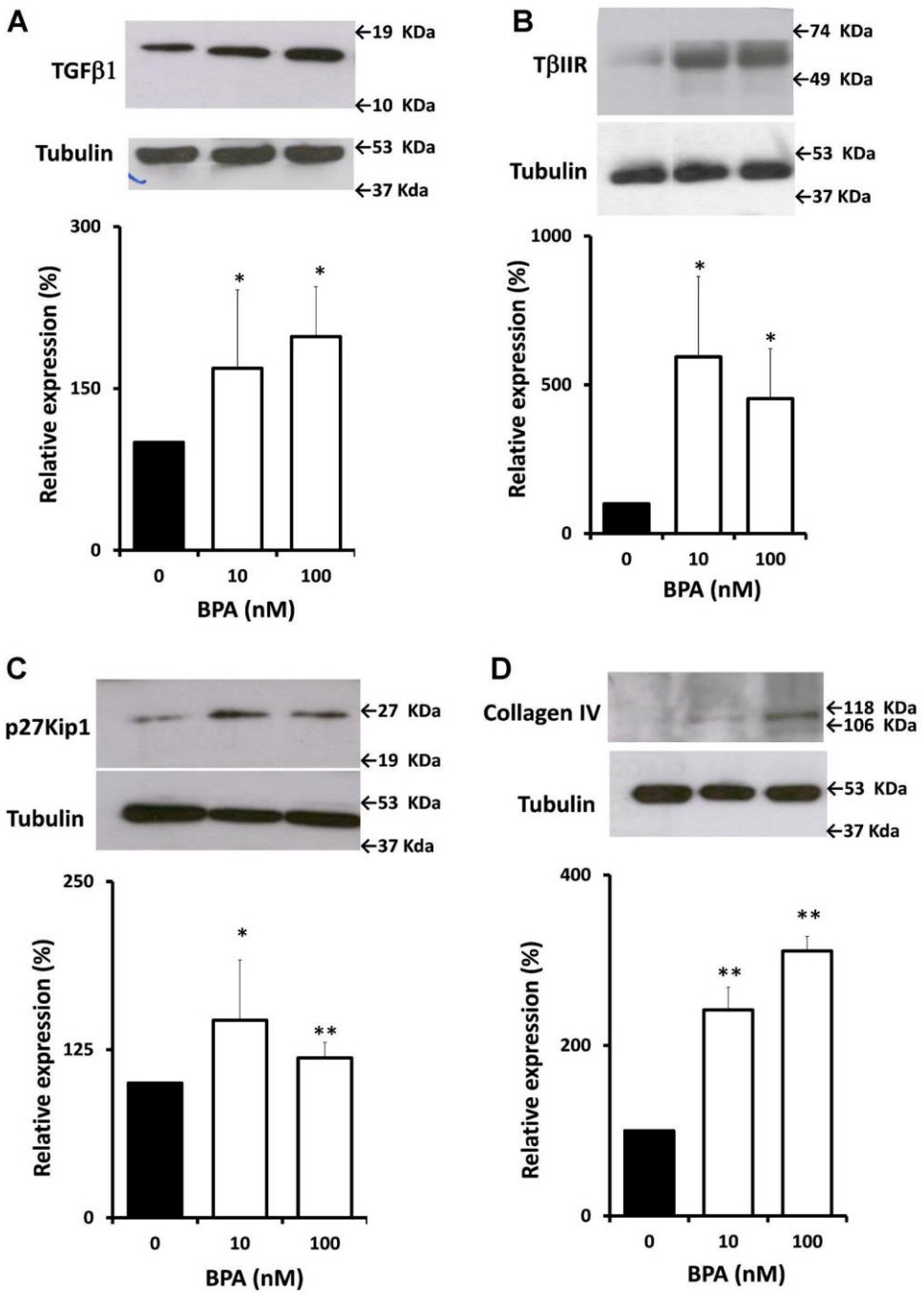

Fig. 3. Effects of BPA on the expression of TGFβ1 system, p27Kip1 and collagen IV on cultured podocytes. Protein TGFβ1 expression (A), TβIIR expression (B), p27Kip1 expression (C), and collagen IV expression (D) were analized by Western blot. Data are the means ± SD of three different experiments, each performed in duplicate. *$P < 0.05$ and **$P < 0.01$ using ANOVA test for the comparison between control and BPA-treated cells.

blue. We observed that BPA-injected mice showed a significant increase in the glomerular mesangial matrix area (Fig. 5A,B,C).

At ultrastructural level, the kidney of BPA-injected mice displayed podocyte cytoplasmic and foot processes enlargement (Fig. 5C and E), as well as the presence of condensed chromatin, suggesting apoptotic images. This figure also shows the presence of abundant collagen fibers (Fig. 5F).

Quantification of podocyte cell numbers, using WT-1, as specific podocyte marker, showed a statistically significant decrease of these cells in the renal corpuscles of BPA-injected mice (Fig. 6A and C, respectively).

Using the TUNEL assay, the kidney of BPA-injected mice showed the presence of apoptotic cells in the glomeruli of these animals; besides showing both podocyte morphology and location, the presence of apoptotic podocytes was further demonstrated by the costaining with podocin (a specific podocyte marker). Other epithelial cells were also affected, which might require future studies (Fig. 6B and C). These data

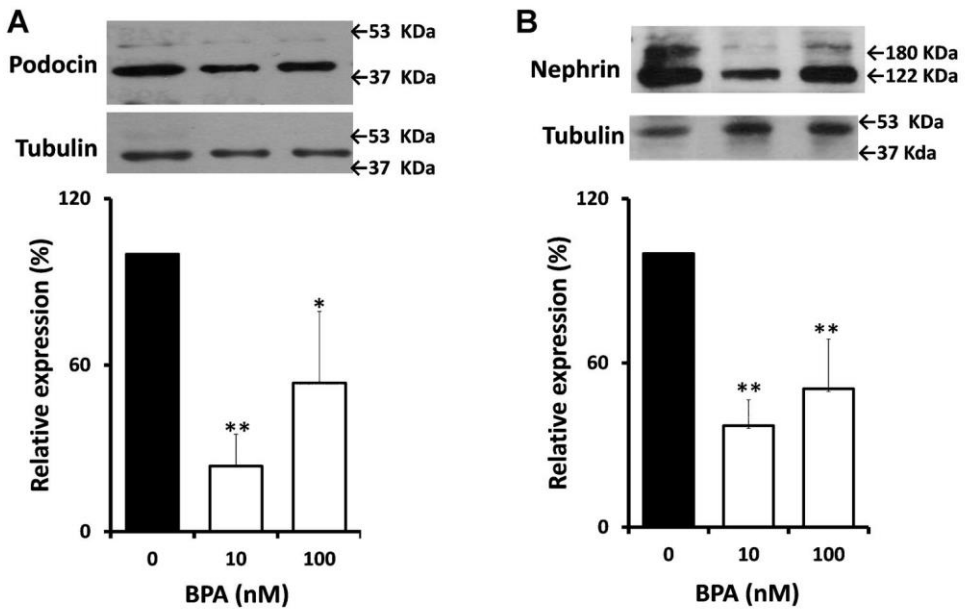

Fig. 4. Podocin and nephrine expression induced by BPA. Protein podocin expression (A) and nephrine expression (B) were measured by Western blot. Data are the means ± SD of three different experiments, each performed in duplicate. *$P < 0.05$ and **$P < 0.01$ using ANOVA test for the comparison between control and BPA-treated cells.

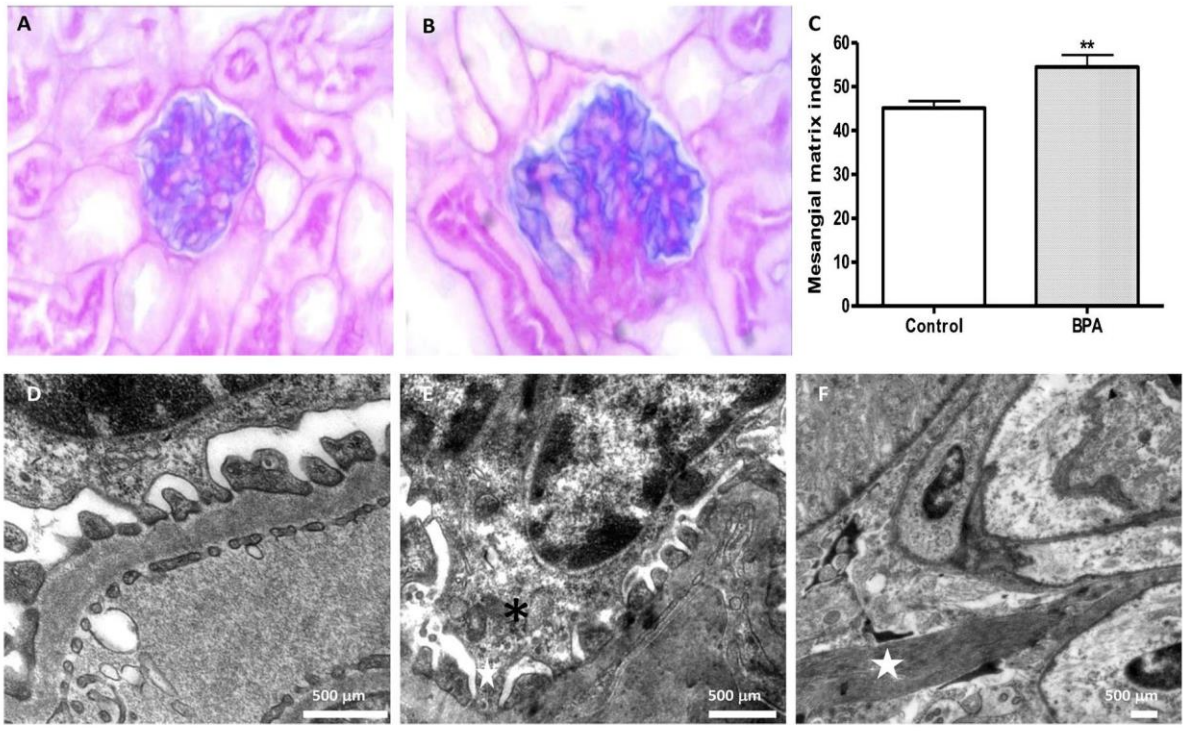

Fig. 5. Renal histological changes in BPA-injected mice. (A,B) Sections from kidney of control and BPA-injected mice stained with PAS-Alcian blue. The renal corpuscles from BPA mice showed higher staining with PAS and alcian blue than controls (pink and blue colors) (X300). (C) Mesangial matrix index. Data are means ± SD. **$P < 0.01$, ***$P < 0.0001$, using ANOVA test for the comparison between control and BPA-treated cells. (D) Electron micrographs of podocytes from control mice. (E) podocytes from BPA-injected mice showing an enlarged cytoplasm (asterisk) as well as broadening of foot processes (white star). (F) Presence of collagen fibers (star) between two podocytes (arrows) with light cytoplasm and condensed chromatin suggesting apoptotic images.

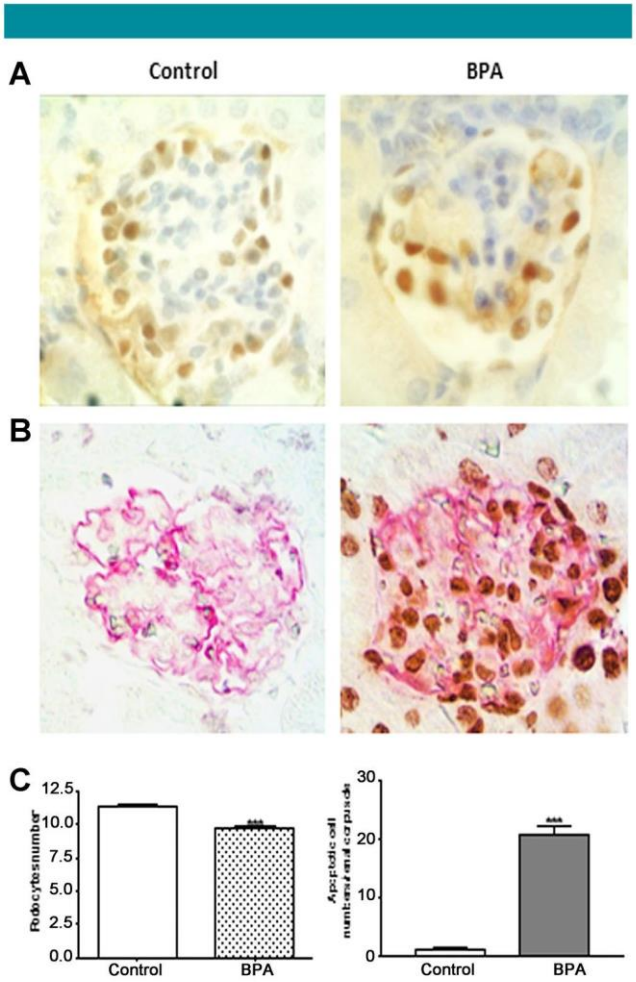

Fig. 6. Podocytopenia in BPA-injected mice. (A) WT-1 labelling. In BPA-injected mice the podocyte cell number (brown nuclei) was decreased with respect to control mice. X300. (B) TUNEL assay (brown nuclei) combined with podocin immunohistochemistry, a marker of podocyte foot processes (red). The renal corpuscle from BPA-mice treated showed higher numbers of apoptotic podocytes than controls (X300). (C) Left, graph representing the statistical analysis for the podocyte cell number. Right, histogram representing the apoptotic cell number in control and BPA-mice. ***$P < 0.001$ using ANOVA test for the comparison between control and BPA-treated cells.

suggest that apoptosis might be involved in the mechanisms of the observed podocytopenia in BPA-injected mice.

Changes in renal protein expression induced by BPA

As expected from our in vitro data, BPA-injected mice display the renal protein upregulation of the TGF-β1 system, and an increase in the cyclin-dependent kinase inhibitor p27Kip1 as well as in collagen IV (Fig. 7).

Discussion

Recent data re-emphasize the utility of rodent models to analyze BPA toxicity, and the fact that BPA has equal potency in both rodent and human cells (Taylor et al., 2011). It has been shown that BPA exerts pleiotropic cellular and tissue-type specific effects at the cellular and intracellular levels at relatively low physiological concentrations (Wetherill et al., 2007). In some experiments we found that low BPA dose tended to be more effective at increasing podocyte protein synthesis, albeit without statistical significance. In this regard, qualitative changes in response to a wide range of doses of BPA have been repeatedly reported, in vitro and in vivo studies, where an increase in dose does not necessarily correspond to an increase in response (Kundakovic et al., 2013). These U-shaped (or inverted U-shaped) dose response curves are considered "non-monotonic". Although these dose response curves were initally ignored due to the lack of a mechanism to explain the appearance of this phenomenon, this is no longer the case, thanks to recent studies which have gained insight into possible causes. These mechanisms include cytotoxicity (mentioned above), cell and tissue-specific receptors and cofactors, receptor selectivity, receptor downregulation and desensitization, receptor competition, and endocrine negative feedback loops (Vandenberg et al., 2009; Vandenberg et al., 2012). This non-monotonic response, which could also explain some of our present data on podocytes, clearly shows that dose selection is critical in studies of chemicals such as BPA (Kundakovic et al., 2013). For this reason, in our in vivo experiments we used a dose of BPA that had been previously shown to affect renal redox control systems in vivo (Kabuto et al., 2003). In any case, long term studies—particularly in humans—are needed in order to establish safe levels of BPA.

Herein we first studied whether BPA could affect renal cell biology, specifically the development of renal cell hypertrophy, a condition known to occur in several podocytopathies. We have found that BPA was able to induce podocyte hypertrophy. Besides the previously mentioned finding on the BPA-induced TGF-β1 system expression, we also studied the effect of BPA on the podocyte expression of the cycline-dependent kinase inhibitor p27Kip1, which is known to play a key role in the mechanism of renal cell hypertrophy (Wolf and Ziyadeh, 1999; Iglesias-de et al., 2002; Iglesias-de la Cruz et al., 2002; Griffin and Shankland, 2008; Ziyadeh and Wolf, 2008; Romero et al., 2010). We found that BPA was also capable of stimulating the podocyte production of p27Kip1. Taken together, these findings suggest that BPA is able to induce hypertrophy of the podocyte by a mechanism which involves both the TGF-β1 system and p27Kip1.

We then investigated whether BPA affects podocyte viability, which is known to occur in several podocytopathies including decompensated podocyte hypertrophy (Wiggins et al., 2005). We found that over time BPA significantly reduced podocyte viability. We then observed that the BPA effect on podocyte viability involved apoptosis. In this regard, it is interesting to mention that evidence from animal models suggests that critical perturbations in the balance between proapoptotic and antiapoptotic factors promote podocyte depletion and progressive glomerulosclerosis. Proapoptotic podocyte factors, such as TGF-β1, also possess prohypertrophy and prosclerotic properties, providing a possible synergistic link to sclerosis (D'Agati, 2008). The TGF-β1 transgenic mouse is a particularly valuable model that has shed mechanistic light on the interrelationship between podocyte apoptosis and glomerulosclerosis, given that these animals develop podocyte apoptosis and glomerulosclerosis concurrently (Schiffer et al., 2001; D'Agati, 2008). Supporting these notions, herein we found that BPA was able to stimulate, on podocytes, the expression of both TGF-β1 and its receptor.

It is known that proteins of the slit diaphragm—"gaps" between adjacent podocyte foot processes—are not only simple structural protein barriers, but also function to signal to other proteins within the body of the podocyte (Shono et al., 2007; D'Agati, 2008). For example, nephrin and podocin also provide a survival function for podocytes. Thus, alterations in the structure and/or function of these proteins lead not only to abnormalities in the size barrier, but also to the overall integrity

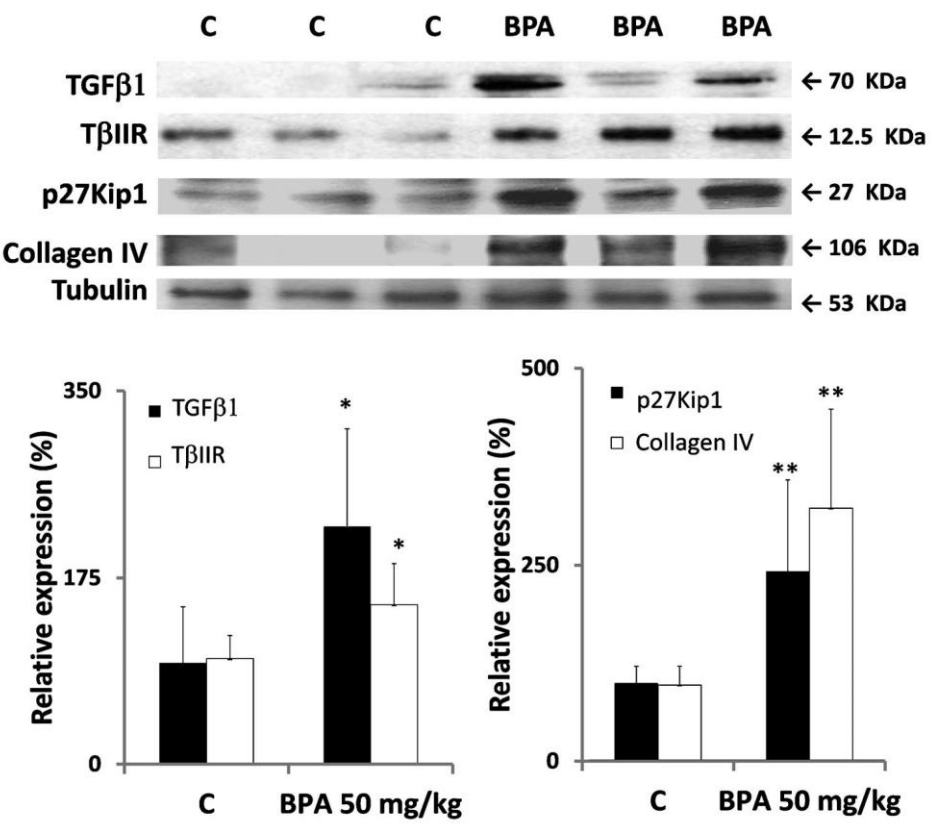

Fig. 7. Renal protein expression in BPA-injected mice. The TGFβ1 system, p27Kip1 as well as collagen IV were analyzed by Western blot. Data are the means ± SD of three different experiments, each performed in duplicate. *$P < 0.05$ and **$P < 0.01$ using ANOVA test for the comparison between control and BPA-injected mice.

of podocytes (Shono et al., 2007; D'Agati, 2008; Jefferson et al., 2008). Therefore, we studied the effect of BPA on the expression of these proteins in cultured podocytes. We found that BPA induced a downregulation of both nephrin and podocin. These data suggest a mechanism by which BPA might affect podocyte viability.

To further explore the significance of BPA-induced TGF-β1 system expression on podocytes, in these cells we also studied the effect of BPA on the production of collagen IV, a molecule which is known to play a key role in the mechanism of TGF-β1-related glomerulosclerosis (Wolf and Ziyadeh, 1999; Ziyadeh and Wolf, 2008). We found that BPA was able to increase collagen IV expression on the podocyte in a dose-dependent manner. Collectively, our results strongly suggest that podocytes are a target for BPA, which might induce a podocytopathy characterized by hypertrophy, diminished expression of slit diagram proteins as well podocyte survival.

Although further studies are needed to assess the mechanism of the observed effects of BPA on podocytes, it is worth mentioning recently reported mechanisms involved in BPA damage on other cell types, such as Leydig cells, and germ cells from mice, where BPA induces apoptosis by a mechanism which involves the upregulation of Fas/FasL and Caspase-3 expression (Li et al., 2009). Furthermore, it has been reported that BPA could induce damage by promoting cytokine release as well as stimulating electron transfer and oxidative stress in target cells, including renal tissue (Kabuto et al., 2003).

To determine whether the observed BPA-induced podocytopathy might also occur in vivo, mice were intraperitoneally injected with BPA for 5 weeks. We observed that these animals developed an increase in both urinary albumin excretion and endogenous creatinine clearance. Renal immunostaining with a specific podocyte marker showed a significant reduction in glomerular podocyte number in BPA-injected mice. Moreover, these animals also displayed an expansion of the glomerular mesangium area. Furthermore, electron microscopy and the TUNEL assay showed podocyte cytoplasmic enlargement as well as the presence of apoptosis, respectively. Interestingly, as observed on cultured podocytes, the kidney of BPA-injected animals showed an increase in the protein expressions of the TGF-β1 system, and an increase in the cyclin-dependent kinase inhibitor p27Kip1 as well as in collagen IV. Collectively, these findings show that BPA-injected mice develop a podocytopathy with proteinuria. Although there are limitations when using mouse models for assessing renal failure or long-term histomorphological changes (Breyer et al., 2005), our findings may have pathophysiological implications, since the amount of proteinuria is a reliable predictor of the progression of renal disease (D'Amico and Bazzi, 2003).

Although it is difficult to discern, with certainty, a potentially dangerous BPA concentration in body fluid, it is important to point out a recent report from Carwile et al. (2011) showing that consumption of 1 serving of canned soup daily over 5 days was associated with a more than 1000% increase in urinary BPA in adult Americans. Moreover, BPA was routinely detected in human blood, including maternal and umbilical cord blood in an ng/ml range (Vandenberg et al., 2010; Chou et al., 2011; Sriphrapradang et al., 2013), a range also found in our experimental animals.

In agreement with the data herein we found high BPA levels in the blood of control animals, which might reflect a "progressively" high BPA exposure, as previously suggested (Huang et al., 2012). In any event, this data is difficult to analyze in experimental animal studies since BPA levels from non-BPA treated animals are not regularly reported, albeit a warning for the potential BPA exposure in experimental animals has been reported (Howdeshell et al., 2003). In any case, this might be due to the animal housing, a counterpart to environmental sources of high BPA levels of exposure in humans.

In addition, it is known that blood levels of conjugated-BPA could vary depending on whether the exposure is oral or parenteral, resulting in a smaller fraction when BPA is injected (Pottenger et al., 2000). This might be the case in our BPA-treated mice. Even though it is possible that the levels of conjugated-BPA were below LC-MS detection limits in control mice, the above-mentioned authors did not detect plasma conjugated-BPA in control animals.

Finally, it is worth mentioning other forms of BPA exposure, such as dermal exposure, inhalation of household dust, and exposure through medical devices (e.g., dental sealants) (Huang et al., 2012). BPA has also been detected in river waters contaminated by industrial effluents in Japan as well as in Spain (Huang et al., 2012). Of note is the fact that several investigators have reported inconsistencies between the currently estimated exposure level of BPA from the ingestion of food and beverages in the United States with findings in biomonitoring studies. Indeed, this discrepancy has led some to speculate about the existence of large unidentified sources of human exposure to BPA (Vandenberg et al., 2010; Taylor et al., 2011; Doerge et al., 2012).

All the available data, together with our present findings, strongly suggest that even low-grade proteinuria associated with BPA exposure might involve podocyte damage of an uncertain (or as yet unexplored) outcome, indicating the need for future studies and raising a red flag of caution against increasing BPA exposure.

In conclusion, on cultured podocytes, BPA is able to induce apoptosis, hypertrophy, upregulation of the TGF-β1 system, p27Kip1, as well as collagen IV, while diminishing the expression of both slit diaphragm proteins and podocyte survival. BPA-injected animals develop proteinuria, glomerular hyperfiltration, and podocytopenia. Further studies are needed to clarify the potential role of BPA in the pathogenesis as well as in the progression of renal disease.

Acknowledgments

We thank Professors A Izquierdo from Universidad Rey Juan Carlos, MA Pérez-Albarsanz and P López-Aparicio from the Department of Biochemistry, University of Alcala, for their comments and suggestions. We thank A Rodgers for proofreading the manuscript. We also thank Professor C Barbas, Director of CEMBIO Metabolomic Unit, San Pablo-CEU University, Madrid, for LC-MS analysis. N. Olea is recipient of a research contract from Comunidad Autónoma de Madrid -Programa de Actividades I+D en Biociencias 2010-(S2010/BMD-2378). This work was supported in part by grants from Ministerio de Ciencia e Innovación (SAF2009-12009-C02-01), Instituto de Salud Carlos III (PI12/02825) and The Eugenio Rodríguez Pascual Foundation.

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IV. CAPÍTULO 1

COMPARISON OF THE RENAL EFFECTS OF BISPHENOL A IN MICE WITH AND WITHOUT EXPERIMENTAL DIABETES. ROLE OF SEXUAL DIMORPHISM.

BIOCHIMICA ET BIOPHYSICA ACTA - MOLECULAR BASIS OF DISEASE

(5.187, Q1 BIOPHYSICS [2020]).

DOI:10.1016/J.BBADIS.2021.166296

BBA - Molecular Basis of Disease 1868 (2022) 166296

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Comparison of the renal effects of bisphenol A in mice with and without experimental diabetes. Role of sexual dimorphism

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ARTICLE INFO

Keywords:
Bisphenol A
Mouse
Diabetes
Diabetic nephropathy

ABSTRACT

Bisphenol-A (BPA), a chemical -xenoestrogen- used in the production of the plastic lining of food and beverage containers, is present in the urine of almost the entire population. Recent studies have shown that BPA exposure is associated with podocytopathy, increased urinary albumin excretion (UAE), and hypertension. Since these changes are characteristic of early diabetic nephropathy (DN), we explored the renal effects of BPA and diabetes including the potential role of sexual dimorphism. Male and female mice were included in the following animals' groups: control mice (C), mice treated with 21.2 mg/kg of BPA in the drinking water (BPA), diabetic mice induced by streptozotocin (D), and D mice treated with BPA (D + BPA). Male mice form the D + BPA group died by the tenth week of the study due probably to hydro-electrolytic disturbances.

Although BPA treated mice did not show an increase in serum creatinine, as observed in D and D + BPA groups, they displayed similar alteration to those of the D group, including increased in kidney damage biomarkers NGAL and KIM-1, UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli, as well as TGF-β, CHOP and PCNA upregulation. UAE, collapsed glomeruli, PCNA staining, TGF-β, NGAL and animal survival, significantly impaired in D + BPA animals. Moreover, UAE, collapsed glomeruli and animal survival also displayed a sexual dimorphism pattern.

In conclusion, oral administration of BPA is capable of promoting in the kidney alterations that resemble early DN. Further translational studies are needed to clarify the potential role of BPA in renal diseases, particularly in diabetic patients.

1. Introduction

Diabetes mellitus (DM) is a growing disease that affects an increasing number of people. In 1980 it affected 108 million people globally [1], increasing to 171 million in 2000 [2]. Interestingly, in 2014, the number of patients augmented to 422 million [1]. According to studies conducted by the International Diabetes Federation, it is estimated that almost 500 million people in the world are currently living with diabetes. That number is projected to reach 578 million in 2030 and 700 million in 2045 [3]. Of all of them, it is estimated that about half of patients with type 1 diabetes develop diabetic nephropathy (DN) throughout their lives, being 30–50% in the case of patients with type 2

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https://doi.org/10.1016/j.bbadis.2021.166296
Received 25 May 2021; Received in revised form 19 October 2021; Accepted 21 October 2021
Available online 27 October 2021

diabetes [4].

In the same way, an increase in the prevalence of diabetic kidney disease has also been observed. Gheith et al. [4] determined an increase from 7.1% (1988–1994) to 10.7% (2005–2008) in people over 65 years of age. DN is also the most common cause of end-stage renal disease, having been observed in the United States to cause up to 80% of cases [5]. DN has been recognized as a worldwide medical catastrophe and is now the most important etiology of end-stage renal failure in developed countries [6]. However, since not all diabetic patients suffer renal alterations, it is considered that there must be numerous environmental and biological factors yet to be determined.

Parallel to the increase in chronic diseases, an increase in exposure to xenobiotic compounds, such as bisphenol A (BPA), has been observed [7,8]. BPA is an essential element in manufacturing PVC and epoxy resins, used in numerous sectors such as the food industry, medical-surgical material, thermal paper cash register receipts, or even clothes [9–12]. In this way, BPA levels have been detected in the vast majority of foods, which manifests as significant levels of BPA in almost the entire human population studied, reaching percentages of 95% in the United States [13], 97% in Spain [14], 90–100% in China [15,16] or even 100% in Canada [17].

BPA is known to act as an endocrine disruptor, and due to its physical properties, it is capable of crossing any physiological barrier, such as the blood-brain or placental barrier [18]. In the last 20 years, various studies have determined that BPA can affect numerous organs and systems, such as the female and male reproductive system, the cardiovascular system and the endocrine system, or even the correct fetal development, to name a few examples [19–22]. In this sense, a pattern of sexual dimorphism has been observed during the gestational or perinatal period, compared to the same exposure to BPA, reaching differences on hepatic [23,24], adipogenic [25], neurological [26–28], endocrine [29], immunological [30], and renal development [31]. Pollock et al. [32] determined that the kidney is one of the organs where more BPA can be quantified, and this concentration rises before prolonged exposure over time, which also suggests the existence of a possible accumulation.

Epidemiological studies in humans suggest the possible association between high urinary BPA levels and predisposition to albuminuria [33,34] or hypertension [35,36]. Moreover, Olea et al. [37] in an experimental animal model have shown the development of podocytopathy and increased urinary albumin excretion (UAE) in mice after five weeks of i.p. exposure of BPA. Furthermore, BPA exposure has been associated with hypertension in mice [22].

Since these changes are characteristic of early DN, herein we explored the hypothesis that oral BPA exposure, being the most important one in mammals, might resemble DN and promote the progression of kidney damage. Moreover, we also studied well-characterized molecules involved in the pathophysiology of DN, such as the proapoptotic and profibrogenic TGF-β, and the well-known inflammatory mediators MCP-1 and TNF-α.

2. Material and methods

2.1. Animal model

CD1 mice (25–30 g) were used in all the experiments. All the studies were performed following guidelines established by Institutional Animal Care and Use Committees at the University of Alcalá. Mice were housed in a temperature-controlled room (21 ± 2 °C) on a 14/10 h light/dark cycle under pathogen-free conditions and food ad libitum. Mice were given free access to water or BPA-containing water at a dose of 150 μg/mL (Sigma, Saint Louis, MO).

Four different animal groups were studied: Control (C), non-diabetic animals with BPA in the drinking water (BPA) 150 μg/mL, (21.2 mg/kg/day), diabetic animals without BPA (D), and diabetic animals with BPA in the drinking water (D + BPA).

In total forty non-diabetic mice were used, 20 mice as control and 20 mice treated with BPA. Diabetes was induced by three daily consecutive intraperitoneal injections of streptozotocin (STZ) (Sigma), 65 mg/kg body weight in citrate buffer, pH 4.5 (vehicle) [38]. Mice with blood glucose >300 mg/dL were included in the study ($n = 45$). Male and female mice were used in approximately equal number with the exception of the male D + BPA group (17 male and 8 female) due to the mortality of the former group at 10 weeks. For this reason, the period of the study was limited to eight weeks using 5 to 7 animals per group except for the Kaplan-Mayer survival table.

Mice were placed in metabolic cages, and 24-h urine was collected for biochemical measurement as previously reported [39]. After eight weeks of treatment and development of the diabetic model, the animals were sacrificed under isoflurane. Blood was taken by cardiac puncture under ether anesthesia for biochemical measurements. The right kidney of each animal was removed, weighed, frozen in liquid nitrogen, and stored at −80 °C for subsequent total RNA extraction. The left kidney was weighed and fixed in 10% buffered formaldehyde for morphological and immunohistochemistry studies. The degree of renal hypertrophy was expressed as an index, the ratio of kidney weight to total body weight.

2.2. Determination of biochemical test

Quantifications of plasma sodium, potassium, albumin, creatinine, and urine albumin and creatinine were performed with an ADVIA Chemistry system (Siemens Healthineers).

2.3. Immunohistochemistry

Serial sections (4 μm thicknesses) from mice kidneys formalin-fixed and embedded in paraffin were deparaffinized with xylene, rehydrated, and placed in 10 mM sodium citrate buffer, pH 6.0, and heated in a conventional pressure cooker for 2 min. The sections were allowed to cool for 20 min. After rinsing with distilled water, the sections were washed twice in Tris buffer saline (TBS), pH 7.6, for 5 min. Endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2 for 20 min. Sections were subsequently washed with distilled H_2O and TBS and incubated with 3% normal donkey serum plus 0.05% Triton X-100 in TBS (blocking solution) at room temperature for 30 min to prevent nonspecific binding of the first antibody. Afterward, sections were incubated overnight at 4 °C with primary antibodies diluted in blocking solution, which was diluted 1:9 in TBS. (WT-1, 1:1000; CHOP, 1:300; PCNA, 1:1500; MCP-1, 1:400; 4-Hydroxynonenal (4-HNE) 1:250). Then, the sections were washed and incubated in primary antibodies amplifier Quanto (Ultravision Quanto detection system–peroxidase, Master Diagnóstica, Granada, Spain) for 10 min. After an extensive wash in TBS, the sections were incubated in polymer Quanto for 10 min. The peroxidase activity was detected using the DAB kit (Master Diagnóstica). Tissue sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in Entellan®.

The immunoreactivity of each focus of interest was measured using the stereological software Motic Images Advanced 3.2 (Motic China Group Co, Ltd). This program allows the selection of fields to be studied by systematic random sampling after the input of an appropriate sampling fraction. An average of 10 light microscopic fields per section was scanned using the ×20 objective. The staining intensity was measured using a grey level scale (0: white; 255: black) with a negative image. All results are expressed as mean ± SEM.

2.4. TUNEL assay

For the detection of apoptotic cells, the DeadEndTM Fluorometric TUNEL System was applied (Promega, Madison, WI, USA) to paraffin-embedded sections according to the manufacturer's instructions. Then, the sections were incubated with an anti-FITC-HRP for 30 min. and the reaction product in nuclei was developed with DAB.

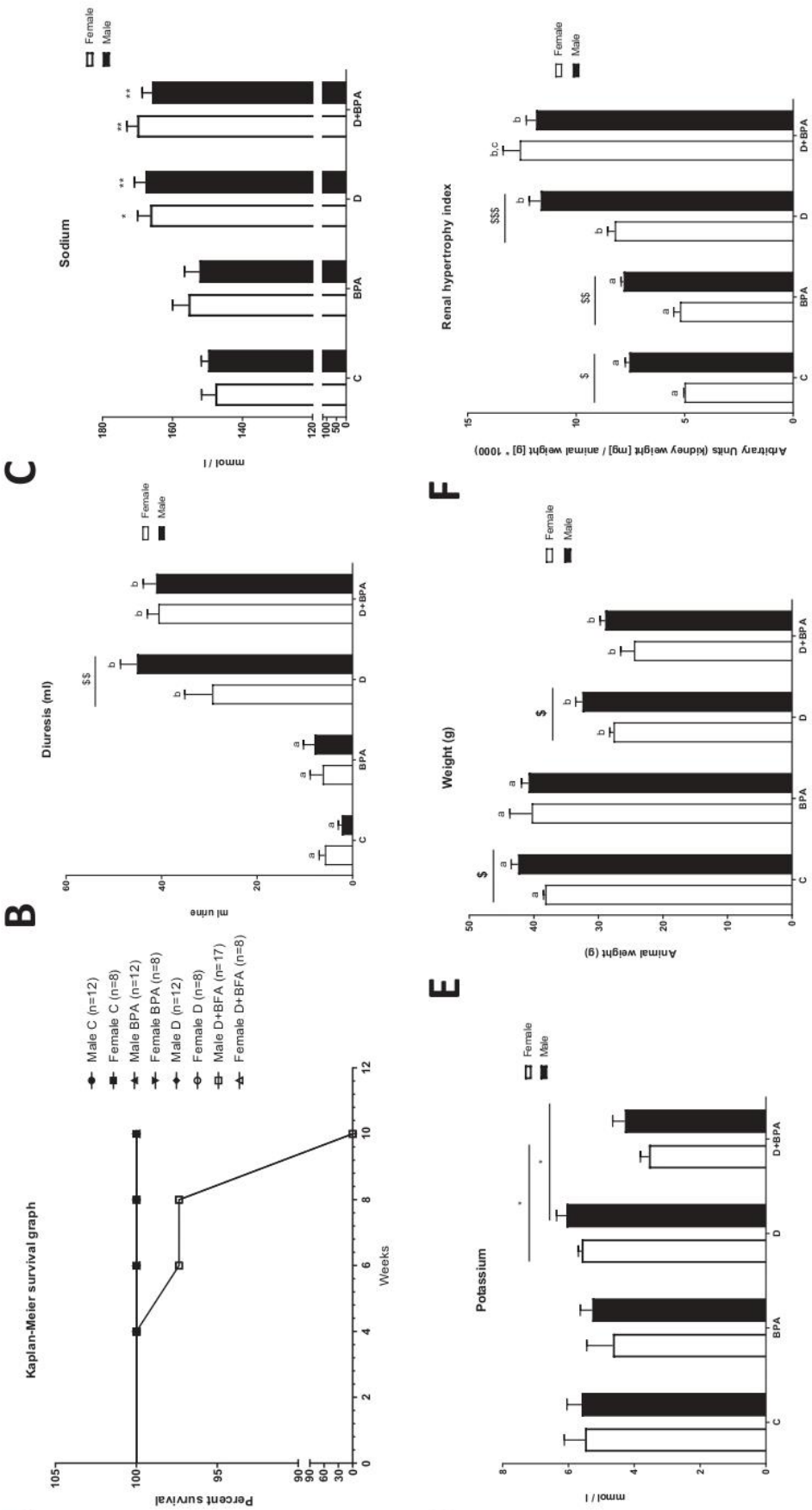


Fig. 1. Survival, plasma ions, and physiological study. A. Kaplan-Meier survival graph. Note that the survival of all groups is 100%, except for the D + BPA group. For this reason, all symbols and lines overlap at the same height, preventing correct viewing. B. 24-hour diuresis, in mL. All diabetic animals excreted volumes of urine between 5 and 10 times higher than control and BPA groups. C. Plasma sodium, in mmol/L. All diabetic animals showed elevated plasma sodium concentrations. D. Plasma potassium, in mmol/L. Only the D + BPA group showed low plasma potassium concentrations. E. Body weight, in grams. Diabetic animals showed a significant decrease in body weight than C and BPA groups. Note that only C and D groups showed sexual dimorphism. F. Renal hypertrophy index (kidney weight [mg] / animal weight [g] * 1000). All diabetic animals showed a significant increase in renal hypertrophy index. Note that C, BPA, and D groups showed sexual dimorphism. All groups with different letters have significant differences ($p < 0.01$). Letter c represents significant differences with diabetic female ($p < 0.0001$). $^{*}p < 0.05$; $^{**}p < 0.01$ comparing to their respective control group. $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ comparing between males and females of the same group. The graph represents mean values ± SEM. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

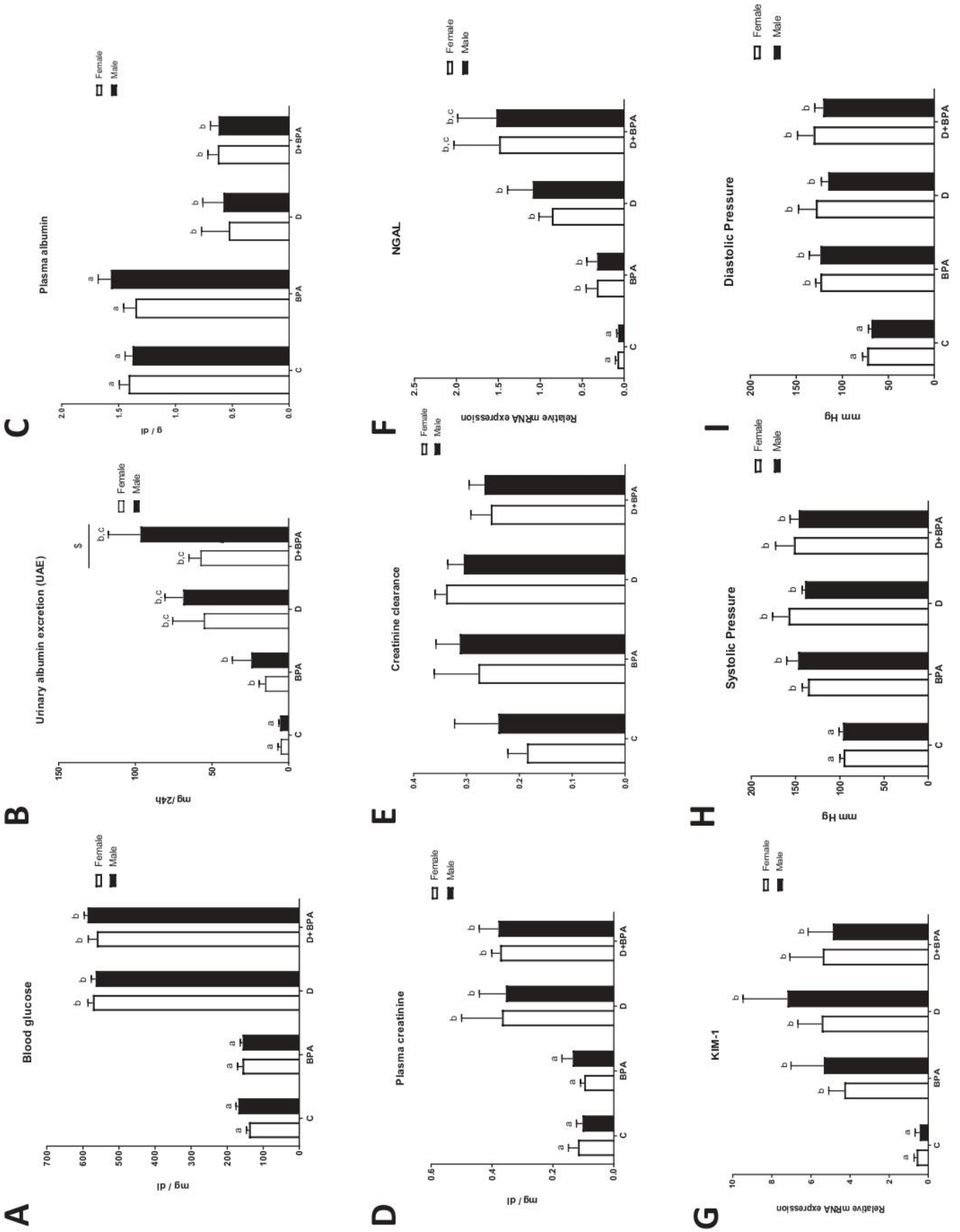

A
Blood glucose
mg / dl
B
Urinary albumin excretion (UAE)
mg /24h
C
Plasma albumin
g / dl
D
Plasma creatinine
mg / dl
E
Creatinine clearance
F
NGAL
Relative mRNA expression
G
KIM-1
Relative mRNA expression
H
Systolic Pressure
mm Hg
I
Diastolic Pressure
mm Hg
Female
Male
C
BPA
D
D+BPA

Fig. 2. Biochemical study of the kidney and arterial pressure. A. Blood glucose. All diabetic animals showed higher blood glucose at the end of the experiment (8 weeks). B. Albuminuria, expressed in mg of albumin excreted in 24 h. Note that all groups showed elevated levels of albuminuria compared with the control group. C. Plasma albumin, in g/dL. All diabetic animals showed low values of plasma albumin, in the nephrotic range. D. Plasma creatinine, in mg/dL. All diabetic animals showed high plasma creatinine values. E. Creatinine clearance. All groups showed similar creatinine clearance values. F. NGAL qPCR. G. KIM-1 qPCR. H. After eight weeks of treatment, all groups showed a significant increase in systolic pressure than the control group. I. Diastolic pressure showed the same pattern than systolic pressure. $^{\$}p < 0.05$ comparing between males and females of the same group. All groups with different letters have significant differences ($p < 0.05$). Letter c represents significant differences with BPA group ($p < 0.05$). The graph represents mean values ± SEM. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

2.5. Total RNA extraction and quantitative RT-PCR

Total RNA was isolated from each mouse kidney homogenate obtained using TriReagent (Sigma-Aldrich). Total RNA was quantified by Nanodrop 1000 Spectrophotometer (Thermo Scientific). A total of 500 μg of RNA was reverse transcribed into cDNA using the High Capacity cDNA Promega Kit according to the manufacturer's instructions (5 min 65 °C and 1 h 37 °C). PCR was performed duplicated for each sample using an adequate dilution of cDNA as a template for different genes (see supplementary material Table S1 for SYBR Green primers and TaqMan probes). β-Actin and 36B4 were used as housekeeping genes. The amplification was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by using the following conditions: 2 min 50 °C, 10 min 95 °C, 40 cycles (15 s 95 °C, 1 min 60 °C). Reagents were from Applied Biosystems (for TaqMan probes) and SYBR Green (for SYBR Green primers). We used a standard curve method, using the untreated samples as a calibrator, to calculate the relative quantity of gene expression. We used the BestKeeper software tool (http://www.gene-quantification.info/) to validate housekeeping genes [40].

2.6. Measurement of blood pressure (tail cuff method)

We used a non-invasive pressure gauge (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain) to measure the blood pressure. The measurements were always made at the same time of day, with the least possible stress for the animal (acoustic and luminous). Being nocturnal animals and having a high inactivity peak at 8 a.m., they were not manipulated until at least 2 h later. The animals were prewarmed to 30 °C with a heater (LE5660/6, Letica Scientific Instruments). The sphygmomanometer and the pulse transducer were never placed in the area near the animal's body because they can cause damage.

2.7. Statistical analysis

All results were expressed as mean ± SEM. $p < 0.05$ was considered statistically significant. To determine the effects of treatments, one-way ANOVA or Kruskal-Wallis followed by a Bonferroni or Dunns test, respectively, were carried out. To determine sex differences in the BPA and diabetes treatment, two-way ANOVA followed by a Tukey's multiple comparisons test or Sidak's multiple comparisons test were carried out. The *p*-values presented in figures and tables corresponded to post hoc test. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Animals display a sexual survival dimorphism pattern

As shown in Fig. 1A, the Kaplan-Meier animal survival analysis showed that all-male D + BPA mice died by the tenth week of treatment while no animal loses were found among the other experimental animal groups. In order to obtain all the biological samples needed to complete the animal's studies, some male animals were included in the studied groups as described in the methods section.

Besides dehydration, no other potential cause of death was observed in the bodies of dead mice. As shown in Fig. 1B, both D and D + BPA groups showed a significant increase in urinary output than the control littermates. However, only D group showed significant differences between males and females. As expected, these animal groups display an increased value of plasma sodium together with a decreased potassium (Fig. 1C and D), albeit these values did not display a sexual dimorphism pattern. Unfortunately, one limitation of the study is the lack of biochemical data beyond the study's eight weeks. Therefore, although the cause of the male D + BPA group's death cannot be determined with certainty, we can only assume that it was related to hydroelectrolytic

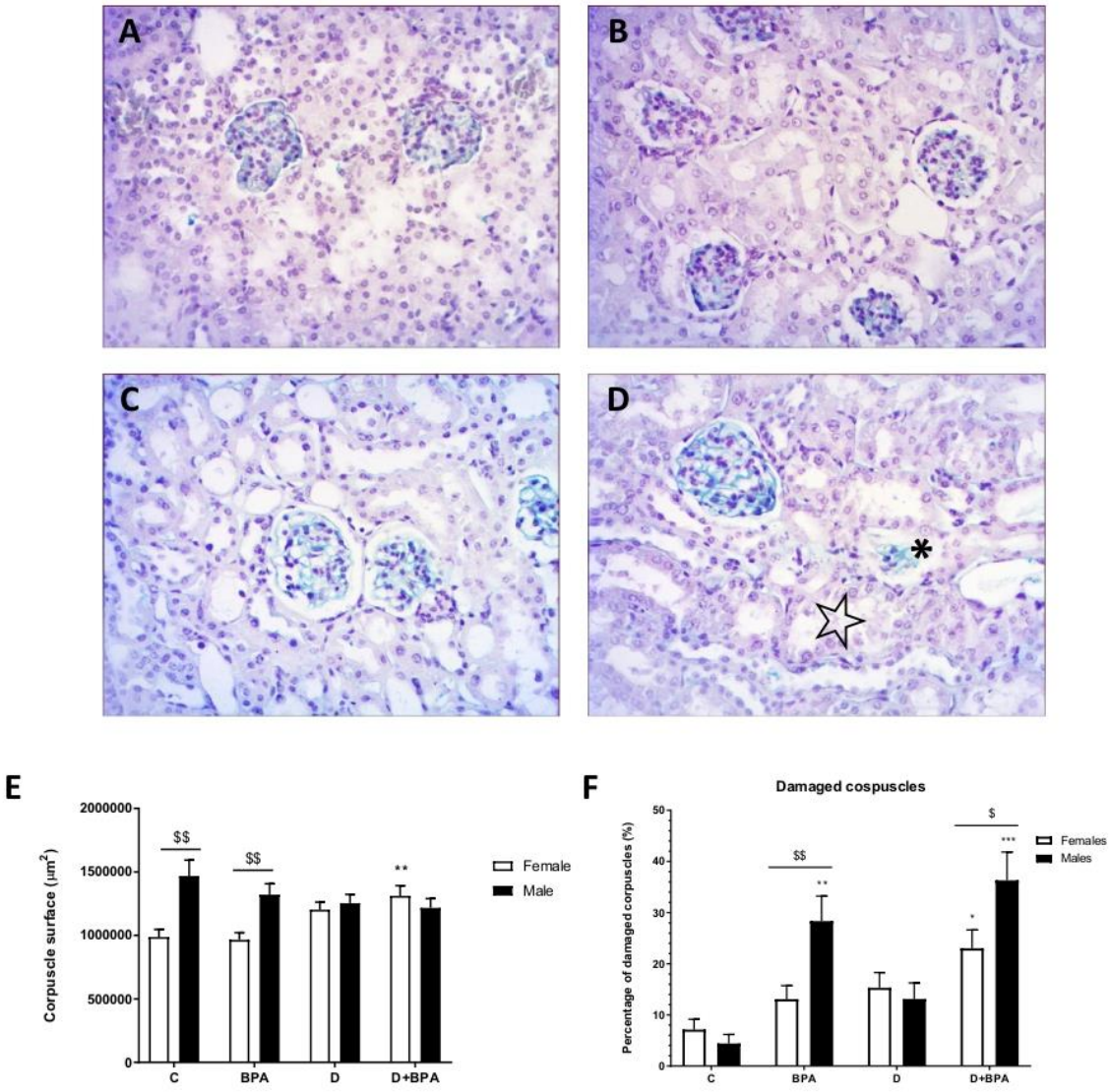


Fig. 3. Histological examination (PAS-Alcian blue staining). A. Control kidney. B. Mice BPA-treated showed altered renal corpuscles and dilated convoluted tubules. C. The kidney of the diabetic mice showed both Bowman's capsules and convoluted tubules extremely dilated. D. D + BPA mice showed mesangial expansion, some corpuscles were destroyed (*), and the epithelium in some tubules was damaged (star) (×300). E. Histogram compares the corpuscular surface in each group observing statistically significant differences between females and males in both control and BPA-treated groups. F. Histogram represents the collapsed renal corpuscles percentage, showing a sexual dimorphism pattern in BPA and D + BPA groups. $^{*}p < 0.05$; $^{**}p < 0.01$ using one-way ANOVA or Kruskal-Wallis test for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes. $^{\$\$}p < 0.01$ comparing between males and females of the same group.

disturbances.

3.2. Animal body weight and renal functional parameters

As previously described [41–44], control male mice showed a significant increase in body weight and renal hypertrophy compared to females' littermates (Fig. 1E and F). As shown in Fig. 1E, compared to control mice, the average animal body weight was not affected in the BPA group, while both D and D + BPA groups showed a significant decrease in this parameter. Moreover, both control male and D male mice showed a significant increase in body weight compared to females' littermates. The D and D + BPA mice showed a significant increase in the renal hypertrophy index compared to the control group. Of note is that the females of the D + BPA group develop an increase in this parameter similar to males. In the rest of the groups, the females showed a lower hypertrophy index than their male littermates (Fig. 1F).

At 8 weeks of treatment, the glycemia of diabetic animals remained above 400 mg/dL in all animals, while BPA group were not significantly different from those of the C group (Fig. 2A).

As expected, all animal groups studied display a significant increase in UAE compared to the C group (Fig. 2B). UAE was significantly higher in the D group compared to the BPA group. Interestingly, male D + BPA mice display a significant increase in UAE compared to the BPA group, demonstrating a sexual dimorphism pattern compared to their female littermates.

Moreover, a significant decrease in plasma albumin was observed in D and D + BPA (Fig. 2C). Although endogenous creatinine clearance not showed significant differences among animal groups studied, plasma creatinine displayed a statistically significant increase in both D and D + BPA groups compared to their correspondent's controls (Fig. 2D and E). To further investigate BPA effects at the molecular level on the kidney, the gene expression of the kidney damage biomarkers Havcr-1 and Lidocalpin-2, which codify NGAL and KIM-1, respectively, were evaluated [45,46] (Fig. 2F and G). Both mRNAs were significantly increased

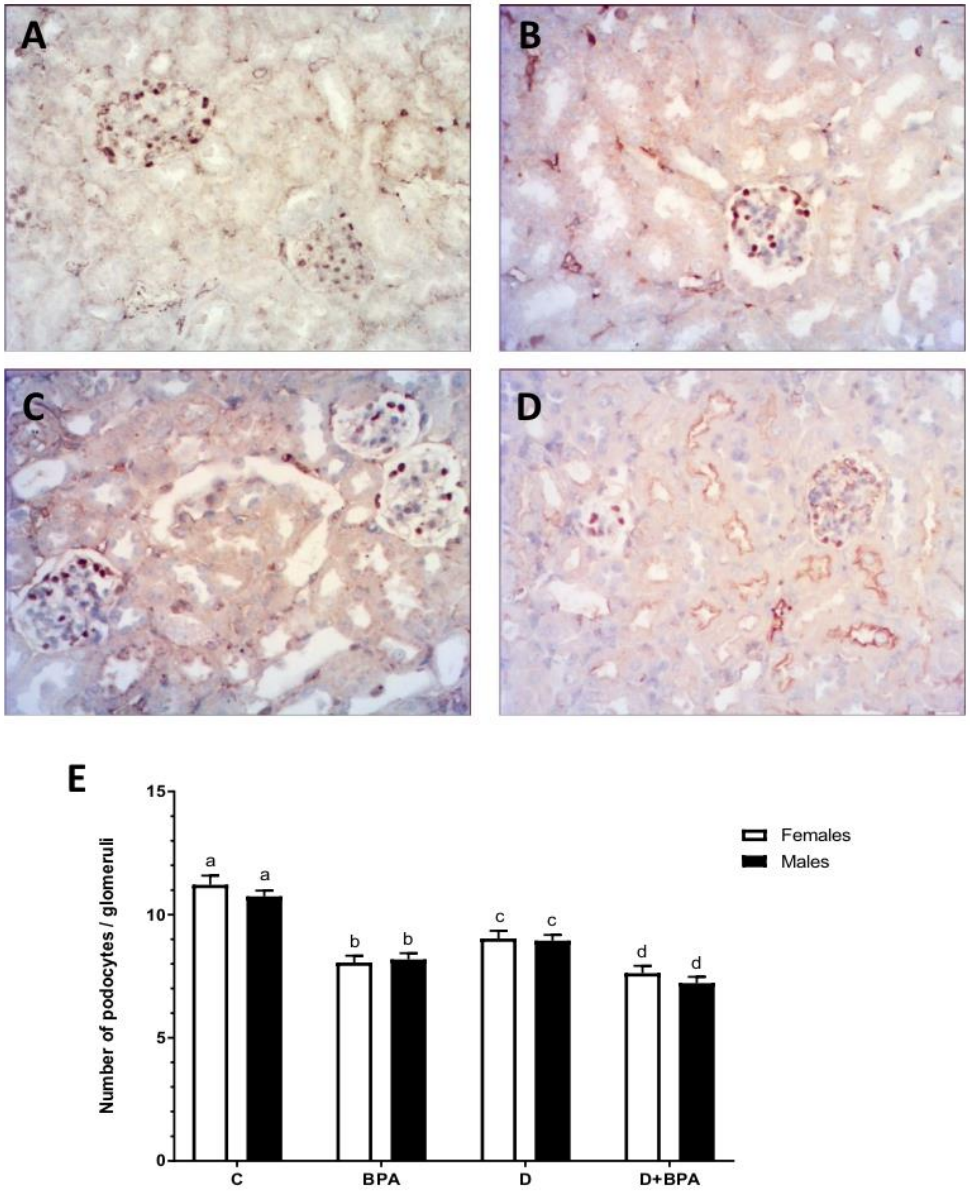


Fig. 4. WT-1 immunostaining. A. Control kidney. B. Mice BPA-treated is showing a reduction in the podocytes number vs. control animal. C. Diabetic mice presented a higher podocyte number than BPA-treated animals but lower than control. D. D + BPA mice had the lowest number of podocytes (×300). E. The histogram shows statistically significant differences between all experimental groups. All groups with different letters have significant differences ($p < 0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

in all treated mice in comparison to their control littermates. Interestingly, NGAL also showed a significant further increase in diabetic animals receiving BPA. Thus, kidney damage biomarkers supported the notion that BPA exposures to mice induced kidney damage in non-diabetic mice and exacerbated injury in diabetic mice. No sexual dimorphism was observed in these parameters.

As shown in Fig. 2H and I, systolic and diastolic arterial blood pressures were substantially higher in the three experimental models. No sexual dimorphism in this parameter was observed.

3.3. Histological, immunohistochemical, and Q-PCR studies

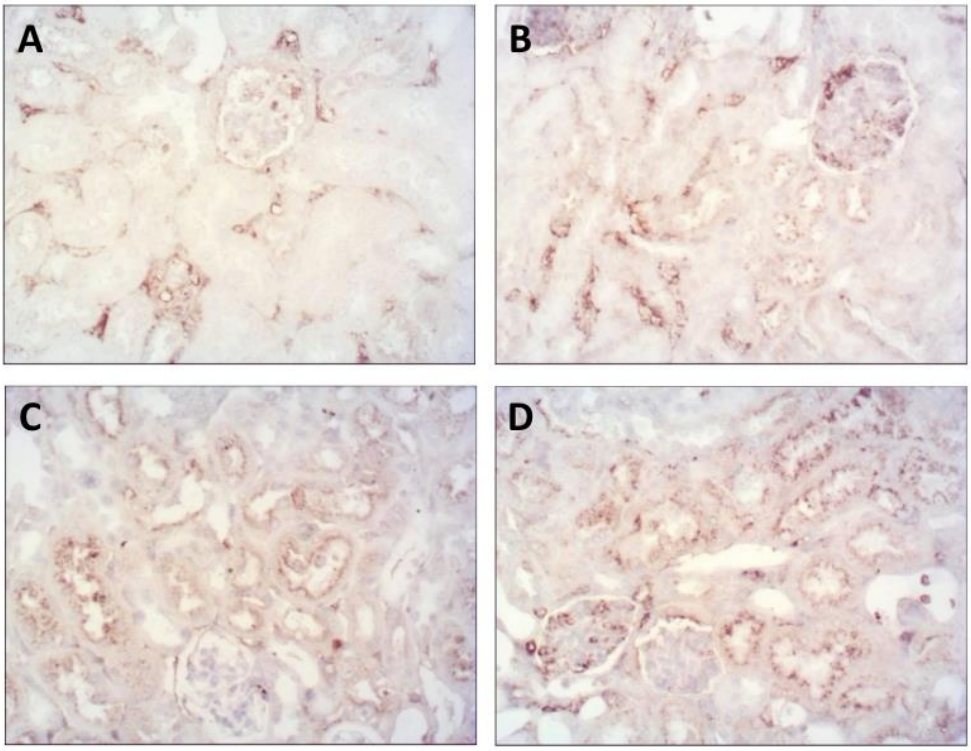
BPA and D groups display similar renal histological changes at both glomerular and tubular compartments, including collapsed glomeruli, tubular dilatation, sloughing off the tubuloepithelial cells, and hyaline casts in the tubules' lumen. Diabetic mice treated with BPA showed similar histological alterations observed in these groups. Of note, glomerular damage analyzed as collapsed corpuscles percentage showed a sexual dimorphism pattern in BPA and D + BPA groups being male mice significantly impaired (Fig. 3).

Immunolabeling with WT-1, a specific marker of podocytes, demonstrated that podocytes cell number was significantly decreased in all groups compared to their corresponding control littermates (Fig. 4). Interestingly, D + BPA animals display a further decrease in this parameter compared to both BPA and D groups. No sexual dimorphisms were observed on this parameter.

Cell death, including apoptosis, as analyzed by CHOP immunohistochemistry and TUNEL assay, respectively, were significantly increased in all group studies in comparison to their control littermates. D group presented the highest optical density in CHOP immunostaining (Fig. 5). D + BPA group presented the highest number of apoptotic cells (Fig. 6).

To explore if there was a compensatory mitotic response to the observed increase in cell death, cell proliferation was analyzed by PCNA immunostaining. Both BPA and D groups develop a significant proliferative response, even higher in female mice than their male littermates (Fig. 7). Interestingly, D + BPA group also showed PCNA-upregulation compared to their control littermates, albeit without sexual dimorphism.

We then explored the potential role of oxidative stress as an inflammatory mediator by analyzing lipid peroxidation as 4-HNE staining (Fig. 8). Control and BPA animals showed a sexual dimorphism pattern



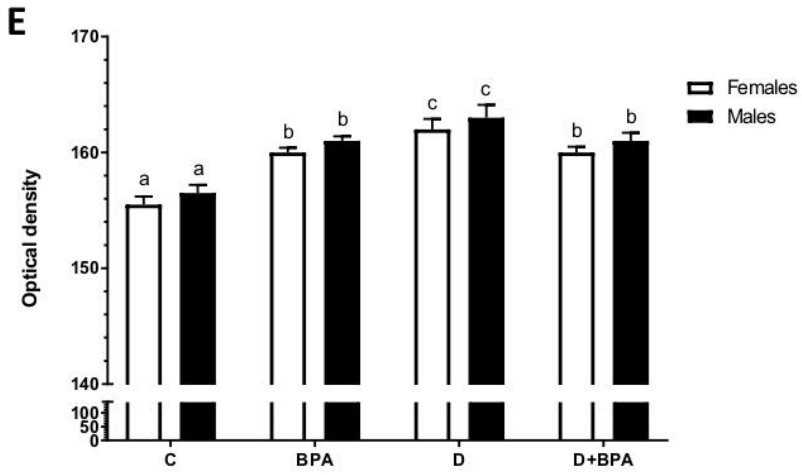


Fig. 5. CHOP immunostaining. A. In the control group, the CHOP labeling was observed in the blood vessel endothelial cells. B. In BPA-treated kidneys, the immunolabeling was increased and appeared in the apical cytoplasm of the tubuloepithelial cells. C. Diabetic mice presented the highest immunoreaction to CHOP antibody. D. D + BPA mice presented elevated immunostaining but lower than in diabetic mice (X300). E. Histogram showing the optical density of CHOP immunostaining. All groups with different letters have significant differences ($p < 0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

being significantly higher in the male. All treated mice, except D + BPA male displayed a significant increase in comparison to their control littermates.

We finally explored the potential role of several inflammatory mediators in the present experimental model (Figs. 9 and 10). No significant changes in the expression of TNF-α, MCP-1, and IL-1β were observed in BPA-treated groups (Figs. 9A, B, C). The same expression pattern was observed in MCP-1 immunolabeling (Fig. 10). By contrast, all of them were significantly upregulated in D animal group. Moreover, D + BPA group showed a significant upregulation of MCP-1. Fig. 9D showed a significant upregulation of IL-10 restricted to BPA animal group. TGF-β and its receptor, a well-characterized system involved in both BPA and renal diabetic damage, were analyzed by Q-PCR. TGF-β was significantly upregulated in all animal groups, being significantly higher in the D + BPA group. The latter observation suggests that BPA might potentiate TGF-β upregulation in diabetic mice (Fig. 9E). Interestingly, the D + BPA group also displays the upregulation of the TGF-β receptor (Fig. 9F). No sexual dimorphisms were observed on these parameters.

4. Discussion

Experimental and human studies have demonstrated a significant association between urinary excretion of BPA and albuminuria, a well-known factor involved in the mechanism of renal disease progression [33,34,47,48]. In this regard, Hu et al. [47], in a prospective study of 302 patients followed for six years, have demonstrated serum BPA as a predictor of chronic kidney disease in primary hypertension. Serum BPA was also described as a risk factor in the progression of diabetic nephropathy in patients with type 2 diabetes [48]. Our present study provides an experimental animal model to support these findings since we describe that the oral administration of BPA is capable of promoting

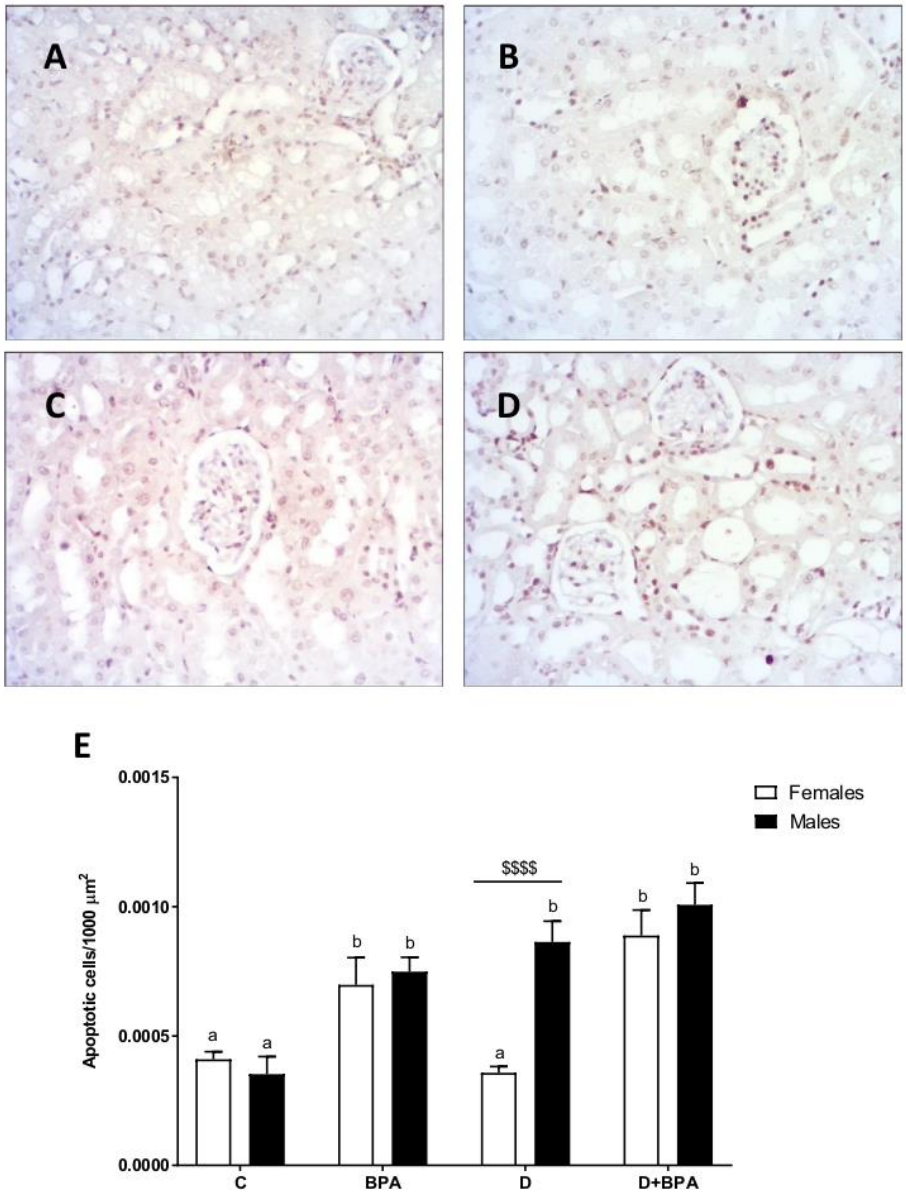


Fig. 6. TUNEL assay. A. Kidneys from control the group presented a small number of apoptotic cells. B. BPA-treated mice presented a higher number of apoptotic cells than controls. C. In diabetic mice, kidneys from males had a high number of dead cells; however, the female mice were similar to the control group. D. D + BPA mice showed the highest number of apoptotic cells (×300). E. Histogram shows the gender differences in each group. $^{\$\$\$\$}p < 0.0001$ females vs. males. All groups with different letters have significant differences ($p < 0.05$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

in the kidney pathophysiological and molecular alterations that resemble early DN, such as increased in UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli as well as CHOP and TGF-β system upregulation.

We presently used the STZ model, which is considered the 'work horse' for experimental studies in diabetic nephropathy [49,50]. Consistent with previous findings in this model, diabetic mice developed renal hypertrophy and an increase in UAE, hypertension, podocytopenia, apoptosis [39,51–55], and the upregulation of CHOP, PCNA, MCP-1, and TGF-β throughout this study [51,52].

Regarding BPA doses used in our study's, animals received 150 μg/mL (21.2 mg/kg). Currently, it is accepted that the "no observed adverse effect level", known as NOAEL, in the renal system is 50 mg/kg [56,57], which is at least twice times higher than the dose used. European Food Safety Authority (EFSA) currently uses this NOAEL dose to estimate the tolerable daily intake (TDI) of 4 μg/kg in humans [57–59]. In the EFSA 2015 report, it is estimated that the total exposure to BPA in the population could reach almost half of the TDI (around 1.5 μg/kg) [57], which is consistent with using an equivalent concentration half of the NOAEL in animal models.

BPA-treated mice did not display significant polyuria as expected from previous publish [37] and unpublished observations using BPA concentration higher than renal NOAEL. Therefore, polyuria and the risk of higher BPA exposure were restricted to D + BPA mice. In any case, the BPA group developed similar renal changes to those of the diabetic group. Although this represents a limitation in our studies, it emphasizes the role of BPA exposure and the importance of maintaining strict metabolic control of diabetic patients to avoid osmotic diuresis and BPA overexposure.

One unexpected finding of this study was that all male mice from the D + BPA group died by the tenth week of the experimental procedure. Unfortunately, a limitation of the study is the lack of data beyond eight weeks. Therefore, albeit the cause of the death cannot be determined with certainty, we can only assume that it was related to hydro-electrolytic disturbances in animals with impaired renal function.

In accordance with previous works [41–44], control male mice showed increased in body weight and renal hypertrophy compared to female littermates. Besides the observed sexual survival dimorphism pattern, other significant pathophysiological differences between male and female animals were found. Treated male animals display

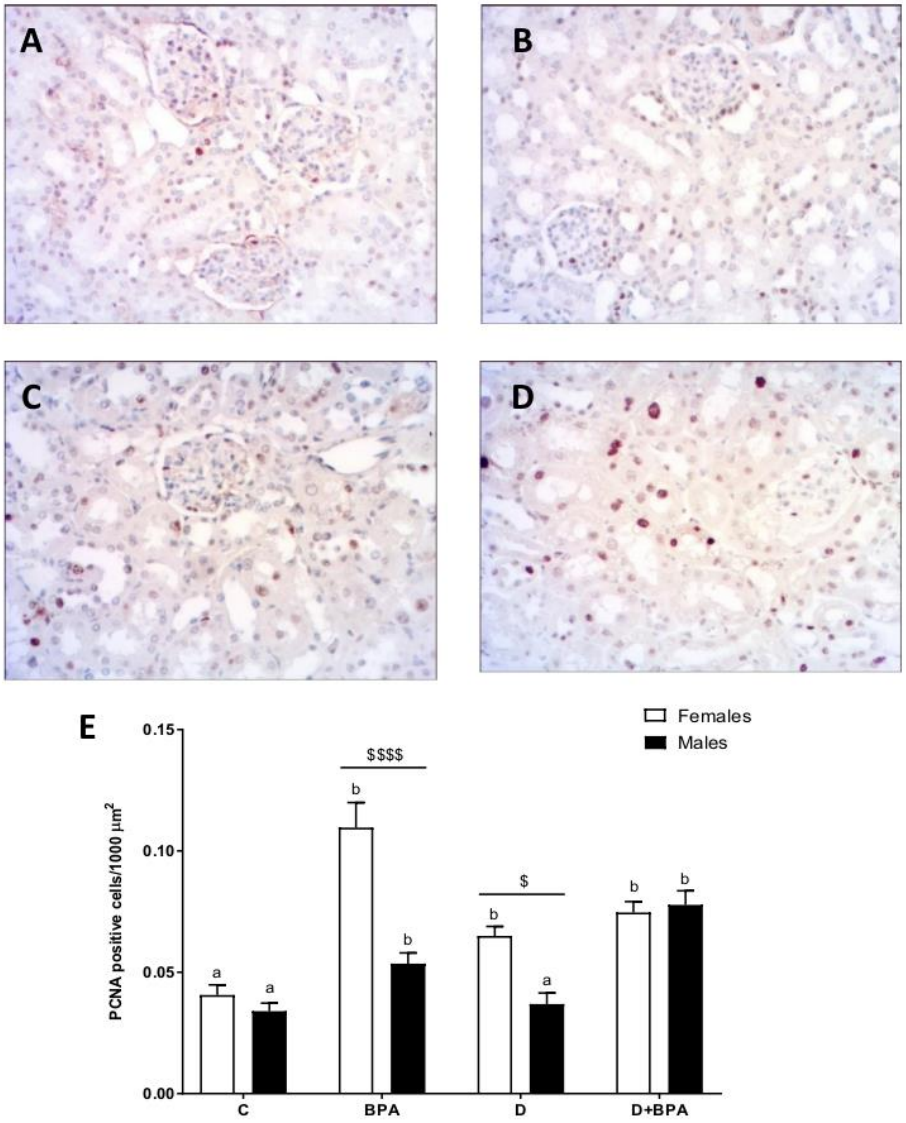


Fig. 7. PCNA immunostaining. A. Control groups showed a low number of proliferating cells. B. Kidneys from BPA-treated mice presented a higher number of PCNA positive cells than control groups, especially females. C. Diabetic mice showed a number similar to control in males and higher in females. D. D + BPA mice presented a high number of proliferating cells without any difference between gender (×300). E. Histogram representing the gender differences in each group. All groups with different letters have significant differences ($p < 0.05$). $^{\$}p < 0.05$; $^{\$\$\$\$}p < 0.0001$ females vs. males. One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

significant increases UAE, and renal damage analyzed as collapsed glomeruli. Renal cell proliferation, as analyzed by PCNA staining, was the only parameter found to be significantly higher in female animals than in the males' littermates.

Although endogenous creatinine clearance showed no significant differences among animal groups studied, serum creatinine displayed a statistically significant increase in both D and D + BPA groups compared to their correspondent's controls. It is known that to a variable degree, tubules secrete creatinine, which, by itself, would lead to an overestimation of renal function [60]. To further investigate BPA effects at the molecular level on the kidney, the gene expression of the kidney damage biomarkers Havcr-1 and Lidocalpin-2, which codify KIM-1 and NGAL, respectively, were evaluated [45,46]. Both genes were significantly increased in all treated mice in comparison to their control littermates. Interestingly, NGAL also showed a significant further increase in diabetic animals receiving BPA. All renal functional data studied supported the notion that BPA exposures to mice induced kidney damage in control mice and exacerbated injury in diabetic mice.

All treated male and female mice showed similar impairment of renal function. In contrast, male mice displayed a significant increase in urinary output restricted to D animal group, and female mice showed a significant increase in cell proliferation in this animal group. It is thus tempering to speculate, albeit not probed in the present setting, that increased urinary output could predispose to hydro electrolyte disturbances while increased in renal cell proliferation could, at least in theory, partially explain differences in sexual survival dimorphism pattern. On the other hand, D + BPA animal group did not display an additional effect on cell proliferation.

Several studies have demonstrated that most renal cells grown in high glucose conditions initially present a self-limited proliferation, followed by cell cycle arrest in the G1-phase, undergoing cellular hypertrophy [51,61]. These cellular events require the combined effect of mitogen-induced entry into the cell cycle and subsequent arrest at the G1 modulated by cell cycle regulatory proteins, including TGF-β1. Therefore, it is possible to speculate that the intense hypertrophy response observed in the D + BPA group may abrogate the mitogenic effect induced by BPA.

Sexual dimorphism could be due to different susceptibility to BPA, physiological differences, including sexual hormones, particularly androgen [62], or a combination of both. Indeed, different responses in males and females to the same exposure to BPA have been found. Most of the studies have been carried out mainly on the gestational or perinatal effect, where it has been determined that BPA can exert a different effect in males and females at the hepatic [23,24], adipogenic [25],

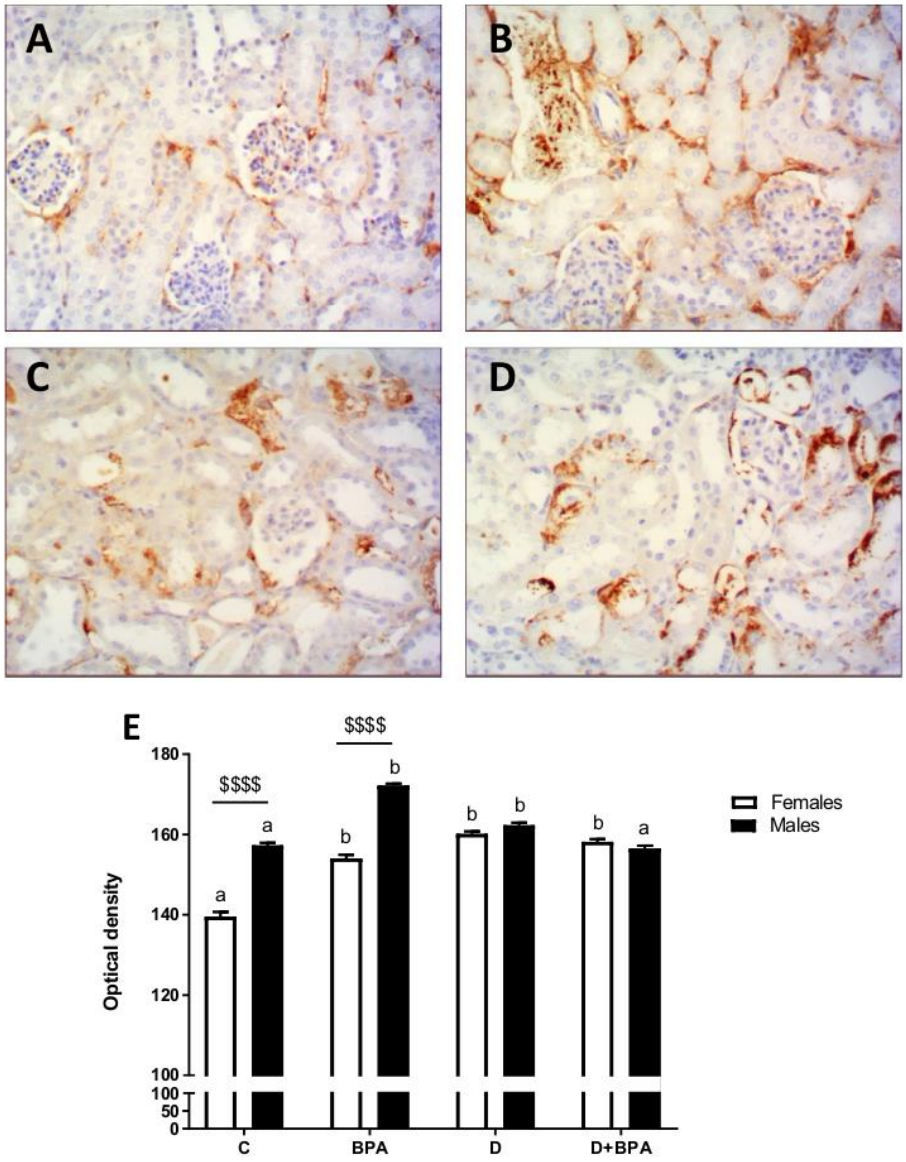


Fig. 8. 4-HNE immunolabeling. A. Control group. B. BPA-mice showed an important 4-HNE elevation, compared to their control group. Furthermore, BPA was the only treated group with sexual dimorphism pattern, similar than control. C. D group showed an important elevation without sexual dimorphism pattern. D. Only females D + BPA showed higher 4-HNE staining. E. Histogram represents the optical density of 4-HNE immunostaining. All groups with different letters have significant differences ($p < 0.01$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

neurological [26–28], endocrine [29], immunological [30], or renal level [31]. Furthermore, it has been observed that BPA is capable of inducing an agonist action on the estrogen receptor and an antagonist on the androgen receptor [63].

On the other hand, there is evidence that suggests that females have higher nephroprotection than males. In experimental animal models, female rats have increased protection against post-ischemic renal failure [64], doxorubicin treatment [65], and cisplatin treatment [66]. Epidemiological studies in humans also suggest the existence of nephroprotection in women. In this sense, one of the most extensive studies carried out is that of Neugarten et al. [67], where after performing a meta-analysis with 68 different studies and a total of 11,345 patients, they determined that men with chronic kidney disease caused by various etiologies show a much faster decline in kidney function than women. In another epidemiological study with 27,805 patients with type I diabetes, a statistically significant relationship was observed between the male sex and microalbuminuria development, an early marker of kidney disease [68].

Furthermore, probably because a BPA dose equivalent to half the NOAEL was used, the BPA group results do not show remarkable differences to the control group at the biometric and biochemical levels. However, an increase in albuminuria (less than in the diabetic groups, but equally significant) and a significant increase in blood pressure were observed. On the other hand, the histological studies showed interesting differences to the control group by showing the collapsed glomeruli, and a reduction in the number of cells, with the subsequent confirmation by TUNEL of the increase of apoptotic cells and a reduction in the number of podocytes labeled with WT-1.

Regarding sexual dimorphism, were observed differences in the renal hypertrophy index and albuminuria, with males presenting a higher number in both parameters. Although podocyte (WT-1) immunostaining showed no sexual dimorphism, particularly in the D + BPA group, the number collapsing glomerulus did show sexual dimorphism. Thus, damaged glomeruli could account for the observed increase in the UAE in the male D + BPA group.

In general, the results show that the route of administration of BPA and the dose used after eight weeks of treatment can exert an effect on the renal system similar to that described in the early stages of the development of diabetic nephropathy [69,70] at a dose lower than that considered "NOAEL" of the renal system [56].

Although there are limitations when using mouse models for assessing renal failure or long-term histomorphological changes [71],

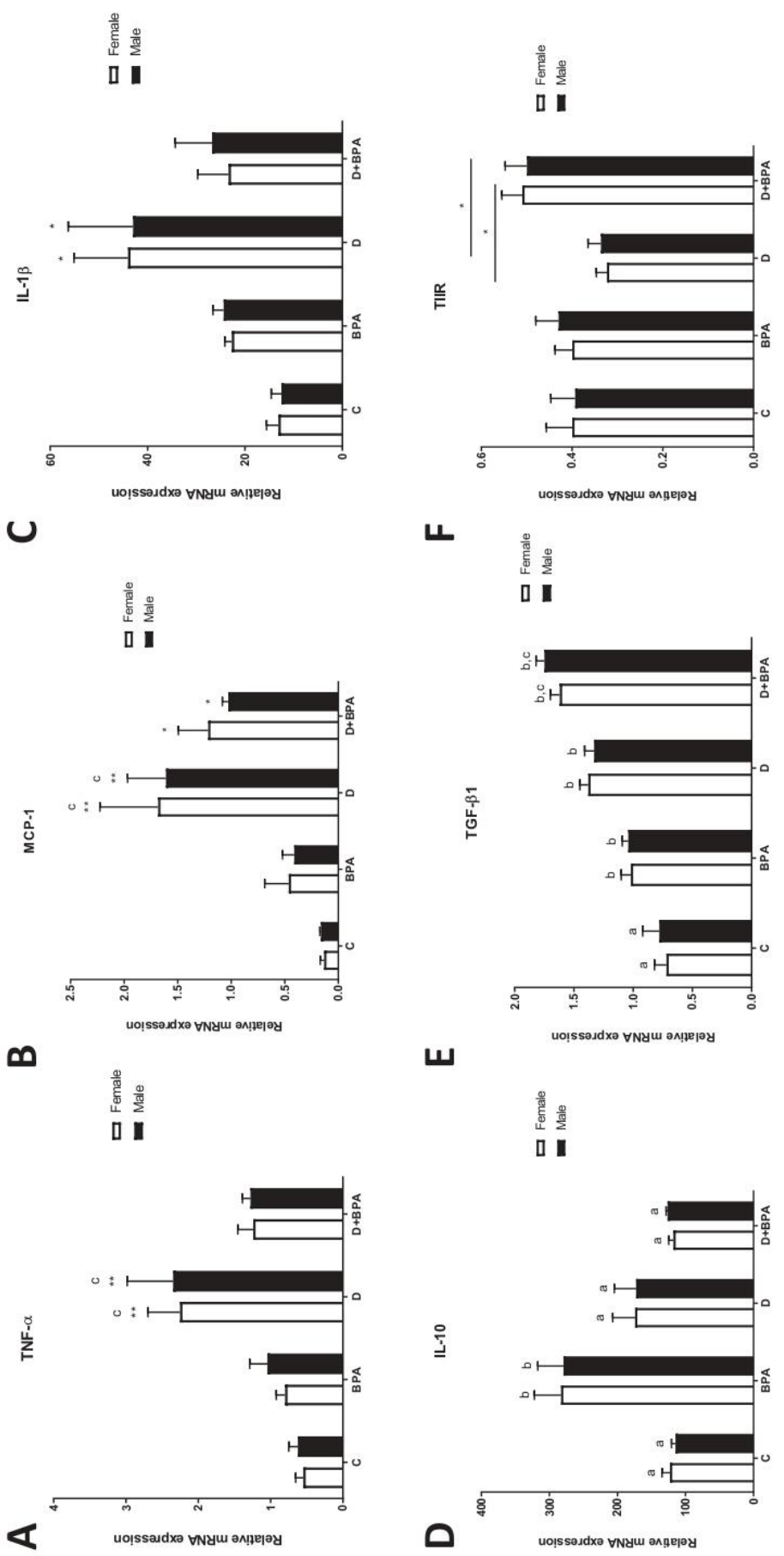


Fig. 9. Quantitative RT-PCR. A. TNF-α. The highest relative expression of the inflammatory messenger corresponds to the D group. B. MCP-1. As in the previous case, the highest relative expression of the inflammatory marker MCP-1 corresponds to the D group. C. IL-1β. The same expression pattern was observed as in the other two pro-inflammatory cytokines. D. IL-10. The expression of this anti-inflammatory cytokine only increased in the BPA group E. TGF-β1. D + BPA group showed the highest relative expression of TGF-β1 mRNA. F. TIIR. The only differences are observed between groups D and D + BPA. $^{*}p < 0.05$; $^{**}p < 0.01$ using the Kruskal-Wallis test for the comparison between groups. All groups with different letters have significant differences ($p < 0.05$). Letter c represents significant differences with BPA group ($p < 0.05$). A two-way ANOVA test was performed for the comparison between sexes.

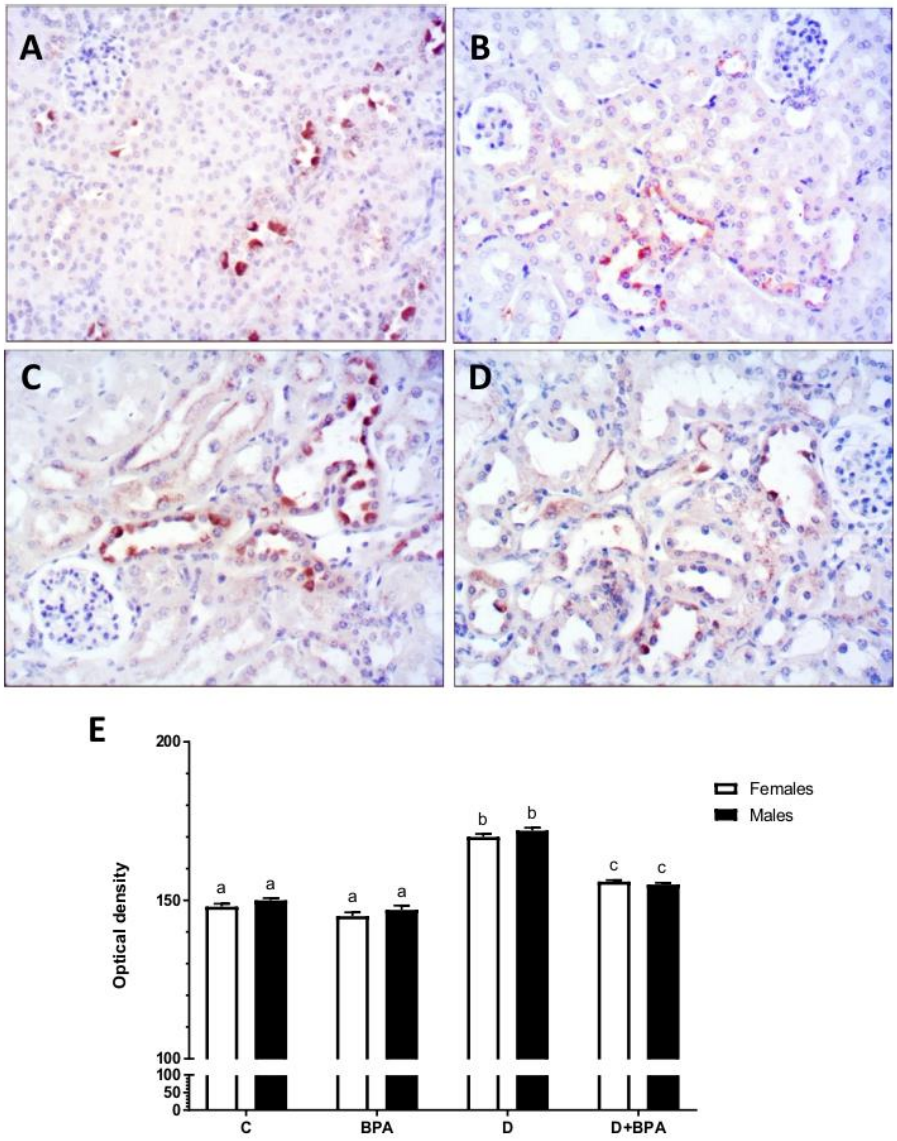


Fig. 10. MCP-1 immunolabeling. A. In control kidneys, MCP-1 was observed in the cytoplasm of some epithelial cells of the collecting tubules. B. In the BPA group, the labeling was similar to the control group. C. Diabetic mice showed a high number of labeled cells and higher intensity in the MCP-1 expression. D. Kidneys from D + BPA mice presented an elevated immunolabeling to MCP-1 antibody but with less intensity than diabetic animals (×300). E. Histogram represents the optical density of MCP-1 immunostaining. All groups with different letters have significant differences ($p < 0.0001$). One-way ANOVA or Kruskal-Wallis test was performed for the comparison between groups. A two-way ANOVA test was performed for the comparison between sexes.

our findings may have pathophysiological implications since the amount of proteinuria, podocyte number and the upregulation of kidney damage biomarkers are reliable predictors of the progression of renal disease [37,72–75]. Together with our present findings, all the available data strongly suggest that even low-grade proteinuria associated with BPA exposure might involve podocyte damage of an uncertain (or as yet unexplored) outcome, jointly with the biomarkers mentioned above are indicators of the need for future studies and raising a red flag of caution against increasing BPA exposure. Our data agree with Ruiz-Priego et al. [76], supporting the use of NGAL and KIM-1 as biomarkers of BPA-nephropathy.

Animals treated with BPA showed a significant increase in the expression of the CHOP protein. The kidney is one of the organs with the highest ER stress susceptibility because the fractional protein synthesis rates are almost half the total body load daily [77]. The possibility that ER stress is involved in the development of diabetic nephropathy has been described. Among the three signaling pathways that make up the ER stress response system, two of them are protective, and the remaining one, ERK-ATF4-CHOP, induces apoptosis in some kidney diseases [78]. It is interesting to note that both diabetic animals (as in other publications [79]) and those treated with BPA showed high CHOP levels. In this way, the hypothesis that BPA can induce similar damage to diabetic nephropathy is reaffirmed.

In order to get inside into the molecular mechanisms involves in the renal changes observed, we first analyzed the proapoptotic and profibrogenic TGF-β system. As expected, diabetic and BPA groups display the upregulation of TGF-β, a cytokine involved in hypertrophy, apoptosis, and renal fibrosis. Interestingly, this upregulation was significantly higher in the D + BPA group, thus suggesting a molecular mechanism for the observed renal parameters impairment in the latter group.

We then analyzed the renal expression of MCP-1, TNF-α and IL-1β well-known pro-inflammatory mediators in DN [80]. As expected, these molecules were found to be upregulated in D mice. On the contrary, BPA mice did not show changes in the expression of these pro-inflammatory mediators. It is well stablished that BPA can promote an inflammatory response by upregulating inflammatory mediators such as IL-1β, TNF-α, or MCP-1 [76,81,82], it is also known that it can also trigger the expression of anti-inflammatory molecules like IL-10 or TGF-β [83,84]. Herein we observed a significant upregulation of IL-10 restricted to BPA-treated mice. This finding could account, at least in part, for the lack of activation of proinflammatory mediators in this animal group.

In any case, recent observations using a BPA concentration higher than renal NOAEL, demonstrate the upregulation of several

proinflammatory mediators (including MCP-1, RANTES or IL-6) [76]. All data available strongly suggest that the level of BPA exposure might be critical to promote renal inflammation.

In conclusion, we observed that oral administration of BPA is capable of promoting in the kidney alterations that resemble early DN, such as increased in kidney damage biomarkers NGAL and KIM-1, UAE, hypertension, podocytopenia, apoptosis, collapsed glomeruli as well as TGF-β, CHOP and PCNA upregulation, albeit they did not develop changes in serum creatinine as observed in D mice. Moreover, UAE, collapsed glomeruli, PCNA staining, TGF-β (TIIR), and animal survival, significantly impaired in diabetic animals receiving BPA. Furthermore, UAE, collapsed glomeruli and animal survival also displayed a sexual dimorphism pattern.

Collectively, these data show that further translational studies are needed to clarify the potential role of BPA in renal diseases, particularly in diabetic patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2021.166296.

Funding

This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02139) -Fondo Europeo de Desarrollo Regional (FEDER), European Union-.

R. Moreno-Gómez-Toledano is recipient of a research contract from CAM (B2017-BMD-3686).

CRediT authorship contribution statement

R.J.B. conceived and designed research;
R.J.B. and M.S., provided grant funding;
R.M.G.T., M.I.A., C.M.M., N.O.H., P.R., A.I.L., A.A.C., M.G.S. and C.Z. performed experiments;
R.M.G.T., M.I.A., A.I.L., C.M.M., P.R. and N.O.H. analyzed data;
R.M.G.T., M.I.A., M.S., and R.J.B., interpreted results of experiments;
R.M.GT. and M.I.A., prepared figures;
R.J.B., R.M.G.T., and M.I.A., drafted manuscript;
R.J.B., R.M.G.T., M.I.A. and M.S. edited and revised manuscript;
All authors reviewed the manuscript and approved the final version.

Declaration of competing interest

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

Acknowledgments

We want to thank M. Bosch for proofreading of the manuscript. We also want to thank Dr. Jordi Bover (Nephrology Service, Germans Trias i Pujol Hospital, Universitat Autònoma de Barcelona) for his suggestions and comments.

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V. CAPÍTULO 2

BISPHENOL A IMPAIRED CELL ADHESION BY ALTERING THE EXPRESSION OF ADHESION AND CYTOSKELETON PROTEINS ON HUMAN PODOCYTES.

SCIENTIFIC REPORTS (4.379, Q1 MULTIDISCIPLINARY SCIENCES).

DOI:10.1038/s41598-020-73636-6

SCIENTIFIC REPORTS
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Bisphenol A impaired cell adhesion by altering the expression of adhesion and cytoskeleton proteins on human podocytes

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Bisphenol A (BPA), a chemical -xenoestrogen- used in food containers is present in the urine of almost the entire population. Recently, several extensive population studies have proven a significant association between urinary excretion of BPA and albuminuria. The alteration of glomerular podocytes or "podocytopathy" is a common event in chronic albuminuric conditions. Since many podocytes recovered from patients' urine are viable, we hypothesized that BPA could impair podocyte adhesion capabilities. Using an in vitro adhesion assay, we observed that BPA impaired podocyte adhesion, an effect that was abrogated by Tamoxifen (an estrogen receptor blocker). Genomic and proteomic analyses revealed that BPA affected the expression of several podocyte cytoskeleton and adhesion proteins. Western blot and immunocytochemistry confirmed the alteration in the protein expression of tubulin, vimentin, podocin, cofilin-1, vinculin, E-cadherin, nephrin, VCAM-1, tenascin-C, and β-catenin. Moreover, we also found that BPA, while decreased podocyte nitric oxide production, it lead to overproduction of ion superoxide. In conclusion, our data show that BPA induced a novel type of podocytopathy characterizes by an impairment of podocyte adhesion, by altering the expression of adhesion and cytoskeleton proteins. Moreover, BPA diminished production of podocyte nitric oxide and induced the overproduction of oxygen-free metabolites. These data provide a mechanism by which BPA could participate in the pathogenesis and progression of renal diseases.

Podocytes in the kidney glomerulus -also known as glomerular visceral epithelial cells- form the final barrier to protein loss, which explains why a podocyte injury or podocytopathy was accompanied by proteinuria[1,2]. Podocytes are mesenchymal-like differentiated cells and have a unique cellular architecture consisting of a cell body, major and foot processes[1,2]. They also contain the three major components of the eukaryotic cytoskeleton, i.e., intermediate filaments, microtubules, and microfilaments, or actin fibers. About 100 actin-associated proteins have been described in podocytes (i.e., alfa-actinin-4, ezrin, kindlin-2, filamin-D, cofilin-1, vinculin, profilin-1). Podocytes are anchored to specific matrix proteins of the glomerular basement membrane (i.e., VCAM-1, tenascin-C) and cell–cell binding (i.e., vinculin, E-cadherin, nephrin). Alterations in cell–matrix and cell–cell adherence lead to podocyte detachment[3–6].

Because of podocytes´ inability to proliferate adequately, podocytopenia follows when cells undergo apoptosis, detachment, necrosis, and altered autophagia in response to injury. This leads to progressive glomerular scarring[1–3]. Podocytopathy could be the result of an increasing list of conditions such as genetic, infectious, immune, and toxic aminoglycosides, including diabetes mellitus, with diabetic nephropathy (DN) being the most common cause of end-stage renal disease in developed countries. Interestingly, loss of podocytes into the urine (podocyturia) has been detected in many glomerular diseases[7–10].

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Bisphenol A (BPA) or 2,2, -bis (4-hydroxyphenyl) propane is a molecule used to synthesize polycarbonate plastics and epoxy resins extensively used in the production water and soft drinks bottles, and as the inner coating of cans and other food and drink containers (reviewed in[11]). It is well established that BPA belongs to the increasing list of endocrine disruptor agents (xenoestrogen). As such, BPA has been implicated with several endocrine and metabolic abnormalities, including hepatic and thyroid disorders, obesity, cardiovascular diseases, and increased susceptibility to diabetes[12–16].

Numerous studies have demonstrated that more than 95% of the population in the USA, Japan, and Spain have detectable urinary levels of BPA[11,17–19]. Even more concerning is the fact that studies conducted in several countries have shown environmental levels of BPA in water, dust, and air[12,20–24]. The environmental exposure is particularly intense among workers in the plastics industry, as well as people exposed to thermal paper[25–27]. BPA is conjugated by the liver, particularly after oral exposure, losing its estrogenic activity, and is then excreted mainly through the intestine. Both non-conjugated (bioactive) and conjugated BPA (inactive) are excreted in the urine[28–30].

Although several large population studies have demonstrated a significant association between urinary excretion of BPA concentration and albuminuria[31–34], there are no direct studies on the potential effect of BPA on human podocytes. Since several investigators have reported that many podocytes recovered from patients' urine are viable[35–39] herein, we hypothesize that BPA could impair podocyte ability to remain attached to the underlined glomerular basement membrane. To test this hypothesis, we performed an in vitro adhesion assay to measure the anchorage of cultured human podocytes to the culture flask. We also studied the potential cellular mechanism by which BPA promotes a novel type of podocytopathy characterized by cell adhesion impairment.

Results

BPA impaired podocyte adhesion. MTT assays showed a decrease in viability at concentrations of BPA higher than 50 μM. No significant differences were observed with lower concentrations (Fig. 1A).

Cell–cell contacts and the adherence of podocytes to the extracellular matrix of the glomerular basement membrane are crucial for podocyte function[3–6]. Herein, adhesion assays were performed to investigate the effect of low concentration of BPA on human podocyte anchorage to the culture flask. As shown in Fig. 1B, we first observed that a concentration of 100 nM BPA decreased podocyte adhesion by 50%. No significant effects were observed at lower BPA concentration (data not shown).

Because most of the BPA is glucuronidated in the liver, where it loses its estrogenic activity, we wanted to explore if liver detoxification can prevent podocyte injury. For this reason, human podocytes were incubated with 100 nM and 1 μM glucuronide BPA for 48 h. No significant effects on the podocyte adhesion were observed. Because BPA is capable of activating the estrogen receptors (ER), we next wanted to investigate if the observed effect of BPA on podocyte adhesion was due to the estrogenic effect of BPA. To this end, we used Tamoxifen and ICI 182,780 (Fulvestrant), compounds capable of blockade ER[40,41]. We pretreated podocytes with either Tamoxifen 100 nM or ICI 100 nM for 1 h before cells were treated with BPA 100 nM for 48 h. We observed that Tamoxifen, but not ICI, was able to abrogate the effect of BPA on podocyte adhesion.

Transcriptomic and proteomic effects of BPA on human podocytes. To identify the mechanism by which BPA impaired podocyte adhesion, we performed a transcriptomic and a proteomic analysis on human podocytes.

The transcriptomic analysis of human podocytes treated with BPA revealed a decrease in the transcription of genes that codified for proteins that interact and stabilize nephrin (involved in podocyte-podocyte binding). Moreover, the Gene Set Enrichment Analysis (GSEA) also revealed an increase in the transcription of genes that encode for proteins involved in the formation of tubulin folding intermediates (Table 1). Other proteins involved in cell adhesion, such as annexin-A2, filamin-C, as well as galectin-1, were also affected (data not shown).

Due to the growing list of molecules involves in the mechanism of cell adhesion, we performed a proteomic analysis of human podocytes incubated with BPA. Overall, the proteomic analysis showed significant changes in the expression of 75 proteins related to lipid and glucose metabolism, calcium-binding, cytoskeleton, and adhesion. Only the corresponding proteins with a q-value < 0.05 were considered when contemplating p-value < 0.05. The list of statistically significant proteins (with a higher False Discovery Rate) reached 363 proteins (data not shown).

Table 2 shows a list of 27 proteins -related to cell adhesion function- in which BPA significantly altered the pattern of expression, of which 21 of them proved to be downregulated while the remaining six were upregulated. Although the transcriptomic analysis showed less sensitivity in-display genomic changes induced by BPA compared to the proteomic studies, we found interesting functional coincidences in the altered pattern expression of proteins related to cell adhesion and the cytoskeleton.

Moreover, an increase in the superoxide dismutase, NADPH adrenodoxin oxidoreductase, and glutathione synthetase expression was also observed, which suggest an oxidative stress response. Herein, the potential effect of BPA on SO and NO production on human podocytes was also studied.

BPA affected the expression of cell–cell adhesion and cytoskeleton proteins as assessed by Western blot and immunocytochemistry. Based upon our main findings obtained in both transcriptomic and proteomic analysis, we proceeded to fully confirm significant changes in protein expression by Western blot as well as immunohistochemistry using the most specific commercially available antibodies.

We found that BPA induced changes in the expression of several critical functional podocyte proteins capable of regulating podocyte cytoskeleton, cytoskeleton stabilization, cell–cell adhesion, and cell–matrix adhesion.

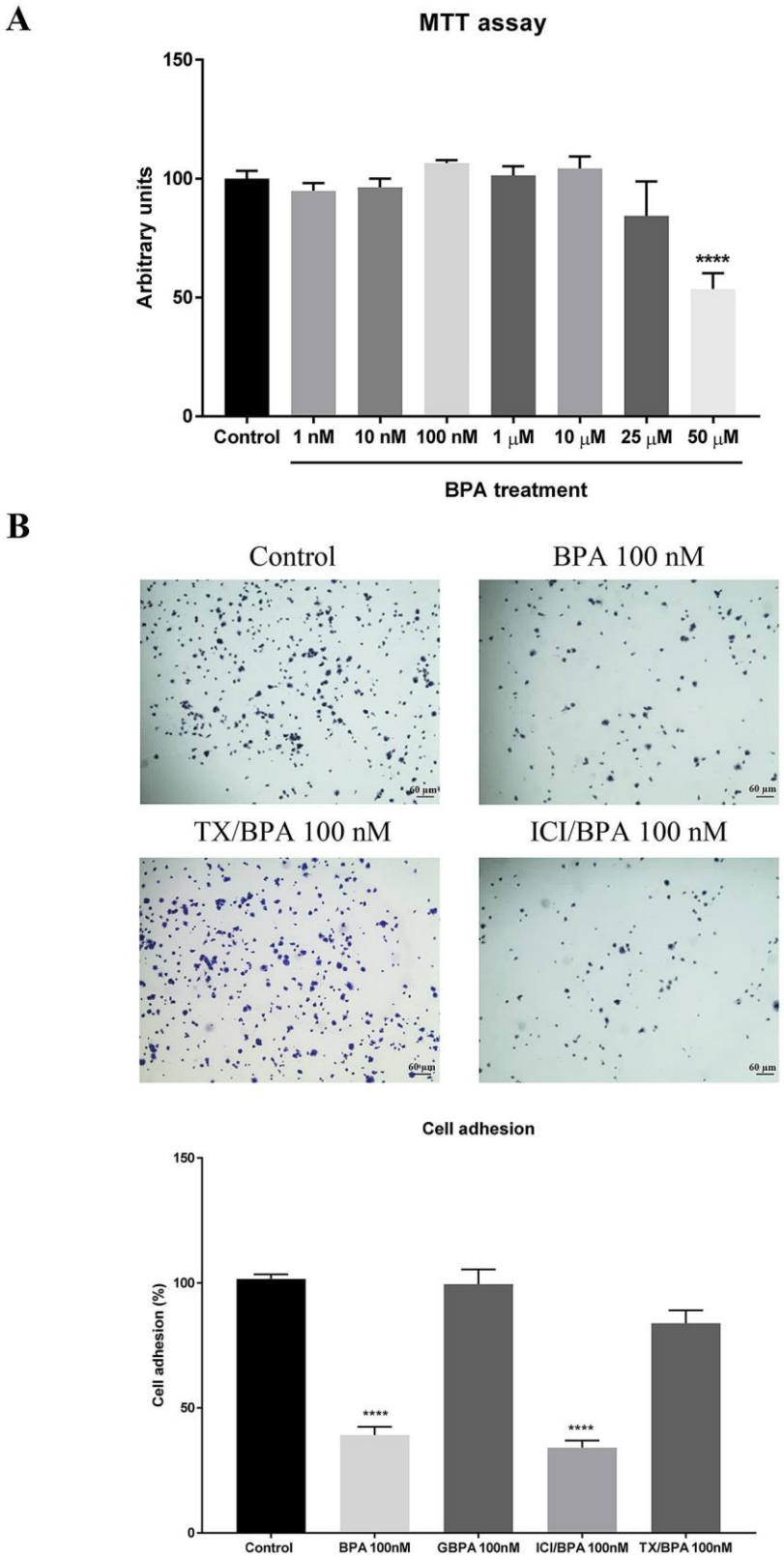

Figure 1. BPA impaired podocyte adhesion. (**A**) MTT assay showing the percentage of cell viability with BPA treatment to different concentrations. (**B**) Adhesion assay: micrographs of podocytes (×40) incubated with BPA as well as pre-treatment 1 h with estrogen receptor blockers Tamoxifen (TX) 100 nM or ICI 100 nM. Note that the result of GBPA, a metabolized form of BPA, has been included in the graph. Data are the means ± SEM of three different experiments, each performed in duplicate. ****$p < 0.0001$ using ANOVA test.

GS	SIZE	ES	NES	NOM *p* val	FDR *q* val	FWER *p* val	RANK AT MAX	LEADING EDGE
Reactome Nephrin interactions	19	− 0.38	− 2.03	0.004	0.030	0.661	10,941	tags = 74%list = 35% signal = 114%
Reactome formation of tubulin folding intermediates	19	0.40	2.07	0.004	0.011	0.571	7222	tags = 63%list = 23% signal = 82%

Table 1. Transcriptomic analysis of cell–cell adhesion and cytoskeleton clusters. For primary data processing, image analysis, per-cycle base calling, and quality score assignment was performed with Illumina Real-Time Analysis software (https://emea.illumina.com/).

Protein	Fold-change	*p* value	*q* value
Nesprin	−0.958	0.00004	0.004
FRAS1-related extracellular matrix protein 3	−0.736	0.000709993	0.026
Transgelin	−0.519	0.00001	0.002
Collagen alpha-1(I) chain	−0.385	0.00004	0.004
Tropomyosin beta chain	−0.298	0.001379986	0.041
Cell surface glycoprotein MUC18	−0.251	0.000419996	0.02
Cofilin-1	−0.241	0.000559994	0.024
Annexin A1	−0.223	0.00002	0.002
Elongation factor 2	−0.217	0.00001	0.001
Vimentin	−0.215	0.00001	0.004
Profilin-1	−0.199	0.000199998	0.011
Galectin-1	−0.196	0.000549995	0.024
Ezrin	−0.185	0.00097999	0.032
Annexin 2	−0.183	0.00001	0.001
Elongation factor 1-gamma	−0.174	0.001779982	0.048
T-complex protein 1 subunit gamma	−0.173	0.000679993	0.026
L-lactate dehydrogenase A chain	−0.17	0.000889991	0.03
Glyceraldehyde-3-phosphate dehydrogenase	−0.159	0.000859991	0.03
AHNAK	−0.152	0.00001	0.002
Vinculin	−0.144	0.000149999	0.009
Filamin-C	−0.134	0.000489995	0.023
Microtubule-associated protein 4	0.154	0.000499995	0.023
Transforming growth factor-beta-induced protein ig-h3	0.193	0.000659993	0.025
Glutathione synthetase	0.197	0.00064999	0.025
Src substrate cortactin	0.206	0.00009	0.007
Fibronectin	0.302	0.00001	0.005
Lactadherin	0.307	0.00009	0.007
Tenascin-C	0.314	0.00001	0.002
NADPH:adrenodoxin oxidoreductase, mitochondrial	0.451	0.00001	0.003
Superoxide dismutase	0.546	0.00001	0.001

Table 2. Proteomic analysis of cell adhesion, cytoskeleton and oxidative stress proteins.

First, we observed that on podocytes BPA promoted a downregulation in the expression of proteins of the cytoskeleton like F-actin, vimentin, tubulin and podocin (Fig. 2). We observed a significant reduction in the number of F-actin filaments (stained with phalloidin), which were mainly located in the borders of the cells; however, vimentin was concentrated around the nuclei; tubulin staining showed a significant decreased of the tubulin levels in the podocytes after the BPA treatment. Interestingly, podocin, a critical functional protein of fully differentiated podocyte, also displayed a decreased staining on BPA treated cells.

Second, BPA also induced a significant decreased in the cytoskeleton-binding (stabilization) proteins cofilin-1 and vinculin (Fig. 3).

Third, we found that BPA induced the downregulation of key proteins related to cell adhesion such as E-cadherin, nephrin, and VCAM-1 (Fig. 3).

Fourth, we found that BPA induced the upregulation of two proteins known to be associated with loss of cell adhesion such as tenascin-C and β-catenin (Fig. 4).

Finally, we observed that Tamoxifen was able to abrogate the changes in protein expression (analyzed by Western blot) of VCAM-1, vinculin, E-cadherin, nephrin and podocin induced by BPA on human podocytes (Fig. 5).

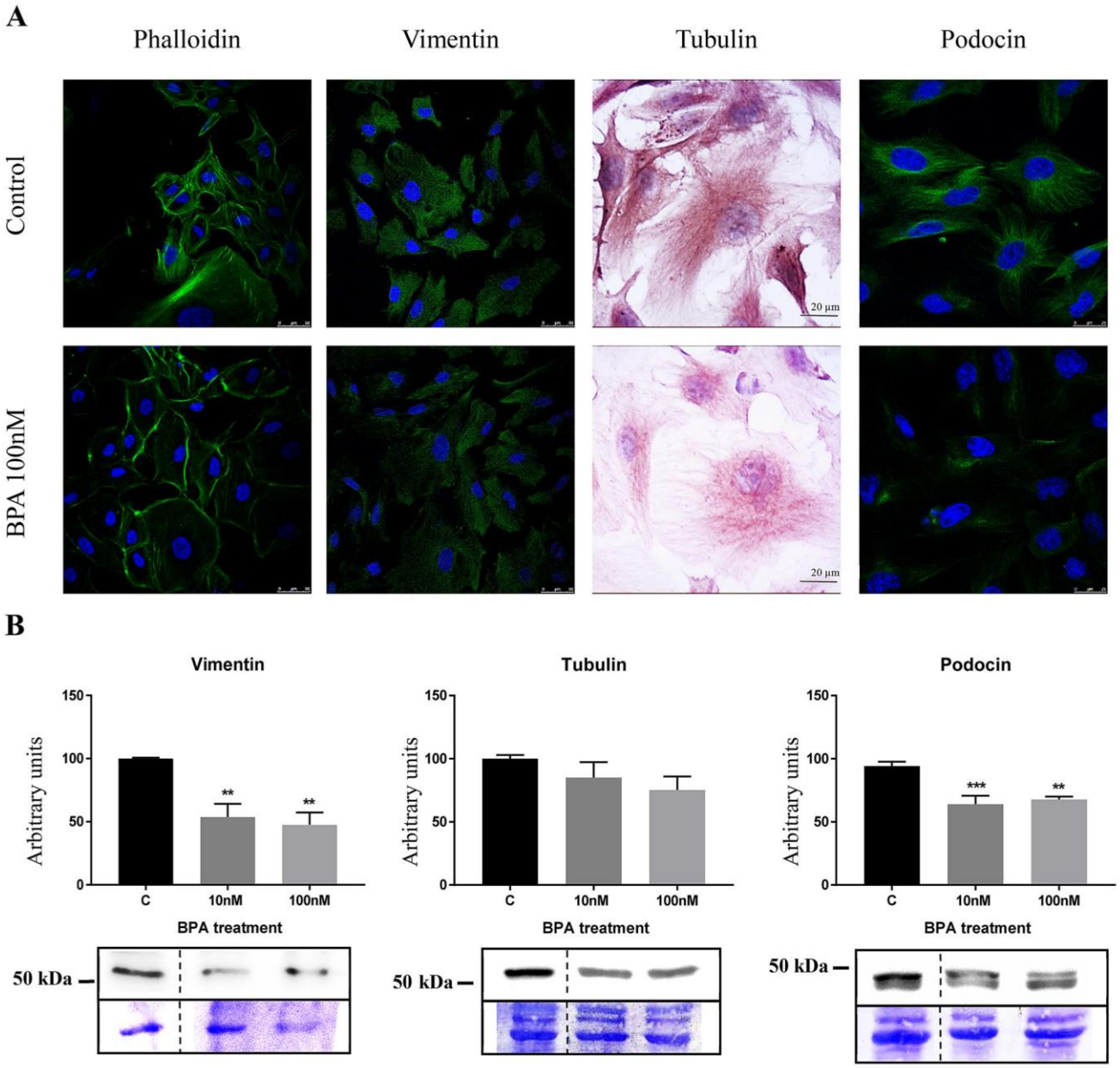

Figure 2. BPA downregulated proteins of podocyte cytoskeleton. (**A**) Immunocytochemistry assay of F-actin (phalloidin), vimentin, tubulin and podocin. (**B**) Western blot of vimentin, tubulin and podocin. Data are the means ± SEM of three different experiments, each performed in duplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ using ANOVA test for the comparison between control and BPA-treated cells. Due to BPA modified the housekeeping proteins (actin, tubulin, etc.) we used coomassie-blue staining to normalized total protein amount. Discontinue line indicates the absent lane of 1 nM BPA treatment. Full-length blots/gels are presented in Supplementary Figs. 1–3.

BPA affected NO and SO production on human podocytes. We measured NO production to explore if BPA activated vasoregulatory genes in human podocytes, observing that BPA decreased its production (Fig. 6). We then measured the output of different free radicals after the BPA exposition by flow cytometry finding that the SO production was significantly increased (Fig. 6).

Discussion

Experimental animal data have shown that BPA could promote podocyte apoptosis and proteinuria[43]. In humans, several extensive population studies have demonstrated a significant association between urinary excretion of BPA and albuminuria, a well-known factor involved in the mechanism of renal disease progression[31–34]. In this regard, Hu et al.[33], in a prospective study of 302 patients followed for 6 years, have demonstrated serum BPA as a predictor of chronic kidney disease in primary hypertension. Serum BPA was also described as a risk factor in the progression of diabetic nephropathy in patients with type 2 diabetes[32]. Our present study provides a cellular

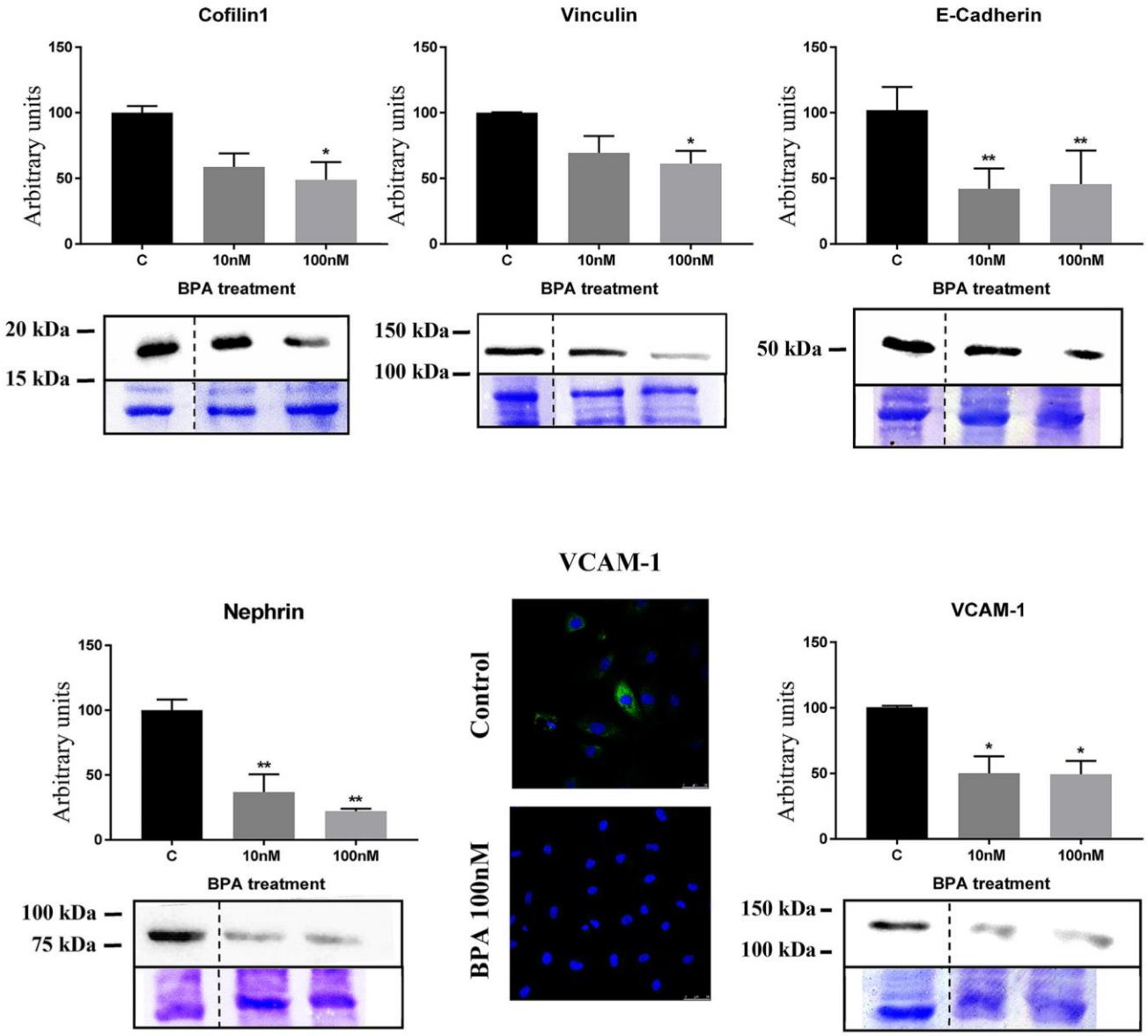

Figure 3. BPA treatment originated a decrease in the protein expression of cofilin-1, vinculin, E-cadherin, nephrin and VCAM-1. Data are the means ± SEM of three different experiments, each performed in duplicate. $^{*}p<0.05$ and $^{**}p<0.01$ using ANOVA test for the comparison between control and BPA-treated cells. Due to BPA modified the housekeeping proteins (actin, tubulin, etc.) we used coomassie-blue staining to normalized total protein amount. Discontinue line indicates the absent lane of 1 nM BPA treatment. Full-length blots/gels are presented in Supplementary Fig. 4–8.

mechanism of these findings since we describe a novel type of podocytopathy characterized by an impairment of podocyte adhesion capabilities because of a direct BPA alteration on the expression of crucial podocyte functional proteins.

We first performed a reliable and reproducible in vitro adhesion assay to measure the anchorage of cultured human podocytes to the culture flask.

We found that 100 nM BPA significantly impaired podocyte adhesion by 50%. Although no effect was observed at lower BPA concentration, this finding is relevant because such concentration can usually be found in people whose jobs have high exposure to BPA, such as the plastics or thermal papers industry[25–27]. Furthermore, it has been shown that higher concentrations can be found in patients with kidney disease in advanced stages undergoing hemodialysis[44,45], as well as in neonates undergoing surgical interventions where BPA levels can be even higher[46].

It is worth noting that the ER antagonist Tamoxifen could abrogate the effect of BPA on podocyte adhesion. Interestingly, glucuronidated BPA (without estrogenic activity) showed no effect on cell adhesion. These findings agree with previous studies showing the adverse effect of BPA through the activation of the ER in other cell types[40,41].

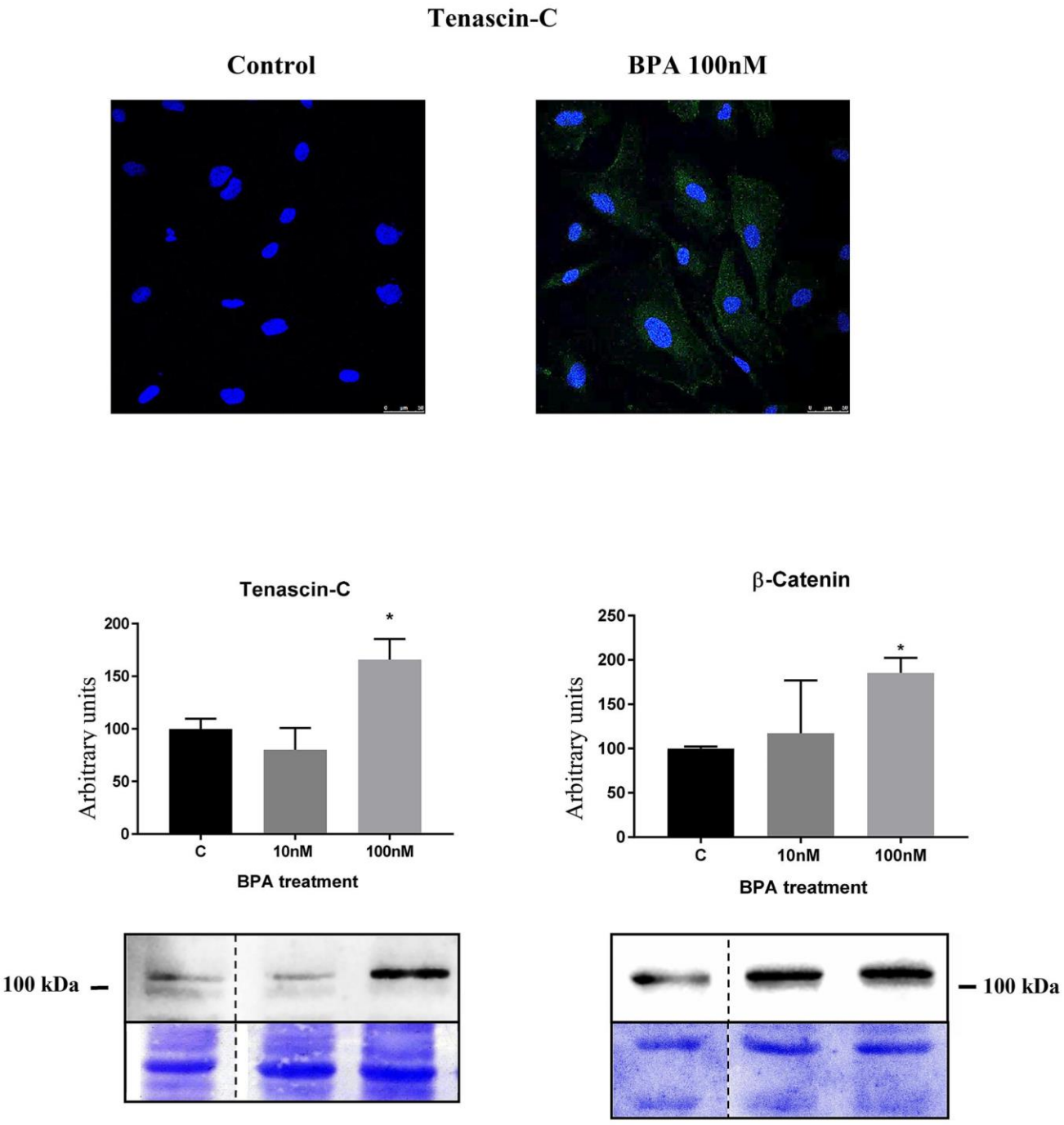

Figure 4. BPA treatment originated an increase in the protein expression of tenascin-C and β-catenin. Data are the means ± SEM of three different experiments, each performed in duplicate. *$p < 0.05$ using ANOVA test. Due to BPA modified the housekeeping proteins (actin, tubulin, etc.) we used coomassie-blue staining to normalized total protein amount. Discontinue line indicates the absent lane of 1 nM BPA treatment. Full-length blots/gels are presented in Supplementary Figs. 9–10.

To investigate the mechanism of BPA´s observed detrimental effect of BPA on human podocyte adhesion, we performed both transcriptomic and proteomic analysis followed by a further verification by Western blot or immunocytochemistry. We found that BPA was able to change significantly the expression of proteins involved in several essential physiological and pathophysiological pathways involved in cell–cell interaction and adhesion.

Firstly, we found that BPA promotes downregulation in the expression of podocytes cytoskeleton proteins such as tubulin, vimentin, and podocin. In this regard, George et al.[47] have shown that tubulin is a direct target of BPA in embryonic and somatic cells where BPA promotes disruption of the microtubule organization. Moreover, vimentin filaments that make up a substantial portion of the cytoskeleton of podocytes cell body and primary and secondary foot processes; it is also likely to be important for cell adhesion and spreading through interactions with actin and filamin trafficking[48]. Furthermore, podocin is a membrane protein that interacts physically and

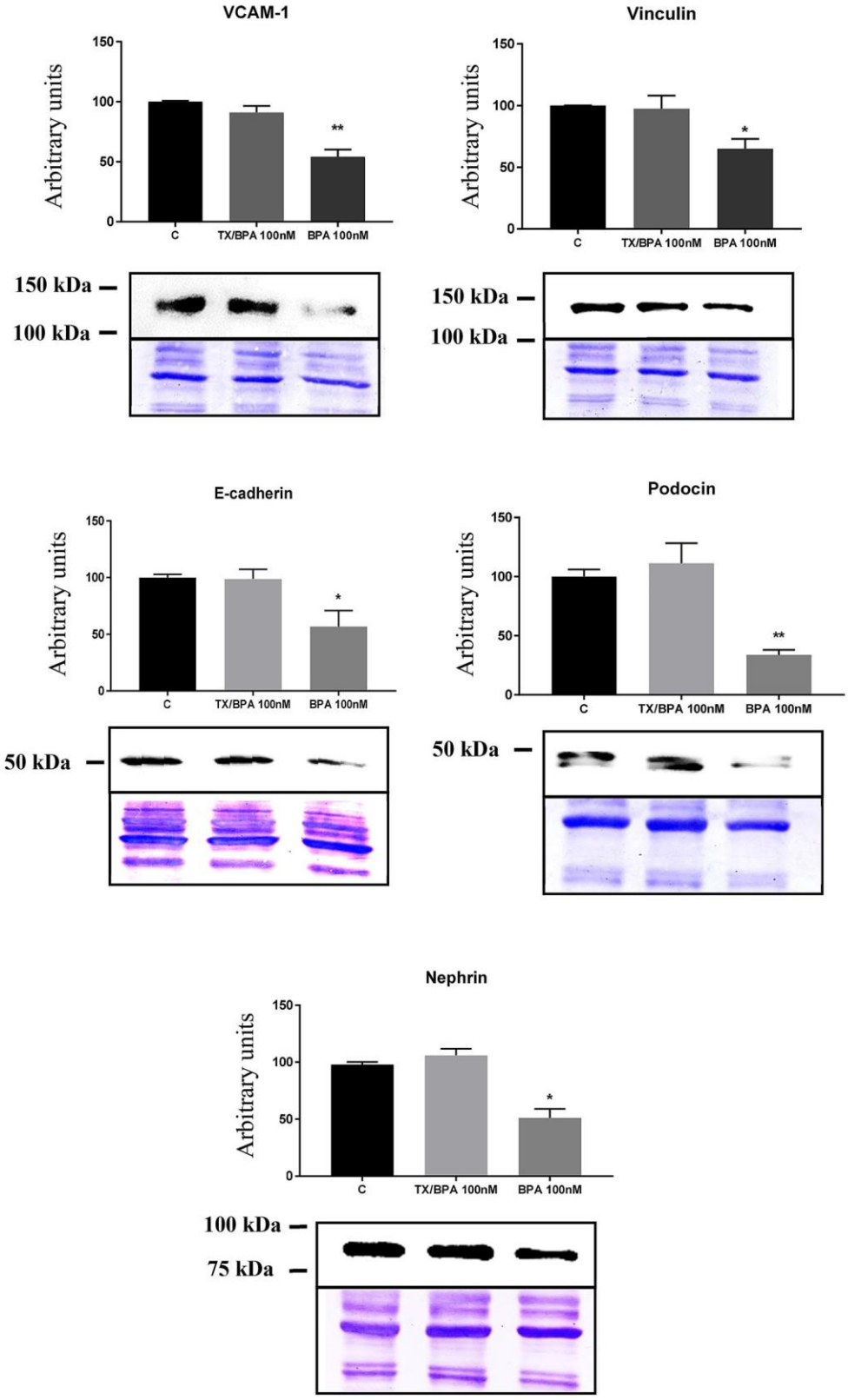

Figure 5. Pre-treatment with Tamoxifen (Tx) inhibited the effect of BPA on adhesion proteins. Data are the means ± SEM of three different experiments, each performed in duplicate. *$p < 0.05$ and ** $p < 0.01$ using ANOVA test. Due to BPA modified the housekeeping proteins (actin, tubulin, etc.) we used coomassie-blue staining to normalized total protein amount. Full-length blots/gels are presented in Supplementary Figs. 11–15.

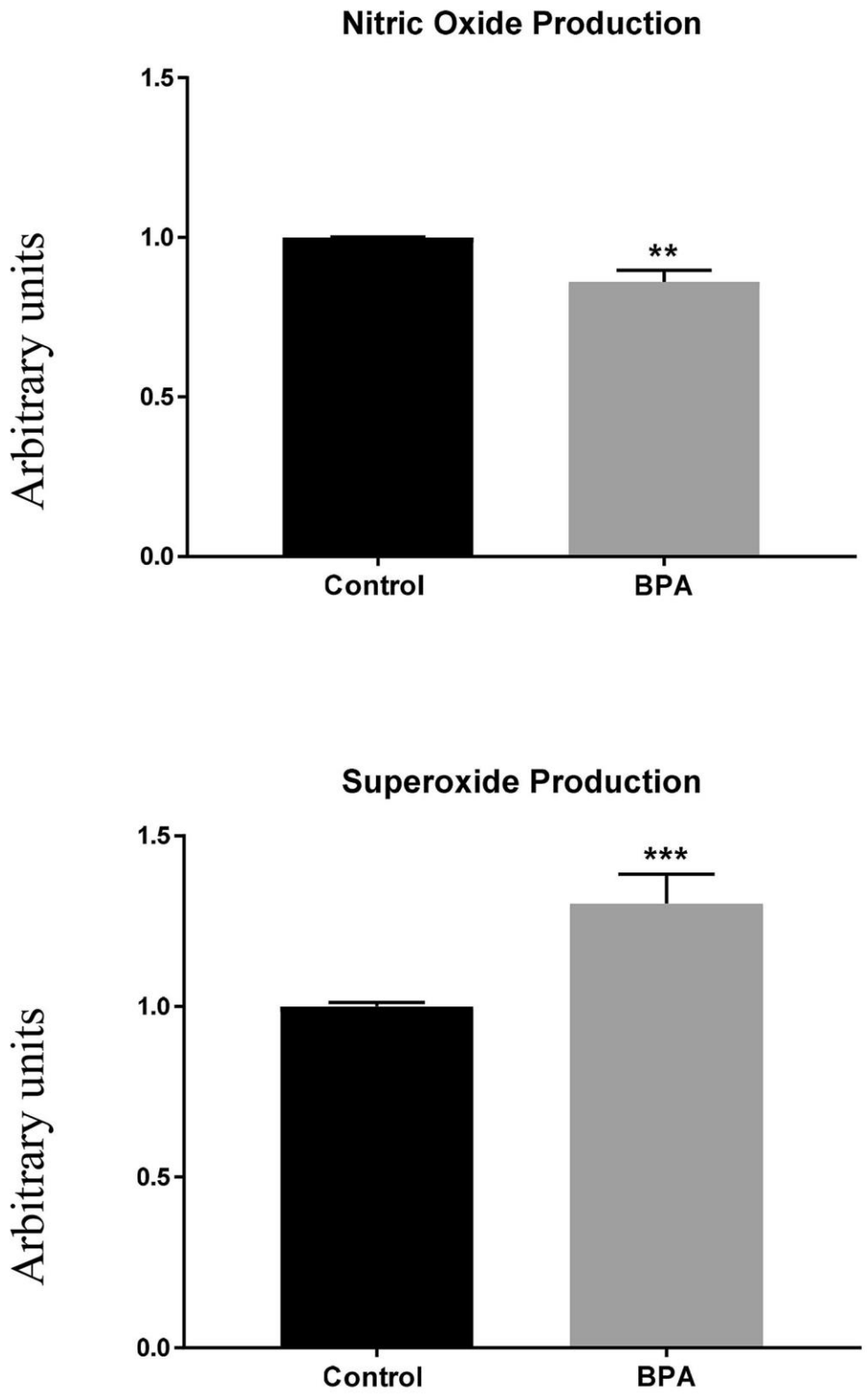

Figure 6. BPA reduced NO production and promoted an increase in the SO concentration. Data are the means ± SEM of three different experiments, each performed in duplicate. ** $p < 0.01$ and *** $p < 0.001$ using ANOVA test.

functionally linking the cell membrane to the cytoskeleton and directing its reorganization[49,50]. Downregulation of podocin has been associated with both proteinuria and podocyte survival. These data are in agreement with our previous observation in rodents receiving BPA[43].

Secondly, BPA also induced a significant decreased in the cytoskeleton-binding and stabilization proteins cofilin-1 and vinculin. Cofilin-1 is an essential regulator for actin filaments recycling that is required for the dynamic nature of podocyte foot processes[51]. Interestingly, higher H_2O_2 concentration, an event related to BPA[52], induces oxidation of cofilin cysteines (C139, C147), resulting in an inhibition of cofilin activity[53,54], an effect that could lead to proteinuria[55].

Vinculin, a cytoplasmic protein, couples actin filaments to integrin-mediated cell–matrix adhesions and cadherin-based intercellular junctions[56–58]. Vinculin is an adapter protein that localizes at cell–matrix adhesions and cell–cell junctions required to maintain glomerular barrier integrity[58]. Vinculin has a well-characterized function of stabilizing cell–matrix adhesions by orchestrating the recruitment and release of other cell–matrix adhesion proteins, thereby controlling the strength of adhesion binding to the extracellular matrix. Besides being more susceptible to glomerular injury, mice lacking podocyte vinculin develop altered adhesion and signaling, including albuminuria. Finally, vinculin levels are reported to be altered in human glomerular diseases[58].

Thirdly, BPA induced the downregulation of essential proteins related to cell adhesion such as E-cadherin, nephrin, and VCAM-1. E-cadherin is a calcium-dependent cell–cell adhesion molecule responsible for the primary cell adhesion system in epithelium[58]. This multiprotein complex interacts with the actin cytoskeleton and physically links cells to each other[59]. This is in accord with our previous findings showing that BPA was able to downregulate nephrin and podocin protein expression in mouse podocytes in culture[43]. VCAM-1 is another protein involved in cell adhesion[60] that we found to be downregulated by BPA.

Fourth, we found that BPA induced the upregulation of two proteins known to be associated with loss of cell adhesion such as tenascin-C and β-catenin. Tenascin-C is an extracellular matrix glycoprotein that modulates adhesion of cells to fibronectin and thus can be classified as an anti-adhesive, adhesion-modulating extracellular matrix protein[61]. β-catenin is the critical component in the highly conserved canonical Wnt pathway, regulating cell–cell adhesion[62] as well as serving as a transcription co-factor[63]. Widely expressed, β-catenin is also found in podocytes and plays a pivotal role in cell adhesion and differentiation. Previous studies showed that β-catenin activity is explicitly upregulated in glomerular podocytes in various proteinuric kidney diseases[64,65]. Herein our results clearly indicated that BPA significantly increased the protein expression of tenascin-C as well as β-catenin. An interaction between β-catenin and tenascin-C[66,67] is known to occur and may also trigger podocytopathy as described in other cell types[68,69].

As previously mentioned, the proteomic analysis revealed an upregulation of the enzymes superoxide dismutase, glutathione synthetase, and NADPH adrenodoxin oxidoreductase, suggesting that BPA could also trigger the production of damaging oxygen-free radicals. In previous work, we demonstrated that in the endothelium BPA could activate vasoregulatory genes such as angiotensin II and calcium-calmodulin kinase II responsible for endothelial dysfunction and hypertension, through a mechanism involving the uncoupling of endothelial nitric oxide synthase promoting a decreased of NO production and overproduction of oxygen free radicals[42]. Interestingly we found that BPA also promotes a decrease in NO production and an overproduction of superoxide on human podocytes.

Since Tamoxifen was able to abrogate BPA´s effect on podocyte adhesion, we analyzed if this protective effect was associated with the alteration of the expression of adhesion and cytoskeleton proteins promoted by BPA. Herein we observed that Tamoxifen was capable to prevent the alteration of fundamental structural proteins such as VCAM-1, vinculin, E-cadherin, nephrin and podocin on human podocytes.

In conclusion, our data show that BPA promoted a novel type of podocytopathy characterizes by an impairment of podocyte adhesion by altering the expression of adhesion and cytoskeleton proteins. Moreover, BPA diminished podocyte NO production and induced the overproduction of oxygen-free metabolites. Although further translational studies are needed to clarify BPA´s potential role in the pathogenesis and the progression of renal diseases, these data provide a mechanism by which BPA could promote renal damage.

Methods

Human podocyte culture. Conditionally immortalized human podocytes (a generous gift from Dr. M Saleem, University of Bristol and Dr. J Nornan, Royal Free Hospital, London, UK) were cultivated as previously described by Saleem et al.[70]. In brief, cells were grown in standard RPMI 1640 medium containing 10% FBS and supplements at the permissive temperature of 33 °C (in 5% CO_2) to promote cell propagation to 50–80% confluence. After that, cells were shifted to the non-permissive temperature of 37 °C (in 5% CO_2) to allow terminal differentiation for 15 days. Next, cells were treated with different concentrations of BPA (Aldrich chemistry) and BPA-glucuronide (G-BPA, a conjugated form by the liver) for 1, 2, or 3 days. In all cases, BPA and G-BPA were dissolved in ethanol and then added to the culture medium.

We used a BPA concentration of 10 and 100 nM, within the range that many authors consider as low dose[71,72] and within the concentration range to which the human being is exposed. It has been described that the general population has a concentration of BPA that ranges between 1 and 10 nM, while workers with high exposure to BPA can reach a concentration of 100 nM[73–75]. Furthermore, it has been described that surgical interventions or hemodialysis in the hospital environment can increase the exposure to BPA even more[44–46].

We also use 100 nM Tamoxifen (H6278 Sigma-Aldrich) and 100 nM ICI 182,780[76–78] (dissolved in ethanol) as ER blockers 1 h before adding the BPA treatment.

MTT cell viability assay. After BPA treatment (3 days), 50 µl of MTT (5 mg/ml) were added to each well in 500 µl of the medium, and the plates were incubated for 1 h 30 min at 37 °C. Then, DMSO (Sigma Aldrich) was

added to solubilize the formazan crystals. The absorbance was measured at a test wavelength of 570 nm with a reference wavelength of 690 nm[43].

Adhesion assay. In order to perform an adhesion cell assay that would allow quantifying the number of cells able to reattach to the culture dish after 48 h of BPA exposition, Human Podocytes were treated with 1, 10 and 100 nM BPA, 100, 1000 nM Glucuronide-BPA and 100 nM Tamoxifen and ICI or the respective control for 48 h. The medium was removed, and cells were exposed to Accutase (Thermo Fisher Invitrogen) until all cells were suspended, which was optically controlled. Hereafter, 25,000 cells were left to settle again in standard RPMI 1640 medium containing 10% FBS for 60 min. Then the medium was removed, the P24 was washed with PBS, and attached cells were measured determining MTT cell viability or count by simple staining with violet crystal[79].

Transcriptomic and proteomic studies on human podocytes. For both transcriptomic and proteomic analyses human podocytes incubated 48 h with 100 nM BPA.

Microarray analysis. *Library construction protocol.* 1 µg of total RNA samples, containing ERCC ExFold RNA Spike-In Mixes (Ambion 4456739), was used. The average sample RNA Integrity Number was 9.8 when assayed on an Agilent 2100 Bioanalyzer. PolyA+ fraction was purified and randomly fragmented, converted to double-stranded cDNA and processed through subsequent enzymatic treatments of end-repair, dA-tailing. This was followed by the ligation to adapters as in Illumina's "TruSeq Stranded mRNA Sample Preparation Part # 15031047 Rev. D" kit (this kit incorporates dUTP during 2nd strand cDNA synthesis, which implies that only the cDNA strand generated during 1st strand synthesis is eventually sequenced).

PCR completed the Adapter-ligated library with Illumina PE primers (10 cycles). The resulting purified cDNA library was applied to an Illumina flow cell for cluster generation and sequenced on an Illumina instrument (Illumina HiSeq2500) by following the manufacturer's protocols[80].

For primary data processing, image analysis, per-cycle base calling, and quality score assignment was performed with Illumina Real-Time Analysis software. Conversion of Illumina BCL files to bam format was performed with the Illumina2bam tool (Wellcome Trust Sanger Institute—NPG).

Proteomic analysis: iTRAQ assay. *Protein digestion and tagging with TMT simplex reagent.* For digestion, 40 µg of protein from each condition were precipitated by methanol-chloroform method. Protein pellets were resuspended and denatured, as previously described by Méndez et al.[81]. The resulting peptides were subsequently labeled using TMT-six-plex Isobaric Mass Tagging Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions as follows: 126: C-1; 127: BFA-1; 128: C-2; 129: BFA-2; 130: C-3; 131: BFA-3. Three biological replicates of each condition were analyzed in this study. After labeling, the samples were pooled, evaporated to dryness and stored at − 20 °C until the LC–MS analysis.

Nano-liquid chromatography and mass spectrometry analysis. A 1 µg aliquot of the labeled mixture was subjected to 1D-nano LC ESI-MSMS analysis using a nano-liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, SCIEX, Foster City, CA) coupled to high-speed Triple TOF 5600 mass spectrometer (SCIEX, Foster City, CA) with a Nanospray III source. The analytical column used was a silica-based reversed-phase Acquity UPLC M-Class Peptide BEH C18 Column, 75 µm × 150 mm, 1.7 µm particle size, and 130 Å pore size (Waters). The trapping column was a C18 Acclaim PepMap 100 (Thermo Scientific), 100 µm × 2 cm, 5 µm particle diameter, 100 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 2 µl/min. The nano-pump provided a flow-rate of 250 nl/min and was operated under gradient elution conditions. Peptides were separated using a 250 min gradient ranging from 2 to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). The injection volume was 5 µl.

Data acquisition was performed with a TripleTOF 5600 System (SCIEX, Foster City, CA). Data were acquired as previously described by Méndez et al.[81].

Data analysis. MS/MS spectra were exported to mgf format using Peak View v1.2.0.3 following by proteomic search engines (Mascot Server 2.5.1, OMSSA 2.1.9, X!TANDEM 2013.02.01.1, and Myrimatch 2.2.140) against a composite target/decoy database built from the 71,785 Homo sapiens sequences at UniProt (proteome ID UP000005640, January 2018) plus some commonly occurring contaminants. Correctly identified peptides from an initial X!TANDEM search with a mass error tolerance of 35 ppm was used to recalibrate parent ion mass measurements in all spectra using linear models. All search engines were then configured as previously described by Méndez et al.[81]. All analyses were conducted using software from Proteobotics (Madrid, Spain).

Western blot. After electrophoresis of total cell proteins, samples were immunoblotted as previously reported[82,83]. Membranes were then incubated overnight at 4 °C with the following polyclonal antibodies [dilution, -fold]: anti-VCAM1 antibody (Abcam,) [5000], anti-E-Cadherin antibody (BS Transduction Laboratories) [1000], anti-tenascin-C antibody (Santa Cruz Biotechnology, Santa Cruz CA) [500], anti- NPHS2 antibody (Abcam) [5000], anti-nephrin antibody (Abcam) [5000] and anti-tubulin (Sigma–Aldrich, Saint Louis, MO [5000]. Anti-vimentin (Santa Cruz Biotechnology) [1000], anti-cofilin-1 (Santa Cruz Biotechnology) [1000], anti-vinculin (Santa Cruz Biotechnology) [1000], anti-ILK (Santa Cruz Biotechnology) [1000], anti-β-catenin (Santa Cruz Biotechnology) [2000].

Coomassie staining (Sigma) of the membrane was used as an internal loading control. Blots were analyzed by densitometric scanning with Image J. Western blot studies in cultured cells were performed in at least three independent experiments, and a representative figure is shown.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde for 10 min and rinsed in phosphate buffer saline (PBS). Cells were then incubated for 30 min with 5% normal donkey serum in PBS to block nonspecific binding. Afterward, cells were incubated overnight at 4 °C with primary antibodies [1:200] and then washed with PBS. Finally, cells were incubated with α-rabbit-Alexa-Fluor 488 or α-mouse-Alexa-Fluor 488, both diluted 1:1500 for 1 h in the darkness. Slides were then washed and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Detection was performed by confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany). In some cases, protein detection was performed with DAB chromogen; then, after primary antibodies incubation, the cells were washed and incubated in primary antibodies amplifier Quanto (Ultravision Quanto detection system–peroxidase, Master Diagnóstica, Granada, Spain) for 10 min. After an extensive wash in PBS, the cells were incubated in polymer Quanto for 10 min. The peroxidase activity was detected using the DAB kit (Master Diagnóstica). Cover slides were counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in Entellan.

Nitric oxide production by human podocytes. Nitric oxide production was measured in podocytes loaded with diaminofluorescein diacetate (DAF-DA; 2 μM), and propidium iodide (1 μg/sample) was used to determine cell viability as previously described (58). After exposure to different experimental conditions; PBS (control) and BPA (100 nM) for 24 h, cells were and labeled with the fluorochrome at 37 °C for 1 h 30 min, trypsin dispersed and followed by cytofluorometric analysis with a fluorescence-activated cell sorter (FACS) scanner (Becton Dickinson, New York, NY, USA). Ten thousand events were analyzed for each condition.

Superoxide anion production. Superoxide reacts with dihydroethidium (DHE), forming 2-hydroxyethidium. Podocytes were incubated with PBS (control) and BPA (100 nM) for 24 h and treated with 10 μM apocynin for 1 h to inhibit NADPH oxidase. After exposure to different experimental conditions, cells were trypsin dispersed and labeled with the fluorochrome (DHE) for 30 min, followed by cytofluorometric analysis with a FACS scanner. A total of 10,000 events were analyzed for each condition[42].

Statistical analysis. Data were reported as the mean ± SEM. The Kolmogorov–Smirnov test assessed normal distribution. To determine the effects of BPA, one-way ANOVA or Kruskal–Wallis followed by a Bonferroni or Dunn´s test, respectively, was carried out. The p values presented in figures and tables corresponded to post hoc test. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$. In the case of the proteomics test, the corresponding proteins data were analyzed using the q value < 0.05[84,85].

Received: 9 March 2020; Accepted: 11 September 2020
Published online: 06 October 2020

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Acknowledgments

We want to thank Dr. Orlando Dominguez from the National Cancer Research Center (CNIO), Madrid, Spain, for helping with the analysis of the transcriptomic data, and Miguel Bosch for proofreading the manuscript. We also want to thank Dr. Guillermo Bodega, from the University of Alcala, and Dr. Sergio Ciordia, from the National Center for Biotechnology (CNB) Madrid, Spain, for the analysis of the proteomic data.

Author's contributions

R.J.B. conceived and designed research; R.J.B. and M.S., provided grant funding; R.M.G.T., M.I.A., C.G.M., N.O.H., P.R., M.D.N., and S.S.E., performed experiments; R.M.G.T., M.I.A., C.G.M., N.O.H., P.R., M.D.N., and S.S.E., analyzed data; R.M.G.T., M.I.A., C.G.M, E.A.F., M.S, and R.J.B., interpreted results of experiments; R.M.GT., M.I.A., and C.G.M., prepared figures; R.J.B., E.A.F., and M.I.A., drafted manuscript; R.J.B., M.I.A., E.A.F., and M.S., edited and revised manuscript; R.J.B. and M.I.A., approved final version of manuscript. All authors reviewed the manuscript.

Funding

This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02,139) -Fondo Europeo de Desarrollo Regional (FEDER)-. N. Olea-Herreo was recipient of a research contract from Comunidad Autónoma de Madrid (CAM)—Programa de Actividades I + D en Biomedicina 2010 (S2010/BMD-2378). R. Moreno-Gómez-Toledano is recipient of a research contract from CAM (B2017-BMD-3686). P. Reventun is a fellow of the program "Ayuda para contratos predoctorales para personal investigador en formación" from UAH.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-73636-6.

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VI. CAPÍTULO 3

BISPHENOL A EXPOSURE AND KIDNEY DISEASES: SYSTEMATIC REVIEW, META-ANALYSIS, AND NHANES 03–16 STUDY. **BIOMOLECULES** (4.879, Q2 BIOCHEMISTRY & MOLECULAR BIOLOGY [2020]).

DOI:10.3390/BIOM11071046

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Bisphenol a Exposure and Kidney Diseases: Systematic Review, Meta-Analysis, and NHANES 03–16 Study

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Abstract: Bisphenol A (BPA) is a compound that is especially widespread in most commonly used objects due to its multiple uses in the plastic industry. However, several data support the need to restrict its use. In recent years, new implications of BPA on the renal system have been discovered, which denotes the need to expand studies in patients. To this end, a systematic review and a meta-analysis was performed to explore existing literature that examines the BPA-kidney disease paradigm and to determine what and how future studies will need to be carried out. Our systematic review revealed that only few relevant publications have focused on the problem. However, the subsequent meta-analysis revealed that high blood concentrations of BPA could be a factor in developing kidney disease, at least in people with previous pathologies such as diabetes or hypertension. Furthermore, BPA could also represent a risk factor in healthy people whose urinary excretion is higher. Finally, the data analyzed from the NHANES 03-16 cohort provided new evidence on the possible involvement of BPA in kidney disease. Therefore, our results underline the need to carry out a thorough and methodologically homogeneous study, delving into the relationship between urinary and blood BPA, glomerular filtration rate, and urine albumin-to-creatinine ratio, preferably in population groups at risk, and subsequently in the general population, to solve this relevant conundrum with critical potential implications in Public Health.

Keywords: bisphenol A; kidney; systematic review; meta-analysis

Citation: Moreno-Gómez-Toledano, R.; Arenas, M.I.; Vélez-Vélez, E.; Coll, E.; Quiroga, B.; Bover, J.; Bosch, R.J. Bisphenol a Exposure and Kidney Diseases: Systematic Review, Meta-Analysis, and NHANES 03–16 Study. *Biomolecules* **2021**, *11*, 1046. https://doi.org/10.3390/biom11071046

Academic Editor: Jose M. Valdivielso

Received: 8 June 2021
Accepted: 13 July 2021
Published: 16 July 2021

1. Introduction

Bisphenol A (BPA) is a phenolic compound widely distributed in many everyday objects due to its multiple uses in the plastic industry [1]. Fundamentally used in the manufacture of polycarbonates and epoxy resins [2], BPA can be found in food and beverage containers, bottles and cans, as well as capsule coffee makers, tap water pipes, toys, electronic devices, and even in the composites used in dental restorations [3–5]. Increasing concerns over BPA as an endocrine-disrupting chemical and its possible effects on human health have prompted BPA removal from consumer products, often labeled "BPA-free".

Currently, the European Food Safety Authority (EFSA) has delimited a tolerable daily intake (TDI) up to 4 µg/kg body weight/day, based on studies by Tyl et al. (2008) on the ability of BPA to affect the kidneys [6,7]. However, only a small percentage of publications study the BPA-kidney paradigm [8–13], compared to other organs and systems such as the reproductive or endocrine systems [14–20]. In this sense, previous studies developed

by our team determined that mouse podocytes (cells that are part of the glomerular filtration barrier) treated with BPA (10 and 100 nM) underwent apoptosis followed by podocytopenia, albuminuria [12], and hypertension. Interestingly, when treating cultured human podocytes at the same doses of BPA, we observed a podocytopathy characterized by impaired cell adhesion due to alteration in the expression of the podocyte's adhesion and structural proteins [13]. Furthermore, epidemiological studies in humans have found interesting relationships between BPA concentration in blood or urine and the risk of developing kidney disease [21–24].

In parallel, other works by our team determined that BPA could influence the development of arterial hypertension, a disease closely related to kidney pathologies. When mice were treated with BPA in drinking water (20–25 mg/kg/day), significant increases in blood pressure were observed even at low doses [25]. The results are consistent with epidemiological studies, such as Shankar et al. [26], in which they observed a positive relationship between urinary BPA excretion and hypertension, independent of other factors. Furthermore, Bae et al. [27] observed that people who consumed canned drinks vs. glass bottles had up to 1600% urinary BPA and a higher variation in systolic pressure.

These data suggest that BPA could play a role in the pathogenesis and progression of renal diseases. Consequently, it is essential to study the possible implications of BPA in the renal system of the healthy population and patients with kidney disease. A preliminary search was conducted to verify whether there is any systematic review or meta-analysis in the academic literature, using the keywords "bisphenol AND (systematic review OR meta-analysis) AND (renal OR kidney)".

The preliminary analysis only showed one relevant article [28]. The publication only studied the potential effect of BPA on the pediatric population, which means that the approach of the present work is novel in the academic literature. Therefore, a thorough review was carried out using the methodology of systematic reviews to analyze potentially deleterious renal effects of BPA and describe potential sources of bias. Next, the available data were analyzed to carry out a meta-analysis that combined all possible studies. Finally, in the absence of publications that study the data set of the NHANES 2003–2016 cohort, a brief analysis was carried out that could provide new evidence on the BPA-kidney paradigm.

2. Materials and Methods

2.1. Study Selection

Studies that reported the association between high exposure to BPA in humans (in urine or blood) and risk of kidney disease were eligible for inclusion. Due to the small number of existing publications, all available population groups and age ranges were studied. To carry out the meta-analysis, all those studies with an available odds ratio (OR) and confidence interval (CI) or, at least, with sufficient data allowing calculation of the latter parameters were selected. All publications with any degree of relationship between BPA and kidney disease were selected.

2.2. Information Sources and Search Strategy

This search was carried out during July 2021 using the reference academic search engines PubMed (pubmed.ncbi.nlm.nih.gov, accessed on 5 July 2021) and Web of Science (apps.webofknowledge.com, accessed on 5 July 2021). To optimize the search strategy, we combined the terms "Bisphenol AND (urine OR urinary OR serum OR kidney OR renal) AND (albuminuria OR microalbuminuria OR nephropathy OR glomerular OR interstitial OR vascular OR CKD)", without year restriction.

After eliminating the repeated articles, using Mendeley (Mendeley Desktop, V. 1.19.4, Mendeley Ltd., Elsevier, London, United Kingdom) manager and organizer, we evaluated the resulting academic papers by title/abstract (Discarding all publications that do not fit the review context, such as in vitro research, animal models, or the study of compounds other than BPA, such as BADGE or BPS. Subsequently, after conducting the first

preliminary study, each of the academic articles selected was evaluated, with the following eligibility criteria:

- Original data (excluding bibliographic reviews) accepted and published.
- Studies in human populations (adults or children).
- Quantification of BPA in urine or serum and its correlation with any predisposition or susceptibility marker of kidney damage or disease.
- Studies published in English.

Two independent reviewers (RMGT and RJB), whose decisions in each of the bibliographic search and evaluation steps were determined by consensus, evaluated all the publications.

2.3. Data Collection and Quality Assessment

Once the publications of interest were selected, they were classified according to the study variable. Three studied BPA in the blood, while the rest analyzed it in the urine. Simultaneously, other important parameters for determining kidney damage were also considered since two articles explored the concept of "low-grade albuminuria," while others investigated the estimated glomerular filtration rate (eGFR). Notably, the studies that evaluated blood BPA were carried out on diagnosed or developing pathologies, while the urinary studies mainly presented data from the healthy population.

2.4. Analytical Method

At the statistical level, the objective was to perform the inverse analysis of variance to unify the studies related to each other, introduced in a random-effects model. The steps followed for the analysis of each subgroup are detailed below.

2.4.1. BPA in Blood

In order to calculate the OR, we analyzed the following articles: Krieter et al. [24], Hu et al. [29], Hu et al. [22], and Shen et al. [30]. In the case of Krieter and Shen, their studies show the mean BPA in plasma depending on kidney damage. In contrast, in the case of Hu's, it shows the OR of the probability of developing kidney damage in the presence of high concentrations of serum BPA in patients with diabetes [29] and hypertension [22]. As there were already 2 ORs, the first step that has been carried out has been to calculate it for Krieter et al. [24] and Shen et al. [30].

Review Manager (RevMan 5.3, Cochrane, London, United Kingdom)was used to calculate the standardized mean difference comparing the control group with the different concentrations of BPA in stages 3 to 5 of chronic kidney disease (CKD) (in Hu et al. [22,29], the patient was considered to have kidney disease when he reaches stage 3, that is when clearance is below 60 mL/min/1.73 m^2). The four subgroups analyzed correspond to stages 3, 4, 5, and stage 5 undergoing hemodialysis (Figure 1).

Once the standardized mean difference has been obtained, the OR can be obtained by applying the formula [31]:

$$\text{Standardized mean difference} = (\sqrt{3}/\pi) \times \ln \text{Odds Ratio},$$

Since the standard error is also required, the following formulas were applied to the CI obtained [31]:

$$\text{Standard deviation (SD)} = \sqrt{N} \times (\text{upper limit} - \text{lower limit})/3.92,$$

$$\text{Standard error (SE)} = \text{Standard deviation}/\sqrt{N}$$

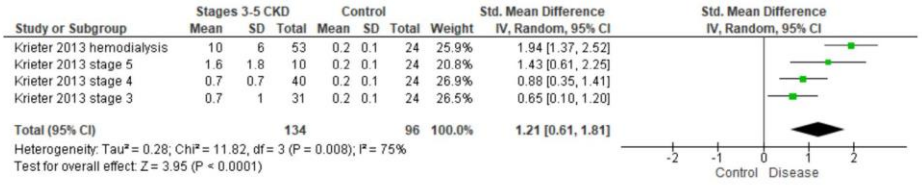

Figure 1. Mean difference analysis using the RevMan program calculates a value to use in the inverse variance analysis [24].

In the case of Shen et al. [30], patients with moderate renal injury [30 ≤ eGFR < 60 mL/min/1.73 m^2], and severe renal injury [eGFR < 30 mL/min/1.73 m^2] were included (Figure 2). In this case, as a control BPA value could not be found, the average of the NHANES cohort was used (see Table 1).

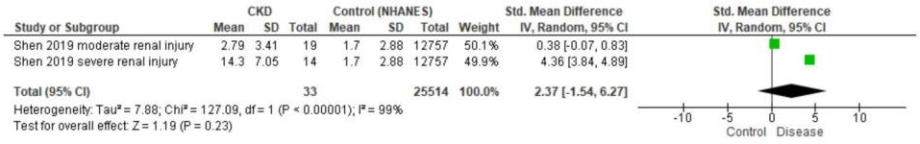

Figure 2. Mean difference analysis using the RevMan program calculates a value to use in the inverse variance analysis [30].

2.4.2. BPA in Urine

Several analyses were carried out, depending on the parameter related to kidney damage. Firstly, two publications studied the concept of low-grade albuminuria; it is understood as an increase in urinary albumin excretion but consistently below the limit considered pathological (30 mg/g creatinine). In this regard, Li et al. [23] and Trasande et al. [21] considered that when studying the "healthy" population, those individuals who were in quartile 4 of urinary BPA have a higher probability of presenting with a higher concentration of albuminuria. Li et al. [23] provides an OR that can be used, but Trasande et al. [21] shows a standardized mean difference, so the formulas previously described must be applied.

On the other hand, in the following two studies conducted by Malits et al. [22] and Kang et al. [32], Lee et al. [33], and Kang et al. [34] the association between BPA and urine albumin-to-creatinine ratio (ACR) were studied. From the work data, an OR could be obtained with CI in all cases, so the logarithms and the corresponding standard errors were calculated.

Secondly, the relationship between BPA and the eGFR has also been studied in the studies by You et al. [35], Malits et al. [36], Lee et al. [33], Jacobson et al. [37], and Kang et al. [34]. Malits et al. [36], Lee et al. [33], Jacobson et al. [37], and Kang et al. [34] offer enough data, but in the work of You et al. [35], OR must be calculated from the standard mean difference of its two study groups. They use the Modification of Diet in Renal Disease (MDRD-4) and the "Chronic Kidney Disease Epidemiology Collaboration" (CKD-EPI) equations to estimate GFR (Figure 3).

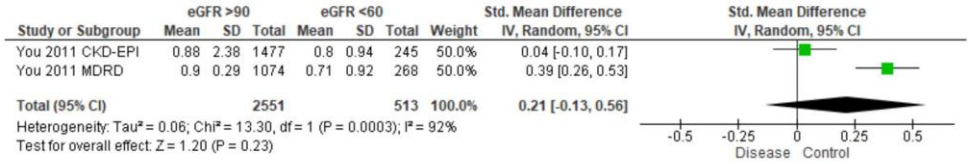

Figure 3. Difference of means of the groups elaborated from the MDRD and CKD-EPI formulas for the estimation of creatinine clearance [35].

Next, the OR with its 95% CI was used to calculate the estimated pooled effect. Heterogeneity between studies was calculated by applying the Chi^2 and I^2 tests. The I^2 statistic was calculated as a percentage, and they were interpreted as low, medium, or high heterogeneity, reaching 25, 50, and 75%, respectively [38]. The p-value < 0.05 was considered statistically significant for all the analyses performed. The statistical program used to carry out the analyses was Review Manager (RevMan 5.3).

2.5. Study of the NHANES 03-16 Cohort

After studying the academic literature, the absence of studies using all the BPA values in the NHANES 2003–2016 cohort was observed, and the analysis of the subpopulations classified as kidney disease during the patient evaluation process undiagnosed kidney disease whose eGFRs are abnormal. Therefore, this section aims to unify in a single database all the American populations' urinary BPA measurements studied from 2003 to 2016 (data currently available) to carry out the statistical study on the relationship between BPA and kidneys with the largest sample size described to date.

Patient files containing elements like urinary bisphenol, albumin and creatinine levels, plasma creatinine, sex, weight, age, kidney diseases, or dialysis patient were first collected to correlate urinary BPA with renal function. All files can be found in Xps Transport File Format (.XPT) and downloaded from "https://wwwn.cdc.gov/ (accessed on 16 July 2021)" Once the data had been extracted, unified, and classified according to each patient's numerical code, a table was obtained with 72,697 people (numerical code 21,005 to 93,702). The adults (age > 18 years) were selected, and all those in whom urinary BPA was not quantified were eliminated, obtaining a sample size of 12,757 people.

Spot urine samples are unable to determine urinary volume or albuminuria excreted within 24 h. Therefore, the ACR (mg of albumin/g of creatinine) was used to estimate albumin excretion. Similarly, the excretion of BPA to urinary creatinine (µg BPA/g creatinine) was also adjusted for the same reason. The MDRD-4 and the CKD-EPI equations (the most widely used formulas) are used to calculate the eGFR [39–41].

NHANES data were analyzed using the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed using the D'Agostino & Pearson and Kolmogorov–Smirnov normality tests. As they did not follow a normal distribution, the data are expressed as median (95% CI) in the tables and the graphs. The Kruskal–Wallis test followed by Dunn´s multiple comparisons test or Mann–Whitney test was performed. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Selection of Academic Articles

The initial search identified 102 articles in PubMed and 86 in Web of Science. After exporting all references to the Mendeley application and eliminating duplicates, 134 items were placed. The selection process was carried out based on the information contained in the title/abstract (in vitro studies, in vivo, reviews/comments). Publications related to quantification methodologies were eliminated, as well as those that studied organs/compounds different from kidney/BPA. After the first screening, 38 articles were identified, and the full texts were reviewed. Of these, twelve were included in the systematic review (Figure 4).

3.2. Study Characteristics

We will proceed to briefly describe the most interesting aspects of the academic publications used in the meta-analysis (for more information, see Supplementary Table S1). Firstly, the studies evaluating BPA in blood samples correspond to Krieter et al. [24], Hu et al. [22,29], Nie [42], and Shen et al. [30]. In the first one [24], the study group consisted in 152 patients with different CKD stages and 24 healthy subjects. The eGFR was calculated using the MDRD equation and compared with the BPA concentrations in plasma (ng/mL). An inverse relationship between plasma BPA and renal function (GFR) was observed. The highest plasma concentrations were present in patients with the lowest

GFR (stage 5). In Hu et al. [29], they studied the relationship between serum BPA and the predisposition to develop CKD in 121 patients with type 2 diabetes over six years. Patients with high serum BPA concentrations had almost seven times the risk of developing CKD than patients with lower BPA concentrations (OR 6.65). In the 2016 publication [22], after studying 302 patients with primary hypertension, they determined that those with higher concentrations of serum BPA were five times as likely (OR 4.79) to develop stage 3 CKD or higher. In both studies, they observed an interesting negative correlation between serum BPA and estimated glomerular filtration rate ($\beta = -0.371$ and -0.132, in the 2015 and 2016 studies, respectively). Finally, in the work of Shen et al. [30], a negative correlation between serum BPA and eGFR was also observed, as well as a significant elevation of plasma BPA in those patients with severe CKD.

Secondly, the academic publications that studied BPA in urine can be subdivided according to the renal parameter studied. The first group of interest corresponds to the two publications that use the concept of low-grade albuminuria. When classifying the "healthy" population (ACR below 30 mg/g) into four quartiles, it has been observed that those subjects who were in quartile four could have a greater predisposition to specific pathologies, such as cardiovascular disease [43]. Within this group were the works of Li et al. [23] and Trasande et al. [21]. In the first of them, they selected from among 3455 Chinese adults, all with ACR lower than 30 mg/g and considered those located in quartile four as presenting low-grade albuminuria. Finally, multivariate stepwise linear regression analysis determined that urinary BPA concentration was an independent predisposing factor to low-grade albuminuria. The other publication studied 710 American children from the American NHANES cohort (National Health and Nutrition Examination Survey). Children were categorized according to their urinary BPA concentration into four quartiles, observing that those in the 4th quartile had a significantly higher ACR than the individuals in the first quartile. They determined a 0.28 mg/g ACR increase for each log unit increase in urinary BPA after performing multinomial regression models.

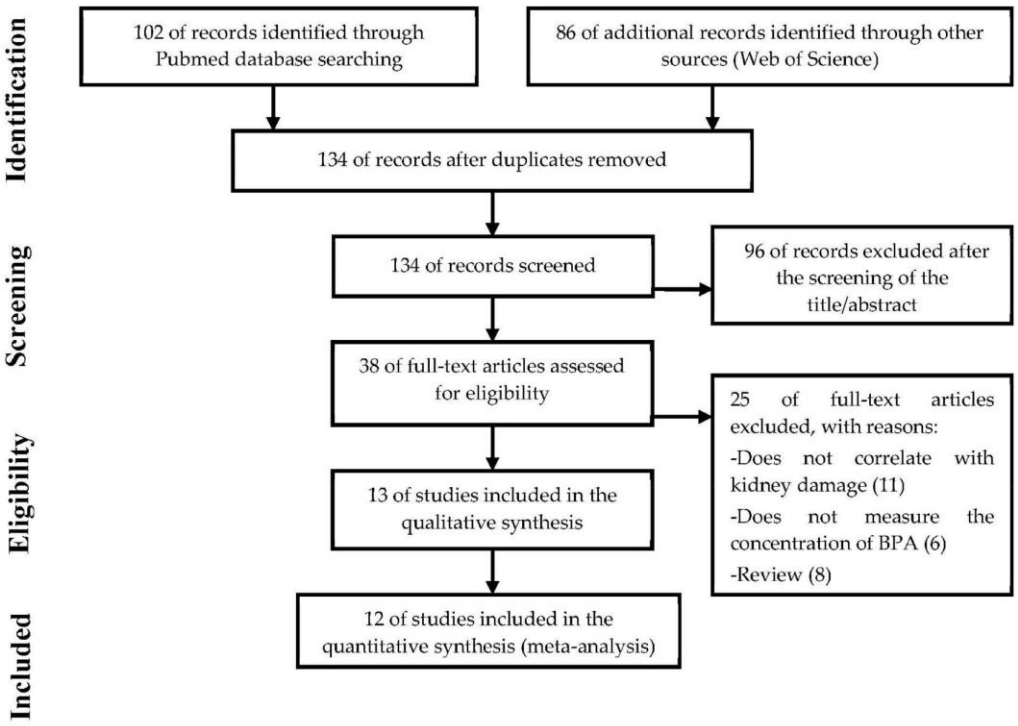

Figure 4. Schematic representation of the methodology used based on The PRISMA Statement [44].

Within the other group can be found the works of You et al. [35], Malits et al. [36], Kang et al. [32], Lee et al. [33], Jacobson et al. [37], and Kang et al. [34]. The first of them [35], studied kidney function by calculating the eGFR using the MDRD and CKD-EPI equations. They subdivided the population into three categories for each equation: $\geq$90 mL/min/1.73 m^2, normal kidney function; 60–90 mL/min/1.73 m^2, mild alteration; $\leq$60 mL/min/1.73 m^2, CKD. They performed regression models and adjusted for factors related to health, socioeconomic factors, and urinary creatinine. The results showed a positive and statistically significant relationship between urinary BPA excretion and the eGFR so that pathological situations, those with a lower GFR, presented lower concentrations of urinary BPA. The second publication compared a cohort of children with CKD with a general cohort (NHANES), determining that children with CKD have urinary BPA concentrations lower than those observed in healthy children. However, multivariate linear regression (corrected for health and social factors) showed a negative relationship between urinary BPA and eGFR.

Kang et al. [32] shows a statistically significant negative relationship between creatinine-corrected BPA and ACR in different regression models when studying a population of 441 Korean adult women (adjusted for age, region, education, and diabetic, obesity and cardiovascular factors). Lee et al. [33] determined a statistically significant negative relationship between BPA and eGFR in the general Korean population. Jacobson et al. [37] observed, in children with kidney disease, a negative relationship between BPA and eGFR (linear mixed-effects models). Interestingly, they also determined a positive and statistically significant relationship between urinary BPA and kidney damage markers, such as NGAL and KIM-1. The work of Kang et al. [34] also determined a strong negative correlation between BPA and eGFR after using a procedure of covariate-adjusted creatinine standardization. Finally, the last publication should be mentioned that did not provide usable data in the urine meta-analysis but is relevant in the BPA-kidney paradigm, is Jain [45]. In this study, it was observed that the association between urinary BPA and ACR could vary depending on kidney function, determining positive or negative relationships depending on the stage of kidney disease.

3.3. Meta-Analysis

As previously mentioned, the BPA-kidney damage paradigm comprises a small number of quite heterogeneous but potentially significant publications.

3.3.1. BPA in Blood

Once all the ORs and their respective standard errors were obtained, the inverse variance method was calculated. As shown in Figure 5 [OR 6.94, 95% CI 3.60–13.36], higher concentrations of BPA were related to a greater risk of developing kidney damage. The heterogeneity tests demonstrated that the data were excellently homogeneous.

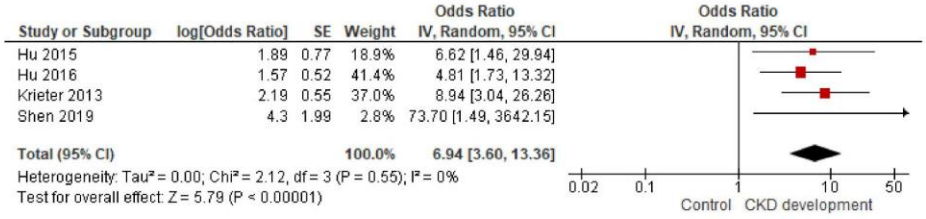

Figure 5. Analysis of the inverse variance of the four publications that study BPA in blood and kidney disease [22,24,29,30].

3.3.2. BPA in Urine

The unified works by Li et al. [23] and Trasande et al. [21], based on patients with low-grade albuminuria, showed high heterogeneity. The OR was positive, but the CI was

vast, with its range including values below 1 (Figure 6). There was a combined probability of 2.2-fold for developing low-grade albuminuria with high urinary concentrations of BPA.

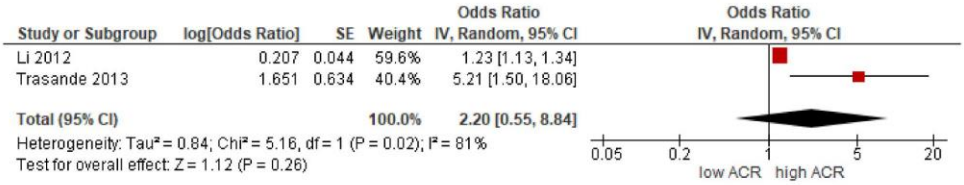

Figure 6. Analysis of the inverse of variance of the two publications that study BPA in urine and low-grade albuminuria [21,23]. ACR: urine albumin-to-creatinine ratio.

In the study between urinary BPA and ACR, the combined effect is slightly positive. There are large SEs in some studies, so the weight of the study falls on three works [33,34]. The combined results show a slight positive relationship between urinary BPA and albuminuria (Figure 7).

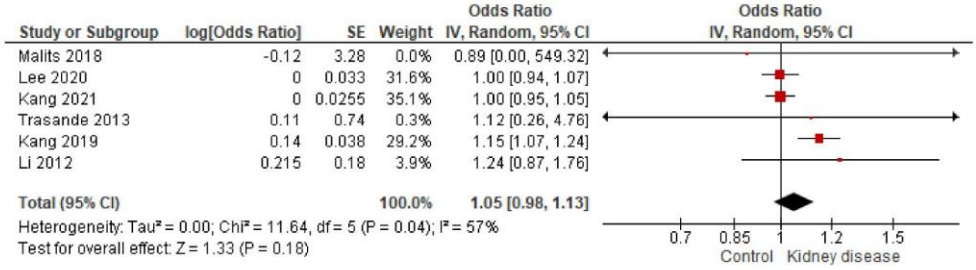

Figure 7. Analysis of the inverse variance of the publications that study BPA in urine and ACR [21,32–34,36].

Finally, in studying the relationship between BPA and eGFR, four studies show a negative relationship between both parameters in their respective multiple linear regression studies (corrected with demographic, social, and health parameters). Only the work of You et al. [35] showed an inverse result (remember that the OR was calculated through the difference of means). Figure 8 shows the combined effect of the four studies with the same methodology, evidencing the negative relationship observed in all publications. In this case, the reduction in eGFR is related to a loss of kidney function. By including the work of You et al. [35], a combined OR (95% CI) of 0.59 (0.30, 1.16) is obtained. In any case, it could be considered that there is sufficient evidence to suggest that BPA could influence the development of CKD, reducing eGFR.

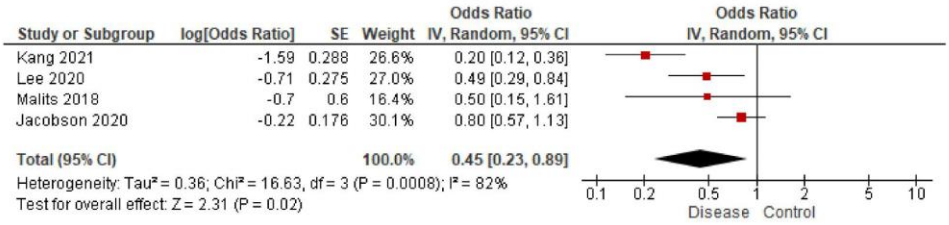

Figure 8. Study of the inverse variance in publications that determine the relationship between BPA and eGFR [33,34,36,37].

3.4. NHANES 03-16 Cohort

As can be seen in Table 1, more than 12,000 data from adult individuals were analyzed. The proportion of men and women was practically the same. The urinary BPA

concentration was relatively low and consistent with the data observed in the general population [46]. Men had significantly higher BPA values per mL of urine but significantly lower BPA corrected for creatinine. On the other hand, men presented significantly lower values of eGFR and ACR but higher values of serum creatinine and albumin.

Table 1. Summary of the adult population studied in NHANES 03-16. The data were analyzed using the D'Agostino & Pearson and Kolmogorov–Smirnov normality tests. Data were represented using the median (95% CI). For statistical analysis, the Mann–Whitney test was performed.

	N (%)	Age, Years	Urinary BPA, ng/mL	Urinary BPA, µg/g Creat.	MDRD-4 (eGFR)	CKD-EPI (eGFR)	Ratio alb./Creat.	Serum Creat., mg/dL	Serum Albumin, g/dL
Male	6238 (48.49%)	47 (46–48)	1.8 (1.7–1.9)	1.4 (1.37–1.44)	88.52 (87.92–89.12)	95.25 (94.55–96.05)	6.06 (5.9–6.21)	0.98 (0.97–0.99)	4.4 (4.4–4.4)
Female	6519 (51.10%)	46 (45–46)	1.6 (1.5–1.6)	1.75 (1.71–1.78)	90.75 (89.88–91.57)	99.94 (99.18–100.7)	7.98 (7.78–8.15)	0.73 (0.72–0.74)	4.2 (4.2–4.2)
Total	12757 (100%)	46 (46–47)	1.7 (1.6–1.7)	1.57 (1.55–1.6)	89.96 (88.97–90.21)	97.48 (96.99–98.09)	7.06 (6.95–7.18)	0.85 (0.84–0.85)	4.3 (4.3–4.3)
p-value		0.0642	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

By analyzing the data based on BPA/creatinine quartile, a significant increase in the ACR was manifested when comparing the group with higher levels of urinary BPA (Q4) with those with the lowest amount (Q1) (Figure 9A). Similarly, when analyzing all people with a ratio below 30 mg/g, the same trend was observed (Figure 9B); people belonging to Q4 showed a significant increase in the ratio compared to those in Q1. The estimated clearance study using the CKD-EPI and MDRD-4 equations can observe significant differences between Q4 and Q1 only in the CKD-EPI equation (Figure 9C,D).

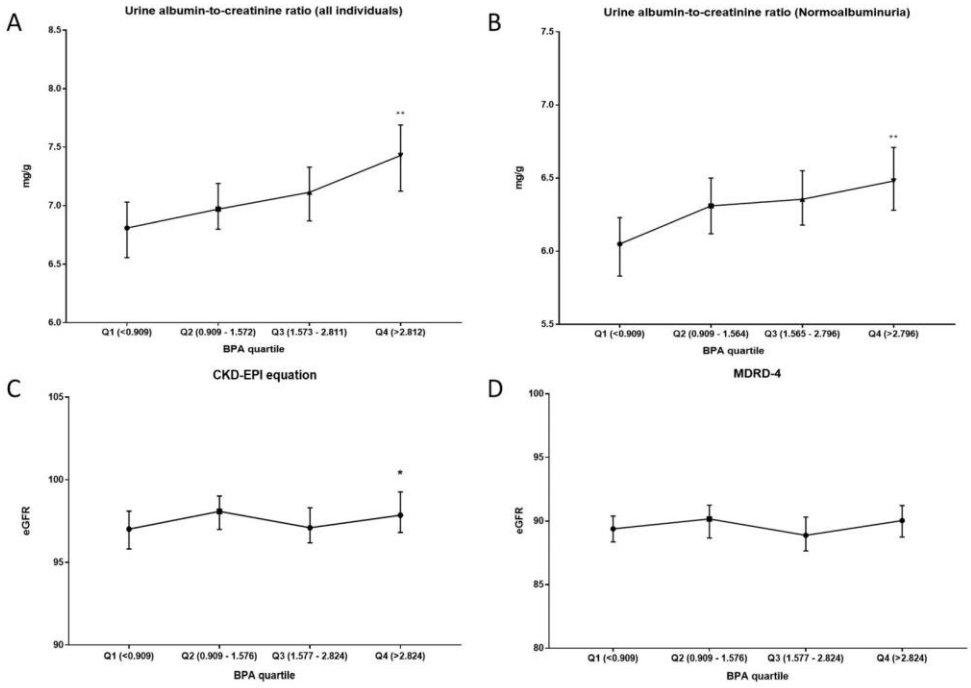

Figure 9. Schematic representation of the results obtained in the parameters related to renal function according to the BPA quartile. (**A**) Urine albumin-to-creatinine ratio (mg/g) in all individuals of the cohort. (**B**) Urine albumin-to-creatinine ratio (mg/g) only in normoalbuminuric individuals (<30 mg/g). (**C**) CKD-EPI equation for eGFR. (**D**) MDRD-4 equation for eGFR. All results were expressed as median (95% CI). Kruskal–Wallis test followed by Dunn´s multiple comparisons test or Mann–Whitney test was performed. * *p*-value < 0.05; ** *p*-value < 0.01.

When analyzing urinary BPA concentrations as a function of ACR, no differences were observed between individuals with non-pathological values (<30 mg/g, normoalbuminuria) vs. individuals with pathological values (>30 mg/g, micro/macroalbuminuria) (Figure 10A). However, when classifying individuals with normoalbuminuria into four quartiles, those in quartile 4 (theoretically they would present low-grade albuminuria [21,23]) had a higher concentration of urinary BPA than individuals in quartile 1 (Figure 10B).

Among the individuals in the cohort were patients with kidney disease and some on dialysis. When studying BPA corrected for creatinine, significant increases were observed in patients with kidney disease vs. healthy subjects, and patients undergoing dialysis vs. patients without treatment (Table 2).

Table 2. Urinary BPA/creatinine in diabetic and kidney patients vs. healthy population. Data were represented using the median (95% CI). For statistical analysis, Mann–Whitney test was performed.

Group	Urinary BPA, µg/g Creatinine	n	*p*-Value
Kidney disease	1.784 (1.5–2)	320	0.016
The rest of the cohort	1.563 (1.538–1.591)	11,572	
Dialysis treatment	2.9 (1.744–3.75)	24	0.031
Non-dialysis kidney patient	1.677 (1.458–1.942)	296	

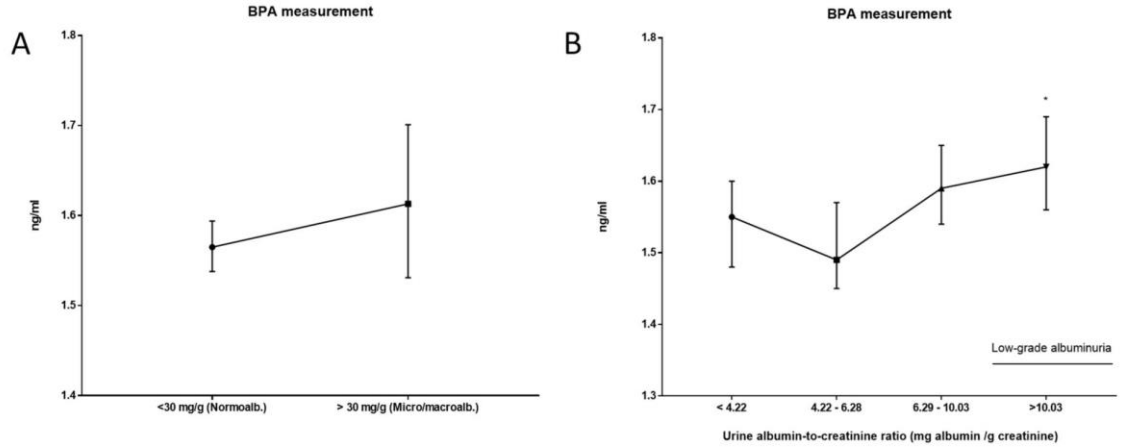

Figure 10. Schematic representation of the results obtained in the urinary BPA according to the urine albumin-to-creatinine ratio. (**A**) < 30 mg/mL (normoalbuminuria) vs. >30 mg/Dl (micro/macroalbuminuria). (**B**) Normoalbuminuria. The population was classified into four quartiles. Those in Q4 are considered to have low-grade albuminuria. All results were expressed as median (95% CI). Kruskal–Wallis test followed by Dunn´s multiple comparisons test was performed. * *p*-value < 0.05.

4. Discussion

Firstly, the systematic review shows an evident scarcity of publications that study the BPA-kidney paradigm in humans. However, it is interesting to note that the limitations on exposure to BPA proposed by the EFSA base their model on animal models that study how this compound affects the kidney [6,7].

Secondly, the meta-analysis shows that the high concentration of BPA in the blood could be a warning sign for kidney disease. Increasing the concentration of a compound in the blood due to the kidney's reduced filtering capacity is logical from a functional point of view. If we only look at the work of Krieter et al. [24] and Shen et al. [30], it could be deduced that this increase is a logical consequence of the kidney's inability to perform its work naturally. However, the two studies by Hu et al. [22,29], both longitudinal, analyze their study group over time, which gives greater strength to the hypothesis that elevated

BPA in the blood could be a predisposing factor to kidney damage, at least within patients with previously diagnosed pathologies such as hypertension or diabetes. Furthermore, these authors identified high levels of serum BPA as a predictor of CKD in two of the most common renal conditions, such as hypertension and diabetic nephropathy.

On the other hand, a high urinary BPA concentration could be related to an increase in the excretion of albuminuria in the healthy population. Perhaps it would be possible that in the healthy population, whose filtering capacity is standard, the increase in excreted BPA could be related to a greater predisposition to increased albuminuria, which could be an early indication of kidney damage. However, some publications suggest that BPA could bind to proteins [47], which would be a consequence of increased albuminuria and not a cause. In the general study of the ACR, a positive relationship was observed. The results' limitations show the need to deepen these aspects with larger sample groups or focus on specific pathologies and carry out new longitudinal studies. In this sense, it would be interesting to observe BPA's urinary excretion in patients in advanced stages of CKD.

BPA is a compound whose metabolism is fundamentally characterized by phase II reactions, biochemical mechanisms capable of modifying its structure to facilitate its excretion [48]. This detoxification capacity can be altered in diseases such as obesity or diabetes [49]. Glucuronidation is the majority reaction, mediated by uridine diphosphate glucuronosyltransferase (UGT) [48,50]. Pharmacokinetic studies have determined that BPA is excreted exclusively through the urine in non-human primates and humans [51–53]. For this reason, the correlation between pathologies and urinary BPA is a powerful tool since the actual exposure to BPA is theoretically taken into account.

Within the study's limitations, it must be considered that the blood studies were carried out on specific population groups, such as patients with kidney disease, type 2 diabetes, and hypertension. In contrast, the urinary studies were carried out in the general population, except that of Malits et al. [36] and Jacobson et al. [37], where they employed a group of children with CKD.

The analysis of eGFR and blood BPA suggests that higher concentrations of BPA present a greater probability of suffering kidney damage (or lower eGFR). However, in the works of You et al. [35] and Malits et al. [36], a lower concentration of urinary BPA is observed in patients with kidney disease. This relationship changes after statistical analysis, which is corrected with social, anthropometric, and health factors, obtaining a negative relationship. It is coherent to think that a damaged kidney with a limited glomerular filtration capacity will not efficiently eliminate BPA from the body, reduce its excretion, and increase its concentration in the systemic circulation. However, a statistically corrected analysis shows that urinary BPA can also be significantly correlated with eGFR. The absolute value of urinary BPA may be affected by parameters such as urinary volume or creatinine, but the statistical relationship is quite strong.

Kidney disease is closely related to diseases such as hypertension. In this sense, it has been observed that BPA could be a predisposing factor for the development of hypertension in animal models [25] or epidemiological studies [26,27]. Recently, in 2021, a study has been published unifying all the available data on BPA and cardiovascular disease, obtaining an OR of 1.13 (95% CI, 1.03–1.23) [54].

The main conclusion that can be drawn from the meta-analysis is the need to carry out a complete study, which delves into the relationship between urinary and plasma BPA, glomerular filtration rate, and albuminuria, both in population risk groups (in patients with diabetes or hypertension) and in the general population. This study must be placing a particular emphasis on the particularities related to the ACR and glomerular filtration (which could be calculated with the main formulas currently used in clinical practice, which are the MDRD-4 and CKD-EPI [55]).

Finally, the data analyzed from the NHANES cohort show new evidence that reinforces the hypothesis that BPA acts as a factor involved in kidney damage. It is interesting to highlight the importance of creatinine correction in studying urinary metabolites, such as BPA. Thus, a significant increase in urinary BPA has been observed in men, expressed in

μg/mL, while women presented higher BPA values corrected for creatinine. In the absence of the absolute volume of urine excreted in 24 h, correction for creatinine allows a better estimation of urinary metabolites, taking into account the renal filtration capacity.

Regarding the analysis of the population, classified according to urinary BPA, evident and significant differences have been observed between people with lower and higher concentrations of urinary BPA (Q1–Q4). In the study of the complete cohort and the study of the healthy population (normoalbuminuria), significant increases in the ACR between Q4 and Q1 have been observed. Similarly, significant increases in urinary BPA were observed in those individuals with low-grade albuminuria. This data is consistent with the results observed by Li et al. [23] and Trasande et al. [21] (Figure 6). On the other hand, only when the CKD-EPI formula was used, significant changes between Q4 and Q1 were observed. Since BPA is a metabolite that constantly enters the body through multiple sources and is constantly excreted in the urine due to its low half-life, it makes sense that with greater glomerular filtration, there is greater BPA excretion. This data is not of great relevance to demonstrate a causal relationship between the BPA-kidney paradigm; however, urinary BPA concentrations in patients with kidney disease in the cohort are of special interest. As observed in Table 2, the kidney disease patients had higher urinary BPA levels than the rest of the subjects in the cohort. Similarly, patients undergoing dialysis treatment had higher BPA concentrations than those who were not yet receiving treatment.

In short, the body of evidence in the present work takes a further step towards the relationship between BPA and the predisposition to develop kidney diseases. BPA may bind to proteins, limiting the correct analysis of parameters such as ACR, and it seems that the glomerular filtration capacity conditions its excretion rate. However, the joint analysis of the studies on BPA in blood, together with the urinary concentrations observed in the NHANES cohort patients, provides new evidence that supports this possible causal relationship. Although experimental data fully support a pathogenic role of BPA exposure on hypertension and renal disease, further epidemiological studies need to clarify this critical issue.

5. Conclusions

- Despite the importance of the BPA-kidney paradigm in the context of human exposure, few works explore the issue.
- In the study of blood BPA and kidney disease, solid evidence correlates high concentrations of BPA in the blood with a greater predisposition to develop kidney disease, at least under pathological conditions.
- In the study of the ACR and urinary BPA, a positive relationship was observed in healthy subjects. The same trend was observed in the NHANES cohort. Similarly, subjects with low-grade albuminuria showed a significant increase in urinary BPA.
- Despite inconsistencies observed in urinary BPA concentration from patients with kidney disease, statistical correlations with eGFR support an important relationship between BPA and glomerular filtration.
- The results, consistent with the experimental models, show interesting evidence that positions BPA as a possible environmental factor inducing kidney damage.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom11071046/s1, Table S1: Summary of the main characteristics of the papers included in the systematic review.

Author Contributions: Conceptualization, R.M.-G.-T. and R.J.B.; methodology, R.M.-G.-T., E.V.-V.; software, R.M.-G.-T. and E.V.-V.; validation, R.M.-G.-T.; formal analysis, R.M.-G.-T., E.V.-V.; investigation, R.M.-G.-T.; resources, R.M.-G.-T.; data curation, R.M.-G.-T.; writing—original draft preparation, R.M.-G.-T., M.I.A., and R.J.B.; writing—review and editing, E.V.-V., B.Q., J.B., and E.C.; visualization, R.M.-G.-T.; supervision, R.M.-G.-T., R.J.B., and M.I.A.; project administration, R.J.B.; funding acquisition, R.J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02139) -Fondo Europeo de Desarrollo Regional (FEDER)-. R. Moreno- Gómez-Toledano is the recipient of a research contract from CAM (B2017-BMD-3686).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We want to thank M. Bosch for proofreading the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. Summary of the main characteristics of the papers included in the systematic review. Abbreviations: CKD, Chronic Kidney Disease; GM, Geometric Mean; CI, Confidence Interval; GSD, Geometric Standard Deviation); SD, Standard Deviation; IR, Interquartile Range; ITR, Intertertile Range; BPA e, BPA exposed; BPA non-e, BPA non-exposed; SRI, Severe Renal Injury; MRI, Moderate Renal Injury.

Study	Year	Country	Study design	Sample size	Sex	Age, Median (Range), years / Mean±SD	Exposure (ng/ml)	Exposure assessment	Study period (years)	Biologic fluid
You [1]	2011	USA	Cross-sectional	2573	Males and females	47.12 (>20 years)	2.04 (1.76, 2.36) – 2.46 (2.41, 2.50) GM (95%CI)	HPLC-MS after deconjugation	2003-2006	Urine
Li [2]	2012	China	Cross-sectional	3455	Males and females	60.8 ± 9.9	0.81 (0.48 - 1.44) Median (IR)	HPLC-MS after deconjugation	2008-2009	Urine
Trasande [3]	2013	USA	Cross sectional	710	Males and females	(6-11) (12-19)	2.25 Mean 1.76 Mean	HPLC-MS after deconjugation	2009-2010	Urine
Krieter [4]	2013	Germany	Cross-sectional (Case-control)	152 CKD patients + 24 healthy	Males and females	69.7 ± 12.6	CKD stage 5 with dialysis 10 ± 6.6 Mean (SD)	ELISA	----	Plasma
Hu [5]	2015	China	Longitudinal study	121 type 2 diabetes patients	Males and females	65.13 ± 10.57	0.40 (0.174, 1.40) Median (ITR)	ELISA	2008-2014	Serum
Hu [6]	2016	China	Longitudinal study	302 hypertensive patients	Males and females	65.29 ± 9.78	0.61 (0.26, 2.44) Median (ITR)	ELISA	2008-2014	Serum
Malits [7]	2018	USA	Cross sectional (case-control)	538 CKD patients	Males and females	10.77 ± 0.18 (1-17)	0.69 (0.61, 0.78) GM (95% CI)	HPLC-MS after deconjugation	2005-2008; 2009-2014	Urine
Kang [8]	2019	Korea	Cross-sectional	441	Female (before menopause)	34.77 (20-48)	0,5 median	HPLC-MS after deconjugation	2015-2016	Urine
Shen [9]	2019	China	Cross sectional	58 CKD,30 healthy	Males and females	63.12 ± 6.31 (BPA e) 60.45 ± 6.95 (BPA non-e)	SRI: 14.30 (8.93, 16.32) MRI: 2.79 (1.01, 4.08) GM (95% CI)	HPLC-MS after deconjugation	----	Serum
Lee [10]	2020	Korea	Cross sectional	1292	Males and females	(19 - >70)	1.31 (0.51, 2.72) Median (IR)	UPLC-MS/MS after deconjugation	2015-2017	Urine
Jacobson [11]	2020	USA and Canada	Cross sectional	618	Males and females	11 (7.6, 14.6) Median (IR)	0.59 (3.49) GM (GSD)	HPLC-MS after deconjugation	2005-2015	Urine
Kang [12]	2021	USA	Cross sectional	9008	Males and females	(20 – 79)	1.5 (0.7, 3) GM (IR)	HPLC-MS after deconjugation	2005 - 2016	Urine

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¡Cuántas veces más hemos asistido a este tipo de donaciones de personas altruistas y generosas que nos ha permitido utilizar muestras biológicas obtenidas en momentos nada fáciles, en algunas ocasiones tan solo a unas pocas horas de haberles comunicado un diagnóstico grave con un futuro incierto! [21]

Nicolás Olea (1954 -)

Las leyes estadísticas
son tan certeras en lo general
como falaces en lo particular [2]

Edward Gibbon (1737-1794)

VII. DISCUSIÓN

La presente tesis doctoral ha pretendido profundizar en el estudio de la posible relación causal entre los contaminantes ambientales, como el BFA, y las patologías nefro-vasculares. De este modo, a lo largo del **prólogo** [271] se ha realizado un modelo experimental en podocitos inmortalizados de ratón, evidenciando de manera novedosa en la literatura el efecto del BFA a dosis bajas sobre esta línea celular. El podocito es conocido por ser el elemento fundamental sobre el que se desarrollan enfermedades como la glomeruloesclerosis, la nefropatía diabética (ND) o la enfermedad renal crónica (ERC) [232]. En esta clase de enfermedades, el podocito suele sufrir mecanismos de hipertrofia celular asociado a diversas causas, bien como acción directa de un entorno con elevada concentración de glucosa [272], bien por mecanismos compensatorios asociados a la pérdida de células colindantes [231, 244].

Partiendo de esta premisa, se evaluó el potencial efecto hipertrófico del BFA sobre el **podocito de ratón** en cultivo, observando un aumento de la superficie celular, lo que fue corroborado con el estudio del índice de hipertrofia celular y el porcentaje de incorporación de leucina, determinando incrementos significativos a las dosis de 10 y 100 nM. Este efecto hipertrófico inducido por BFA se ha observado también en otros modelos animales, como en el trabajo de Veiga-Lopez y cols. [273], en el que observaron hipertrofia de adipocitos de la grasa visceral en un modelo ovino con exposición prenatal a BFA. Del mismo modo, la exposición prenatal a BFA también se ha relacionado con la hipertrofia del músculo esquelético en el trabajo de Jing y cols. [274]. En modelos murinos adultos, Tyl y cols. [104] describieron que el BFA podía inducir hipertrofia del hepatocito, mientras que Bahey y cols. [275] observaron hipertrofia del miocito. Por último, investigaciones recientes publicadas por nuestro equipo colaborativo de investigación nefro-vascular han observado que el BFA también puede estar relacionado con la hipertrofia cardíaca (**Anexo IV y V,** [276, 277]).

La hipertrofia del podocito inducida por una alta concentración de glucosa está mediada por una vía de señalización celular que implica a las proteínas TGF-β y $p27^{Kip1}$ [245–249]. $p27^{Kip1}$ es un inhibidor de quinasa dependiente de ciclina, capaz de regular el ciclo celular inhibiendo la progresión del ciclo celular (mitosis) en la fase G_1 y cuya activación depende de TGF-β [252]. El consecuente estudio de la expresión relativa por

Western blot confirmó la sobreexpresión de dichas proteínas, lo cual reafirmó la hipótesis de que el BFA podría estar relacionada con la ND, estimulando el mismo mecanismo de hipertrofia celular que las condiciones de elevada concentración de glucosa.

A continuación, se investigó el posible efecto del BFA sobre la viabilidad celular debido a que en podocitopatías, como la hipertrofia podocitaria descompensada, se observan fenómenos de pérdida de viabilidad [278]. El ensayo por MTT determinó que el BFA a bajas dosis era capaz de reducir significativamente la viabilidad celular. A continuación, se determinó con la técnica TUNEL que el efecto del BFA sobre la viabilidad incluye fenómenos de apoptosis. Es interesante destacar que la alteración de los fenómenos pro-apoptóticos y anti-apoptóticos influye de manera crítica en la pérdida de podocitos, lo que conlleva irremediablemente el progresivo desarrollo de la glomerulosclerosis. Kopp y cols. [253] observaron, en un modelo de ratón transgénico de TGF-β (con elevada concentración en plasma), que esta proteína contribuye a la progresión de la glomerulosclerosis. Posteriormente, D´Agati y cols. [254] observaron en el mismo modelo transgénico, que la sobreexpresión de TGF-β puede inducir mecanismos de apoptosis del podocito, lo que conduce hacia la progresión de la glomerulosclerosis.

De forma coherente con estas afirmaciones, los resultados mostraron que el BFA puede inducir un aumento de la expresión relativa tanto de TGF-β como de su receptor. Además, se analizó la expresión del colágeno tipo IV, conocido por tener un papel importante en el desarrollo de glomerulosclerosis relacionada con TGF-β [246, 248]. De este modo, se observó que el BFA, de forma dosis-dependiente, era capaz de estimular la expresión de esta proteína. Es importante recordar que el colágeno tipo IV es uno de los principales componentes de la membrana basal glomerular [279]. Otros elementos fundamentales son la red de proteínas que conectan el citoesqueleto de actina con los procesos podales de los podocitos adyacentes, como son la podocina y la nefrina [238–241] Además, ambas juegan un importante papel en la supervivencia del podocito. Cualquier alteración sobre esta clase de proteínas, no sólo implicará alteraciones de la barrera de filtración glomerular, sino que afectará a la integridad estructural del propio

podocito [254, 280, 281]. De este modo, el análisis por Western blot confirmó la pérdida de la expresión relativa de ambas proteínas inducida por BFA.

En conjunto, los datos muestran que el BFA a bajas dosis es capaz de afectar al podocito, induciendo una podocitopatía caracterizada por hipertrofia celular, pérdida de expresión de las proteínas de la barrera de filtración glomerular y reducción de la supervivencia celular. Es interesante destacar que algunos de los resultados observados en el modelo celular no correspondieron con el clásico patrón de respuesta lineal, o dosis-dependiente. En la respuesta hipertrófica, la dosis más baja de BFA (10 nM) presentó una mayor respuesta que la dosis más alta (100 nM), un patrón que se observó en las 3 técnicas de análisis empleado. La dosis de 10 nM también indujo una mayor reducción de la expresión de podocina y nefrina que la dosis de 100 nM. Este interesante resultado puede explicarse mediante la posible acción no monotónica del BFA. Dicha acción, del mismo modo que sucede con diversos estímulos hormonales, puede suponer la aparición de efectos biológicos a dosis bajas, de forma que, en lugar de seguir una relación de linealidad entre la dosis y los efectos observados, se observan curvas cóncavas o convexas, denominadas curvas dosis-respuesta no-monotónicas (NMDRC, por sus siglas en inglés) [37, 189, 190]. Laura Vandenberg, en su revisión publicada en 2014, determinó que las NMDRCs son comunes en la literatura relativa al BFA, llegándose a identificar en más del 20 % de todos los experimentos [191]. Por su parte, el trabajo de Angle y cols. [282] manifiesta la existencia de curvas de dosis-respuesta multimodales, donde se aprecian incrementos y descensos en las mismas, así como variaciones en la respuesta máxima en función del tipo de tejido. Dicha afirmación, en consecuencia, dificulta aún más la correcta valoración del estado de la cuestión acerca de la supuesta inocuidad del BFA en las concentraciones encontradas actualmente en la población.

La dosis empleada en el modelo celular, 10 y 100 nM, se encuentra dentro del rango que en la literatura académica se considera como dosis baja [283–285]. La justificación de la dosis empleada, si bien parte de una sólida búsqueda bibliográfica previa, se reforzó sólidamente durante el proceso de elaboración de un capítulo de libro, que recientemente hemos publicado [64]. En él, mediante la exhaustiva revisión de la

literatura, realizando una revisión sistemática y analizando los datos de las principales cohortes mundiales se pudo obtener una concentración de BFA urinario en la población general muy próximo a la dosis de 10 nM. El subsecuente análisis de los grupos poblacionales con mayor exposición a BFA reveló que los pacientes con enfermedad renal crónica en etapa terminal (diálisis) o los pacientes en cuidados intensivos [68, 69], así como algunos grupos de mujeres embarazadas [63, 101] podrían exponerse a una concentración de BFA más cercana a la dosis de 100 nM. Por último, existen casos extremos de trabajadores de la industria del plástico que podrían incluso superar este rango de concentraciones [208, 286, 287].

A continuación, se procedió a explorar el posible papel del BFA como inductor de enfermedad renal en un **modelo *in vivo*** de administración intraperitoneal (IP) de BFA durante 5 semanas. Los animales desarrollaron un aumento significativo de la excreción de albúmina urinaria (albuminuria), del aclaramiento de creatinina y del índice de hipertrofia renal. El aumento del aclaramiento es un indicativo de que se está produciendo hiperfiltración glomerular, un signo característico de la disfunción renal en la diabetes y la obesidad [288–290]. En los primeros estadios de la nefropatía diabética, se ha descrito tradicionalmente al riñón como un órgano hipertrofiado con un característico aumento de la filtración glomerular [260, 262].

La inmunohistoquímica renal mostró una reducción significativa del número de podocitos, marcados con la proteína WT-1, específica para este tipo de células. El ensayo con la técnica TUNEL reafirmó los resultados observados en el modelo celular, mostrando la presencia de apoptosis en los glomérulos de los animales tratados con BFA. El estudio histológico de la morfología glomerular mostró un incremento del área correspondiente a la matriz mesangial y variaciones en el tamaño glomerular. Además, la microscopía electrónica reveló indicios de hipertrofia celular y apoptosis. El estudio de expresión relativa de proteínas por Western blot mostró el mismo patrón que en el modelo celular, con el aumento de la expresión de TGF-β, $p27^{kip1}$ y el colágeno tipo IV. De este modo, los resultados en conjunto mostraron que los animales expuestos a BFA por vía IP desarrollan podocitopatía con proteinuria. A pesar de la existencia de limitaciones en el uso de animales para evaluar la insuficiencia renal o los cambios

histológicos a largo plazo, el aumento de albuminuria es un marcador fiable de progresión de enfermedad renal [291].

La dosis utilizada en el modelo animal (50 mg/kg) es la misma dosis que Tyl y cols. definieron como NOEL renal [104] (menor dosis a la que no se observaron efectos). En su trabajo administraron concentraciones de BFA desde 0,03 hasta 600 mg/kg/día a ratones a través de la dieta. Los resultados sólo mostraron efectos renales (incremento del peso del órgano) a la dosis más alta, determinando en consecuencia el NOEL renal en la dosis inmediatamente inferior. Por su parte, Kabuto y cols. [154] también utilizaron la dosis de 50 mg/kg, pero con la vía de administración IP. En su trabajo observaron que el BFA podía afectar a la glutatión peroxidasa renal, implicada en la protección frente al estrés oxidativo y cuya expresión se encuentra habitualmente reducida en los pacientes con enfermedad renal crónica, especialmente en la etapa final o enfermedad renal terminal [292]. Esta dosis es de gran relevancia en la actualidad, ya que diversas instituciones lo utilizan como referencia a la hora de estimar las concentraciones de BFA seguras en humanos [56]. En el caso de la EFSA, esta institución europea ha estimado la *Ingesta Diaria Tolerable* siguiendo los siguientes pasos: Utilizando como referente el NOEL renal, calcularon la equivalente *dosis de Benchmark.* Esta dosis representa el punto en que se estima que puede existir una alteración en el 10 % de los animales tratados y es de 9 mg/kg. A partir de ella, aplicaron un factor de corrección para estimar la dosis equivalente en humanos, obteniendo la concentración de 600 µg/kg. Por último, añadieron un factor de incertidumbre de 150, obteniendo el resultado final de 4 µg/kg/día [56].

Esta cifra, a pesar de encontrarse a varios órdenes de magnitud por debajo del NOEL renal murino, sigue estando por encima de las concentraciones estimadas en la población general. Los resultados observados en un reciente análisis de la concentración urinaria mundial publicado por nuestro grupo [64], han determinado que la gran mayoría de individuos presenta concentraciones por debajo de este umbral de seguridad delimitado por la EFSA. Sin embargo, existen excepciones que deberían tenerse en consideración. Por un lado, los pacientes en cuidados intensivos y los enfermos con enfermedad renal crónica en estadio 5 (diálisis) presentan mayores

concentraciones promedio de BFA [63, 64, 68, 69]. Teniendo en cuenta que ciertas patologías pueden afectar a la capacidad de metabolización del BFA [96], y en base a los resultados descritos en el prólogo, se manifiesta la necesidad de reevaluación de los materiales empleados en el entorno hospitalario. Por otro lado, las cifras observadas en los trabajadores de la industria del plástico resultan, en algunos casos, alarmantes, motivo por el cual también deberían reconsiderarse las medidas de protección específicas relacionadas con los trabajadores de estas industrias [64, 208, 286, 287]. Además, también podría existir una gran variabilidad en el rango de exposición dentro de cada grupo. Esta afirmación se basa en el trabajo de Carwile y cols. [293], en el que observaron que el consumo de un bote de sopa durante 5 días podía inducir un aumento del 1000 % en la concentración urinaria de BFA en sujetos sanos.

Paralelamente a estas cuestiones sobre la exposición, también deberían replantearse los dos paradigmas que configuran los modos de estudiar la exposición a BFA. Por un lado, los modelos farmacocinéticos realizados en humanos con moléculas modificadas de BFA (d(16)-bisfenol A) [51, 52], han creado un axioma ampliamente aceptado de que el BFA se metaboliza y excreta rápidamente por la orina, a pesar de que las modificaciones estructurales con deuterio pueden alterar la farmacocinética (y la toxicidad) de cualquier compuesto [294, 295]. Además, existen ciertas contradicciones en la literatura, como el trabajo de Stahlhut y cols. [87], en el que observaron que los niveles de BFA no disminuían con el ayuno, lo que sugería que el BFA podía acumularse en el organismo, poniendo en duda su rápida capacidad de excreción. Por ello, los rangos de exposición humanos, definidos en base a la excreción urinaria de BFA, podrían infravalorar la cuestión. Recordemos que el BFA no sólo se ha identificado en orina, y es que gracias a sus propiedades físicas es capaz de desplazarse por todo el organismo, atravesando barreras fisiológicas como la trasplacentaria o incluso atravesar la propia membrana celular. De este modo, se han llegado a determinar concentraciones de BFA fetal en un rango similar al de la sangre de sus madres [37, 296]. Por otro lado, los límites de seguridad (IDT) calculados por la EFSA se basan en una concentración “inocua” que, sin embargo, afecta al riñón de los modelos experimentales animales desarrollados en la presente tesis doctoral. Esta IDT, además, se desarrolla en un contexto de relación dosis-dependiente. Puesto que el BFA presenta indicios de acción

no monotónica, teóricamente podría afectar a múltiples niveles a concentraciones por debajo de las consideradas seguras [192–194].

Teniendo todo ello en cuenta, puede afirmarse que el presente modelo animal utiliza una dosis de BFA en un rango coherente, dentro de los parámetros aceptados por la comunidad científica, que además se utiliza para definir el rango de concentraciones seguros en humanos. No obstante, existen diversos factores que dificultan la comprensión completa del paradigma. Es importante también tener en cuenta que la vía de administración de BFA puede influir en su metabolismo [88], y que las principales formas de entrada al organismo en humanos son la oral y la dérmica [56, 77].

En conclusión, el BFA es capaz de inducir hipertrofia, sobreexpresión del sistema TGF-β, $p27^{kip1}$, colágeno tipo IV y apoptosis, reduciendo a su vez las proteínas estructurales y de supervivencia podocina y nefrina. Por su parte, los animales tratados con BFA intraperitonealmente desarrollaron proteinuria, hiperfiltración glomerular, hipertrofia renal y podocitopenia, de manera similar a los cambios descritos en la ND temprana.

A continuación, considerando el conjunto de resultados descritos, se procedió a desarrollar un nuevo modelo experimental animal (**Capítulo 1** [297]), en el que se administrara BFA por la vía oral, ya que es la forma mayoritaria de entrada al organismo en humanos. Para ello, se administró en el agua a través de biberones de vidrio. Además, las evidencias que relacionaron la exposición a BFA con la ND introdujeron la cuestión de si el BFA podría agravar la enfermedad renal del paciente diabético. Teniendo en cuenta que menos de la mitad de los pacientes que padecen diabetes desarrollan ND, quizás el BFA podría ser un factor ambiental capaz de promover el desarrollo de esta enfermedad. Para ello, se desarrolló un **modelo de diabetes experimental** inducida por estreptozotocina (STZ), generando un estado fisiológico similar a la diabetes tipo I [266–268]. La administración de sucesivas dosis bajas de STZ indujeron incrementos significativos en los niveles de glucosa en sangre en poco tiempo, con una tasa de supervivencia del 100 %. Al inicio del tratamiento con BFA, la glucemia de todos los animales tratados se encontraba por encima de 400 mg/dl. El modelo experimental

incluyó 4 grupos: control (C), animales sanos que beben BFA (BPA), diabéticos que beben agua sin tratar (D) y diabéticos tratados con BFA (D+BPA).

Es interesante destacar que los animales del grupo BPA desarrollaron albuminuria, al igual que el anterior modelo de inyección IP. En estudios transversales humanos se ha observado una relación positiva entre el BFA urinario y la albuminuria, tanto en niños [124] como en adultos [123]. La misma relación se ha observado en los estudios longitudinales de Hu y cols. [200, 201], en los que determinaron un mayor riesgo a desarrollar enfermedad renal en los individuos con mayor concentración de BFA en sangre y patologías previas como diabetes e hipertensión. El modelo animal desarrollado en el presente capítulo ha aportado nuevas evidencias que correlacionan la exposición oral a BFA con el desarrollo de alteraciones moleculares y fisiopatológicas que recuerdan a la ND temprana, como la albuminuria, hipertensión, reducción en el número de podocitos (podocitopenia) y aumento de células apoptóticas, mayor presencia de glomérulos colapsados y mayor expresión relativa de CHOP y TGF-β. No obstante, algunos cambios descritos en el modelo anterior no se desarrollaron en los animales que bebieron BFA, como son la hiperfiltración glomerular, hipertrofia renal o poliuria [271]. Este hecho podría estar condicionado por la vía de administración y/o por la dosis empleada, la mitad del NOEL renal (50 mg/kg) [56, 104] (recordar que el NOEL se utilizó por la EFSA para calcular la IDT de 4 μg/kg en humanos [56, 103, 298]). En la dosis del presente modelo experimental se utilizó como referente un informe de la EFSA [56] en el que estimaron, mediante el análisis de BFA liberado por alimentos y objetos, que la exposición podría encontrarse en un valor cercano a la mitad del IDT en algunos grupos. Por este motivo resultaba coherente utilizar concentraciones de BFA cercanas a la mitad del NOEL renal.

Por otro lado, los animales del grupo D, de forma consistente con los resultados publicados por otros autores, desarrollaron poliuria, hipertrofia renal, incremento de albuminuria, hipertensión, podocitopenia, apoptosis, y aumento de la expresión relativa de CHOP, PCNA, MCP-1 y TGF-β [245, 258, 299–301]. Al administrar BFA a los animales diabéticos (grupo D+BPA) sucedió un hecho inesperado, y es que todos los machos de

este grupo murieron a las 10 semanas. Desafortunadamente, este hecho condicionó la duración del modelo experimental, limitándolo a 8 semanas.

La mortalidad exclusiva de los machos D+BPA, si bien fue un hecho inesperado, no resulta incoherente, pues manifiesta la existencia de dimorfismo sexual, ya sea por la susceptibilidad al BFA, por las diferencias que se manifiestan en el sistema renal, o incluso ambas. En el grupo C se observó la presencia de dimorfismo sexual en el peso corporal y el índice de hipertrofia renal, mayor en los machos y acorde con los resultados de otros autores [302–305]. Los machos diabéticos mostraron un aumento significativo del volumen de diuresis con respecto a las hembras diabéticas. Además, las hembras de este grupo manifestaron un incremento significativo de la proliferación celular. En consecuencia, resulta coherente especular con que el aumento de la producción de orina podría predisponer a alteraciones hidroelectrolíticas, lo que unido al aumento de la proliferación celular podría, al menos en teoría, explicar parcialmente las diferencias en el patrón de dimorfismo sexual observado en la supervivencia de los animales. Por su parte, en el grupo D+BPA también se observó dimorfismo en la excreción urinaria de albúmina y en el número de corpúsculos colapsados (mayor en los machos).

En la literatura académica se ha descrito que existen diferentes respuestas en machos y hembras ante la misma exposición al BFA. Este tipo de estudios se han realizado fundamentalmente sobre el efecto gestacional o perinatal, donde se ha llegado a determinar que el BFA puede ejercer un efecto diferente en machos y hembras a nivel hepático [306, 307], adipogénico [308], neurológico [309–311], endocrino [312], inmunológico [313], sobre el desarrollo renal [144] e incluso sobre los mecanismos de metilación de determinadas secuencias de DNA implicadas en el desarrollo y crecimiento fetal [314]. Además, el BFA, por encontrarse dentro de la categoría de disruptor endocrino o estrógeno ambiental, es una molécula que tiene la capacidad de ejercer un efecto modulador sobre diferentes receptores, produciendo una acción agonista sobre el receptor estrogénico y antagonista en el receptor androgénico [114]. No obstante, al comparar los efectos del BFA con fármacos de potente acción estrogénica como el dietilestrilbestrol, se ha podido determinar que no se producen los mismos efectos, lo cual manifiesta que clasificar al BFA únicamente como un estrógeno

ambiental no refleja todo el rango de posibles efectos que puede ejercer sobre un organismo vivo [282]. Por otro lado, en modelos animales también se han descrito diferencias sobre la concentración de BFA en órganos como intestino, riñón e hígado en machos y hembras, siendo los machos los que mayor concentración del compuesto presentaron [315]. A la hora de estudiar la concentración de BFA urinario en humanos destaca el trabajo de You y cols. [160], en el que encuentran una relación positiva entre BFA urinario y la función renal en mujeres pero no en hombres, lo que le permitió afirmar que el efecto renal del BFA no puede explicarse exclusivamente por diferencias en la dieta, sino que puede existir un elemento ligado al sexo.

Dejando a un lado el efecto del BFA, existen numerosas evidencias que manifiestan la existencia de dimorfismo sexual tanto a nivel experimental como en estudios epidemiológicos humanos, que demuestran que el sexo femenino posee una mayor nefroprotección ante diversos trastornos, comparado con el sexo masculino. En modelos experimentales animales se ha descrito que las ratas hembra poseen una cierta protección frente al fallo renal postisquémico [316]. Del mismo modo, se ha determinado que ante el tratamiento con doxorubicina, los ratones macho presentaban lesiones tubulares y glomerulares mucho más severas, así como una mayor presencia de fibrosis en el riñón [317]. En el estudio de Shi y cols. [318] observaron que, ante el tratamiento con cisplatino, los ratones macho mostraban mayores concentraciones de creatinina y nitrógeno ureico (BUN) plasmático, así como un menor aclaramiento de creatinina. Por último, Clotet y cols. [319] observaron, en un modelo de diabetes inducida por STZ en ratas, que a las 12 semanas los machos presentaban mayores niveles de albuminuria, así como una mayor presión arterial, que las hembras. Existen diferentes hipótesis que intentan determinar la causalidad de las diferencias anteriormente mencionadas, entre las que destacan la dieta, diferencias estructurales, respuestas glomerulares hemodinámicas al estrés, metabolismo lipídico, presión arterial y el efecto directo de las hormonas sobre las células [320, 321]. Esta última posibilidad es la más utilizada para intentar responder a la cuestión, pues se ha determinado que la administración de testosterona sobre un modelo de diabetes inducida por STZ promueve el daño tubular [319]. En modelos *in vitro*, se ha observado que la testosterona puede inducir apoptosis en podocitos mediante una vía andrógeno-

receptor dependiente, de manera independiente al mecanismo de señalización de TGF-β, y que los estrógenos pueden proteger a los podocitos de la apoptosis inducida por testosterona [321]. En la misma línea, se ha demostrado que la orquiectomía en ratas macho puede protegerlos frente a lesiones renales asociadas al envejecimiento [322].

Los estudios epidemiológicos en humanos convergen en la idea de la posible nefroprotección en las mujeres respecto a los hombres. Dichos estudios muestran que, en pacientes con daño renal agudo, los hombres presentan un mayor grado de daño renal, así como un mayor riesgo de progresión hacia enfermedad renal crónica que las pacientes femeninas [318]. En este sentido, uno de los mayores estudios realizados es el de Neugarten y cols. (2000), donde tras realizar un meta-análisis con 68 estudios diferentes y un total de 11.345 pacientes determinaron que los hombres con enfermedad renal crónica originada por diversas etiologías muestran una disminución de la función renal mucho más rápida que las mujeres [323]. En un estudio epidemiológico con 27.805 pacientes con diabetes tipo I se observó una relación estadísticamente significativa entre el sexo masculino y el desarrollo de microalbuminuria, un marcador precoz de enfermedad renal [321].

En definitiva, el mayor dimorfismo sexual evidentemente lo presentó el grupo D+BPA, puesto que las diferencias estaban relacionadas con la supervivencia de los individuos. Curiosamente, el dimorfismo característico que se observó en el peso corporal de los grupos C y D no se observó en ninguno de los dos grupos tratados con BFA (BPA y D+BPA). De manera similar, el dimorfismo observado en el índice de hipertrofia renal tampoco se determinó en el grupo D+BPA. La pérdida de dimorfismo asociada a BFA se ha descrito fundamentalmente en el sistema nervioso y a nivel conductual [324–326], pero también existe un trabajo, de Núñez y cols. [144], en el que observaron dimorfismo en el glomérulo renal de ratones con 30 días de vida, que desaparece en los animales que han sufrido exposición prenatal a BFA.

En el estudio de la función renal, el aclaramiento de creatinina no mostró cambios significativos en ningún grupo, tan sólo se aprecia una tendencia que recuerda a la hiperfiltración observada en el modelo animal anterior. No obstante, en humanos se han desarrollado fórmulas que han disminuido el uso del aclaramiento de creatinina

debido a que generalmente es superior al GFR por la secreción tubular [327]. Por ello, en la práctica clínica se emplean fórmulas como la MDRD o la CKD-EPI, donde las variables cuantitativas corresponden a los parámetros de creatinina plasmática y edad, aplicando a su vez factores de corrección en función de la etnia y el sexo [328]. Debido a que nuestro modelo animal está constituido por animales de la misma edad y todos ellos pertenecen a la cepa de ratones CD1, podría ser una opción viable el estudio de la función renal a partir de la concentración de creatinina plasmática. Sin ir más lejos, numerosos estudios de investigación básica en modelos animales utilizan la excreción urinaria de albúmina y la concentración plasmática de creatinina para realizar el estudio bioquímico de la función renal [329–331]. En este sentido, fueron los grupos D y D+BPA los que mostraron las cifras más elevadas de creatinina plasmática, lo cual unido a la albuminuria, es un claro indicativo de la enfermedad renal incipiente. A continuación, se estudiaron dos conocidos biomarcadores de daño renal, KIM-1 y NGAL [332, 333], mediante el análisis de expresión relativa de ARNm. En todos los animales se observó la sobreexpresión de ambos mensajeros. Es interesante destacar que NGAL mostró el mayor nivel de expresión en el grupo D+BPA, lo que da soporte a la idea de que el BFA puede ejercer daño renal en los animales sanos y ejercer un mayor daño en los animales diabéticos, potenciando la nefropatía diabética.

A pesar de la existencia de limitaciones en el uso de modelos animales para evaluar la insuficiencia renal o los cambios histomorfológicos a largo plazo [334], los resultados del modelo animal presentan importantes implicaciones fisiopatológicas, puesto que los valores de proteinuria, el número de podocitos y la sobreexpresión de los biomarcadores de daño renal son elementos predictivos de la progresión de la enfermedad renal [271, 291, 335–337]. Teniendo todo ello en cuenta, los datos sugieren que incluso la albuminuria de bajo grado asociada a la exposición a BFA podría estar relacionada con el daño podocitario. Además, la expresión de los biomarcadores de daño renal reafirma la cuestión, manifestando la necesidad de futuros estudios y de extremar las precauciones con la exposición al BFA, especialmente en el contexto de la diabetes y la enfermedad renal. El uso de los biomarcadores NGAL y KIM-1 en el contexto de la nefropatía por BFA coincide con el trabajo de Ruiz-Priego y cols. [166], en el que observaron un resultado similar al administrar una dosis de 120 mg/kg.

Probablemente debido a que la dosis empleada era equivalente a la mitad del NOEL, el grupo BPA no llegó a desarrollar diferencias significativas con el grupo control a nivel biométrico (peso corporal, índice de hipertrofia) o bioquímico (creatinina, sodio y potasio en plasma). Sin embargo, sí se produjo un aumento significativo de la proteinuria, de la tensión arterial y de los marcadores de daño renal. Además, el estudio histológico mostró mayor número de glomérulos colapsados, peroxidación lipídica y apoptosis, así como un menor número de podocitos. Respecto al dimorfismo sexual, se observaron diferencias significativas en el índice de hipertrofia renal y la albuminuria, siendo mayor en los machos. A pesar de que el recuento de podocitos no mostró dimorfismo sexual, el número de glomérulos colapsados sí que mostró diferencias en los grupos tratados con BFA, lo que podría ayudar a explicar el incremento significativo de la albuminuria en los machos D+BPA.

Diversos estudios han demostrado que la mayoría de células renales que crecen en condiciones de elevada concentración de glucosa inicialmente presentan un aumento limitado de la proliferación celular, seguido de detención en la fase G1 del ciclo celular y el desarrollo de hipertrofia celular [245, 338]. Estos eventos requieren de la combinación de mitógenos que induzcan la entrada al ciclo celular, con proteínas reguladoras del ciclo celular, como TGF-β1, capaces de detener el ciclo en la fase G1. Por ello, en el análisis de los mecanismos moleculares implicados en los cambios renales se estudió el sistema TGF-β. Como era de esperar, todos los grupos sufrieron un aumento significativo de la expresión de este sistema, involucrado en procesos de hipertrofia, apoptosis y fibrosis. El mayor nivel de expresión relativo correspondió con el grupo D+BPA, lo que permite especular que la intensa respuesta podría anular el efecto mitogénico inducido por BFA.

En términos generales, los resultados mostraron que el BFA, en un rango de dosis inferior al NOEL [104], administrado por la vía oral en el agua, puede ejercer un efecto similar al descrito en los estadios tempranos de la nefropatía diabética [261, 263]. Además, la presencia de hipertensión en los animales tratados con BFA abrió un nuevo campo de estudio: las implicaciones cardiovasculares del BFA. Dicha posibilidad generó una vía de colaboración con el equipo de fisiopatología cardiovascular dirigido por la

doctora Marta Saura, lo que culminó en la publicación académica que figura en el **Anexo III** [339]**.** En este trabajo se determinó que el **BFA oral promueve la hipertensión y la disfunción endotelial de manera dosis-dependiente.** Estos estudios [340] han demostrado que los animales tratados con BFA desarrollan hipertensión arterial y disfunción endotelial en forma dosis dependiente, efecto que comenzó a observarse con dosis menores de la mitad de las consideradas seguras. El análisis de expresión génica por microarrays en células endoteliales murinas tratadas con BFA demostró la activación de genes implicados en la regulación vascular como la angiotensina II y la calcio-calmodulina quinasa II (CaMKII). Posteriormente, se comprobó que esta activación ocurre también *in vivo* y es responsable de la disfunción endotelial e hipertensión inducida por el BFA, dado que la activación de la CaMKII promueve el desacople enzimático de la óxido nítrico sintasa endotelial, lo que conduce a la producción de radicales libres del oxígeno en lugar de óxido nítrico, principal factor vasodilatador y protector del endotelio. Este aumento en la producción de radicales libres del oxígeno indica que el BFA, además de inducir hipertensión, podría participar en los mecanismos de progresión de la lesión arteriosclerótica.

En el presente modelo animal se determinó estrés oxidativo mediante el análisis de 4-HNE, relacionado con la peroxidación lipídica. Esta molécula en 2013 fue reconocida como una toxina urémica, capaz de acumularse en el enfermo renal [341]. Se ha descrito que el 4-HNE puede promover disfunción de la barrera endotelial y además se ha asociado con estrés del retículo endoplásmico [342]. El riñón es uno de los órganos con mayor susceptibilidad esta clase de estrés debido a que presenta un elevado ratio de síntesis proteica [343]. Dentro de las 3 vías de señalización que integran el sistema de respuesta al estrés del retículo endoplasmático, 2 de ellas son protectoras, y la restante, ERK-ATF4-CHOP, es conocida por estar relacionada con mecanismos pro-apoptóticos [344–346] e incluso se ha descrito que puede promover apoptosis en algunas enfermedades renales [347]. En este sentido, es interesante destacar que tanto los animales diabéticos (al igual que en otras publicaciones [348, 349]), como los 2 grupos tratados con BFA, mostraron elevados niveles de CHOP, reafirmando la hipótesis de que el BFA es capaz de inducir un daño análogo a la nefropatía diabética.

En el posterior análisis de MCP-1, TNF-α e IL-1β, conocidos mediadores pro-inflamatorios relacionados con la ND [350], se observó un aumento significativo en los animales del grupo D, de manera similar a la descrita en otros modelos animales [351, 352]. En pacientes con diabetes tipo I se ha observado que la citoquina TNF-α se encuentra elevada en suero, lo cual contribuye a la inflamación sistémica observada en estos pacientes [353]. Sin embargo, los ratones BFA no mostraron cambios en la expresión de dichas citoquinas, e incluso fueron los únicos animales en los que se sobreexpresó la citoquina anti-inflamatoria IL-10. A pesar de que se ha descrito que dosis de BFA superiores al NOEL pueden inducir una mayor liberación de citoquinas pro-inflamatorias en el riñón [166, 175], también se ha observado en otros contextos que el BFA es capaz de promover la liberación de moléculas anti-inflamatorias como IL-10 o TGF-β [354, 355]. En este sentido, los datos apuntan a que la concentración de BFA puede ser crítica en el proceso de inflamación renal. En los animales D, además, se observó una reducción de la expresión del receptor de TGF-β. Este resultado es coherente con los mecanismos inflamatorios observados, pues se ha descrito en un modelo de obstrucción ureteral que la eliminación condicional del gen de TGF-βRII en células tubulares renales puede inducir un aumento de la inflamación renal acompañado de la sobreexpresión de IL-1β y TNF-α [356]. Por último, en los animales D+BPA es interesante destacar que tan sólo se pudo observar un aumento significativo dela citoquina MCP-1. Este hecho quizás se haya visto condicionado por el gran aumento de TGF-β, que, como se ha descrito previamente, es una molécula implicada en procesos de fibrosis, apoptosis, remodelación y anti-inflamatoria. En cualquier caso, trabajos recientes afirman que esta compleja molécula puede tener efectos beneficiosos o perjudiciales en función del contexto patológico [357].

En conclusión**,** los resultados del modelo experimental han mostrado que **la administración oral (a dosis relativamente bajas) de BFA es capaz de promover en el riñón alteraciones que se asemejan a los estadios tempranos de la ND**, como la albuminuria, hipertensión, podocitopenia, apoptosis, glomérulos colapsados, y la sobreexpresión de biomarcadores de daño renal (NGAL y KIM-1), CHOP, 4-HNE, TGF-β y PCNA, pese a que no se observaron cambios en la función renal (ni en el aclaramiento de creatinina ni en la creatinina plasmática). **Los animales diabéticos expuestos a BFA**

sufrieron un empeoramiento notable, destacando el evidente dimorfismo observado en la supervivencia de los individuos, un patrón que también se observó en la albuminuria y el número de glomérulos colapsados, mayor en los machos.

En conjunto, los datos apuntan hacia el paciente diabético como grupo de especial interés y potencial susceptibilidad para el desarrollo o progresión de enfermedad renal inducida por BFA. Es interesante destacar que en las últimas décadas se ha producido un importante aumento de la incidencia de diabetes en el mundo (y complicaciones derivadas como la ND), de forma paralela al incremento sustancial en la demanda y producción de compuestos xenobióticos como el BFA.

La diabetes es una enfermedad en auge que afecta a un número cada vez mayor de personas. De hecho, las cifras actuales han superado las previsiones estimadas, ya que en el año 2000 la cifra ascendió desde los 108 millones de afectados en 1980 [358] hasta 171 millones de personas, por lo que se estimó que se podría alcanzar la cifra de 366 millones para el año 2030 [359]. Sin embargo, en el año 2014 se llegó a los 422 millones [358], y en la actualidad, según los estudios realizados por la Federación Internacional de Diabetes, se estima que casi 500 millones de personas en el mundo viven con diabetes. Las últimas estimaciones apuntan a que la cifra podría llegar a aumentar hasta las 578 millones para el año 2030 y a 700 millones para el año 2045 [360]. Como puede observarse en la figura 12, en España se observa el mismo patrón en el incremento de diabetes en el porcentaje de población total.

De todos ellos, se estima que cerca de la mitad de los pacientes con diabetes tipo 1 desarrollan nefropatía diabética a lo largo de su vida, siendo del 30 – 50 % en el caso de los pacientes con diabetes tipo 2 [361]; y es que la diabetes mellitus se ha convertido en la principal causa de insuficiencia renal crónica terminal en los países desarrollados [259, 362–364]. De forma coherente, también ha aumentado la prevalencia de la enfermedad renal diabética, determinándose un incremento desde el 7,1 % (1988-1994) hasta el 10,7 % (2005 – 2008) en mayores de 65 años [361]. La Sociedad Española de Nefrología estima que alrededor del 21 % de los pacientes con enfermedad renal crónica padecen nefropatía diabética [340] en España. No obstante, debido a que no todos los

pacientes diabéticos sufren alteraciones renales, se considera que deben existir numerosos factores ambientales y/o biológicos aún por determinar.

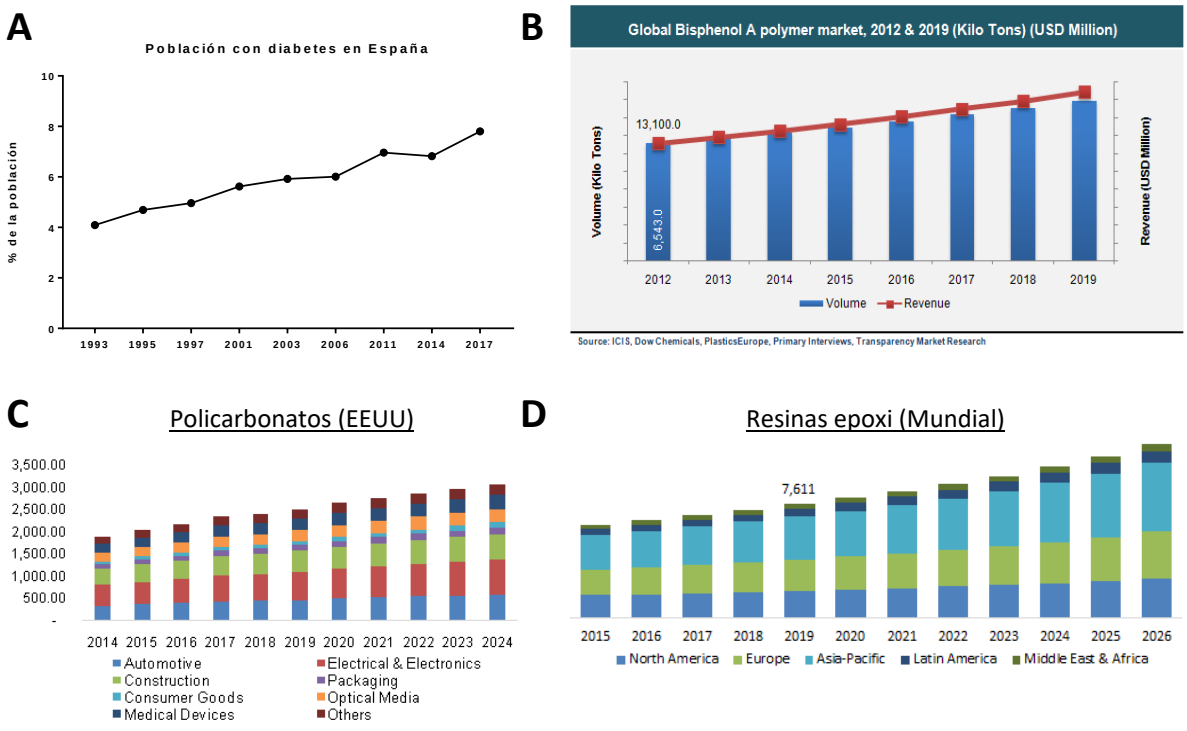

Figura 12.- A) Evolución de la prevalencia de la diabetes en España. Gráfica propia realizada con los datos recopilados en www.epdata.es (Fuente: Ministerio de Sanidad). B) Evolución del mercado de BFA en el mundo (producción y valor de mercado). Adaptado de www.transparencymarketresearch.com. C y D) Estimación gráfica del flujo económico generado por la demanda estadounidense de policarbonato (izquierda) y demanda mundial de resinas epoxi (derecha), en millones de dólares. Adaptado de [365, 366].

De forma llamativamente paralela, la producción de BFA se ha incrementado a lo largo del tiempo, y se prevé que siga aumentando en los años venideros. Los estudios de mercado han previsto que el mercado mundial de BFA sufra una *Tasa compuesta de crecimiento anual* del 3 % anual, alcanzando un volumen de producción de 7348 kilotoneladas (KT) para finales del 2023 [33] (en 2018 se estimó en 7200 KT [32]). En España se ha producido un considerable aumento de la producción de BFA en las últimas dos décadas, posicionándose como uno de los principales países europeos productores de BFA junto con Alemania y Holanda. De hecho, la fábrica situada en La Aljorra, Cartagena, es la mayor factoría de BFA de Europa, con una capacidad de producción anual de 500 KT [367, 368].

Las previsiones económicas estiman que para 2026 se habrá incrementado sustancialmente el valor del mercado del BFA, debido a la importante demanda de este compuesto en la industria automovilística [34] (figura 12). El mismo patrón de crecimiento se puede observar en productos derivados del BFA, como los policarbonatos [32, 35] (figura 12). Por otro lado, en el mercado del fenol se estima que se producirá un aumento de producción desde los 13,4 millones de toneladas en 2020 hasta los 16 millones de toneladas en 2025 [36]. Para comprender la magnitud de la situación, se calcula que cada año se produce una liberación de más de 100 toneladas de BFA a la atmósfera [37].

En definitiva, existen sólidas evidencias que sugieren que el BFA podría ser un factor ambiental implicado en el desarrollo y/o la progresión de la nefropatía diabética. Sin embargo, serán necesarios futuros estudios traslacionales que terminen de clarificar la relación de causalidad entre ambas variables.

Una vez realizados los estudios en células y modelos animales murinos, determinando el efecto potenciador del BFA en el desarrollo y progresión de la nefropatía diabética, el siguiente paso fue la aproximación traslacional a una **línea celular humana** (**Capítulo 2** [369]).

Se ha descrito que el podocito tiene una escasa o nula capacidad de replicación [231], por lo que cualquier situación que provoque pérdida de adhesión y, en consecuencia, reducción en el número de células glomerulares, producirá los mismos efectos a nivel renal que la muerte celular. De hecho, existen numerosos estudios que han determinado la presencia de podocitos viables excretados en la orina de pacientes en estadios iniciales de enfermedad renal, lo cual se ha sugerido que podría ser un marcador adicional a tener en cuenta junto con la microalbuminuria [370, 371], y que además se ha visto especialmente útil en patologías como la preeclampsia [372]. De hecho, debido a que la reducción del número de podocitos (podocitopenia) se ha relacionado con fenómenos de glomeruloesclerosis, se ha llegado incluso a considerarlo como un mejor marcador que la albuminuria en este tipo de patologías [373]. Por todo ello, se procedió a realizar estudios sobre la capacidad de adhesión celular en el rango de concentración nanomolar, lo cual demostró que los podocitos sufren alteraciones en

su capacidad de adhesión tras ser sometidos a una exposición de BFA de 100 nM. En consecuencia, se procedió a confirmar los efectos descritos en la prueba funcional, con el estudio de proteínas relacionadas con la adhesión a nivel de transcriptómica, proteómica, Western blot e inmunocitoquímica.

En primer lugar, el estudio transcriptómico manifestó los primeros indicios de alteración de la adhesión, mostrando diferencias significativas en los ARNm relacionados con nefrina y tubulina. El posterior estudio proteómico mostró una alteración significativa de una treintena de proteínas implicadas en procesos de unión intercelular, unión a matriz extracelular, así como de proteínas estructurales. Los resultados de Western blot mostraron alteraciones en la expresión relativa de proteínas involucradas en la formación del citoesqueleto, como actina, tubulina, vimentina y podocina, así como en proteínas estabilizadoras del citoesqueleto como vimentina y cofilina-1 [374]. El citoesqueleto de los procesos podales primarios está formado por microtúbulos y filamentos intermedios de vimentina, mientras que el citoesqueleto de los procesos secundarios se encuentra formado exclusivamente por filamentos de actina [214, 234], por lo que los resultados sugieren que el BFA puede inducir alteraciones en todo el citoesqueleto celular. Por su parte, la podocina, además de participar en los procesos de unión célula-célula junto con la nefrina [241], también participa en la reorganización del citoesqueleto de actina de los procesos podales secundarios [375, 376]. De forma coherente con la reducción de la expresión de podocina, la nefrina mostró una alteración similar, lo cual sugiere que el mecanismo de unión intercelular se encuentra comprometido tras la exposición a una concentración de BFA de 100 nM. De forma análoga a los resultados en las proteínas implicadas en las uniones intercelulares, otras proteínas involucradas en los mecanismos de unión intercelular o a la membrana basal, como son la E-cadherina, vinculina o VCAM-1 [377–380], también mostraron alteraciones importantes en su expresión. A su vez, cabe destacar una proteína cuya sobreexpresión induce alteraciones en la adhesión, la tenascina C, ya que ha sido descrita como una proteína anti-adhesiva [381]. En este caso, y de forma coherente con todo lo anteriormente expuesto, la expresión relativa de la tenascina se encontraba significativamente elevada.

Una vez confirmadas las alteraciones en la expresión de las proteínas involucradas en el mantenimiento estructural y/o de la adhesión celular, se procedió a determinar si el efecto biológico producido por el BFA pudiera estar mediado por la acción de un receptor estrogénico. Puesto que el BFA es ampliamente conocido por sus propiedades como disruptor endocrino, resulta lógico comenzar por el estudio de dichos receptores. Por ello, mediante el uso de dos conocidos inhibidores estrogénicos, ICI 182,780 (también llamado fulvestrant) y tamoxifeno (su forma metabólicamente activa, 4-OH tamoxifeno, o 4-OHT), se replicaron los ensayos de adhesión usando la concentración de BFA de 100 nM. Los resultados mostraron que el pretratamiento con ICI no afectaba al efecto en la adhesión promovido por BFA. Sin embargo, tras el pretratamiento con tamoxifeno el número de células adheridas fue similar al control, revertiendo de este modo la acción del BFA sobre la adhesión del podocito humano. Ambos inhibidores poseen similitudes relativas a la capacidad de unirse y afectar a las vías de señalización mediadas por los receptores estrogénicos α y β (ERα y ERβ, por sus siglas en inglés). Sin embargo, las publicaciones que estudian el posible efecto inhibitorio de estos compuestos sobre la acción del BFA han observado que en el sistema nervioso central sólo el tamoxifeno parece afectar al daño mediado por BFA, lo cual se atribuye a las diferencias de acción sobre el receptor de estrógenos relacionado con el receptor gamma (ERRγ, por sus siglas en inglés) [382, 383]. De hecho, en el trabajo de Delfosse y cols. [384] se describe al 4-OHT como un agonista inverso y al BFA como un agonista del ERRγ. En la misma línea, el trabajo de Prossnitz y cols. [385] deteminó que el BFA tiene una mayor afinidad por el ERRγ (en el rango nanomolar) que por el ERα y ERβ (micromolar).

En último lugar, el estudio por citometría de óxido nítrico (NO, por sus siglas en inglés) y anión superóxido (SO) mostró que bajas concentraciones de BFA pueden inducir una reducción significativa en la cantidad de NO, así como un incremento proporcional de SO. Dicho resultado resulta coherente puesto que la biodisponibilidad de NO depende de la concentración local de SO debido a que al combinarse se produce la formación de peroxinitritos, una reacción 3 veces más rápida que la que cataliza la

superóxido dismutasa[19] [386]. Se ha descrito que la disminución de NO en la circulación puede afectar de forma negativa al podocito, favoreciendo el desarrollo de daño renal [387], así como que el NO protege al riñón frente a las acciones del SO [386]. Es interesante destacar que la proteína con mayor sobreexpresión observada en el estudio proteómico fue la superóxido dismutasa, lo que reafirma la hipótesis de que el daño podocitario pudiera estar relacionado con un desequilibrio entre NO y SO. Del mismo modo, también se observó una importante sobreexpresión de proteínas como la glutatión sintetasa, implicadas en los mecanismos de respuesta al estrés oxidativo [388]. El NO participa en la regulación de los procesos de remodelación de actina, al intervenir en la expresión de proteínas como HIF-1 [389], lo cual sostiene y reafirma los estudios previamente descritos que manifiestan alteraciones tanto en las propias proteínas del citoesqueleto, como en las proteínas estabilizadoras vimentina y cofilina. De este modo, los resultados relativos al NO pueden encajar dentro del conjunto de alteraciones estructurales y de la adhesión inducidos y/o mediados por la exposición al BFA.

Los resultados sugieren que el BFA a dosis bajas es capaz de inducir alteraciones en la capacidad de unión celular y en la integridad estructural de los podocitos humanos, lo cual constituye un sólido argumento a favor de **considerar al BFA como un agente ambiental capaz de promover un nuevo tipo de podocitopatía caracterizada por la pérdida de adhesión celular, favoreciendo el desarrollo de podocitopenia y de enfermedad renal.** Estos datos sugieren la necesidad de realizar futuros estudios traslacionales que permitan correlacionar el BFA urinario con la pérdida de podocitos urinarios, especialmente en el contexto de la ND. Por otro lado, en los estudios paralelos realizados sobre el sistema cardiovascular, se observó que **el BFA puede jugar un importante papel en el desarrollo de un novedoso tipo de muerte celular (necroptosis), así como en la aceleración del envejecimiento celular en el endotelio murino (Anexo IV** [276] **y V** [277]**).** También serán necesarios futuros estudios que exploren las posibles implicaciones de estos descubrimientos sobre la enfermedad renal.

[19] La enzima superóxido dismutasa detoxifica el anión superóxido en peróxido de hidrógeno y oxígeno [386].

Finalmente, el **capítulo 3** [64] se ha desarrollado a partir de la necesidad transformadora de las metodologías de investigación en el contexto de una crisis sanitaria, y su objetivo fue la unificación de todos los estudios humanos que estudiaran el paradigma BFA-riñón, con el fin de realizar un **meta-análisis** [82]. Debido a la heterogeneidad de las publicaciones, se tuvieron que subclasificar en función del fluido biológico y la forma de determinación de la enfermedad o el daño renal. De este modo, se pudo calcular un odds ratio (OR) combinado entre BFA en sangre y riesgo de desarrollo de enfermedad renal muy significativo, con una probabilidad de desarrollar enfermedad renal de 6,94 por cada incremento logarítmico de la concentración de BFA. De forma análoga, el resultado de BFA urinario y filtrado glomerular estimado manifestó una relación inversa y significativa, demostrando que mayores concentraciones de BFA tenían un mayor riesgo de reducir la capacidad funcional del riñón. Por otro lado, los resultados relativos a la excreción de albúmina urinaria, tanto en la población general (ACR), como en la población sana (low-grade albuminuria) no llegaron a mostrar un resultado significativo.

El estudio mostró que la presencia de elevados niveles de BFA en sangre podría ser un biomarcador, una señal de aviso frente a la predisposición a la enfermedad renal. No obstante, resulta coherente pensar que, ante una reducción significativa de la capacidad funcional renal, con una limitada capacidad de filtración glomerular, pueda existir acumulación de elementos en sangre que no hayan sido capaces de excretarse correctamente. Teniendo en cuenta únicamente los estudios transversales en sangre [195, 199] podría deducirse que el BFA elevado en sangre podría ser una consecuencia y no una causa de la enfermedad. Sin embargo, los estudios longitudinales de Hu y cols. [200, 201], realizados a lo largo de varios años, confieren robustez al estudio, reafirmando que el BFA elevado en sangre podría ser un factor independiente de predisposición a la enfermedad renal, al menos en pacientes con patologías previas como la diabetes o la hipertensión.

Por otro lado, también podría sugerirse que la elevada excreción urinaria de BFA podría estar relacionada con un aumento en la excreción de albúmina en la población general sana. De hecho, existen autores que sostienen que el BFA puede unirse a

proteínas [76], lo que podría también suponer una consecuencia de la albuminuria y no una causa de ella. En este aspecto del meta-análisis no se ha encontrado una relación significativa, y sería necesario realizar estudios longitudinales que exploren la relación entre el BFA urinario y la albuminuria en estadios avanzados de enfermedad renal crónica (ERC).

A pesar de no encontrar una relación significativa entre el BFA urinario y la albuminuria, sí que se observó un resultado significativo en el análisis del eGFR, lo que sugería que las mayores concentraciones de BFA urinario presentan una mayor probabilidad de desarrollar ERC. En el análisis de las publicaciones académicas, cabe destacar los trabajos de You y cols. [160] y Malits y cols. [202], puesto que observaron menores concentraciones de BFA urinario en pacientes con ERC. Sin embargo, una vez corregidos los modelos de regresión con factores antropométricos, sociales y relacionados con el estado de salud, se pudo observar esta relación significativa. Resulta coherente la idea de que un riñón dañado, con una reducida capacidad de filtración glomerular, no sea capaz de eliminar eficientemente el BFA del organismo, reduciendo su excreción y acumulándose en sangre. Sin embargo, el análisis estadístico ha mostrado que el BFA urinario es un factor independiente relacionado negativamente con la capacidad de filtración glomerular.

Las principales conclusiones que pueden extraerse del meta-análisis son, por un lado, la existencia de una asociación significativa entre el BFA en sangre y el riesgo de desarrollar ERC y entre el BFA urinario y la pérdida de función renal. Por otro lado, se manifiesta la necesidad de desarrollar un estudio completo longitudinal, que profundice en la relación entre el BFA urinario y el BFA en sangre, observando los cambios derivados de la pérdida de función renal tanto en la población general como en grupos de riesgo.

Por su parte, el análisis estadístico de la **cohorte NHANES** americana 2003 – 2016 mostró interesantes resultados relacionados con la albuminuria. En primer lugar, la estadística descriptiva mostró que todos los individuos con mayor cantidad de BFA urinario presentaban mayor excreción de albúmina en la orina, tanto en individuos sanos (normoalbuminuria) como en toda la cohorte, un hecho acorde a los resultados

descritos por Li y cols. [123] y Trasande y cols. [124]. La corrección de BFA urinario por creatinina, un elemento necesario en el análisis de orina espontánea, mostró diferencias entre hombres y mujeres, siendo superior la concentración media de BFA absoluto (ng/ml) en hombres, mientras que las mujeres presentaron un mayor valor de BFA corregido por creatinina. La corrección permite obtener un valor más realista de la excreción de metabolitos urinarios, que tiene en cuenta la capacidad de filtración renal en los casos en los que no se ha podido obtener el volumen de orina en 24 horas.

Por otro lado, en el análisis del BFA urinario en función del filtrado glomerular estimado sólo se observaron diferencias significativas al utilizar la ecuación CKD-EPI, aunque no fueron concluyentes. En cualquier caso, los valores urinarios de BFA fueron significativamente superiores al comparar a los sujetos diagnosticados con enfermedad renal, siendo aún mayor en el caso de los pacientes sometidos a hemodiálisis, obteniendo nuevas evidencias en la búsqueda de la relación de causalidad entre el BFA y la enfermedad renal.

Por último, se realizó un **estudio propio**, cuantificando el BFA urinario de población hospitalaria y población control (**Anexo I).** El estudio comparativo determinó una mayor presencia de este compuesto en la orina de la población enferma. A pesar de que no se hallaron diferencias significativas entre los pacientes con diferentes patologías, el conjunto de individuos presentó mayores concentraciones de BFA que la población control. Por su parte, el BFA urinario de los controles no mostró diferencias significativas al compararlo con otros trabajos académicos realizados en poblaciones españolas, o incluso con otras cohortes mundiales, lo que validó y respaldó los resultados obtenidos.

Por otro lado, se observaron diferencias en el porcentaje de bisfenol conjugado, lo cual podría deberse a alteraciones metabólicas relacionadas con el contexto patológico del paciente. Es interesante destacar que estos porcentajes eran muy superiores al de los estudios farmacocinéticos, que utilizan modificaciones estructurales del bisfenol. Otras publicaciones académicas que realizaron estudios similares al nuestro [390–392] también hallaron elevados porcentajes de BFA libre, validando los resultados y poniendo en cuestión el uso de formas deuteradas de BFA en la realización de modelos

farmacocinéticos. Serán necesarios futuros estudios longitudinales, preferiblemente con un mayor tamaño muestral, que permitan profundizar en la posible relación entre el BFA y el riesgo de desarrollo de enfermedad renal crónica.

En definitiva, la presente tesis doctoral ha avanzado en la búsqueda de la relación entre el contaminante ambiental BFA y el desarrollo y progresión de las enfermedades renales. Se han identificado importantes efectos deletéreos a nivel celular inducidos por bajas dosis de BFA, de gran relevancia en la capacidad funcional del riñón. Se han descrito numerosas analogías entre los efectos renales inducidos por BFA y eventos asociados a la nefropatía diabética, potenciándose su combinación hasta el extremo de causar la muerte en ratones de sexo masculino. Los estudios poblacionales han demostrado que la exposición a BFA en la población general se acerca peligrosamente a las dosis empleadas en nuestros modelos experimentales, especialmente en grupos de especial susceptibilidad como el paciente con enfermedad renal crónica. Además, se ha evidenciado una fuerte relación entre el BFA en sangre y el desarrollo de enfermedad renal, así como entre el BFA urinario y la reducción de la capacidad funcional del riñón.

Todo ello, unido a los avances a nivel cardiovascular, donde se ha observado una mayor sensibilidad a la hipertensión inducida por BFA, la promoción de mecanismos de muerte celular como la necroptosis, e incluso la aceleración del envejecimiento celular asociado con una vía de señalización especialmente implicada en procesos patológicos, han demostrado que el BFA no es sólo un disruptor endocrino capaz de afectar al sistema reproductor, sino que tiene la potencialidad de afectar a numerosos órganos y sistemas biológicos diferentes.

En conclusión, los resultados sugieren que la nefropatía inducida por BFA, clínicamente indistinguible de la ND, podría explicar el aumento en la incidencia de esta patología. De este modo, se manifiesta la necesidad de estudios transversales y/o longitudinales que profundicen en el papel del BFA en las enfermedades renales, con especial énfasis en el paciente diabético.

La sociedad actual ha normalizado la presencia de compuestos xenobióticos en la orina de la población general, puesto que se ha aceptado la existencia de rangos "seguros", a pesar de las evidencias contradictorias. Es el momento de romper el ciclo y acabar con la normalización, pues el bienestar endocrino y correcto desarrollo fisiológico de las futuras generaciones depende de las decisiones que tomemos hoy.

Al mirar el contorno de un mapa,
el estudiante ve líneas sobre un papel,
mientras que el cartógrafo
ve una fotografía de un terreno [393]

Thomas Kuhn (1922 – 1996)

La ciencia siempre está en falta.
Nunca soluciona un problema
sin crear otros diez [2]

George Bernard Shaw (1856 – 1950)

VIII. CONCLUSIONES

1. En podocitos de ratón, el BFA ejerce un efecto citotóxico a bajas concentraciones, manifestando reducción de la viabilidad y aumento de apoptosis, generando en consecuencia mecanismos hipertróficos compensatorios mediados por el sistema de proteínas TGF-β y $P27^{kip1}$, de forma análoga a la hipertrofia inducida por glucosa.

2. En modelos animales, la administración intraperitoneal de BFA produce un aumento de la albuminuria y del aclaramiento de creatinina, así como expansión mesangial e hipertrofia renal y glomerular, características similares a las que se encuentran en la nefropatía diabética temprana.

3. Los modelos animales con nefropatía diabética inducida por STZ presentan un peor pronóstico y evolución al ingerir BFA por vía oral, destacando la mortalidad precoz asociada a los machos, probablemente relacionada con alteraciones en los mecanismos fisiológicos que regulan el equilibrio hidroelectrolítico y una mayor atrofia corpuscular y albuminuria.

4. En podocitos humanos, el BFA a bajas concentraciones afecta a los mecanismos de adhesión celular y mantenimiento estructural. La pérdida de densidad celular glomerular supone el progresivo deterioro de la función renal y, en consecuencia, favorece el desarrollo de enfermedad renal crónica.

5. Los estudios estadísticos combinados han evidenciado una importante relación positiva entre el BFA en sangre y el riesgo de desarrollar enfermedad renal crónica. También se ha identificado una relación negativa entre el BFA urinario y la capacidad de filtración renal, lo que reafirma la hipótesis inicial de que el BFA es un contaminante ambiental capaz de afectar al sistema renal. Finalmente, el análisis cuantitativo de BFA urinario ha confirmado un mayor grado de exposición en la población enferma.

A mí mismo me parece haber sido como un muchacho que juega a la orilla del mar, divirtiéndose ahora y entonces buscando una piedra escondida o una pequeña concha, mientras el gran océano de la verdad permanecía ignorado frente a mí [2]

Sir Isaac Newton (1642 – 1727)

Un científico es un hombre tan endeble y humano como cualquiera; sin embargo, la búsqueda científica puede ennoblecerle, incluso en contra de su voluntad [2]

Isaac Asimov (1920 – 1992)

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X. ANEXO I

ESTUDIO TRASLACIONAL DE LA EXPOSICIÓN HOSPITALARIA A BFA

Estudio traslacional de la exposición hospitalaria a BFA

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I. Introducción

Las nuevas evidencias aportadas a lo largo de la presente tesis han demostrado que el BFA es capaz de afectar al sistema nefro-vascular en un rango de concentraciones bajo. En el contexto de los modelos experimentales *in vitro*, la concentración nanomolar puede ejercer acciones lesivas, induciendo hipertrofia y pérdida de viabilidad en podocitos de ratón [271] y alteraciones de la capacidad de adhesión y el mantenimiento estructural del podocito humano [369]. Los modelos animales han demostrado que la exposición oral a concentraciones inferiores al NOEL renal también pueden ejercer un efecto nefrotóxico, con manifestaciones análogas a la nefropatía diabética [297]. El uso de modelos celulares humanos [369], meta-análisis y análisis de la cohorte NHANES [82] han aportado interesantes evidencias desde el punto de vista traslacional. Sin embargo, resulta necesario un estudio con una cohorte propia que permita estimar la exposición potencial a la que se encuentra expuesta la población de la Comunidad de Madrid y Castilla La Mancha, grupos poblacionales con escasa representatividad en los estudios cuantitativos del BFA urinario.

Para ello, es importante destacar que todos los estudios farmacocinéticos humanos convergen en la idea de que el BFA tiene una vida media reducida (unas 6 horas) y su excreción se produce exclusivamente por la vía urinaria [51–53, 394]. De este modo, se asume que la cuantificación urinaria de BFA permitiría realizar una aproximación fidedigna de la exposición real a BFA. Además, existen evidencias en la literatura que respaldan la idea de que los materiales médico-quirúrgicos empleados en

el entorno hospitalario podrían incrementar la exposición a BFA. Se ha descrito que los niños en la unidad de cuidados intensivos neonatal [61, 62, 65], adultos en cuidados intensivos [69] o pacientes con enfermedad renal crónica terminal sometidos a diálisis [66, 68] presentan niveles superiores de BFA urinario a la población general.

Este hecho, ya de por sí preocupante, resulta de especial interés al analizarlo dentro del contexto de la presente tesis, pues podrían ser grupos poblacionales con especial sensibilidad a los efectos deletéreos inducidos por BFA. Por ello, se realizó un estudio comparativo de la concentración de BFA en sujetos sanos y pacientes del Hospital Príncipe de Asturias (Alcalá de Henares, Comunidad de Madrid) y del Hospital de Guadalajara (Guadalajara, Castilla-La Mancha).

II. Material y métodos

Población de estudio: 24 Pacientes del Hospital de Guadalajara, 102 pacientes del Hospital Príncipe de Asturias y 22 sujetos sanos. El estudio se completó con el análisis de la literatura académica, con el fin de comparar los resultados con los valores medios descritos en España y en el mundo.

Fases de estudio: En una primera fase se recogieron muestras de sangre, orina de 24 horas y micción espontánea de 24 pacientes con el fin de estudiar la variabilidad entre los diferentes fluidos biológicos. Puesto que no se observaron diferencias significativas entre la orina espontánea y la orina de 24 horas, en la segunda fase se recogieron 102 orinas espontáneas de pacientes y 22 de sujetos control.

Recogida de muestras: Todas las orinas se recogieron en recipientes estándar de orina espontánea o de 24 horas. En el momento de la recepción se centrifugaron a 1500 rpm, se recogió el sobrenadante y se congeló a -20 °C. Las orinas se procesaron siguiendo un protocolo similar al descrito en otras publicaciones [51], incubando a 37 °C toda la noche con enzimas procedentes de *Helix pomatia*. De este modo, al desconjugar las formas glucuronizadas y sulfatadas se pudo determinar la concentración de BFA total. Por otro lado, también se procesaron las muestras sin incubar con la enzima para determinar la concentración de BFA libre (BFA sin metabolizar). La sangre se recogió en un tubo estándar para recogida de suero, sin anticoagulante. A continuación, una vez

formada la coagulación sanguínea, centrifugó a 1500 rpm durante 5 minutos para separar el suero.

Cuantificación de BFA: La extracción de BFA fue realizada según el protocolo de Vela-Soria y cols. [395], con algunas modificaciones. Se añadieron 1200 µl de acetona con el fin de precipitar proteínas y compuestos solubles en solventes orgánicos. La solución se agitó 30 segundos y centrifugó a 3500 rpm durante 15 minutos. Después se añadieron 9360 µl de NaCl al 5 % y 240 µl de HCl 2M. A continuación, se añadieron 600 µl de acetona y 900 µl de cloroformo lo más rápido posible, desplazando el BFA hacia el cloroformo. Ambos solventes son miscibles entre sí, pero sólo la acetona es miscible en agua. Por ello, tras la adición de la mezcla, la acetona se mezcla con la fase acuosa, y el BFA se desplaza hacia la fase formada por el cloroformo. Tras mezclar durante un minuto y centrifugar a 3500 rpm durante 20 minutos, se transfirió la fase orgánica a un tubo limpio de cristal y se evaporó la muestra con un evaporador de vacío. El residuo se reconstituyó en 150 µl de agua/metanol (6,6:1 v/v) para el posterior análisis.

La separación y cuantificación se llevó a cabo con un sistema de HPLC Agilent 1100, equipado con un detector de fluorescencia. Se utilizó una columna Kromaphase C18 de 250 mm x 4,6 mm y 5 µm de tamaño de partícula. Las muestras fueron separadas utilizando una mezcla de agua y acetonitrilo (50:50) con un flujo de 1 ml / min en una fase móvil en modo de elución isocrático. El volumen de inyección fue de 50 µl. Las longitudes de onda de excitación y emisión fueron de 275 y 300 nm, respectivamente. El tiempo total de análisis fue de 15 minutos.

Análisis estadístico: Para la realización de estadística descriptiva y análisis comparativo se utilizó el programa GraphPad Prism 7 (GraphPad, San Diego, CA, USA). Tras la realización de los test de normalidad (D'Agostino & Pearson, Shapiro-Wilk y Kolmogórov-Smirnov), las variables se expresaron como media aritmética (desviación estándar) en el caso de presentar distribución normal, y como media geométrica (95 % de intervalo de confianza), en el caso de distribuciones no paramétricas. En el análisis comparativo se utilizaron los test ANOVA o Kruskal-Wallis, según procedía. Por último, para el modelo de regresión logística binomial se utilizó el programa IBM SPSS Statistics for Windows, versión 27 (IBM Corp., Armonk, N.Y., USA). Se consideró la variable

dependiente la presencia o ausencia de enfermedad renal crónica (variable dicotómica, 0=sano y 1=enfermo), y covariables la concentración urinaria de BFA, edad, sexo, hipertensión y diabetes. Se consideraron significativos todos aquellos resultados cuyo p-valor se encontrara por debajo de 0,05.

III. Resultados

Como puede observarse en la figura 13, en la primera aproximación comparativa no se observaron diferencias significativas entre la orina de 24 horas y la micción espontánea, lo que facilitó enormemente el proceso de recogida de muestras. Por otro lado, ambas muestras urinarias presentaron diferencias estadísticamente significativas respecto a la concentración de BFA en suero. En el análisis de BFA total (BFAt), se identificó este compuesto en el 100 % de las muestras analizadas. Sin embargo, en el análisis de BFA libre (BFAl) sólo se identificó este compuesto en el 45,84 % en suero, 70,84 % de orina espontánea y 66,67 % de orina de 24 horas.

Teniendo en cuenta los resultados obtenidos, se analizó exclusivamente la orina espontánea, al no observarse diferencias significativas con la orina de 24 horas. En consecuencia, se recogieron y analizaron 102 muestras más de pacientes, comparándose los resultados con 22 sujetos control. Para completar dicho estudio, además, se realizó un análisis de la literatura académica que permitiera obtener un valor de BFA urinario en España y en el mundo. La concentración de BFA urinario mundial se calculó en una revisión sistemática previamente publicada por nuestro grupo [64] en la que, utilizando los valores de las mayores cohortes mundiales, se obtuvo un valor medio (desviación estándar) de 1,56 (0,74) ng/ml, y un promedio (teniendo en cuenta el tamaño muestral de cada estudio) de 1,55 ng/ml. Durante la realización de dicho análisis sistemático se identificaron 14 publicaciones que analizaban BFA urinario en diferentes cohortes o grupos poblacionales en España [106, 396–408]. 12 de ellas representaron los valores de BFA urinario en ng/ml, pero dos de ellas utilizaron el valor corregido por creatinina. Para homogeneizar resultados, se utilizaron los valores medios de creatinina de las 3 mayores cohortes mundiales de BFA, utilizando la metodología previamente utilizada [64]. En dicho trabajo, mediante la realización de un modelo de regresión lineal se determinó una relación lineal entre media geométrica y mediana, con un valor de R^2

de 0,9919, y una ecuación de la recta de Y = 0,9855 X. Por ello, se utilizó por defecto el valor correspondiente a la media geométrica del BFA urinario, pero en caso de no hallarse, se utilizó el valor de la mediana corregido con dicha ecuación de la recta.

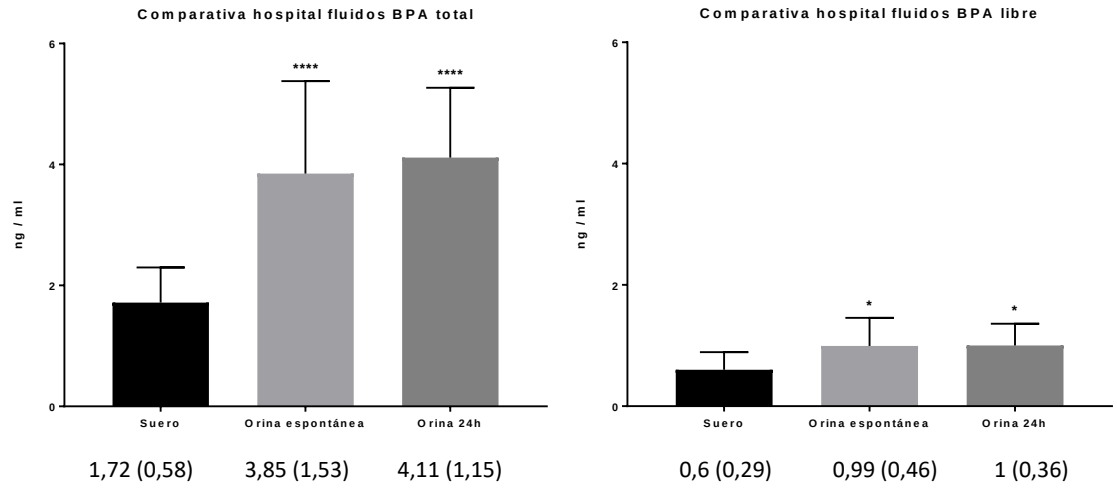

*Figura 13.- Estudio comparativo de la concentración de BFA total y libre en diferentes fluidos biológicos de pacientes del Hospital de Guadalajara (24 pacientes). Los test de normalidad mostraron una distribución normal, por lo que se representan como media (desviación estándar) tanto en la gráfica como en el valor numérico inferior. El análisis estadístico se realizó mediante el test ANOVA de una vía. * representa un p-valor <0,05; **** representa un p-valor < 0,0001.*

Como puede observarse en la figura 14, el estudio comparativo de BFAt mostró importantes diferencias entre los pacientes del entorno hospitalario y el resto de grupos, no observándose diferencias significativas entre el ellos (Control, España y Mundo). Los datos se representaron en ng/ml para simplificar la comparativa con el resto de grupos, puesto que la mayoría de publicaciones expresan la concentración de BFA urinario en dichas unidades. Sin embargo, debido a la naturaleza de los sujetos del hospital, algunos de ellos tendrán alteraciones importantes de la filtración glomerular y, en consecuencia, de los valores de creatinina urinaria. Por ello, se calcularon los valores de BFA urinario corregidos y sin corregir por creatinina, y se analizaron utilizando el test estadístico Mann-Whitney, no hallándose diferencias significativas (p-valor = 0,2383).

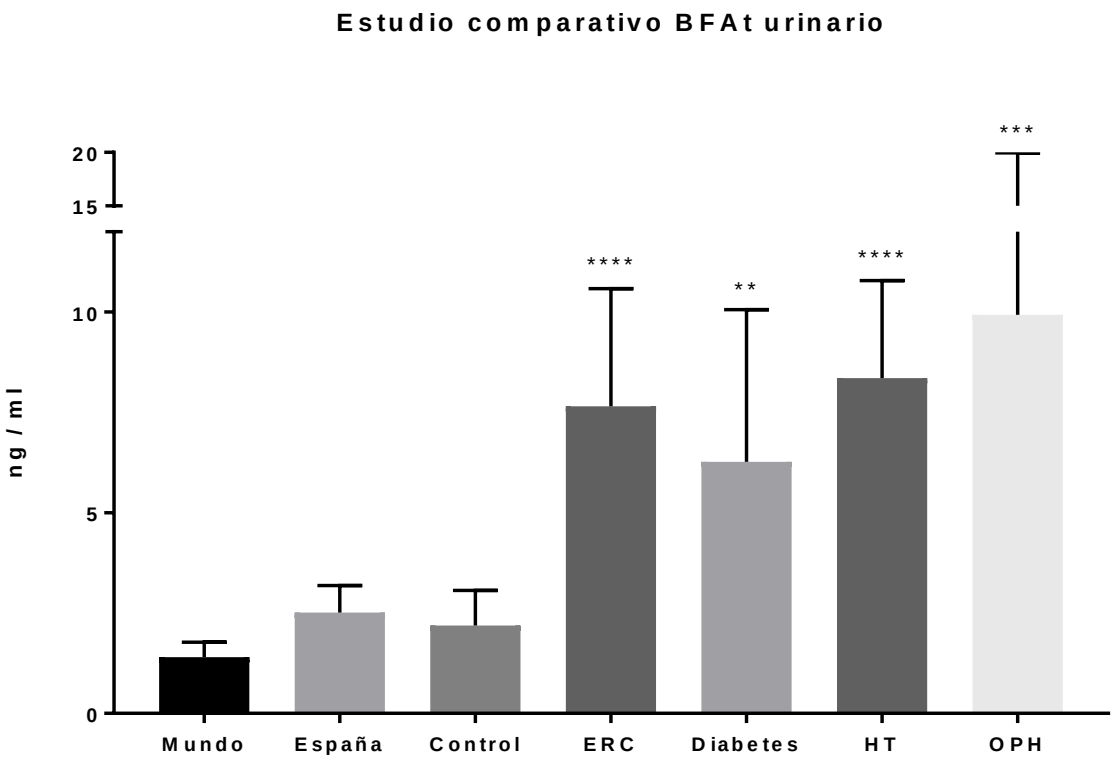

*Figura 14.- Estudio comparativo de la concentración urinaria de BFA total en diferentes patologías, sujetos control, cohortes españolas [16–29] y principales cohortes mundiales [15]. Puesto que muchos resultados mostraron distribución no paramétrica se representaron como media geométrica (95 % de intervalo de confianza). El análisis estadístico Kruskal-Wallis sólo mostró diferencias significativas entre el grupo hospitalario y el resto de grupos, no observándose diferencias entre los grupos Control, España y Mundo. ** representa un p-valor <0,01; **** representa un p-valor < 0,0001. Abreviaturas: ERC, Enfermedad renal crónica; HT, Hipertensión; OPH, Otras Patologías Hospitalarias.*

Por último, gracias a la obtención de los valores urinarios de BFA, y teniendo en cuenta que los modelos de estudio farmacocinéticos avalan que el 100 % del BFA se excreta por la orina en un espacio de tiempo relativamente corto [51–53, 394], se calculó la exposición estimada diaria (EED) de BFA. Para ello, se utilizaron valores de referencia de excreción diaria de orina y peso medio de adultos de la literatura académica [409] (1400 ml de orina diarios y un peso medio de 66,5 kg). Teniendo todo ello en cuenta, se aplicó la siguiente fórmula para calcular la EED de BFA:

$$EED = \frac{BFA\ urinario\left(\frac{ng}{ml}\right) x\ vol.urinario\ (ml)}{peso\ corporal\ (Kg)}$$

De este modo, se estimó que los sujetos control, así como los valores medios de España y del mundo, se encuentran entre 1,4 y 2,5 ng/ml, lo que supone una exposición diaria estimada de entre 30 y 40 ng/kg de peso y día. Estos valores equivalen a una molaridad de BFA de entre 6 y 11 nM. Por su parte, los pacientes presentaron concentraciones superiores de BFA, con una exposición estimada de entre 132 y 209 ng/kg, o una molaridad urinaria de 27 – 43 nM (Tabla 1).

GRUPO	N	MG (95 % IC) - ng/ml	EED - ng/kg	MOLARIDAD - nM	MEDIANA (RI) – ng/ml
MUNDO	19	1,4 (1,11 - 1,77)	29,47	6,13	1,6 (0,9 - 1,96)
ESPAÑA	19	2,51 (1,98 - 3,19)	52,84	10,99	2,3 (2,04 - 4,26)
CONTROL	22	2,19 (1,56 - 3,06)	46,11	9,59	2,15 (1,17 - 3,82)
ERC	56	7,65 (5,53 – 10,58)	161,05	33,51	8,6 (3 – 16,53)
DIABETES	30	6,27 (3,90 – 10,06)	132,00	27,47	6,93 (2,11 – 13,7)
HT	83	8,35 (6,47 – 10,78)	175,79	36,58	8,66 (4,06 – 18,15)
OPH	11	9,93 (4,94 – 19,96)	209,05	43,50	10,56 (5,25 – 13,09)

Tabla 2. Análisis de la concentración urinaria de BFA y estimación de la exposición diaria. Significado de abreviaturas: N, tamaño muestral (Hospital y Control) o número de cohortes y subgrupos analizados (España y Mundo). Abreviaturas: ERC, Enfermedad renal crónica; HT, Hipertensión; OPH, Otras Patologías Hospitalarias; MG, Media Geométrica; IC, Intervalo de Confianza; EED, Exposición Estimada Diaria; RI, Rango Intercuartil.

IV. Discusión

En la actualidad existen unos límites de exposición a BFA bien definidos, dentro de los cuales se asume que no existen riesgos para la salud. En Europa este umbral, denominado TDI, se encuentra en 4000 ng/kg, y en América, la FDA lo ha situado en 50000 ng/kg [103, 207]. Desde esa perspectiva, los resultados obtenidos en este trabajo no supondrían un riesgo para la salud humana. Sin embargo, hay varios puntos que deben tenerse en cuenta a la hora de abordar la cuestión.

En primer lugar, Los cálculos de la EFSA se basan en modelos animales que determinan la dosis de 50mg/kg como la menor concentración a la que no observan efectos sobre el riñón [56, 104]. Sin embargo, nuestros modelos animales han determinado efectos renales a la misma dosis [271] e incluso a la mitad [297], y nuestros modelos celulares han determinado importantes efectos deletéreos sobre el podocito en cultivo en el rango nanomolar [271, 369], en concentraciones cercanas a las determinadas en la orina de la población.

En segundo lugar, la posible existencia de efectos no-monotónicos inducidos por BFA [192–194] supone una dificultad añadida en el cálculo de concentraciones seguras al BFA, pues teóricamente podría ejercer efectos lesivos tanto a dosis bajas como a dosis elevadas.

En tercer lugar, los individuos con patologías previas podrían tener una mayor susceptibilidad al daño inducido por BFA, además de tener una mayor exposición media. En los trabajos de Hu y cols. [200, 201] se observó que, en los pacientes con diabetes o con hipertensión, aquellos individuos con mayores niveles de BFA sérico tenían entre 6 y 7 veces más riesgo de desarrollar enfermedad renal crónica.

En cuarto lugar, los modelos farmacocinéticos humanos afirman que se metaboliza más del 90 % del BFA, excretándose por la orina un porcentaje muy reducido de BFAl (no conjugado). Sin embargo, dichos modelos se realizan con formas modificadas de BFA, lo que podría suponer variaciones en la farmacocinética del compuesto [294, 295]. En nuestro estudio se observa que en la orina de todos los pacientes se detecta BFAl en un 83 % de ellos y en el 50 % de los sujetos control. La media geométrica de BFAl - BFAt es de 2,84 - 7 en los pacientes, y de 0,64 - 2,19 en los controles, lo que supone un 40 % y un 30 %, respectivamente (ver figura 15). Estos resultados son sustancialmente diferentes a los descritos en los modelos farmacocinéticos, que administraron d6-BFA a sujetos voluntarios. En el trabajo de Thayer y cols. [53] determinaron que la proporción de BFAl en suero es menor al 1 % tras la administración oral, mientras que Sasso y cols. [99] afirmaron que el porcentaje es superior al 8 % tras la administración dérmica. Las enormes diferencias hacen sospechar de la posible contaminación en el presente modelo, lo que supondría una

sobreestimación de las concentraciones de BFA. Sin embargo, en la comparativa con otras cohortes de España y del mundo no se han observado diferencias significativas en el BFA urinario, un hecho que refuerza los resultados descritos. Otras publicaciones que analizan BFA libre y total en cuidados intensivos pediátricos [390], adultos con /sin diabetes [391] y embarazadas con/sin defectos en el desarrollo fetal [392] determinaron unas proporciones de BFAl - BFAt más próximas a las descritas en el presente manuscrito que en los estudios con formas deuteradas de BFA, lo que valida y reafirma la coherencia de los resultados presentados.

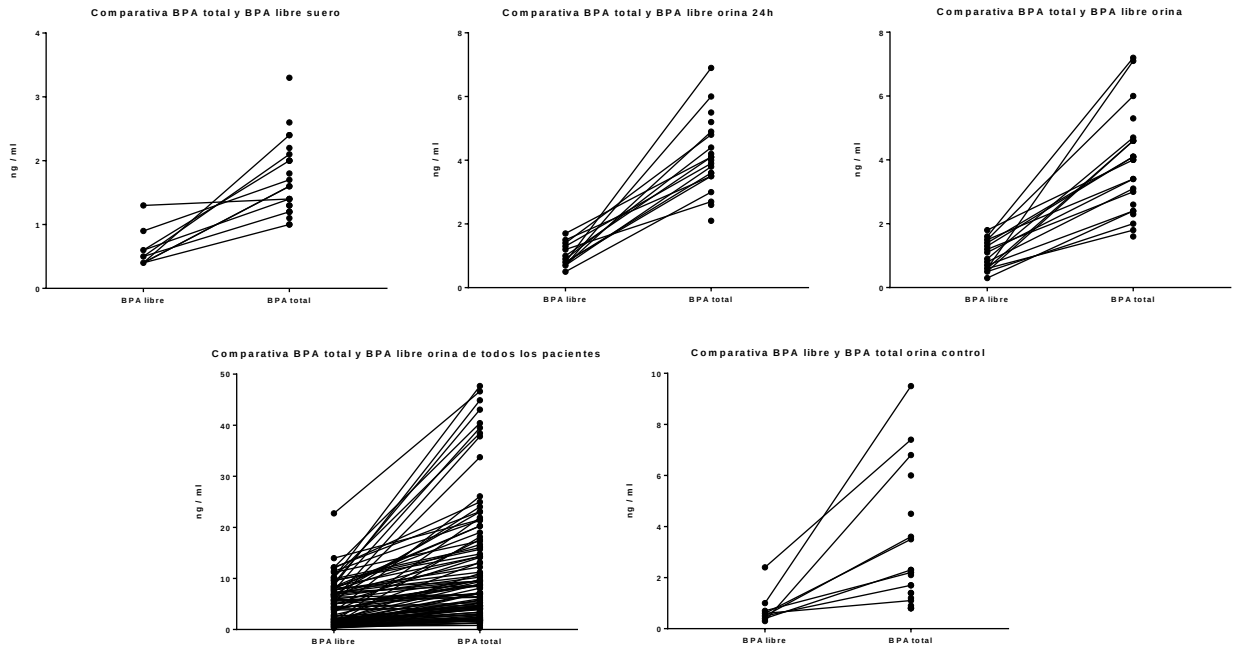

Figura 15.- Relación entre el BFA libre (BFAl) y el BFA total (BFAt) en cada uno de los pacientes del estudio preliminar (N=24) y en la orina espontánea de todos los pacientes (N=126) y controles (N=22). En la gráfica de todos los pacientes se redujo la proporción del eje Y para facilitar el visionado, desplazando fuera del rango 4 puntos.

Por otro lado, la existencia de un menor número de individuos control con niveles detectables de BFAl podría deberse a dos causas: quizás existan alteraciones metabólicas asociadas a las patologías hospitalarias, lo cual impediría metabolizar correctamente el BFA [96], o bien que sus valores estuvieran por debajo del límite de detección, al tener una menor concentración promedio del compuesto. Estas diferencias metabólicas asociadas a patologías podrían ayudar a explicar el porcentaje de BFAl entre grupos, aunque tampoco puede descartarse la existencia de variaciones asociadas a la vía de entrada en el organismo [88].

Comparativamente a otras cohortes mundiales, en las que se analizan miles de pacientes, el presente estudio utiliza un número reducido de individuos, por lo que debe considerarse como una aproximación preliminar. Las diferencias cuantitativas entre el grupo hospitalario y el resto son evidentes, lo que, al menos, reafirma la existencia de una mayor presencia de BFA en sujetos con patologías. No se ha podido correlacionar estadísticamente la enfermedad renal con la concentración urinaria de BFA, ya que el tamaño muestral reducido puede suponer una limitación en el desarrollo de modelos de regresión logística. En definitiva, el presente estudio ha determinado que la presencia de ciertas patologías se encuentra relacionado con una mayor concentración de BFA. Serán necesarios futuros estudios longitudinales, preferiblemente con un mayor tamaño muestral, que permitan profundizar en la posible relación entre el BFA y el riesgo de desarrollo de enfermedad renal crónica.

XI. ANEXO II

ESTUDIO PRELIMINAR DE LA PODOCITURIA EN LA ENFERMEDAD RENAL CRÓNICA Y SU RELACIÓN CON EL BISFENOL-A

Estudio preliminar de la podocituria en la enfermedad renal crónica y su relación con el bisfenol-A

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I. Introducción

Las evidencias desarrolladas en el capítulo 2 de la presente tesis han demostrado que el bisfenol-A (BFA) es capaz de afectar a la capacidad de adhesión de los podocitos humanos en cultivo. El podocito es el principal responsable del desarrollo y progresión de las enfermedades glomerulares que conducen a la enfermedad renal crónica [410]. De hecho, se ha relacionado la reducción del número de podocitos glomerulares con la enfermedad renal crónica terminal [411].

En los últimos años se ha comenzado a estudiar la posible relación entre la pérdida de podocitos a través de la orina (podocituria) y diversas enfermedades como la nefropatía diabética [412, 413], nefritis lúpica [414], nefropatía membranosa idiopática [415], enfermedad de Fabry [416] o la preeclampsia [411, 417].

El presente trabajo propone explorar dos cuestiones. En primer lugar, el estudio de la podocituria en pacientes con enfermedad renal crónica avanzada (ERCA), es decir, aquellos pacientes con la ratio de filtración glomerular menor a 30 ml/min. Y, en segundo lugar, la excreción de BFA urinario podría confirmar los resultados observados en los modelos *in vitro*. Para ello, se analizarán orinas de 12 horas, permitiendo extrapolar fácilmente la diuresis diaria y la excreción real de BFA y podocitos urinarios.

II. Material y métodos

1. Determinación de podocituria

Recogida de muestras: El método de determinación de podocituria se basa en los trabajos de Pérez-Hernández y cols. [414] y Mella y cols. [415], con algunas modificaciones. Ambos utilizaron volúmenes de entre 30 y 50 ml de orina espontánea de primera hora de la mañana. En el presente trabajo se recogió orina de 12 horas y se procesó inmediatamente después de su llegada al laboratorio. Se seleccionaron 25 individuos sin patologías de cualquier edad como sujetos control. Los pacientes seleccionados fueron todos aquellos individuos con un aclaramiento de creatinina menor o igual a 30 ml / minuto, es decir, con enfermedad renal crónica avanzada (n=20). Por último, hubo una consideración añadida en el caso de las mujeres, con el fin de prevenir alteraciones de los resultados ocasionadas por la menstruación se utilizaron las orinas de aquellas que se encontraban aproximadamente en la mitad del ciclo menstrual.

Procesado de muestras para la determinación de podocituria por citometría: Una vez anotada la diuresis de 12 horas, se centrifugaron 100 ml de orina (2 Falcon de 50 ml, para hacer cada ensayo por duplicado) a 1500 rpm durante 5 minutos (temperatura ambiente). Se recogió una parte del sobrenadante para la posterior cuantificación de bisfenol urinario. El resto se desechó apropiadamente. A continuación, se lavaron los pellets con 3 ml de PBS, centrifugando de nuevo con las mismas condiciones y desechando de nuevo el sobrenadante.

Al pellet resultante se añadieron 350 microlitros de PBS + 20% medio RPMI + 5% FBS + 0,1% acida sódica y se dividió el contenido en dos tubos de citómetro. A uno de ellos se le añadieron 5 µl de podocalixina-1 (PDX-1, ref. IC2419A) conjugado con aloficocianina (APC). Al segundo tubo de citómetro se le añadieron 5 µl de IgG2B de ratón conjugado con APC, (ref. IC0041A) con el fin de detectar uniones inespecíficas del anticuerpo. Todos los tubos se incubaron durante 30 minutos en oscuridad a 4 °C.

Posteriormente se lavaron los pellets, centrifugando los tubos a 1500 rpm durante 5 minutos (temperatura ambiente), desechando el sobrenadante y añadiendo 200 µl de solución de incubación. Por último, se analizaron en el citómetro de flujo, donde se fijó un volumen final de 200 µl, de los que se utilizaron 100 µl. Se empleó una velocidad media, identificando un total de 50 000 eventos.

Optimización de la técnica utilizando podocitos humanos en cultivo: Los podocitos se cultivaron a 33 °C, una temperatura permisiva para su proliferación. Al alcanzar una confluencia del 80-90%, se realizó un pase 1:7 y los Flask resultantes se incubaron a 37 °C, una temperatura que impide su proliferación, favoreciendo la diferenciación celular. Esta clase de células se encuentra diferenciada entre el día 11 y el 15.

Una vez diferenciadas, las células se tripsinizaron, lavaron y se incubaron de acuerdo al protocolo de citometría. Los resultados se utilizaron para delimitar las regiones correspondientes a podocitos marcados con PDX-1 unida a APC. Las regiones delimitadas en los correspondientes dot plots se utilizaron para el posterior análisis de las orinas humanas, aplicando los mismos parámetros para todas las muestras. En la figura 16 se representa la configuración con mayor sensibilidad y menor cantidad de uniones inespecíficas.

2. Cuantificación de BFA urinario

Se realizó el mismo procedimiento que en el anexo I

3. Análisis estadísticos

Para la realización de estadística descriptiva y análisis comparativo se utilizó el programa GraphPad Prism 7 (GraphPad, San Diego, CA, USA). En el caso de los modelos de regresión lineal simple se empleó de manera complementaria el programa IBM SPSS Statistics for Windows, versión 27 (IBM Corp., Armonk, N.Y., USA). Tras la realización de los test de normalidad (D'Agostino & Pearson, Shapiro-Wilk y Kolmogórov-Smirnov), las variables se expresaron como media aritmética (error estándar de la media) en el caso

de presentar distribución normal, y como media geométrica (95 % de intervalo de confianza), en el caso de distribuciones no paramétricas. En el análisis comparativo se utilizaron los test ANOVA o Kruskal-Wallis, según procedía. Se consideraron significativos todos aquellos resultados con un p-valor igual o inferior a 0,05.

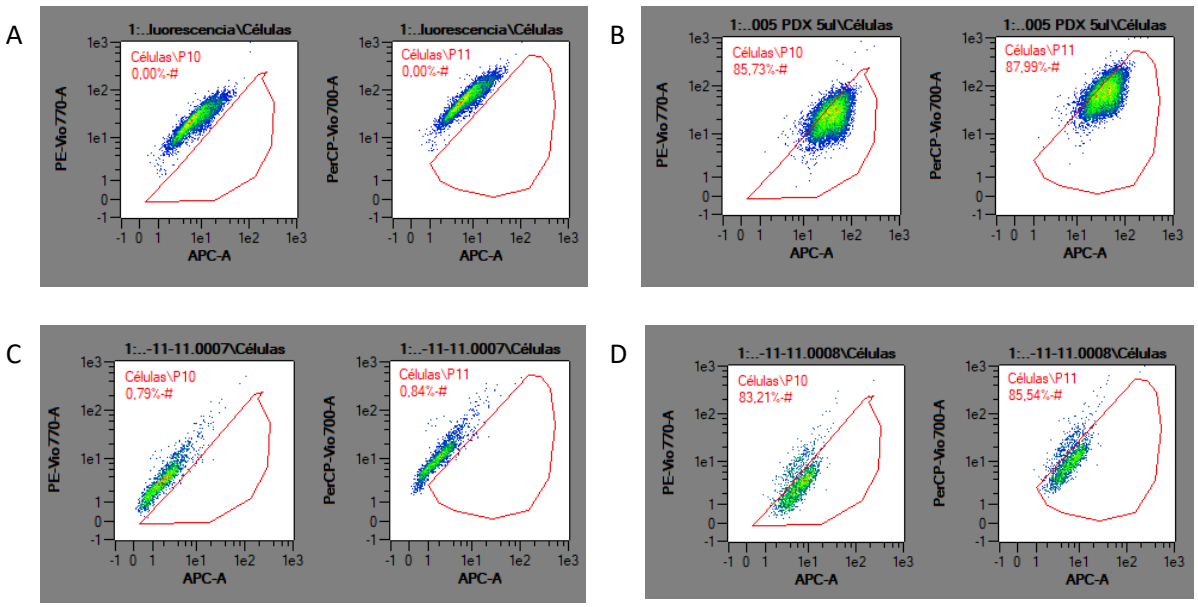

Figura 16.- Estudio por citometría de flujo de células marcadas con PDX-1. A) Podocitos humanos en cultivo marcados con IgG2B (autofluorescencia y uniones inespecíficas). B) Podocitos humanos en cultivo marcados con PDX-1. C) Orina humana de paciente con enfermedad renal crónica avanzada (ERCA) incubada con IgG2B. D) Orina de paciente con ERCA incubada con PDX-1.

III. Resultados

Los resultados mostraron diferencias significativas entre los individuos control y los pacientes con ERCA en ambos sexos en la podocituria de 24 horas, y en las mujeres en el caso de podocitos por ml (figura 17). A pesar de que las gráficas muestran diferencias cuantitativas entre sexos, existen similitudes en la proporcionalidad observada entre controles y pacientes. Por ejemplo, en el caso de podocitos en 24 horas, los pacientes tienen 4,21 veces más podocitos. En el caso de las mujeres la cifra es de 4,33 veces. A continuación, se analizó el BFA urinario, observando diferencias significativas entre el grupo control y los pacientes de ERCA, con valores de media geométrica (95% intervalo de confianza) de 0,34 (0,19 – 0,6) y 5,02 (1,97 – 12,82) respectivamente. Los modelos de regresión lineal simple mostraron significación

estadística en la comparativa entre podocituria y BFA corregidos por ml de orina (p-valor = 0,0384), mientras que el resultado no llegó a ser significativo en el caso del análisis de los parámetros en 24 horas (p-valor = 0,1204) (figura 18).

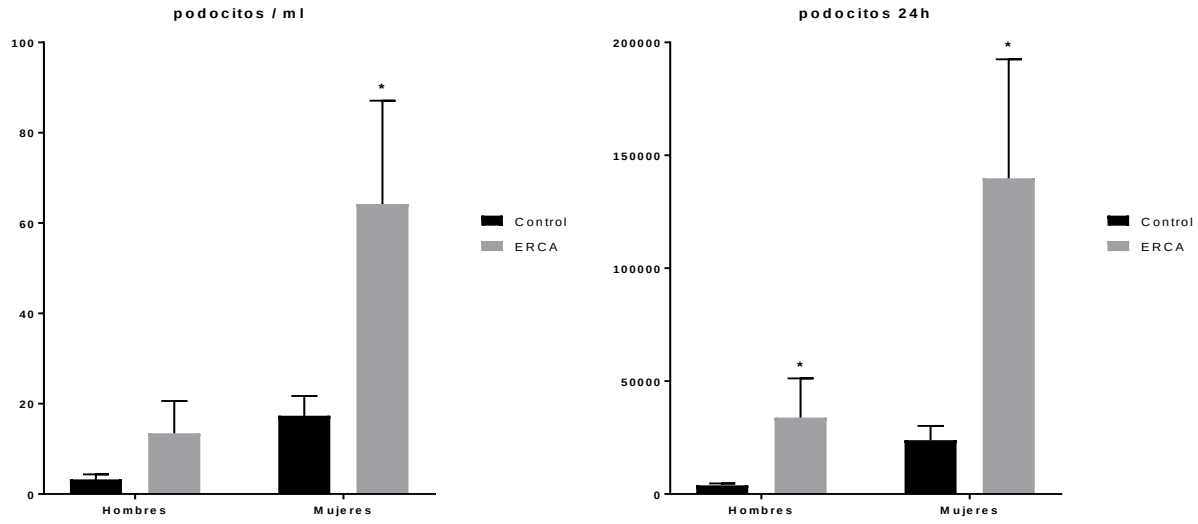

Figura 17.- Estudio comparativo de podocitos por ml de orina (izquierda) o en 24 horas (derecha) entre sujetos control y pacientes con enfermedad renal crónica avanzada (ERCA). El asterisco representa diferencias significativas en el estudio comparativo entre pacientes y controles del mismo sexo, con un p-valor ≤ 0,05.

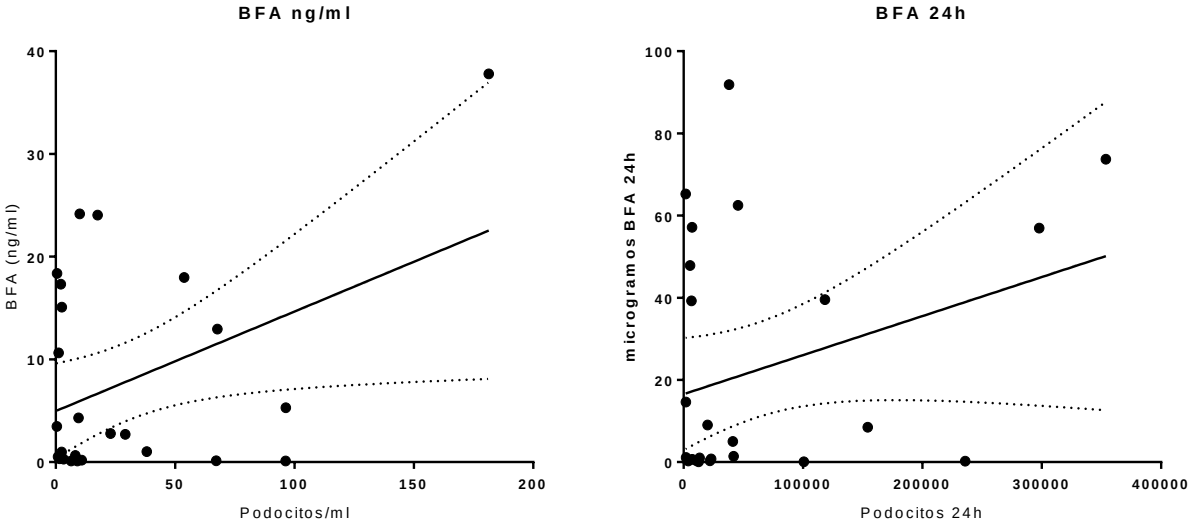

Figura 18.- Análisis de regresión lineal simple entre podocitos y BFA urinario corregidos por ml (izquierda) o en 24 horas (derecha).

IV. Discusión

El presente trabajo ha explorado por primera vez el estudio de podocituria en pacientes con ERCA mediante citometría de flujo y su relación con la exposición al contaminante ambiental BFA.

El podocito es uno de los principales elementos que conforman la barrera de filtración glomerular, y cuya alteración está fuertemente relacionada con la progresión de las alteraciones glomerulares que conducen a la enfermedad renal crónica, llegando incluso a relacionarse su pérdida con la enfermedad renal crónica terminal [410, 411].

Trabajos de otros autores, como Hara y cols. [418], Pérez-Hernández y cols. [414] o Mella y cols. [415] utilizan la podocalixina para identificar podocitos urinarios, puesto que se encuentra predominantemente localizada en la superficie apical de los podocitos. Sin embargo, existen otros autores que utilizan el concepto de eventos positivos o células positivas a sinaptopodina, puesto que aunque consideran que es el marcador más inclusivo, existen evidencias de que podría expresarse en otros tipos celulares [413, 419]. En este sentido, los análisis de podocituria por citometría de flujo han mostrado diferencias significativas entre pacientes y controles, y a pesar de las diferencias cuantitativas observadas entre sexos se observó la misma proporcionalidad dentro de cada sexo. Es interesante destacar que se observó un mayor número de eventos en el sexo femenino que en el masculino, lo cual podría deberse a que las mujeres eliminan células epiteliales vaginales en la orina [420]. Por esta misma razón, por la presencia de un mayor número de eventos en los dots plots de la citometría, o quizás por la presencia de podocalixina en algunas células epiteliales, se han observado diferencias entre sexos. Deberán realizarse futuros estudios que profundicen en la cuestión, analizando el ARNm de los pellets urinarios, o realizando inmunocitoquimicas con otros marcadores específicos del podocito, como la podocina o la nefrina.

Por otro lado, la cuantificación de BFA urinario ha sido coherente con los resultados observados en el Anexo I. Las diferencias entre los pacientes y controles eran evidentes, desde un punto de vista cuantitativo. No obstante, con el fin de resolver la

relación de causalidad entre el BFA y la enfermedad renal, serán necesarios futuros estudios longitudinales que permitan estudiar en profundidad la relación entre el BFA urinario, sanguíneo y el riesgo a desarrollar ERC y/o la velocidad de deterioro de la capacidad de filtración renal.

Por último, el modelo de regresión lineal simple ha mostrado una relación positiva entre el BFA y los podocitos urinarios, tanto al corregirlos por ml de orina como en su valor absoluto de excreción en 24 horas. Sin embargo, los resultados sólo han mostrado valores de p-valor inferiores a 0,05 en el caso de los parámetros corregidos por ml. Probablemente este hecho se deba al reducido número de sujetos del estudio, lo cual es una evidente limitación del modelo.

En cualquier caso, los resultados observados en el presente estudio preliminar muestran prometedoras perspectivas en el estudio de la podocituria y su relación con el BFA. A pesar del reducido número de sujetos, se ha observado una relación significativa y positiva entre los podocitos urinarios (o células podocalixina positivas) y el BFA urinario, lo que sugiere que los resultados observados en el capítulo 2 de la presente tesis, realizados con podocitos humanos en cultivo, podrían extrapolarse a la población humana.

XII. ANEXO III

ORAL ADMINISTRATION OF BISPHENOL A INDUCES HIGH BLOOD PRESSURE THROUGH ANGIOTENSIN II / CAMKII-DEPENDENT UNCOUPLING OF ENOS. **FASEB JOURNAL** (5.043, Q1 BIOCHEMISTRY & MOLECULAR BIOLOGY)

DOI: 10.1096/FJ.14-252460

Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS

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ABSTRACT **Bisphenol A (BPA) is found in human urine and fat tissue. Higher urinary BPA concentrations are associated with arterial hypertension. To shed light on the underlying mechanism, we orally administered BPA (4 nM to 400 μM in drinking water) to 8-wk-old CD11 mice over 30 d. Mice developed dosage-dependent high blood pressure (systolic 130±12 *vs.* 170±12 mmHg; EC_{50} 0.4 μM), impairment of acetylcholine (AcH)-induced carotid relaxation (0.66±0.08 *vs.* 0.44±0.1 mm), a 1.7-fold increase in arterial angiotensin II (AngII), an 8.7-fold increase in eNOS mRNA and protein, and significant eNOS-dependent superoxide and peroxynitrite accumulation. AngII inhibition with 0.5 mg/ml losartan reduced oxidative stress and normalized blood pressure and endothelium-dependent relaxation, which suggests that AngII uncouples eNOS and contributes to the BPA-induced endothelial dysfunction by promoting oxidative and nitrosative stress. Microarray analysis of mouse aortic endothelial cells revealed a 2.5-fold increase in expression of calcium/calmodulin-dependent protein kinase II-α (CaMKII-α) in response to 10 nM BPA, with increased expression of phosphorylated-CaMKII-α in carotid rings of BPA-exposed mice, whereas CaMKII-α inhibition with 100 nM autocamptide-2-related inhibitor peptide (AIP) reduced BPA-mediated increase of superoxide. Administration of CaMKII-α inhibitor KN 93 reduced BPA-induced blood pressure and carotid blood velocity in mice, and reverted BPA-mediated carotid constriction in response to treatment with AcH. Given that CaMKII-α inhibition prevents BPA-mediated high blood pressure, our data suggest that BPA regulates blood pressure by inducing AngII/CaMKII-α uncoupling of eNOS.—Saura, M., Marquez, S., Reventun, P., Olea-Herrero, N., Arenas, M.I., Moreno-Gómez-Toledano, R., Gómez-Parrizas, M., Muñóz-Moreno, C., González-Santander, M., Zaragoza, C., Bosch, R.J. Oral administration of bisphenol A induces high blood pressure through angiotensin II/CaMKII-dependent uncoupling of eNOS. *FASEB J.* 28, 4719–4728 (2014). www.fasebj.org**

Key Words: BPA · hypertension · endothelial dysfunction · superoxide · AngII

CHEMICAL POLLUTANTS ARE now considered as contributing factors for many diseases, including arterial hypertension (AH; ref. 1). The xenoestrogen bisphenol A [BPA; 2,2-*bis*-(4-hydroxyphenyl) propane], a chemical used for manufacturing products containing polycarbonate plastics and epoxy resins, is extensively used in the food industry as a barrier coating for food and drink containers (2). Exposure to BPA is widespread, and BPA is detectable in urine and fat tissue, as well as in serum of pregnant women, breast milk, and amniotic

Abbreviations: AcH, acetylcholine; AH, arterial hypertension; AIP, autocamptide-2-related inhibitor peptide; AngII, angiotensin II; apo, apocynin; BPA, bisphenol A; CaMKII-α, calcium/calmodulin-dependent protein kinase II-α; DAB, diaminobenzidine; DAF, diaminofluorescein; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; FACS, fluorescence activated cell sorter; HRP, horseradish peroxidase; i.v., intravenous; L-NAME, L-NG-nitroarginine methyl ester; MAEC, murine aortic endothelial cell; NO, nitric oxide; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species; SNP, sodium nitroprusside

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doi: 10.1096/fj.14-252460

fluid and fetal tissues. BPA is metabolized in the liver and is present in the urine of 95% of U.S. adults (3–5).

Exposure to BPA might lead to abnormal metabolism of glucose and lipids and exacerbate the development of cardiovascular diseases (5). Recently, the U.S. National Health and Nutrition Examination Survey (NHANES) found that higher urinary BPA concentrations were associated with cardiovascular diseases and type 2 diabetes (6–8). To date, no studies have examined the underlying mechanisms by which BPA promotes AH.

Nitric oxide (NO) is a potent vasodilator produced by the endothelial NO-synthase isoform endothelial NO synthase (eNOS; ref. 9). Accordingly, the absence of NO leads to endothelial dysfunction and vasoconstriction and increased adhesion of circulating monocytes and platelets to the endothelium (10, 11). Uncoupling of eNOS enzymatic activity results in a significant production of superoxide anion and contributes to the release of reactive oxygen and nitrogen species and peroxynitrite ($ONOO^-$; refs. 12, 13), which magnifies the dysfunctional phenotype. Several lines of evidence have shown that eNOS uncoupling is a principal source of hypertension (14). Among different factors implicated in this uncoupling, angiotensin II (AngII) is thought to play a pivotal role (15), in part through activation of the calcium signaling mediator, calcium/calmodulin-dependent protein kinase II-α (CaMKII-α; ref. 16).

In the present study, the vascular effects of orally administrated BPA were investigated by measurement of blood pressure and real-time noninvasive vascular reactivity in mice *in vivo*, together with *in vitro* cell culture analysis. Our data provide the first experimental evidence of BPA-induced eNOS uncoupling in the vascular endothelium, which leads to an impairment of vascular function and development of hypertension.

MATERIALS AND METHODS

Reagents

General cell culture supplies were purchased from BD Biosciences (San Jose, CA, USA); calf serum was from BioWhittaker (Verviers, Belgium). Cell culture-grade gelatin, trypsin, antibiotics, and hematoxylin-eosin, were from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and liquid diaminobenzidine (DAB) substrate were from Dako (Carpinteria, CA, USA). Anti-eNOS antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Ang antibody was from LifeSpan Biosciences (Seattle, WA, USA). Dihydroethidium (DHE) and TRIzol reagent were from Invitrogen (Carlsbad, CA, USA). The miRNeasy mini kit was from Qiagen (Valencia, CA, USA). High Capacity cDNA Achieve kit was from Applied Biosystems (Foster City, CA, USA). Collagenase was from Worthington Biochemical Corp. (Lakewood, NJ, USA). Collagenase type IA, A23187, AcH, sodium nitroprusside (SNP), L-NG nitroarginine methyl ester (L-NAME), apocynin (apo), HRP-conjugated anti-rabbit secondary antibody, anti-nitrotyrosine anti-GADPH antibodies, BPA, and KN93 were from Sigma-Aldrich. Amersham ECL detection kit was from GE Healthcare Life Sciences (Madrid, Spain).

Animals

Wild-type CD1 mice were purchased from Charles River (Wilmington, MA, USA) and housed in our animal facilities in isolated rooms. All animal procedures were approved by the Research Ethics Committee at the Centro Nacional de Investigaciones Cardiovasculares (CNIC) and conformed to EU Directive 86/609/EEC and recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes (enacted under Spanish law 1201/2005). CD1 mice received the following dosages of BPA in the drinking water: 4 nM, 40 nM, 0.4 μM, 4 μM, 40 μM, and 400 μM. Unless otherwise specified, a dosage of 0.4 μM was selected. *In vivo* AngII and CaMKII-α inhibition was performed by feeding mice with 0.5 mg/ml losartan and 0.24 μg/ml KN93, respectively, in the drinking water. Mice were given free access to water or BPA-containing water at a dose of 5 ml/d/mouse, a value in accordance with the findings of other researchers (17).

Blood pressure

Indirect measurements of blood pressure were obtained in conscious animals by means of a tail-cuff sphygmomanometer (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain). The animals were trained for 5 d before starting the measurement to prevent stress and were prewarmed to 30°C with a heater (LE5660/6, Letica Scientific Instruments).

Arterial pressure was several times between 9:00 and 12:00 AM, and pressure values were considered acceptable at ≥10 consecutive measurements.

Micro-ultrasound imaging

Ultrasound imaging parameters of the right carotid artery were measured with the Vevo 2100 system (VisualSonics, Toronto, ON, Canada). Images were captured with a 55 MHz scan head with a 4.5 mm focus and an axial resolution of 30 μm. Mice were anesthetized with 2% isoflurane gas, yielding a heart rate of ~500 beats/min during imaging. A long axis view in B mode, in combination with color Doppler ultrasound, was used to identify the carotid artery by its characteristic flow pattern. M-mode ultrasound was used to determine aortic diameter, and power Doppler ultrasound mode was used to measure blood velocity. Probe position was optimized to show clear vessel wall/lumen interfaces, allowing automated recognition by the analysis software. Experiments were initiated after a 15-min equilibration period to allow achievement of a stable core body temperature. Flow dilatation was measured before and after infusion of AcH or SNP. After obtaining baseline readings for diameter and heart rate, control injections (saline) were administered *via* tail-vein catheter (30 gauge; BD Biosciences). Readings were taken immediately after injection (0 min; $t=0$) and after 1, 2, 4, and 10 min. At 15 min after the last measurement, a bolus of 10^{-8} M Ach was administered, and recordings were taken at the same times as before. To test endothelium-independent vasorelaxation, SNP was infused at 10^{-7} M (30 μl) and recordings were taken as above. Each treatment was injected in a 30 μl final volume. Carotid diameter and blood velocity analyses were performed offline from recorded loops, using the software provided by the manufacturer.

Cells

Mouse aortic endothelial cells (MAECs) were cultured from aortas extracted from anesthetized CD1 mice as described

previously (18). Cells were purified by flow cytometry (Dako Cytometrix, Copenhagen, Denmark). Purification was verified by confocal immunofluorescence with anti-ICAM-2 and anti-PCAM-1 monoclonal antibodies (BD Biosciences). The purity of MAEC cultures used for experiments was >99%.

RNA isolation and gene expression

Total RNA from tissue and cell extracts was extracted with TRIzol as described previously (12). First-strand cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction mixture using the High Capacity cDNA Achieve kit, as recommended by the manufacturer.

mRNA microarray analysis was performed by using the Whole Mouse Genome Microarray Kit 44K (Agilent Technologies, Madrid, Spain), following the manufacturer's instructions. Genes matching the following two criteria were selected: logFC > 2; $P < 0.05$.

Primers for quantitative polymerase chain reaction (qPCR) were as follows: CaMKII-α, forward 5′-TTCAGCATCCCAGCCCTAGT-3′ and reverse 5′-GCTCCCTTTCCCAGTTCCTC-3′; eNOS, forward 5′-GGGGAACAAGCCCAGTAGT-3′ and reverse 5′-AATTCGCCAATGACAAGACG-3′.

Three technical replicates were performed for each experimental condition, and differences were assessed with a 2-tailed Student's *t* test. Results were normalized using the GAPDH housekeeping gene and the ΔΔ cycle threshold (C_t) method and are expressed in arbitrary units.

Protein detection by immunoblot

Immunoblotting was performed as described previously (19). Immunoreactive bands were visualized with the ECL system (GE Healthcare Life Sciences).

Immunohistochemistry

Carotid specimens were harvested and paraffin embedded, cut into 3- to 5-μm serial sections, and incubated for 1 h with the corresponding primary antibodies. Aortic sections were then washed 3 times with PBS and incubated with biotin-conjugated secondary antibodies (Dako), followed by exposure to avidin–peroxidase complex (Dako). Stain was developed by addition of DAB substrate (Dako). Samples were counterstained with Mayer's hematoxylin and mounted with DPX and visualized in a bright field microscope (Eclipse 50i; Nikon, Tokyo, Japan).

NO production

NO production was measured in cells loaded with diaminofluorescein diacetate (DAF-DA; 5 mM), and propidium iodide (1 μg/m) was used to determine cell viability. After exposure to different experimental conditions, cells were trypsin dispersed and labeled with the fluorochrome at 37°C, followed by cytofluorometric analysis with a fluorescence activated cell sorter (FACS) scanner (Becton Dickinson, New York, NY, USA). A total of 10,000 events were analyzed for each condition.

To measure aortic NO production, aortic rings from animals treated with BPA, losartan, KN93, and combinations were divided into 2 experimental groups: control and A23187 10^{-6} M. Arteries were incubated with 2 μM DAF-2 for 45 min, and in the last 15 min, eNOS agonist or saline was added. NO release was measured by spectrofluorimetry (LS50 with FL WINLAB software, excitation wavelength 492 nm; emission wavelength 515 nm; Perkin Elmer Instruments, Wellesley, MA, USA). Some assays were performed in the presence of L-NAME in order to ensure assay specificity. The amount of NO released was expressed as arbitrary units per milligram of tissue.

Superoxide anion production

DHE reacts with superoxide to form 2-hydroxyethidium. Frozen carotid sections embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) were incubated with saline buffer or 10^{-8} M AcH in combination with 500 μM L-NAME and/or 10^{-5} apo to inhibit endogenous NADPH oxidase formation of superoxide. Sections were then incubated with 1 μM DHE for 15 min, and superoxide was determined by confocal microscopy (Leica Microsystems, Wetzlar, Germany) at 575–700 nm. MAECs were incubated with PBS (control) or BPA (1 nM) for 24 h, harvested, and treated with 10^{-5} M apo for 30 min to inhibit NADPH oxidase. After exposure to different experimental conditions, cells were trypsin dispersed and labeled with the fluorochrome at 37°C, followed by cytofluorometric analysis with a FACS scanner. A total of 10,000 events were analyzed for each condition.

Protein oxidation

Protein oxidation was carried out with the Oxyblot protein detection kit (Chemicon, Temecula, CA, USA). Total aortic protein lysates (15 mg) were incubated with 2,4-dinitrophenyl hydrazine (DNPH) for 15 min, as recommended by the manufacturer. Proteins were electrophoresed in 15–4% gradient SDS-PAGE gels with 6 mg of derivatized protein/lane and transferred to nitrocellulose membranes at 100 mA constant current for 90 min. Membranes were subsequently incubated for 2 h at room temperature with anti-DNP (1:100, Chemicon). Blots were washed 3 times and incubated with goat anti-rabbit IgG HRP conjugated, and developed by ECL, as described previously (19). Bands were scanned with an imaging densitometer, and optical densities were quantified.

Statistical analysis

All values are means ± SD. Statistical analysis was performed with the GraphPad Prism software package (GraphPad Software, La Jolla, CA, USA). Unless otherwise specified, assays were repeated ≥3 times, and differences were considered statistically significant at $P < 0,05$, as determined by unpaired 2-sided Student's test (assays with 2 experimental conditions) or 2-way ANOVA followed by Bonferroni's test (assays with >2 experimental conditions).

RESULTS

BPA induces high blood pressure and impairs carotid relaxation in mice

Oral administration of BPA (4 nM to 400 μM in drinking water) to 8-wk-old CD11 mice over 30 d increased both systolic and diastolic blood pressure relative to non-BPA-treated mice (systolic 130±12 *vs.* 196±12 mmHg; EC_{50} 0.4 μM; diastolic 90±15 *vs.* 142±17 mmHg; **Fig. 1*A***), reaching a plateau at dosages of 40 μM BPA.

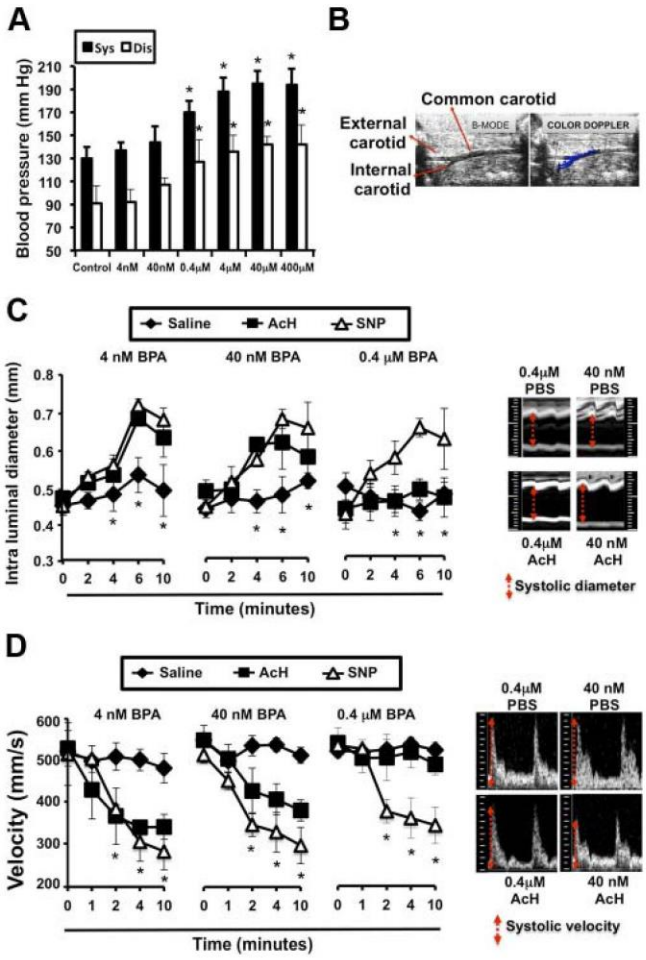

Figure 1. BPA induces high blood pressure and inhibits AcH-induced vasorelaxation. *A*) Mice were given BPA at the dosages indicated or no treatment (control). Blood pressure was evaluated 30 d after drug administration. $*P < 0.05$ *vs.* corresponding control ($n=10$). *B*) Left panel: B-mode ultrasound of carotid arteries. Right panel: color Doppler ultrasound showing blood flow. *C*) Left panel: M-mode ultrasound measurement of arterial diameter in response to i.v. injection of saline, 10^{-8} M AcH, and 10^{-7} M SNP in mice administered 4 nM, 40 nM, and 0.4 μM BPA. $*P < 0.05$ for AcH *vs.* corresponding saline ($n=10$). Right panels: M-mode ultrasound images of the carotids of BPA-treated mice at the doses indicated, 10 min after injection of PBS or AcH. Arrows indicate systolic diameter of the vessels. *D*) Left panel: color Doppler ultrasound measurement of blood velocity through the same carotids as in *B*) $*P < 0.05$ for AcH *vs.* corresponding saline ($n=10$). Right panels: carotid velocity images in the same arteries as in *B*. Arrows indicate systolic blood velocity.

To explore whether BPA may affect vascular relaxation, we performed *in vivo* real-time common carotid high-frequency echo Doppler ultrasound (Fig. 1*B*), in mice treated or not with BPA, and explored the BPA dosage effect on endothelium-dependent AcH-mediated changes in vessel diameter (M mode; Fig. 1*C*), and blood velocity (power Doppler mode; Fig. 1*D*). Thus, mice were administered BPA at the dosages indicated, and real-time M-mode cine loops were recorded to measure intraluminal diameter of the vessels over 10 min, following intravenous (i.v.) administration of 10^{-8} M AcH (Fig. 1*C*, right panels). At 15 min after the last measurement, the same animals were intravenously injected with 10^{-7} M SNP, an NO donor and a positive control of relaxation, to test whether NO-mediated signaling is affected by BPA. No differences were detected between AcH-mediated endothelium-dependent relaxation and SNP-mediated endothelium-independent relaxation at the lower dosages of BPA (Fig. 1*C*). However, mice administered 0.4 μM BPA in water, the same dosage that increased arterial blood pressure in mice, showed impaired AcH-mediated endothelium-dependent carotid relaxation with respect to relaxation measured after injection of 10^{-7} M SNP (SNP *vs.* AcH: 0.66 ± 0.08 *vs.* 0.44 ± 0.1 mm; Fig. 1*C*, left graph). Thus, higher doses of BPA inhibit endothelium-mediated NO-dependent relaxation in mice, but NO downstream signaling remained functional. Similarly, power Doppler ultrasound examination demonstrated that carotid blood velocity of mice administered 0.4 μM BPA was reduced with respect to control mice (Fig. 1*D*, left graph), whereas no differences were detected at lower dosages of BPA (Fig. 1*D*, middle and right graphs).

BPA targets eNOS production of NO

Endothelial dysfunction, as defined by the loss of endothelium-dependent relaxation, and caused by inhibition of eNOS mediated production of NO, is considered a central mechanism of high blood pressure (14). We investigated whether BPA inhibits the expression of eNOS; however, quantitative reverse transcription PCR (qRT-PCR) and immunohistochemistry analysis revealed increased distribution of eNOS throughout the endothelium layer in BPA-treated mice (**Fig. 2*A*, *B***). No significant changes in P-Akt content, indicative of calcium-independent activation of eNOS, were detected (Fig. 2*B*). Consistently, incubation of MAECs with 10 nM BPA for 24 h significantly increased the level of eNOS protein (Fig. 2*C*), although calcium ionophore A23187 stimulation of NO production by eNOS was blunted by coincubation with 10 nM BPA (Fig. 2*D*).

BPA-mediated expression of eNOS induces carotid oxidative stress

Under certain pathological conditions, alterations of eNOS activity can result in uncoupling and the formation of superoxide rather than NO (15). To assess this possibility, mice were given 0.4 μM BPA in drinking water for 30 consecutive days, and the production of superoxide anion was measured by DHE staining of carotid artery rings (see Materials and Methods). Compared with control mice, DHE oxidation was significantly increased in carotid rings taken from BPA-treated mice (**Fig. 3*A***). To study whether BPA administration resulted in uncoupling of eNOS, we measured superoxide production in carotid rings pretreated with apo (10^{-5} M), an inhibitor of endogenous NADPH oxidase (17), a major reactive oxygen species

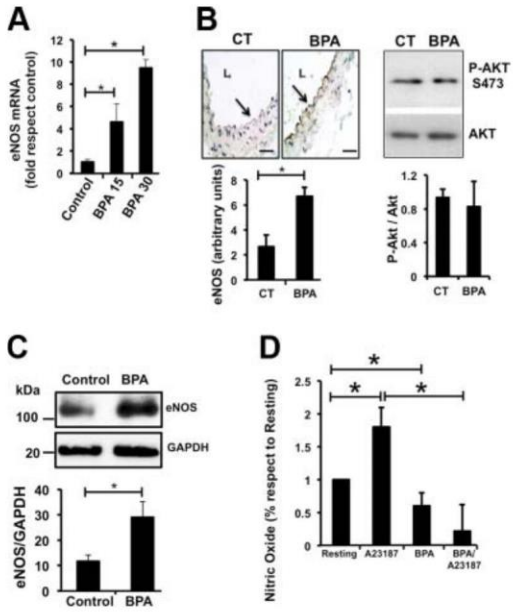

Figure 2. BPA induces the expression of eNOS and inhibits the production of NO. *A*) Detection of eNOS mRNA at 15 and 30 d after administration of 0.4 μM BPA. $*P < 0.05$ (n=10). *B*) Top left panel: immunohistochemical detection of eNOS in aortic rings isolated from mice administered control (CT) water or 0.4 μM BPA, for 30 d. L, lumen. Scale bars = 50 μm. Bottom left panel: densitometric analysis of eNOS staining from 3 independent experiments. $*P < 0.05$. Top right panel: immunoblot detection of P-Akt (top blot) and total Akt (bottom blot) in the same mice. Bottom right panel: densitometric analysis from 3 independent experiments. *C*) Top panel: detection of eNOS in MAECs after 24 h incubation with 10 nM BPA. Bottom panel: densitometry analysis of eNOS/GAPDH (n=3). $*P < 0.05$. *D*) Production of NO in MAECs stimulated with A23187 and incubated with saline buffer (control), 10 nM BPA, or both stimuli. $*P < 0.05$, $**P < 0.05$ (n=3).

(ROS) producer. Interestingly, a significant amount of superoxide was still present in apo-treated carotid rings from mice administered with 0.4 μM BPA, compared with control mice (Fig. 3*B*), which suggests that impaired vessel relaxation could be, in part, due to uncoupling of eNOS. Consistent with this premise, pharmacological inhibition of eNOS activity with L-NAME (500 μM) was sufficient to inhibit BPA-induced DHE staining (Fig. 3*B*). Furthermore, in MAECs incubated with apo, superoxide formation was significantly increased in BPA-treated cells relative to control cells (Fig. 3*C*). Consistent with these findings, protein nitration of carotid sections isolated from mice treated with 0.4 μM BPA for 30 d was further increased (Fig. 3*D*). Collectively, these results suggest that BPA may uncouple eNOS in vascular endothelium.

BPA impairs vascular function and increases blood pressure through AngII

AngII-induced oxidative stress plays a pivotal role in the pathogenesis of hypertension (20). To test whether BPA may regulate uncoupling of eNOS through the expression of AngII, immunohistochemical analysis was performed, which revealed a substantial accumulation of AngII in the carotid arteries of 0.4 μM BPA-treated mice (**Fig. 4*A***). Consistently, administration of a pharmacological inhibitor of the AngII receptor, losartan (0.5 mg/ml), to 0.4 μM BPA-treated mice repressed BPA-induced superoxide production (Fig. 4*B*), suggesting that BPA-induced high blood pressure may result from AngII-mediated uncoupling of eNOS.

Next, we assessed AcH-mediated vessel relaxation and blood velocity by echo Doppler ultrasound in mice treated with BPA, losartan, and a combination of BPA and losartan. We measured intraluminal common carotid diameter in anesthetized mice over 10 min following i.v. administration of 10^{-8} M AcH, or the equivalent volume of PBS. Whereas no differences were detected between PBS and AcH-dependent relaxation in mice treated with 0.4 μM BPA (Fig. 4*C*, second graph), cotreatment with 0.5mg/ml losartan restored AcH-dependent carotid relaxation (Fig. 4*C*, fourth graph) to levels found in non-BPA treated mice (Fig. 4*C*, first graph), and losartan-only treated mice (Fig. 4*C*, third graph).

Similarly, no differences in blood velocity were observed between PBS- and AcH-treated mice administered BPA (Fig. 4*D*); however, treatment with losartan was sufficient to recover the velocity detected in control and losartan-treated mice (Fig. 4*D*), and blood pressure was also reduced to control levels (Fig. 4*E*). Thus, our

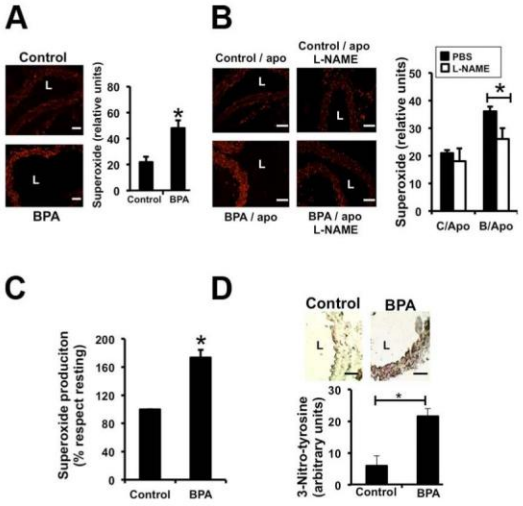

Figure 3. BPA induces eNOS-dependent production of superoxide and peroxynitrite. *A*) Confocal microscopy detection of DHE in aortic rings from control mice or 0.4 μM BPA-treated mice, incubated with saline buffer (PBS). L, lumen. $*P < 0.05$ (n=10). Scale bars = 50 μm. *B*) Aortic rings from control mice or 0.4 μM BPA-treated mice, incubated with 10^{-5} M apo and 500 μM L-NAME as indicated. $*P < 0.05$ (n=10). Scale bars = 50 μm. *C*) Production of superoxide in MAECs incubated with 100 μM apo and treated with 10 nM BPA. $*P < 0.05$ (n=3). *D*) Top panel: immunohistochemical detection of 3-nitrotyrosine in carotid sections from control mice and mice administered 0.4 μM BPA for 30 d. Scale bars = 50 μm. Bottom panel: densitometric analysis of 3-nitrotyrosine from three independent experiments. $*P <$ 0.05

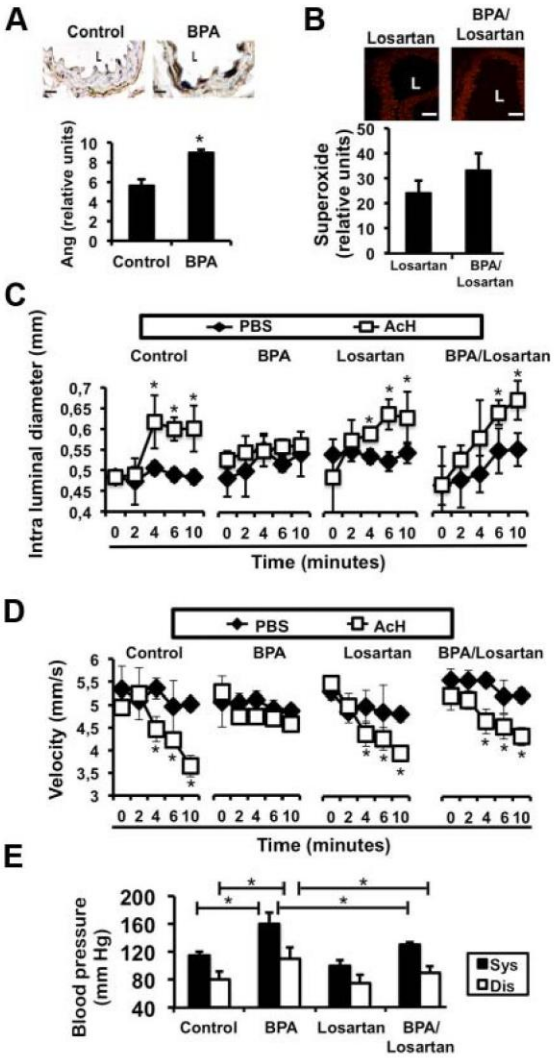

Figure 4. AngII signaling inhibition inhibits vascular superoxide production, improves vascular function, and reduces blood pressure in BPA-treated mice. *A*) Top panel: immunohistochemical detection of Ang in aortic rings from mice treated with 0.4 μM BPA. Bottom panel: densitometry of Ang staining from 3 independent experiments. $*P < 0.05$ (n=10). *B*) Aortic rings from mice treated with 0.5 mg/ml losartan, or a combination of 0.5 mg/ml losartan plus 0.4 μM BPA (n=10). Scale bars = 50 μm. *C*) M-mode ultrasound measurement of carotid diameter, after i.v. tail injection of 10^{-8} M AcH or PBS in control mice and mice treated with 0.4 μM BPA, 0.5 mg/ml losartan, or a combination, as indicated. $*P < 0.05$, $**,^{@@}P < 0.05$, $***P < 0.05$ *vs.* corresponding PBS (n=10). *D*) Color Doppler ultrasound measurement of blood velocity through the same carotids as in *A*. $*P < 0.05$ *vs.* corresponding PBS (n=10). *E*) Blood pressure of mice treated as in *A*, 30 d after BPA administration. $*P < 0.05$ (n=10).

data suggest that AngII-mediated endothelial dysfunction is the primary cause of BPA-induced hypertension.

BPA induces expression and phosphorylation of CaMKII-α

To further study the mechanism of BPA-induced changes in the endothelium that may account for eNOS deregulation, we assessed genome-wide gene expression levels of MAECs treated with 10 nM BPA for 24 h. Whole-genome microarray analysis revealed that from the 89 vascular relaxation/constriction-related genes analyzed (see Materials and Methods for selection criteria), CaMKII-α (logFC 2.5; P=0.03), eNOS (logFC 2.7; P=0.044), and Ang (logFC 2.2; P=0.035) genes were up-regulated by incubation with BPA. Interestingly, CaMKII-α regulates the expression of eNOS under oxidative stress (21), and its inactivation participates in the prevention of AngII-mediated hypertension (16). CaMKII-α expression was increased 2.5-fold following treatment of MAECs with BPA, as confirmed by qRT-PCR (**Fig. 5*A***). To assess whether BPA activates CaMKII-α in the endothelium, carotid rings were isolated from BPA-treated mice, and immunohistochemistry was used to evaluate CaMKII-α phosphorylation. Results showed that, whereas samples from control mice exhibited minimal staining with an anti-phospho-CaMKII antibody, carotid rings from BPA-treated mice had intense phospho-CaMKII-α immunoreactivity (Fig. 5*B*). Notably, mice coadministered 0.4 μM BPA plus 0.5 mg/ml losartan exhibited reduced CaMKII-α phosphorylation (Fig. 5*B*), suggesting that CaMKII-α activation by BPA is mediated through AngII.

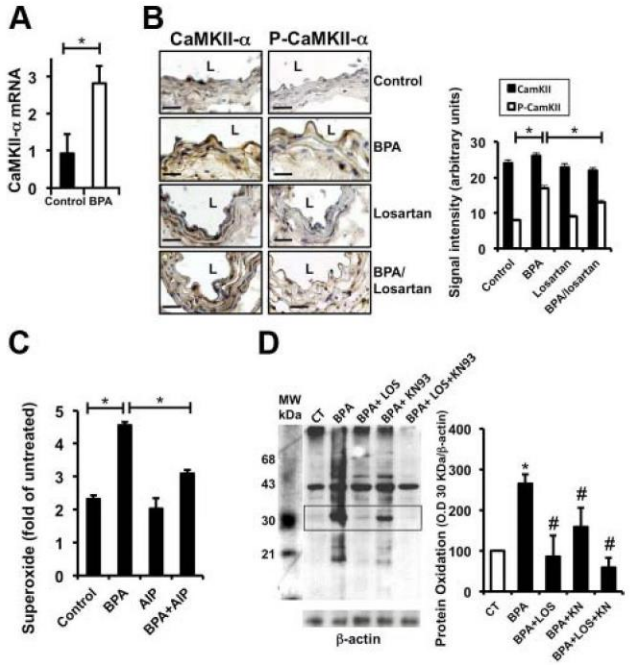

Figure 5. Activation of CaMKII-α is induced by AngII in BPA-treated mice. *A*) qRT-PCR detection of CaMKII-α mRNA in mice administered 0.4 μM BPA for 30 d. $*P < 0.05$ (n=10). *B*) Immunohistochemical detection of CaMKII-α or phospho-CaMKII-α in aortic rings from mice fed as in *A*. L, lumen. $*P < 0.05$ (n=10). *C*) Superoxide (DHE) detection in MAECs treated with 10 μM apo and 10^{-5} M A23187 (control), and incubated with 200 nM AIP, with 10 nM BPA, or both. $*P < 0.05$. *D*) Detection of aortic protein carbonyl formation from mice treated with 0.4 μM BPA, a combination of BPA plus 0.5 mg/ml losartan (los), 0.24 μg/ml KN93, or 0.5 mg/ml losartan/0.24 μg/ml KN93, as indicated. $*P < 0.05$ *vs.* control (CT); $^{\#}P < 0.05$ *vs.* BPA (n=10).

CaMKII-α is a target of AngII-mediated endothelial dysfunction induced by BPA

To address the importance of CaMKII-α activity for BPA-induced formation of superoxide, MAECs were coincubated with 10 μM apo (to inhibit NADPH oxidase activity) and 10^{-5} M A23187 (control) to induce eNOS enzymatic activity. Cells were then incubated with 10 nM BPA; autocamptide-2-related inhibitor peptide (AIP; 200 nM), a specific inhibitor peptide of CaMKII-α; or a combination of both. As anticipated, BPA induced a 2-fold increase in superoxide formation relative to control cells, and a significant reduction of superoxide was observed in MAECs coincubated with AIP (Fig. 5*C*). These data indicate that AngII-mediated activation of CaMKII-α is a critical step for BPA-induced endothelial dysfunction.

To further investigate whether CaMKII-α activity is a target for AngII-induced formation of superoxide in response to BPA *in vivo*, mice were treated with 0.4 μM BPA, 0.5 mg/ml losartan, and 0.24 μg/ml KN93, a specific CaMKII-α inhibitor for *in vivo* studies, as well as a combination of BPA/losartan, BPA/KN93, and BPA/losartan/KN93. After 30 d, the amount of ROS was measured through detection of protein carbonyl formation, a specific measure of protein oxidation, by using an Oxyblot kit (see Materials and Methods). BPA-induced oxidative stress was inhibited by cotreatment with losartan, as previously detected by DHE evaluation (Fig. 4), and by KN93. Cotreatment with both inhibitors almost abolished the effect of BPA on protein oxidation (Fig. 5*D*), which suggests that, as in endothelial cell cultures, CaMKII-α is at least one target of AngII in the formation of superoxide in response to BPA *in vivo*.

We next measured blood pressure in mice treated as above. Inhibition of either AngII or CaMKII-α was sufficient to reduce BPA-induced increase of blood pressure in these mice, and coinhibition of both was even more effective, which suggests that CaMKII-α is a target of AngII in this effect (**Fig. 6*A***). Furthermore, the vasomotor response was assayed in the same mice by high-frequency ultrasound, finding that right common carotid diameter and blood velocity values in both BPA/losartan or BPA/KN93 treated mice within the first 10 min after i.v. injection with 10^{-8} M AcH, were decreased and similar to control mice, whereas coincubation with both inhibitors induced a more pronounced effect (Fig. 6*B*).

To confirm the effect of BPA on AngII-mediated CaMKII-α uncoupling of eNOS, we examined the production of NO and anion superoxide in aortic rings from the same mice stimulated with the calcium ionophore A23187. As expected, A23187 induced the production of NO in aortic rings from control mice, whereas aortas from BPA-treated mice failed to produce such increase in response to A21387. By contrast, the effect of BPA was partially reverted in aortas from mice cotreated with BPA/losartan, or with BPA/KN93, while the combination of BPA with losartan and KN93

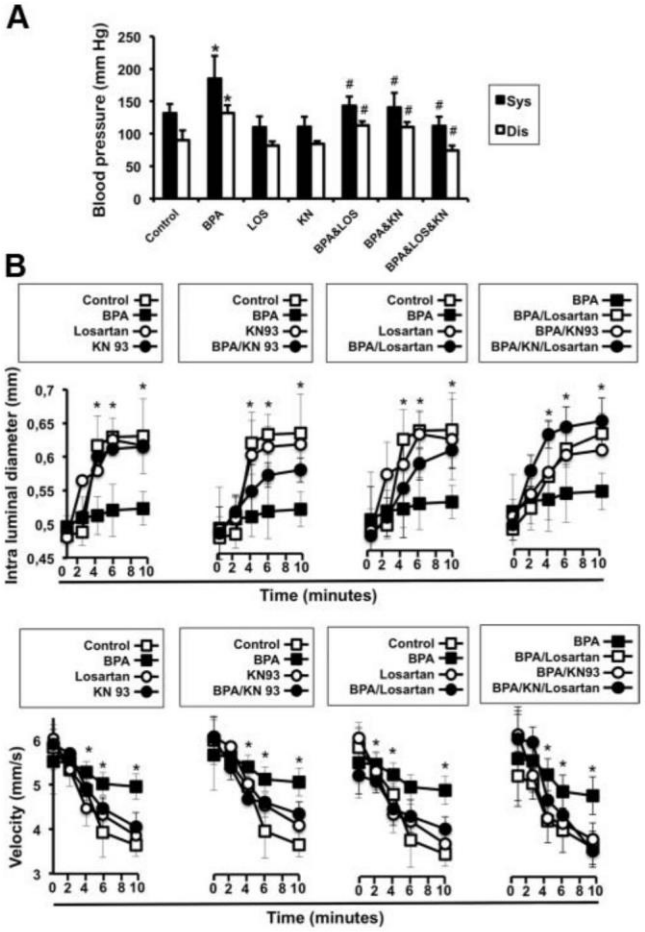

Figure 6. CaMKII-α is a target of AngII in BPA-treated mice. *A*) Mice were given 0.4 μM BPA, 0.5 mg/ml losartan (los), 0.24 μg/ml KN93 (KN) or a combination, as indicated, and blood pressure was evaluated 30 d after drug administration. $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* BPA (n=10 mice/condition/triplicate). *B*) Top panels: M-mode ultrasound measurement of carotid diameter, after i.v. tail injection of 10^{-8} M AcH in the same mice as in *A*. Bottom panels: color Doppler ultrasound measurement of blood velocity through the same carotids as in *A*. $^{*}P < 0.05$ for BPA *vs.* all other groups (n=10).

induced a more significant production of NO (**Fig. 7*A***). Likewise, superoxide production was increased in BPA aortic rings and inhibited when losartan, KN93, or a combination of both was included (Fig. 7*B*). This result was consistent with the phosphorylation (activation) of CaMKII-α in BPA-treated mice, where cotreatment with losartan, KN93, or a combination of both inhibited such effect (Fig. 7*C*). These results point toward BPA as an inducer of AngII/CaMKII-α-mediated uncoupling of eNOS.

Based on the findings that BPA induces AngII-mediated uncoupling of eNOS, BPA induces CaMKII-α activation, and either AngII or CaMKII-α inhibition returns BPA-induced blood pressure and vasomotor function closer to control levels, we suggest that AngII mediated activation of CaMKII-α is at least one of the mechanisms by which BPA induces high blood pressure through uncoupling of eNOS in mice.

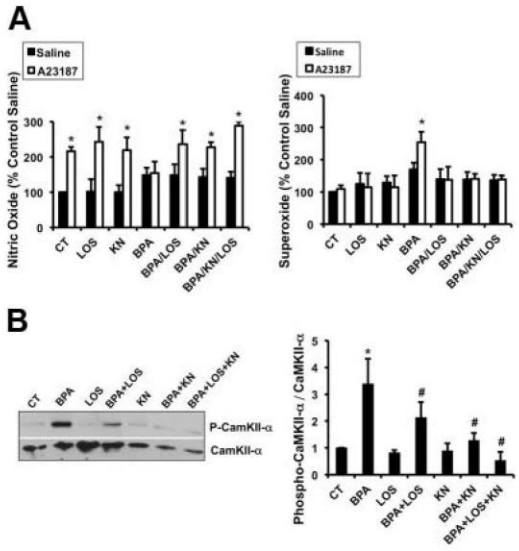

Figure 7. BPA induces endothelial uncoupling of eNOS through AngII-mediated activation of CaMKII-α. *A*) Mice were given 0.4 μM BPA, 0.5 mg/ml losartan (los), 0.24 μg/ml KN93 (KN), or a combination as indicated, and NO (left panel) or superoxide anion (right panel) was evaluated. $^{*}P < 0.05$ *vs.* saline. *B*) Left panel: immunoblot of CaMKII-α and phospho-CaMKII-α in the same mice as before. Right panel: densitometric analysis of 3 independent assays. $^{*}P < 0.05$ *vs.* control (CT); $^{\#}P < 0.05$ *vs.* BPA (n=3 mice/condition/assay).

DISCUSSION

Several epidemiological studies have shown that exposure to BPA may have adverse effects on human health. Our study sheds new light on the mechanism by which BPA may lead to increased blood pressure in a rodent model. We found that BPA induces endothelial dysfunction by uncoupling eNOS activity, impairing vessel relaxation, and significantly increasing blood pressure. Likely, this results from AngII-mediated uncoupling of eNOS, leading to increased oxidative and nitrosative stress through accumulation of superoxide and peroxynitrite free radicals. Moreover, our results suggest that AngII-induced activation of CaMKII-α may play a pivotal role in the endothelium dysfunction induced by BPA.

Recent data reemphasize the utility of rodent models to analyze BPA toxicity, since BPA has equal potency in both rodent and human cells (22). At relatively low physiological concentrations, BPA exerts pleiotropic cellular and tissue type-specific effects (23). As the oral route is the main pathway of BPA accumulation in humans, we established a regime in which BPA was administered in the drinking water of mice. We show that the lowest BPA dosage that can induce both high blood pressure and vascular dysfunction in mice is 0.1 μg/kg/d, corresponding to a daily intake of 20 μg/kg/d. This value represents less than half of the recommended safe daily BPA exposure (50 μg/kg/d; ref. 24).

BPA is routinely detected in human blood in the nanogram per milliliter range (25, 26). Recently, a high BPA concentration (up to 66.91 ng/ml) has been reported in human blood (27). Although it is difficult to discern a potentially dangerous BPA concentration in body fluids, a recent report showed that consumption of one serving of canned soup daily over 5 d was associated with a >1000% increase in urinary BPA (28). A relationship between BPA and cardiovascular disease has been previously reported (29), but the mechanism was not identified. Using *in vitro* and *in vivo* methodologies, we show here that BPA induces high blood pressure through AngII-dependent uncoupling of eNOS. We also provide evidence that implicates CaMKII-α in this process (30).

We and others found that pharmacological inhibition of CaMKII-α reduces blood pressure (31). Also, it also abrogates the effects of oxidative stress associated with ionizing radiation in cancer (32). Furthermore, CaMKII-α inhibition also reduces the production of superoxide in diabetic hearts (33) and inhibits AngII-mediated smooth muscle cell hypertrophy (34). In contrast, activation of CaMKII-α mediates ROS-dependent up-regulation of eNOS, contributing to the formation of superoxide and peroxynitrite (35). We found that treatment with BPA increased the level of CaMKII-α mRNA and protein in MAECs and also increased CaMKII-α activity, suggesting a dual effect of CaMKII-α on BPA-mediated hypertension. First, by increasing the level of eNOS, CaMKII-α contributes to the formation of superoxide and peroxynitrite, which are major risk factors for AH (36–39). Second, BPA-mediated phosphorylation of CaMKII-α might also induce AH by promoting phospholipase A2 (PLA2) and arachidonic acid activation, which has been shown to induce vasoconstriction (16).

The production of NO is tightly regulated by several mechanisms, including substrate availability, cofactor binding, phosphorylation, and protein–protein interactions in the appropriate cellular environment (12). Superoxide production is a limiting factor for NO availability, inducing uncoupling of eNOS through oxidation of BH4, a cofactor required to produce NO. Uncoupled eNOS produces superoxide rather than NO and contributes to the accumulation of ROS (12). A relationship between ROS and BPA has been observed in different scenarios, including infertility (40), diabetes (41), liver toxicity (42), and menopause (43). Our data show that BPA induces AngII-dependent uncoupling of eNOS *in vivo*.

A major consequence of eNOS uncoupling is the generation of the free radical peroxynitrite ($ONOO^-$). Peroxynitrite induces nitration of essential enzymes, including cGMP-dependent protein kinase 1 (PKG1), which is responsible for NO-mediated vasorelaxation, resulting in pulmonary hypertension (44). We found extensive protein nitration in the vessels of BPA-treated mice, suggesting that peroxynitrite produced from eNOS uncoupling may also contribute to the adverse effects on blood pressure. In addition, as a xenoestrogen, BPA interacts with estrogen receptors (2) by yet unknown mechanisms. Interestingly, it was recently proposed that peroxynitrite-modified forms of BPA have stronger binding affinity for estrogen receptors

than estradiol (45). Therefore, we cannot exclude the possibility that the effects of BPA could also be a consequence of peroxynitrite-induce modification of BPA, increasing its affinity for estrogen receptors.

A relationship between estrogen receptors and cardiac and vascular function has been described (46), but little is known about the contribution of these receptors to hypertension mediated by BPA. Interestingly, oral administration of estrogens leads to a >3-fold upregulation of angiotensin in aortic tissue (47, 48), which is similar to our results with BPA-administered mice. Consequently, our data allow us to speculate on a possible molecular relationship between dysfunctional endothelium, impaired in NO-dependent vasodilatation, and peroxynitrite-mediated nitrated forms of BPA binding to estrogen receptors, increasing the levels of angiotensinogen in vascular endothelium.

NADPH oxidase, the major source of superoxide in the vasculature, can further contribute to oxidative stress through oxidation of BH4, a cofactor required for NO production by eNOS (46, 49). We show here that uncoupled eNOS is a source of superoxide in mice administered BPA and that AngII inhibition dramatically reduces ROS generation. Therefore, we cannot conclude at this moment whether enhanced superoxide generation by eNOS is the primary event in BPA-mediated endothelium dysfunction, or whether oxidative stress and uncoupled eNOS act synergistically.

In summary, the present study provides the first evidence that eNOS is a target of BPA during AngII-mediated AH. Thus, strategies focused on restoring NO production by eNOS are central to prevent oxidative stress-related, BPA-mediated, hypertension. FJ

The authors thank Dr. Irene Cuadrado-Berrocal for her technical assistance. This work was supported in part by grants from the Ministerio de Ciencia e Innovación (SAF2009-12009-C02-01), Instituto de Salud Carlos III (PI12/02825), and Eugenio Rodríguez Pascual Foundation to R.B.; Ministerio de Economía y Competitividad (SAF 2012-35141), Spanish Society of Nephrology 2012, to M.S.; and Ministerio de Economía y Competitividad (SAF 2008-04629 and SAF 2011-28375) to C.Z. N.O. is the recipient of a research contract from Comunidad Autónoma de Madrid–Programa de Actividades I+D en Biociencias 2010 (S2010/BMD-2378). The authors declare no conflicts of interest.

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Received for publication March 12, 2014.
Accepted for publication July 21, 2014.

XIII. ANEXO IV

BISPHENOL A INDUCES CORONARY ENDOTHELIAL CELL NECROPTOSIS BY ACTIVATING RIP3/CAMKII DEPENDENT PATHWAY. **SCIENTIFIC REPORTS** (4.379, Q1 MULTIDISCIPLINARY SCIENCES).

DOI: 10.1038/S41598-020-61014-1

SCIENTIFIC REPORTS
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Bisphenol A induces coronary endothelial cell necroptosis by activating RIP3/CamKII dependent pathway

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Epidemiological studies link long term exposure to xenoestrogen Bisphenol-A to adverse cardiovascular effects. Our previous results show that BPA induces hypertension by a mechanism involving CamKII activation and increased redox stress caused by eNOS uncoupling. Recently, CamKII sustained activation has been recognized as a central mediator of programmed cell death in cardiovascular diseases, including necroptosis. However, the role of necroptosis in cardiac response to BPA had not yet been explored. Mice exposed to BPA for 16 weeks showed altered heart function, electrical conduction, and increased blood pressure. Besides, a stress test showed ST-segment depression, indicative of cardiac ischemia. The hearts exhibited cardiac hypertrophy and reduced vascularization, interstitial edema, and large hemorrhagic foci accompanied by fibrinogen deposits. BPA initiated a cardiac inflammatory response, up-regulation of M1 macrophage polarization, and increased oxidative stress, coinciding with the increased expression of CamKII and the necroptotic effector RIP3. In addition, cell death was especially evident in coronary endothelial cells within hemorrhagic areas, and Evans blue extravasation indicated a vascular leak in response to Bisphenol-A. Consistent with the *in vivo* findings, BPA increased the necroptosis/apoptosis ratio, the expression of RIP3, and CamKII activation in endothelial cells. Necrostatin-1, an inhibitor of necroptosis, alleviated BPA induced cardiac dysfunction and prevented the inflammatory and hemorrhagic response in mice. Mechanistically, silencing of RIP3 reversed BPA-induced necroptosis and CamKII activation in endothelial cells, while inhibition of CamKII activation by KN-93 had no effect on RIP3 expression but decreased necroptotic cell death suggesting that BPA induced necroptosis is mediated by a RIP 3/CamKII dependent pathway. Our results reveal a novel pathogenic role of BPA on the coronary circulation. BPA induces endothelial cell necroptosis, promotes the weakening of coronary vascular wall, which caused internal ventricular hemorrhages, delaying the reparative process and ultimately leading to cardiac dysfunction.

Bisphenol A (BPA) is a ubiquitously used chemical with endocrine-disrupting activity. Many of BPA effects are dependent on its ability to interact with estrogen receptors[1,2]. Due to its widespread use, humans are exposed continuously to accumulative doses of BPA. Significant exposure often occurs by consuming food and beverages contaminated with BPA and in dental procedures, so that up to 92.6% of the human population has detectable BPA levels in their bodies[3,4]. Epidemiological studies have demonstrated that higher BPA urine concentrations are associated with increased morbidity and mortality from cardiovascular diseases[5,6], and several studies in humans and animal models have suggested a causative role for BPA in cardiovascular disease including hypertension, atherosclerosis, cardiac hypertrophy and arrythmias[7–10]. Recently, it has been reported that a life long exposure to BPA in rodents produces arrhythmogenic effects[11]. Moreover, it has been demonstrated that acute or chronic exposure to BPA, impaired functional recovery after myocardial infarction due to a reduced ability to induce macrophage polarization from proinflammatory to a reparative state[12,13]. Indeed, there is an increasing concern about BPA higher bioaccumulation in developing organisms and special vulnerable populations such

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as patients exposed to BPA leaching from medical devices, people handling thermal paper receipts, or plastic industry workers[4,13,14].

Despite all the evidence, there is not a consensus about the extent of cardiac damage caused by BPA. Moreover, the mechanisms by which BPA exposure induces cardiovascular disease remains to be fully elucidated. Many reports rely on cardiomyocyte-specific mechanisms to explain the adverse cardiac effects of BPA. So, most studies reported alterations on electrical conduction[15,16], dysregulation of the contractile apparatus[17,18], and metabolic abnormalities on cardiomyocytes[19], but little attention has been focused on the coronary circulation; indeed, BPA can promote the development of atherosclerosis[20]. A connection between urinary BPA and hypertension has been established in human and animal models, and the role of BPA in peripheral disease has been identified[8,21]. Recently, it has been reported that although BPA itself does not affect the coronary response in coronary ischemia/reperfusion, it reduces coronary flow after reperfusion, and when administered together with estrogens, it counteracts their protective effect during reperfusion[22].

Our previous studies demonstrated a direct effect of orally administered BPA in mice, causing hypertension and endothelial dysfunction due to endothelial nitric oxide synthase (eNOS) uncoupling via Angiotensin II/Ca^{2+}-CamKII pathway[23]. In the heart, calmodulin-dependent protein kinase II (CamKII) regulates cardiomyocyte Ca^{2+} handling, contractility, and cell survival; however, sustained CamKII activation is recognized to promote heart failure[24], arrhythmia[25], and sudden cardiac death[26]. Besides, chronic CamKII activation contributes to endothelial-dependent vascular disease in diabetes and hypertension, which can also impact cardiac function. Recently, CamKII sustained activation has been recognized as a central mediator of programmed cell death, including necroptosis, in cardiovascular diseases[27]. CamKII can be a substrate of RIP 3, the main switch in necroptosis, during ischemia-reperfusion resulting in mPTP opening and cardiac cell death[28]. However, the role of necroptosis in cardiac response to BPA had not yet been explored.

In this study, we sought to evaluate if chronic administration of BPA had any effect on cardiac function. Since BPA induced cardiac damage might be secondary to vascular effects, here we explore whether BPA effects are direct, on cardiomyocytes, or indirect, on coronary vascular endothelium. In addition, the underlying molecular mechanism of these effects will be explored. Clarifying the relationships between BPA and coronary vascular function and the molecular mechanism involved in cardiac cell death may widen our understanding of BPA's impact on cardiovascular disease.

Material and Methods

A detailed listing of the reagents and antibodies used through the study are provided in Supplementary Data.

Animals. Wild-type CD1 mice were purchased from Charles River (Wilmington, MA, USA) and housed in our animal facilities with four mice/cage located in isolated rooms. All animal procedures were approved by the University of Alcala Animal Care Committee and Autonomous Community of Madrid (experimental procedure 007/16) and conformed to the EU Directive regarding the protection of animals used for experimental and other scientific purposes (enacted under Spanish law 1201/2005). CD1 male mice of 8 weeks (~30 g weight) were used.

Ethanol dissolved-BPA was added to the drinking water at final concentration of 4×10^{-5} M. CT consisted of an equivalent volume of ethanol (final concentration 0.01%) in drinking water. This value delivers BPA $\leq$ 50 mg/kg animal weight/day considered a *low dose*[29]. Only male mice were included in our study.

To study the BPA effects on cardiac necroptosis, we randomly divided mice into 2 groups: necrostatin- 1 (NEC) or vehicle control group (CT). Some were administered BPA as above for 4 to 8 weeks (BPA + NEC and NEC). Necrostatin-1 was injected intraperitoneally 3 mg/kg animal weight/day, 3 days a week, as reported elsewhere[30].

Cell cultures. Murine aortic ECs (MAECs) were isolated from mouse aorta, as previously reported[31]. Briefly, the aortas were sectioned into 2-mm pieces, deposited in Matrigel solution, and fed with fresh growth medium for seven days [DMEM/HAM´s medium, 20% FBS, 0.05 mg/mL penicillin/streptomycin, and 2.5 μg/mL amphotericin]. The tissue was removed, and 500 μL of Cell recovery solution was added to each culture. The Matrigel layer was removed and poured on ice for one hour. The solution was centrifuged at 4 °C, resuspended in 4 mL of growing medium, and plated. MAECs were selected by their ability to express the intercellular adhesion molecule-2 (ICAM-2) protein and purified with a flow cytometry cell sorter (DAKO)[32]. Purification was verified by confocal microscopy of MAECs double-stained with Von Willebrand factor antibodies.

H9c2 cells, a myoblastic cell line derived from embryonic rat myocardium, were cultured in the L-glutamine Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.05 mg/mL penicillin/streptomycin in a humidified CO_2 incubator with 5% CO2 at 37 °C[33].

Cardiac myocytes and non-myocytes Isolation from the mouse heart. In order to elucidate the effect of BPA in mouse coronary endothelial cells, cardiac myocytes and non-myocytes cellular fractions were isolated from the mouse heart following the method described by Ackers-Johnson M, *et al.*[34]. Briefly, hearts from CT and BPA mice were perfused with a high EDTA buffer, excised from the circulatory system, and transferred to a 60 mm plate containing EDTA buffer. Digestion was achieved by injection of collagenase solution into the left ventricle (LV). When digestion was observed, the atria and right ventricle were carefully removed, and the left ventricle mechanically dissociated. Enzimatic activity was blocked with stop buffer and the cell suspension was passed through a 100-μm filter, followed by four sequential rounds of gravity settling.

The cell pellet in each round was enriched with myocytes and ultimately formed a highly pure myocyte fraction, whereas the supernatant from each round was combined to produce a fraction containing non-myocyte cardiac populations. Non-myocytes cells were characterized by immunofluorescence staining of the endothelial marker CD31 and α-smooth muscle actin. CD31 positive cells quantification was performed, counting six

different homogeneous fields that contain a mean of 60 cells/field. Endothelial cells represents the 73 ± 1.23% of the nonmyocytes fraction. Myocytes and non-myocytes fraction were lysed using protein lysis buffer and analyzed by western blotting.

ECG recordings. Mice were anesthetized with isoflurane (2% in pure O2). Fine needle electrodes (25 G) were inserted subcutaneously at the level of both armpits and left groin and connected to an AC amplifier (Cyberamp, Axon Instruments). The ECG leads were placed initially in the lead II configuration and exchanged when required, to the lead I and lead II at the pre-amplifier. The signals were amplified 500 times and band-pass filtered between 1 Hz and 100 Hz, digitized at 1000 Hz (Power 1401, CED, UK) and stored for off-line analysis using Spike 2 software (CED, UK)[35].

Echocardiography. Mouse hearts were visualized by echocardiography by using a Vivid Q ultrasound system (GE healthcare) equipped with a 12.5 MHz scan head. Mice were anesthetized with 1.5% isoflurane gas, resulting in a heart rate of approximately 400 beats/min. Parasternal short-axis-view images of the heart were recorded in a B-mode to allow M-mode recordings by positioning the cursor in the parasternal short-axis view perpendicular to the interventricular septum and posterior wall of the left ventricle[36]. From these recordings, the following parameters were determined using the on-site software cardiac package: systolic and diastolic Interventricular septum thickness (IVS), systolic and diastolic left-ventricle internal diameter (LVID), systolic and diastolic left-ventricle posterior Wall thickness (LVPW), left-ventricle ejection fraction (EF), left ventricle shortening fraction (FS), heart rate (HR), and cardiac output (CO).

Dobutamine stress test. Mice were injected a maximally effective dose of the β -AR agonist, dobutamine (3 μg/g)[37]. Inotropic and chronotropic responses to this stimulation were verified in all mice by echocardiography and ECG recordings of DI and II for 15 min.

Blood pressure. Indirect measurements of blood pressure were obtained in conscious animals using a tail-cuff sphygmomanometer (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain)[23]. The animals were trained for 5 d before starting the measurement to prevent stress and were pre-warmed to 30 °C with a heater (LE5660/6, Letica Scientific Instruments, Hospitalet, Spain). Arterial pressure was measured several times between 9:00 and 12:00 AM, and pressure values were considered acceptable at ten consecutive measurements.

Vascular permeability assay. Evans blue is vital dye used to study blood vessel and cellular membrane permeability[38]. Evans blue was injected IP, 24 hours before euthanization, to allow circulation. The striking blue color can be identified the EBD-albumin conjugate within the tissue *ex-vivo*[39]. To extract Evans blue dye, 250 μL deionized formamide was added to the dry tissue and incubated overnight in an oven or a heating block at 55 °C, then loaded into a 96-well plate for absorbance reading with a spectrophotometer. Evans blue was also detected by confocal microscopy of the LV sections due to the fluorescence spectrum of this product (620 nm excitation/680 nm emission)[40].

Histology. LV sections were fixed in a 10% formalin solution, dehydrated in ethanol, and then embedded in paraffin as previously described[36]. Tissue sections (5 μm) were obtained in a microtome, were deparaffinized, rehydrated, and stained with Masson's trichrome staining kit (EMD Millipore Corporation, Billerica, MA, USA) and Sirius red staining (Sigma- Aldrich, San Luis, MI, USA) for fibrosis quantification. Hematoxylin/Eosin staining was performed to explore heart morphology (Sigma-Aldrich, San Luis, MI, USA).

Immunoblot. Protein lysates were immunoblotted as previously described[41]. Twenty-five μg of total protein was separated in a 10% SDS-polyacrylamide gel electrophoresis. For protein detection, blocked membranes were incubated with specific antibodies, washed, and incubated with a secondary antibody. The bands were visualized with the Super-Signal detection kit (Pierce, Waltham, MA, USA).

Immunohistochemistry. Samples were boiled in retrieval buffer for 20 min after xylene deparaffinization. Master polymer plus detection system (Master Diagnostica, Madrid, Spain) was used, and antibody incubation was overnight at 4 °C. Sections were incubated with secondary antibodies for 1 hour at room temperature and counterstained with Harry´s hematoxylin, dehydrated and mounted with DPX (Casa Alvarez, Madrid, Spain)[42]. Images obtained of at least five different hearts per condition (6 per animal) were taken for data quantification using bright-field microscope (Eclipse 50i; Nikon, Tokyo, Japan).

Confocal microscopy. Slides containing tissue sections were incubated with the primary antibodies overnight 4 °C. After washing with PBS, the slides were incubated with FITC, Alexa-488, or Alexa-647-conjugated secondary antibodies for 1 hour at room temperature. Nuclei were stained with Hoechst. Images were taken for data quantification using a Leica TCS SP5 confocal microscope (*UAH-NANBIOSIS-CIBER-BNN)*. At least five different fields per condition were obtained.

Analysis of capillaries densities in the myocardium. For blood vessel counting heart sections obtained from CT and BPAtreated mice were stained with CD31 antibody as described previously[43]. Sections (6 μm) were blocked with 5% BSA for 30 min and, incubated with CD31antibody, a specific marker of endothelial cells overnight at 4 °C and the secondary antibody Alexa fluor 647 was added for 1 hour, room temperature light protected. The samples were incubated with FITC–conjugated wheat germ agglutinin (WGA) (Life Technologies, Carlsbad, Ca, USA) to delimit cardiomyocyte plasma membrane for 1 hour and Hoechst was added to localized the nuclei. Images of subepicardial regions of the left ventricular (LV) wall and the interventricular septum (IVS) on the

section were obtained by confocal microscopy (Leica TCS SP5). Positive CD 31 endothelial cells were quantified from at least six random high-power fields from different heart regions (left ventricle, right ventricle, and septum) by a blinded investigator. The capillary count was analyzed with Fiji[44]. The results were presented as capillary density per field for each heart region analyzed.

Cell transfection. MAECs were transfected with 50 nM non-targeting siRNA(si-Scramble) or RIP 3-targeting siRNA (si-RIP 3) (Santa Cruz Biotech, Santa Cruz, CA, USA)[45] using Lipofectamine 2000 transfection reagent and Opti-MEM (Gibco, Waltham, Ma, USA) for 6 hour. Following 24 hours transfection, cells were incubated with BPA in 5% serum DMEM culture medium for 24 hours at 37 °C, 5% CO2.

Survival, apoptosis and necrosis assay. An MTT assay was used to measure cell viability[46]. Briefly, MAEC (6000 cells/well) were seeded in 24-well plates overnight and were then incubated with various concentrations of BPA at 37 °C for 24 h. MTT (0,5 mg/mL) was added to each well. After three hours incubation, supernatants were removed, and 150 µL DMSO was added to each well as a solvent. Absorbance was measured at 492 nm in a spectrometer plate reader (ELx800, Bio-Tek Instruments, Winooski, VE, USA). Non treated cells (control group) were regarded as having 100% viability.

To determinate cell death in tissue, we use DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) following the manufacturer instructions, in combination to Polyclonal Rabbit Anti-FITC/HRP, the stain was developed by addition of DAB substrate (Dako, Santa Clara, CA, USA). Samples were counterstained with Mayer's hematoxylin and mounted with DPX and visualized in a bright field microscope (Eclipse 50i; Nikon, Tokyo, Japan). Images (2 sections, n = 5 animals/group). Five slide fields were randomly examined using a defined rectangular area under 40 × magnifications. Apoptotic cells were counted under 400 × magnifications. Results are expressed as the percentage of TUNEL-positive cells versus the total number of cell nuclei per field[47].

Necrosis and apoptosis determination by flow cytometry were performed in cells treated with BPA and silenced for RIP3 or a siRNA scramble as control. The cells were trypsinized and resuspended in 150 µl of 1 × binding buffer. Then 5 µl of 20 µg/mL Annexin V-FITC conjugated and 5 µl of propidium iodide (PI) were added and cells incubated for 15 minutes in the dark at room temperature according to manufacturer´s instructions. Cells were analyzed by FACS Calibur (Becton, Dickinson Company, NJ, USA). The percentages of cells in each quadrant were analyzed using Cyflogic software (Cyflo Ltd, Turku, Finland). Results of flow cytometry were obtained by calculating the number of necrotic cells (PI^+/$Annexin^-$) and apoptotic cells (PI^-/$Annexin^+$). Double negative were considered live cells and annexin V^+-PI^+ were late apoptotic or necroptotic cells. All the results experiments were performed in duplicate and repeated at least three times.

mRNA expression. Total RNA from cardiac tissue was extracted with TRIzol reagent from Invitrogen Corporation (Carlsbad, CA, USA) following the manufacturer´s instructions. First-strand cDNA was synthesized from 2 µg of total RNA in a 20 µl reaction mixture using the High Capacity cDNA reverse transcription kit, and the qPCR reaction was performed with SYBR select master mix both of Life technologies (Carlsbad, CA, USA). The qPCR conditions and primers that were used are included in the Supplementary Information Table 1.

Statistical analysis. Every experimental condition was duplicated within each experiment, and each experiment was repeated at least three times. For animal studies, n values refer to the number of individual animals used. Comparisons were made by analysis of variance, followed by Dunnett's modification of the t-test when comparisons were made with common control and the unpaired two-tailed Student's t-test for other comparisons. Results are expressed as mean ± SD, and differences were considered statistically significant at $p < 0.05$.

Results

BPA impaired cardiac function and induced cardiac hyperhrophy. Previously, we had reported that oral administration of BPA at different concentrations induced hypertension in mice as early as five weeks starting at 4×10^{-7} M BPA and reaching a maximum effect at 4×10^{-5} M BPA[23]. We tested whether more prolonged exposure to BPA may negatively affect cardiac function. Mice were orally exposed to 4×10^{-5} M of BPA in the drinking water for 4, 8, and 16 weeks and cardiovascular functional abnormalities were examined. To assess the effect of BPA on cardiac conduction, we performed surface ECGs on CT (n = 10), and BPA treated mice (n = 18) (Fig. 1A). Interestingly, BPA treated mice displayed increased heart rate (526.25 ± 49.08 vs. CT = 440 ± 28.28; $p < 0.001$), and a significantly prolonged PQ interval and PR segment suggesting a first degree AV block.

Echocardiography analysis revealed that cardiac contractility was significantly impaired in BPA treated mice, as demonstrated by decreased ejection fraction (EF) (Fig. 1B) and fractional shortening (FS) (Fig. 1C). Besides, diastolic and systolic Interventricular septum thickness (IVSd) were increased, suggestive of cardiac hypertrophy (Fig. 1D). Left ventricular posterior wall thickness was slightly, but not significantly, elevated. However, end-diastolic but especially end-systolic internal diameter was augmented in animals treated for 8 and 16 weeks with BPA (Supplementary Fig. S1A,B). These results indicates that besides a contractile dysfunction, BPA also induced a slight increase in ventricular size, consistent with ventricular hypertrophy. As expected, BPA also increased systolic and diastolic blood pressure (BP) after 4 weeks, and, was further elevated at 16 weeks (Supplementary Fig S1C).

Consistent with the functional findings, the hearts were significantly enlarged after 16 weeks of BPA treatment, as detected by heart weight-to-tibial length ratio and hematoxylin and eosin sections (Fig. 1E). Cardiomyocyte cross-sectional area measured by Wheat Germ Agglutinin (WGA) staining was also increased, especially at the interventricular septum and left ventricle wall, indicating cardiac hypertrophy (Fig. 1F). Cardiac fibrotic remodeling was not found in BPA hearts when compared with CT mice (Fig. 1G **upper pannel**) and Col I expression was modestly increased in cardiac tissue at 8 and 16 weeks of BPA administration (Fig. 1H). However, perivascular

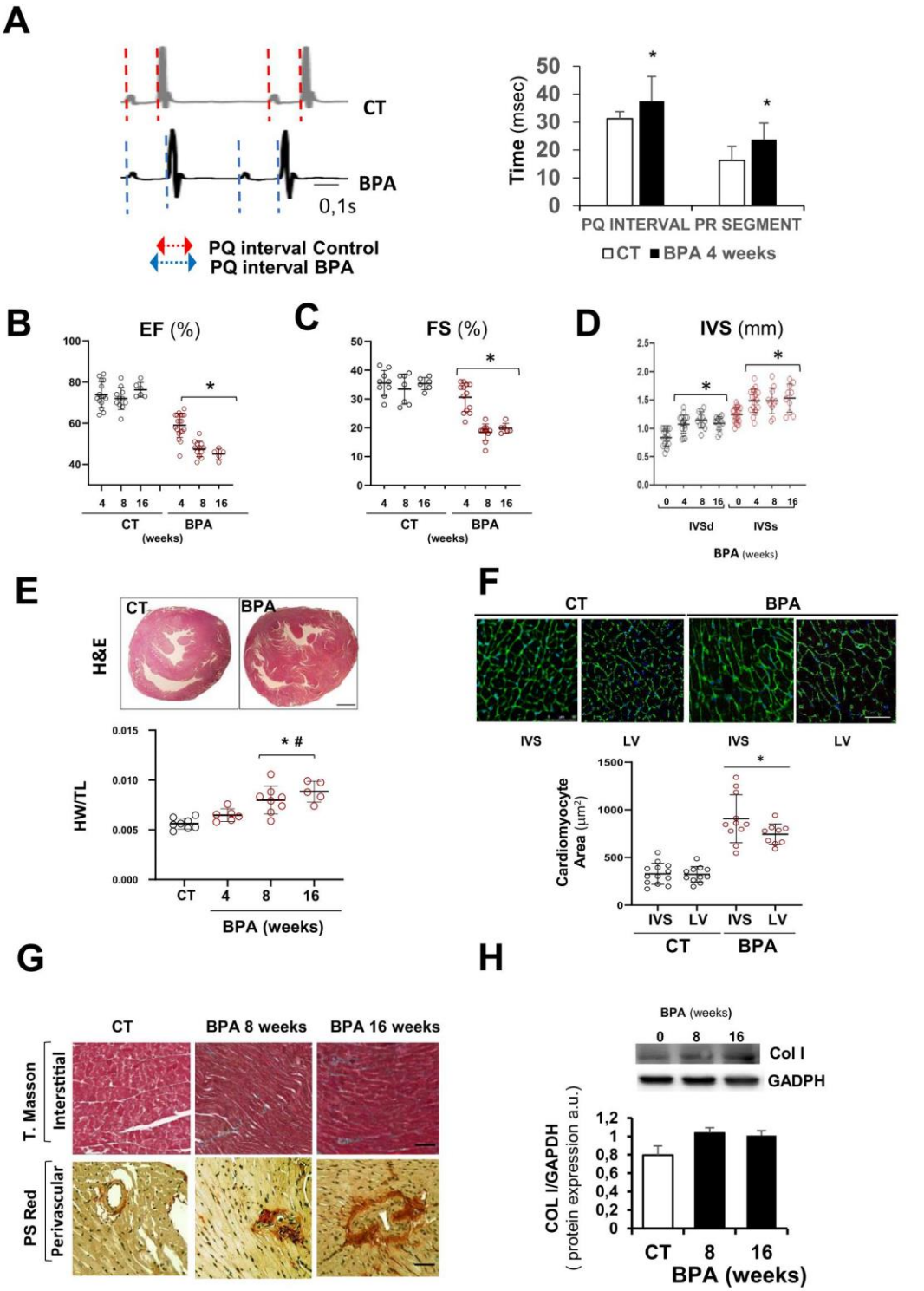

Figure 1. BPA induced impaired cardiac function and cardiac hypertrophy. ECG and echocardiography were performed in CT (vehicle treated) and BPA treated mice for 4, 8, and 16 weeks. (**A**) *Left panel:* Representative ECG recording in DII showing a longer PQ interval in 4 weeks BPA treated mice compared to CTmice. *Right panel* shows mean values for PQ interval and PR segment from ECGs recorded after 4 weeks of treatment (CT n = 10 and BPA n = 18, *$p < 0.05$). (**B**–**D**) shows LV ejection fraction (EF), Fractional shortening (FS) and interventricular septum thickness respectively (CT n = 12 and BPA n = 6–10) *$p < 0.05$ vs. CT; (**E**) *Upper panel:* Representative images of hematoxylin and eosin in heart sections from mouse after 16 weeks of BPA or CT showing IVS enlargement. Scale bar: 1000 μm. *Lower panel:* Quantification of heart weight to tibial length ratio (mg/mm) of CT and BPA treated mice at the indicated time points. (CT n = 12 and BPA n = 6–10 mice per group). *$p < 0.05$ vs. CT; # $p < 0.05$ vs. BPA 4 weeks (**F**) *Upper panel:* Representative images of wheat germ agglutinin (WGA)-fluorescein isothiocyanate-staining in mouse hearts after 16 weeks of treatment showing cardiac myocyte (CM) cross-sectional area at different heart regions (LV wall and interventricular septum, IVS). Scale bars: 20 μm. *Lower panel:* Quantitative data of CM hypertrophy cell surface area (n = 8–12 hearts

per group with 300–600 CMs analyzed per heart). CM size was expressed as μm^2. (**G**) Representative Masson Trichrome and Sirius red-stained sections of CT and BPA mice at 8 and 16 weeks showing perivascular fibrosis but not interstitial fibrosis in BPA treated mice. Scale bar = 60 µm. (**H**) Collagen type I protein expression measured by western blotting in whole heart tissue from CT and BPA treated mice. GADPH is used as loading control. The bar graph shows the average of n = 10 hearts per condition.

fibrosis was significantly increased after eight weeks of BPA (Fig. 1G **lower panel**). Together these results indicate that BPA increased heart rate, impaired cardiac contractility, and induced cardiac hypertrophy.

BPA induces cardiac ischemia under stress and chronic cardiac inflammation. To test the pathophysiological implication of our findings, we performed a dobutamine stress echocardiography study in our BPA treated mice. Following administration of dobutamine (DB), heart rate (HR) increased significantly from baseline values in CT mice, but not in BPA treated mice, suggesting a BPA-mediated impairment of chronotropic responsiveness to β-adrenergic stimulation (Supplementary Fig. S2A). This effects was confirmed by the analysis of surface electrocardiogram in which shorter R-R intervals in response to DB challenge were observed only in CT mice, while R-R intervals failed to decrease further in BPA-treated mice when compared to BPA resting values (Fig. 2A). Moreover, the depressed ST segment, indicative of cardiac ischemia, was evident in most animals treated with BPA, suggesting that the coronary reserve may also be compromised (Supplementary Fig. 2B). Additionally, a DB challenge markedly reduced cardiac contractility after 16 weeks of BPA, as evidenced by the inability to increase the EF (Fig. 2B), the cardiac output (Supplementary Fig. S2C), and the left ventricular diameter, compared with CT mice (Fig. 2C). Altogether, BPA reported abnormal stress test results, which might be indicative of cardiac ischemia.

Cardiac inflammation plays a critical role in adverse cardiac remodeling, as observed in ischemic heart disease, cardiac hypertrophy, and heart failure[48]. Hematoxylin and eosin sections of cardiac tissue from BPA-treated mice revealed an inflammatory response as early as four weeks of treatment, in which localized foci of $CD68^+$ and $Mac\text{-}2^+$ macrophage infiltrates were detected. Increased oxidative stress as detected by 4-Hydroxynonenal (4-HNE) was also present after 16 weeks, which might contribute to BPA-induced myocardial injury (Fig. 2D).

Since mice after 16 weeks of BPA still presented significant inflammatory infiltrates, we explored whether BPA may prevent macrophage polarization toward a phenotype of inflammatory resolution. A qRT-PCR analysis showed that M1 markers (Tumor necrosis factor-alpha (TNF-α) and CCL2 (Fig. 2E,F) and Chemokine (C-C motif) ligand 7 (CCL-7), Chemokine (C-C motif) ligand 12 (CCL-12) (Supplementary Fig. S3A,B), significantly increased in BPA treated mice hearts. CCL2 receptor (CCR2), transcript levels were significantly increased at four weeks of BPA, even before the onset of clinical manifestation of heart disease, and remained elevated after 16 weeks of BPA (Fig. 2G). Confocal analysis showed the colocalization of CCR2 with CD68 macrophages in the hearts of mice at eight weeks of administration of BPA (Supplementary Fig. 3C,D). The accumulation of $CCR2^+$ macrophages may represent a delay in polarization to M2 resolving macrophages[49], and our data indeed indicate that M2 markers, including TFG-β and IL-10, were not increased even after 16 weeks of BPA (Fig. 2H,I).

BPA induces myocardial hemorrhage and vascular leakage. Hematoxylin and eosin staining of heart sections from the BPA mice, exhibited a distinctive phenotype (Fig. 3A). Most of the hearts showed early signs of cardiac interstitial edema with significant disarranged myocardial fibers along with the presence of inflammatory cells (Fig. 3Ab). Interestingly, hemorrhage was apparent in 75 ± 2% of the hearts, as evidenced by the interstitial presence of red blood cells (Fig. 3Ac) with fibrin deposits (Fig. 3Ad), indicative of microvascular damage. Hemorrhagic lesions could be observed as early as four weeks after BPA administration. Large areas of hemorrhagic foci together with fibrin deposits were evidenced after 8 weeks of BPA treatment that were still present in the hearts of mice after 16 weeks, although the hemorrhagic lesions were not as extensive compared to 8 weeks (Fig. 3Ae,f). A similar effect could also be observed in BPA treated mice at lower doses (4×10^{-8} and 4×10^{-7} M), which mimic environmental exposure (Supplementary Fig. 3A). A positive fibrinogen staining near coronary arteries was observed, indicating plasma extravasation (Fig. 3B). In addition, cardiac vascularization in BPA hearts was significantly reduced as detected by decreased number of CD31 positive cells (Fig. 3C). In order to exclude BPA-induced coagulation defects, total platelet counts and coagulation tests (Prothrombin time (PT), Activated partial thromboplastin time (aPTT), Thrombin time(TT) and fibrinogen quantitation) were performed, but the results were similar for both groups CT and BPA. Hence, coagulation abnormalities could be excluded as the origin of cardiac hemorrhages (Supplementary Fig. 3B–E).

To investigate whether BPA could promote structural changes in endothelial cells, we visualized CD31 positive endothelial cells within hemorrhagic areas by confocal microscopy. We observed gaps in the endothelial layer of injured vessels (Fig. 3D), which suggest that BPA could modulate the endothelial barrier function. Next, we analyzed the permeability of endothelial cells by Evans blue (EB) dye extravasation, observing that EB extravasation increased by a 1,5-fold in hearst from 4 and 8 weeks-BPA treated mice compared to CT. Iinterestingly, in the lungs EB extravasation was increased by 3-fold compared to CT (Fig. 3E). EB levels in livers and kidneys were similarly elevated in both BPA and CT mice due to fenestrated endothelium (not shown). EB extravasation in cardiac tissue was also observed by confocal microscopy (red fluorescence of EB), and coronary arteries were stained with α-smooth muscle actin antibody (α-SMA, green). Whilst EB staining was confined to he lumen of the coronary arteries in CT mice Fig. 3F(a,b), in BPA treated mice EB was detected in the outer wall of the coronary artery and intercalated between arterial muscle cells (Fig. 3Fc,d).

Together, these results demonstrate that BPA increased vascular permeability and induced vascular leakage that could account for hemorrhagic damage that, together with a reduced vascularization, might lead to cardiac hypoperfusion and myocardial ischemia.

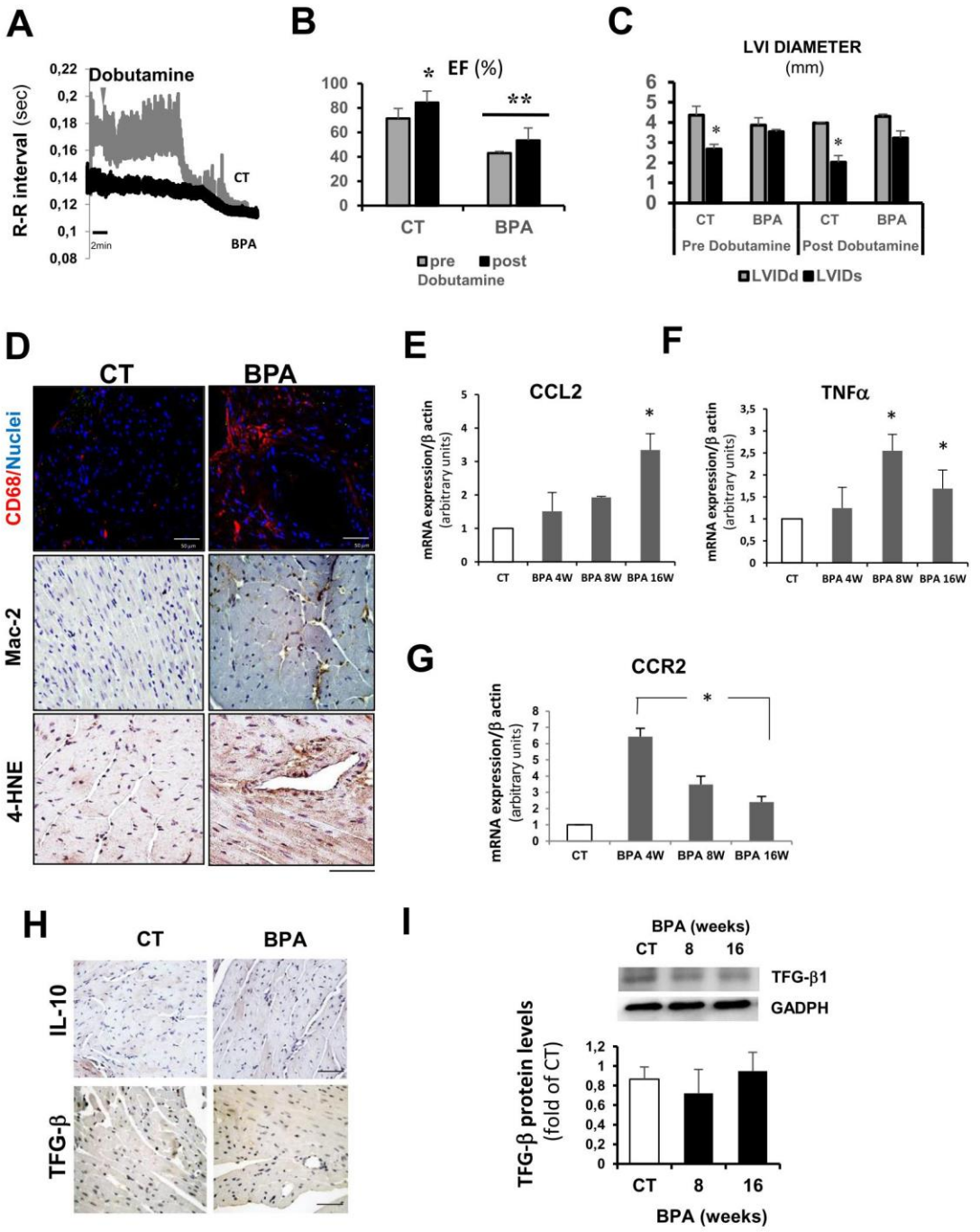

Figure 2. BPA induces cardiac ischemia and chronic inflammation. CT and 16 weeks BPA treated were challenged with an i.p. injection of 3μg/g of dobutamine while recording cardiac activity by EGC for 15 min. (**A**) Graphic representation of the RR interval of CT and BPA mice before and after dobutamine administration (n = 4–6 mice per group). (**B**,**C**) Echocardiogram analysis of CT and BPA mice before and after dobutamine administration showing Left ventricular ejection fraction (EF) and Left ventricular internal diameter (LVI Diameter) respectively. The data are presented as mean ± SD (n = 4 per group, EF: *$p < 0.05$ vs CT pre Dobu and **$p < 0.05$ vs BPA predobu; LVID *$p < 0.05$ vs LVIDd). (**D**) *Upper panel:* Representative confocal images showing infiltration of $CD68^+$ macrophages (red) and nuclear staining with Hoetch (blue) as detected by confocal microscopy in heart sections from CT and 4 weeks treated BPA mice. *Middle and lower panels:* Immunohistochemistry in heart sections from CT and 8 weeks treated BPA mice detecting Mac-2 (macrophage marker), and 4-HNE (oxidative stress marker) at 16 weeks BPA (n = 8 for both groups, Scale bar = 60 μm). (**E**–**G**) RT-qPCR of CT and 4, 8, and 16 weeks BPA treated mice showing cardiac mRNA expression of: (**E**) CCL2 (MCP-1), (**F**) TNF-α and (**G**) CCR2 (CCL2 receptor) (n = 8 per condition with triplicates in each determination, *$p < 0.05$ vs. CT). (**H**) Images representative of immunostaining of IL10 and TFG-β1 in hearts from CT and 16 weeks treated BPA treated mice. Similar results were obtained in triplicate heart sections from n = 4 animals per condition. (**I**) TFG-β1 protein expression measured by western blotting in whole heart tissue from CT and BPA treated mice. The bar graph shows the average of n = 6 hearts per condition.

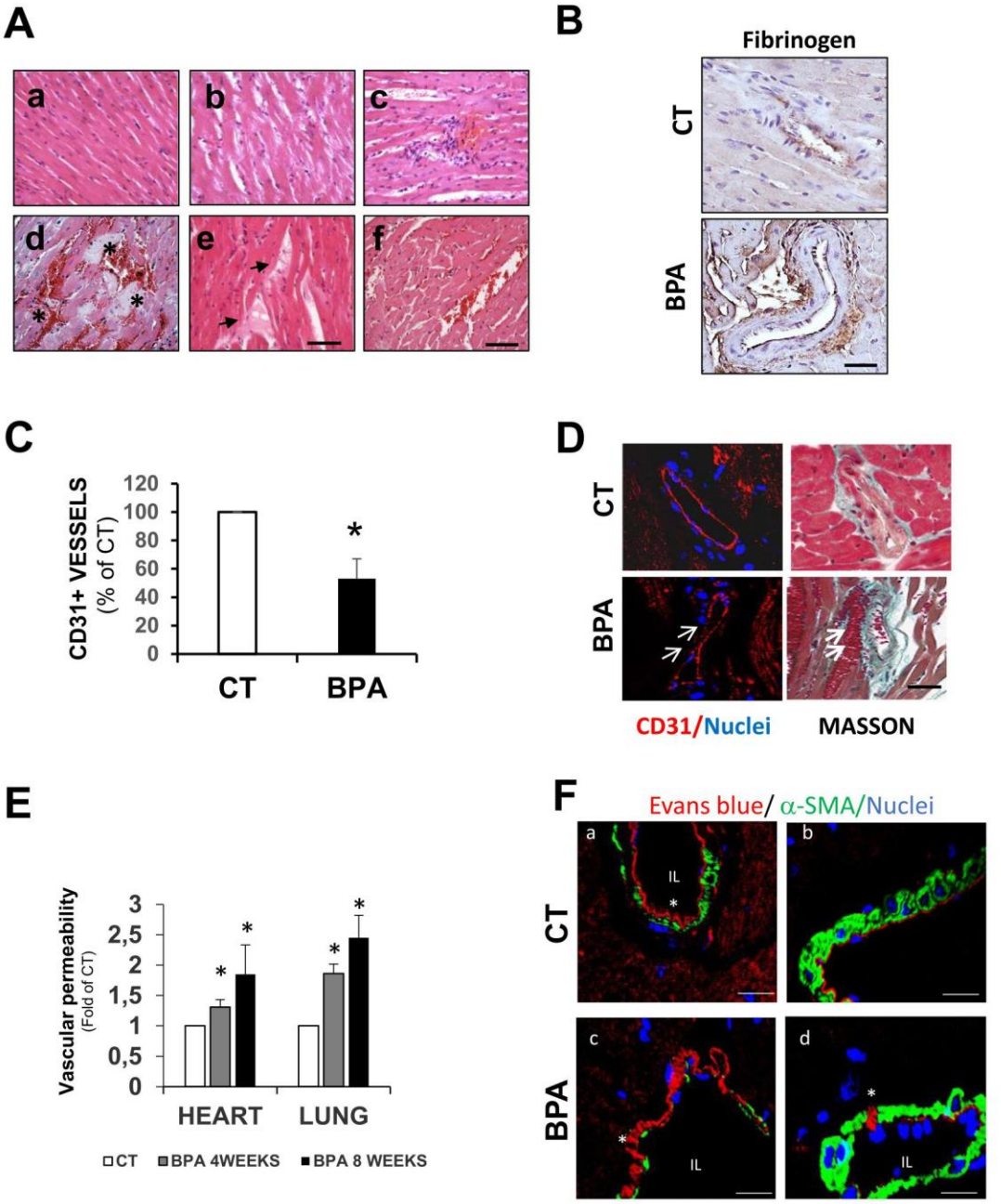

Figure 3. BPA induces hemorrhagic cardiac lesions, decreased vascularization, and vascular leakage. (**A**) Photomicrographs from hearts stained with hematoxylin/eosin of CT (a), BPA 4 weeks (b and c), BPA 8 weeks (d), and BPA 16 weeks (e and f). Asterisk mark extensive hemorrhagic areas with fibrin deposits. Arrows mark fibrin deposit between myocytes fibers (scale bar = 60 μm). (**B**) Immunohistochemistry of heart sections from CT and 8 weeks treated BPA mice stained for Fibrinogen. Scale bar = 25 μm (**C**) Blood vessel quantitation with an anti-CD31 endothelial cell marker in the heart of CT and 8 weeks BPA treated mice. CD31 + cells were labeled in red, and nuclei were labeled with Hoechst in blue. Three different heart regions were used to quantitate: RV, LV and IVS and at least 6 fields of each region were counted (n = 4 hearts per condition. Results are expressed as the number of CD31 + cells/mm^2 and referred to CT). (**D**) *Left panel:* Representative confocal images from heart sections of CT and 8 weeks treated BPA mice showing immunostaining for CD31 (n = 4 mice per condition). *Right panel:* Masson trichrome staining of the same regions. Arrows mark hemorrhagic foci close to the blood vessel in BPA treated mice. Images are representative of n = 4 hearts per condition. (**E**) Vascular permeability in the heart and lungs measured 24 h after injection of Evans blue 2% expressed as ng dye/mg of tissue and referred to CT. The data are presented as mean ± SD, n = 6 mice per group. *$p < 0.05$ vs CT. F) Representative confocal images from heart sections of CT and 8 weeks treated BPA mice after injection with Evans blue (red fluorescence) followed by immunostaining for α-SMA (green). Nuclei were labeled with Hoechst in blue (n = 4 mice per condition). (a and b) CT hearts sections obtained at different magnifications showed no signs of EB extravasation. (c) and (d) are different sections of coronary arteries of BPA hearts with extravasation areas. Scale bar = 25 μm (a and c) and 50 μm (b and d). IL = intraluminal area and (*) marks areas of EB extravasation.

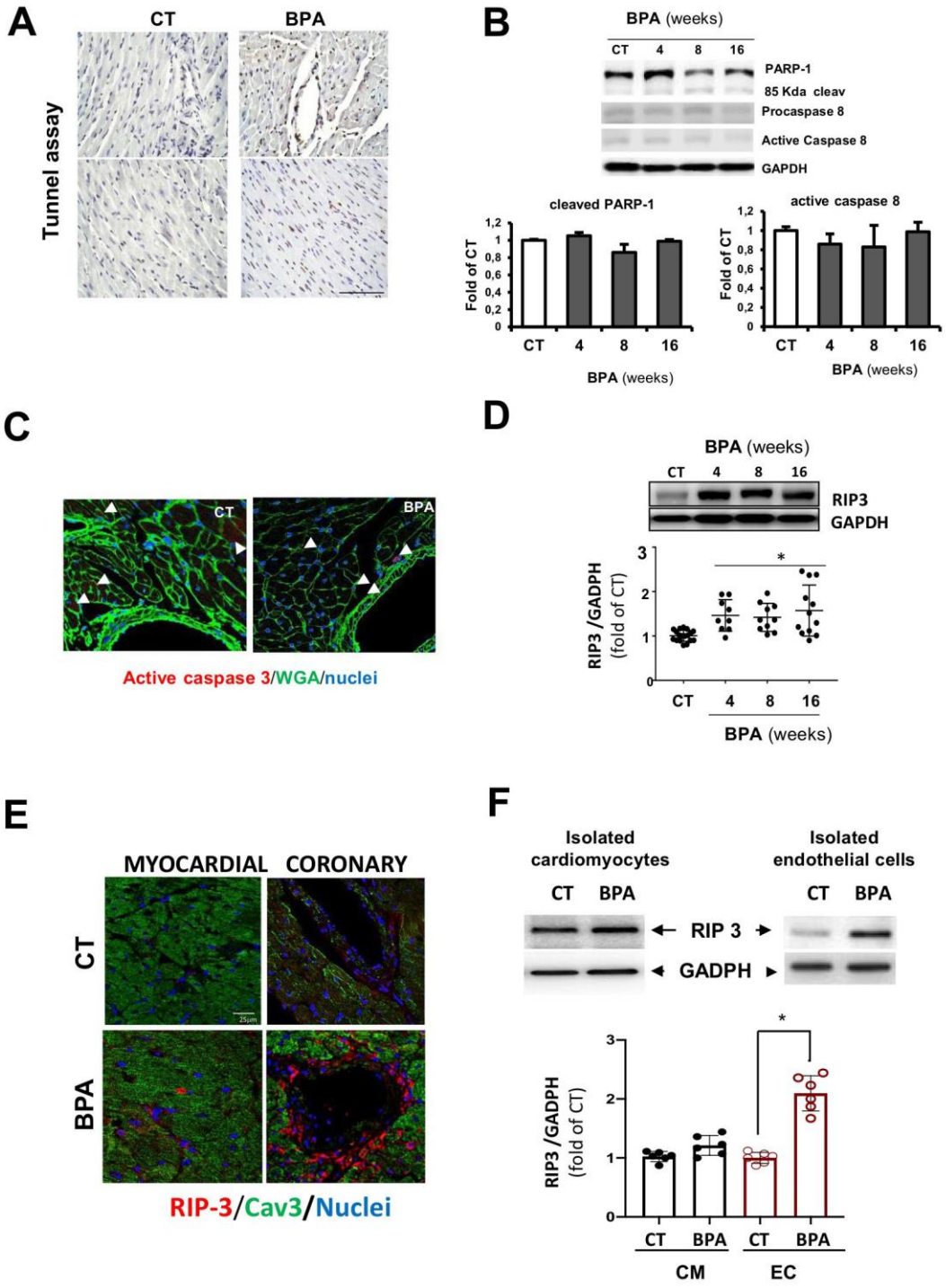

Figure 4. BPA induces cardiac necroptosis. (**A**) Detection of cell death by tunel assay in heart slides of CT and 8 weeks BPA treated mice. Brown staining indicates TUNEL-positive nuclei, and blue indicates living cells. A representative image of two regions of a heart sections is shown. Scale bar = 25 µm. Similar results were obtained in triplicate heart sections from n = 4 animals per condition. (**B**) Immunoblot detection of PARP-1, the 86 KDa cleaved form of PARP-1, procaspase, and 43 KDa active caspase 8 in total heart lysates from CT and BPA treated mice at 4, 8, and 16 weeks. GAPDH was used as a loading control. A representative immunoblot is shown. The densitometric analysis are shown below (data are shown as mean ± SD, n = 4 mice per condition). (**C**) Cardiac cross-sections were examined for activated caspase-3 (red). WGA marked CM are green and Hoechst cell nuclei in blue. Representative images of findings in CT and 8 weeks treated BPA mice are shown (a total of 4 sections per heart were examined; n = 3 to 8 hearts per group. Scale bar = 50 µm). (**D**) RIP3 protein expression measured by western blotting in whole heart tissue from CT and BPA treated mice. GAPDH was used as a loading control. A representative immunoblot is shown. The densitometric analysis is shown below. Data shown represent each mouse cardiac RIP3 expression referred to CT (n = 8–12 mice per group, scale bar = 25 µm). (**E**) Representative confocal microscopy images of RIP3 (shown in red), in regions from CT and 8 weeks treated BPA mice. Scale bar = 25µm. The expression of Caveolin-3 was used as a cardiac myocyte cell marker (Cav3, in green). Nuclei

were stained with Hoechst (blue). Similar results were obtained in n = 4 hearts per condition. (**F**) RIP3 protein expression measured by western blotting in freshly isolated CM or EC from CT and 8 weeks BPA treated mice. The bar graph shows mean ± SD of RIP3/GADPH average refered to CT (n = 6 hearts per condition, *$p < 0.05$ vs CT in ECs).

BPA induced vascular injury is mediated by RIP3/CamKII dependent endothelial cell necroptosis. Inflammation and cell death are associated with the development of heart disease, especially under increased oxidative stress conditions, as observed in BPA treated mice[50,51]. To identify the mechanisms underlying vascular leakage and myocardial hemorrhages induced by BPA, we performed tunel assay in hearts from 8 weeks BPA treated mice, observing increased cell death, especially at the coronary endothelium and within the vascular wall, compared to CT (Fig. 4A). There was a 2.8 ± 1.06 fold increase in total tunel positive cells in heart slides at 8 weeks after BPA treatment, while in areas containing coronary vessels, the increase is 9,89 ± 0.856 indicating a higher susceptibility of vascular cells to BPA induced cell death.

Western blot analysis of whole heart lysate showed neither changes in caspase 3 activity, as reflected by the Poly [ADP-ribose] polymerase 1 (PARP-1) cleavage, nor increased caspase 8 activity (Fig. 4B). Similarly, confocal microscopy analysis of CT and 8 weeks BPA heart sections revealed few differences in active caspase 3 levels (Fig. 4C), suggesting that an apoptosis-independent mechanism is involved in BPA-induced endothelial cell death.

To confirm that endothelial cells were not undergoing apoptotic cell death, we treated mouse aortic endothelial cells (MAEC) with 0-10^{-4} M BPA for 24 h. BPA reduced cell viability from 10^{-7} M (Supplementary Fig. S5A). Therefore MAEC were treated with 10^{-6} M of BPA for 24, 48 and 72 h and the level of caspase 3 activity, as detected by PARP-1 cleavage, and as well caspase 8 were studied by western blot. We found no caspase activation in BPA treated cells compared to vehicle treated cells (CT) (Supplementary Fig. S5B). However, the treatment of cardiomyocyte cells (H9c2) with different concentrations of BPA during 24 h, did no reduce cell viability except at the highest concentration (10^{-4} M). (Supplementary Fig. S5C). Together, these results confirmed that BPA targets endothelial cells by an apoptotic independent form of cell death.

Interestingly, caspase-8 induces apoptosis but also serves as a significant regulator of necroptosis. Active caspase 8 can cleave RIP1 and 3, thus preventing necroptotic signaling[52,53].To explore if necroptosis contributes to BPA induced coronary vessel injury, we investigated RIP3 levels in whole cardiac tissue of BPA treated mice. RIP 3 protein expression, which is critical to initiate necroptosis, was increased in the myocardium of BPA treated animals for 4,8 weeks and 16 weeks (Fig. 4D). Confocal microscopy revealed intense labeling of RIP 3 after 8 weeks of treatment with BPA at the endothelial lining of coronary vessels and around blood vessels. However, RIP3 staining in cardiomyocytes was slightly more intense that in CT hearts (Fig. 4E).

To clarify which cardiac cells were contributing to increased RIP 3 expression, we isolated cardiomyocytes (CM) and non-myocyte cellular fractions from CT and 8 weeks-BPA treated mice. Non-cardiomyocyte cells consisted mostly in endothelial cells, as detected by positive CD31 staining and the absence of α-SMA staining. Although CM from CT and 8 weeks BPA treated mice showed no increase in RIP 3 levels, the endothelial cell fraction showed significant increased RIP 3 expression (Fig. 4F). Similarly, western blot analysis of RIP3 expression on H9c2 cells showed no differences between CT and BPA treated cells (Supplementary Fig. S5D). Together, those results indicate that BPA may initiate a RIP 3-necroptotic pathway in vascular cells.

Recently it has been described that necroptosis can proceed in a RIP 1 independent pathway, activating Ca^{2+}-CamKII to regulate mitochondrial permeability transition pore (mPTP) opening[54,55]. The opening of the mPTP in response to ischemia-reperfusion injury has been linked to cell death in the heart and other organs[56,57]. Here we found that BPA increased CamKII phosphorylation in whole heart homogenates at 4 and 8 weeks of BPA treatment (Fig. 5A).

To better comprehend the relationship between BPA and cardiac necroptosis, we treated mice with BPA and the necroptosis inhibitor Necrostatin-1(NEC-1) for 4 and 8 weeks. Functional analysis revealed that necrostatin was able to partially revert the impairment in cardiac contractility caused by BPA (Fig. 5B). Histological analysis also evidenced a less pronounced cardiac injury compared with BPA treated mice with less oxidative stress as detected by 4-HNE staining (Fig. 5C, lower panels). More importantly, we found smaller hemorrhagic lesions (Fig. 5C H&E upper, and Masson Trichrome middle panels). NEC-1 treatment also produced a reduced inflammatory response compared to BPA alone, as detected by the expression of pro-inflammatory mRNA corresponding to TNF-α, CCR2 and CCL2 (Fig. 5D). RIP 3 levels were studied in isolated EC protein samples, finding a reduction of RIP 3 expression in NEC-1 + BPA compared to BPA alone, which correlates with reduced p-CamKII expression in endothelial cells, indicating a link between BPA and induced endothelial RIP 3, CamKII activation, and cardiac injury (Fig. 5E).

BPA induced endothelial necroptosis is mediated by RIP3/CamKII pathway. We explored the implication of CamKII on endothelial cell necroptosis, using cultured mouse aortic endothelial cells (MAEC). BPA increased the expression of RIP 3 (Supplementary Fig. 5E), similarly to the response induced by BPA in the cardiac endothelial cell fraction. To test whether RIP 3 mediates BPA-induced necroptosis, RIP 3 expression was silenced by siRNA and cell death assayed by flow cytometry. Administration of BPA in RIP3-silenced MAEC had no effect on the number of apoptotic cells (annexin V+/PI−), but necroptosis (annexinV+/PI+) was significantly reduced (Fig. 6A,B and Supplementary Fig. 5F), as well as the BPA-induced activation of CamKII (Fig. 6C). Pharmacological inhibition of CamKII activity with Kn-93 (KN) did not affect BPA induced RIP3 expression (Fig. 6D) but was able to decrease BPA induced necroptosis (Fig. 6E and Supplementary Fig. 6G). Thus, BPA stimulation of endothelial necroptosis proceeds via RIP 3 induction of CamKII signaling.

Taken together, these results suggest that BPA induces necroptosis in endothelial cells, contributing to coronary damage, vascular leakage, and chronic inflammation leading ultimately to myocardial injury.

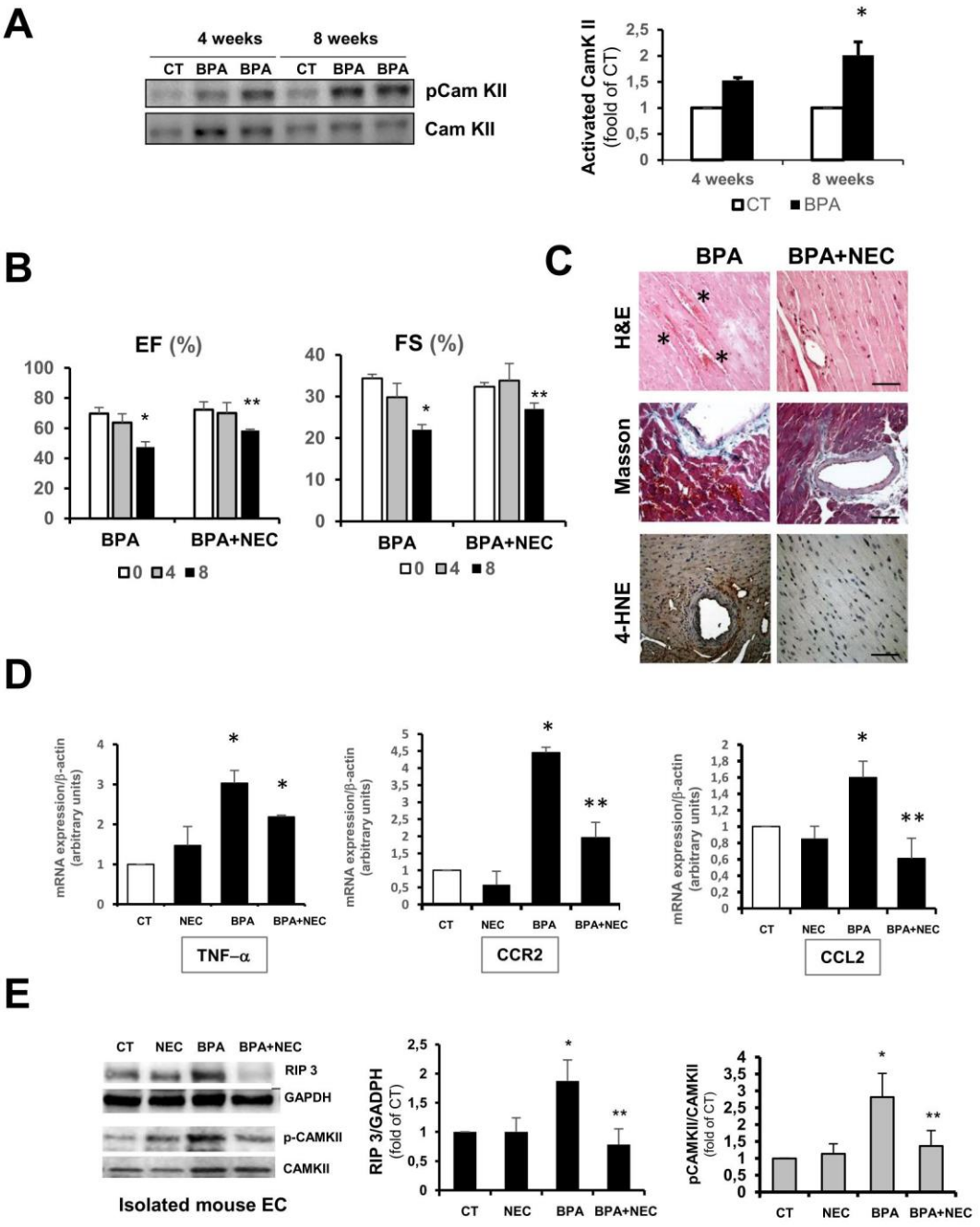

Figure 5. Necroptosis inhibition with necrostatin-1 reduces the adverse cardiac effects of BPA. (**A**) *Left panel:* representative immunoblot showing phosphorylated-CamKII and total CamKII protein expression in whole heart extracts from CT and BPA treated mice at 4 and 8 weeks. *Right panel:* densitometric analysis shown as mean ± SD (n = 4; *p < 0.05 vs control CT). Mice were treated with BPA and BPA and necrostatin-1 (NEC) for 4 and 8 weeks. (**B**) Echocardiography was performed at indicated time points for LV Ejection fraction (EF) and fractional shortening (FS) measurement (BPA n = 8 and BPA + NEC, n = 6 mice per group; *p < 0.05 vs. time 0, **p < 0.05 vs. BPA 8 weeks). (**C**) Representative photomicrographs from hearts stained with hematoxylin/eosin to visualize hemorrhagic lesions, Masson´s trichrome to detect fibrosis, and 4-hydroxinonenal as a marker of oxidative stress (n = 6 animals per group). Asterisk mark extensive hemorrhagic areas. Scale bar = 60 μm. (**D**) RT-qPCR of CT, NEC, BPA and BPA + NEC 8 weeks treated mice showing cardiac mRNA expression of TNF-α, CCL2 and CCR2 (n = 4 per condition with triplicates in each determination, *p < 0.05 vs. CT; **p < 0.01 vs. BPA). (**E**) Immunoblot analysis of RIP 3, phosphorylated-CamKII and total CamKII protein expression in protein extracts from freshly isolated ECs from CT, NEC, BPA, and BPA + NEC treated mice at 8 weeks. A representative immunoblot is shown and densitometric analysis shown as mean ± SD (n = 6; *p < 0.05 vs control CT; **p < 0.05 vs BPA).

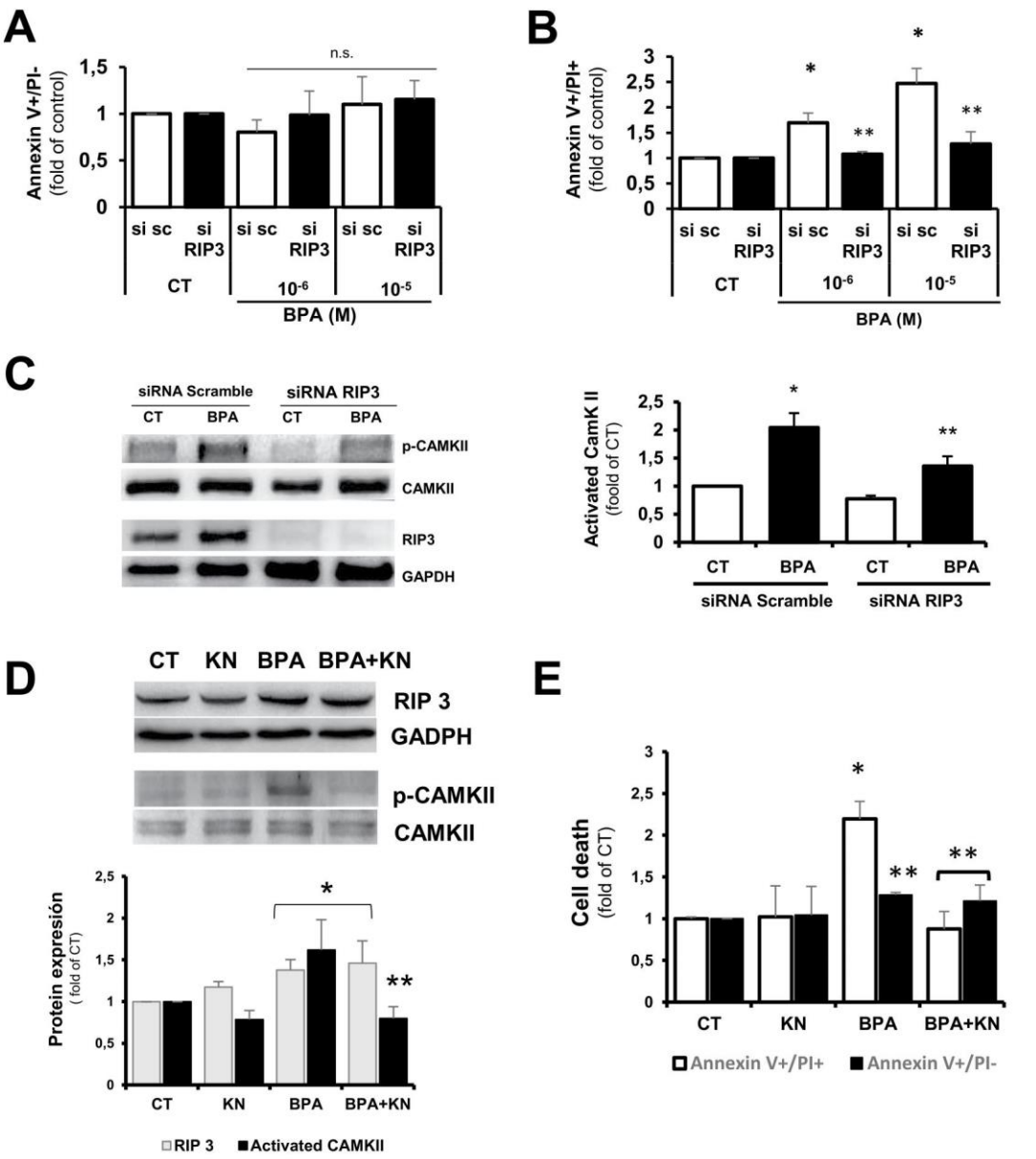

Figure 6. BPA induces endothelial cell necroptosis via RIP3/CamKII. (**A**,**B**) Flow cytometry analysis of MAEC transfected with RIP3-specific siRNA (si RIP3) or non-silencing siRNA scramble (si sc) and treated with BPA at 10^{-6} M and 10^{-5} M for 24 h. Cells were stained with annexin V-FITC and propidium iodide. Data are shown as mean ± SD (n = 4 per condition with duplicates in each determination). (**A**) apoptotic cells annexin V^+/IP^- (ns; non significant) and (**B**) annexin V^+/PI^+ cells. (*$p < 0.05$ vs. CT si sc, **$p < 0.05$ vs. BPA). (**C**) Immunoblot analysis of phosphorylated-CamKII, total CamKII, RIP3, and GADPH protein expression in CT and BPA (10^{-6} M) treated MAEC transfected as above. A representative experiment is shown (n = 4). Data are expressed as mean ± SD and referred to CT, *$p < 0.05$ versus CT siRNA-scramble MAEC; **$p < 0.05$ vs CT SiRNA RIP3. (**D**) Immunoblot analysis of RIP 3 and phosphorylated CamKII and total CamKII protein expression in protein extracts from MAEC treated with vehicle (CT), 10^{-6} M BPA (BPA), KN-93 (inhibitor of CamKII, 10^{-6} M) and BPA + KN for 24 h. A representative experiment is shown (n = 4). Data are presented as mean ± SD and refered to CT; *$p < 0.05$ vs. CT;**$p < 0.05$ vs. BPA. (**E**) Flow cytometry analysis of MAEC treated with vehicle (CT), BPA, KN-93, and BPA + KN for 24 h. Cells were stained with annexin V-FITC and propidium iodide. Data are shown as mean ± SD and referred to CT (n = 4 with duplicates in each determination,*$p < 0.05$ vs. CT or KN and **$p < 0.05$ vs. BPA AnexinV^+PI^+).

Discussion

In this study, we investigated the mechanism of BPA-induced damage in male mice hearts. Our results demonstrate that chronic administration of BPA causes hypertension and cardiac hypertrophy, leading to impaired cardiac function and cardiac hemorrhage. We have uncovered a novel pathogenic role of BPA to induce coronary endothelial cell death by necroptosis through the RIP3-CamKII pathway causing vascular leakage leading to cardiac hypoperfusion.

In our mouse model, BPA induced heart dysfunction with reduced EF, FS, increased IVS thickness, abnormalities on cardiac electric conductivity, hypertension, and cardiac congestion. These results are in agreement with several reports describing a pro-arrhythmogenic effect of BPA in female rat hearts[9,58] and impaired cardiac function and contractility through alterations of myocyte calcium handling initiated by estrogen receptor[15]. Although only male mice were used in our study, an effect mediated by the estrogen receptor cannot be excluded entirely. Nonetheless, our results show that BPA slowed electrical conduction in male hearts with first degree AV block, which is in agreement with BPA effects reported by Posnack NG *et al.* in rat hearts[59,60]. In addition, life long administration of BPA in doses similar to human exposure also demonstrated an arrhythmogenic effect with evidence of cardiomyopathy[61]. This effect is relevant to human health since slowed conduction could represent a mechanism of reentrant arrhythmias, which can also cause tachycardia[62] and could explain the increased heart rate observed in our mice.

Interestingly, the effect of BPA on electric conductivity has been found particularly pronounced under pathophysiological conditions such as stress or ischemic injury[9,10]. Indeed, dobutamine stress tests revealed that chronotropic and inotropic responses were impaired, and the coronary reserve seemed compromised in BPA treated mice. Since BPA may cause vasoconstriction due to a decrease in nitric oxide (NO) production and therefore decreased blood supply to the heart, ischemia could account for the arrhythmogenic behavior found in our study[23]. Besides, BPA treated mice might have a defect on the adrenergic signaling pathway, which could compromise Ca^{2+} homeostasis or in G-protein–coupled receptor signaling[7]. Moreover, BPA treated mice exhibit increased expression and activity of CamKII, which is also increased in animal models of heart failure and in humans in failing or arrhythmia-prone myocardium, pointing to a possible mechanism to explain BPA induced cardiac contractile dysfunction and arrhythmias[55].

Our studies detected the infiltration of macrophages into the heart early after BPA treatment, accompanied by an increased expression of inflammatory cytokines CCL2, TNF-α, CCR2, CCL7 and CCL-12, markers of inflammatory M1 macrophages even at 16 weeks after exposure to BPA. $CCR2^{+}$ macrophages could be detected at 8 weeks. In animal models of pressure overload, $CCR2^{+}$ macrophages play a critical role leading to subsequent cardiac T-cell expansion, pathological LV remodeling, and late heart failure[63]. The presence of $CCR2^{+}$ macrophages, together with reduced myocardial vascularization, may be indicative of defective reparative processes. In agreement with our results, chronic administration of BPA or its substitute, Bisphenol S (BPS), can reduce active cardiac remodeling in response to myocardial infarction, increasing metalloproteinase (MMP) expression in male mice[12]. Moreover, the exposure of resting monocytes to BPA increased polarization to activated macrophages[64]. It has also been reported that positive CCR2 macrophages enriched in the AV node may cause abnormal atrioventricular electrical conduction[65]. Thus, recruitment of $CCR2^{+}$ pro-inflammatory macrophages may also contribute to the arrhythmogenic AV block in response to chronic exposure to BPA.

Most studies about the cardiac effect of BPA focused on electrical conduction and structural or metabolic abnormalities on cardiomyocytes, however, data on its effects on cardiac endothelial cells are still missing. Interestingly, BPA cardiac effects presented accompanied by myocardial hemorrhage. Gear *et al.*, described that female rats exposed to higher doses of BPA for six months developed a cardiomyopathy with hemosiderin containing macrophages, indicative of previous microvasculature damage and hemorrhage[11]. However, the potential causes of BPA induced hemorrhages had not been investigated so far. Our first hypothesis was that BPA targeted the coagulation cascade, but none of the coagulation parameters were altered. Therefore,we speculated that hemorrhages could be secondary to ischemia and hypertension as cardiac hemorrhagic lesions have been described in response to ischemic reperfusion[66]. Here, we observed BPA-induced hemorrhagic foci already at 4 weeks, and although no ischemic events were detected in the ECG in resting conditions, after dobutamine challenge, 70% of the mice showed ST segments depression, indicative of cardiac ischemia. Furthermore, heart rate increased in BPA treated mice and some animals presented even further increases in HR after 8 weeks of BPA treatment. These conditions can contribute to increased oxidative stress, as detected by increased lipid peroxidation, and accumulation of oxidative insults could lead to endothelial wall injury mimicking the conditions present during ischemia-reperfusion. Accordingly, Aboul Ezz *et al.* reported increased lipid peroxidation, decreased glutathione (GSH) levels and catalase activity after 6 to 10-week BPA administration[67]. We also found decreased blood vessel density in BPA treated-mice, which, together with the increased cardiomyocyte mass, could also reduce myocardial oxygen supply leading to ischemia.

The increased Evans Blue extravasation in the hearts and lungs of BPA treated mice support the hypothesis that BPA increases vascular permeability contributing to heart damage. Indeed, the extensive hemorrhagic areas observed at eight weeks also indicate vascular fragility and rupture of coronary microvessels. We have demonstrated that BPA decreases endothelial NO and increases oxidative stress leading to endothelial dysfunction and hypertension[23]. Since BPA can cause hypertension in humans[8] and mice[23], another cause for the hemorrhages may be related to hypertensive degenerative or inflammatory changes affecting small arterioles, which could cause structural weakening and eventually vascular wall rupture. Studies on mouse models of hypertension-induced cerebral microvascular hemorrhages (CMH) demonstrate that pathogenesis of the CMHs involves a weakening of the vessel wall by upregulation of MMPs or oxidative stress-dependent activation of MMPs, such as MMP2 and MMP9[68]. Multiple lines of evidence suggest that NO regulates activation or expression of MMPs, and that decreased NO bioavailability promotes MMP activation in the vascular wall[69,70]. In the present study, we showed that hypertension increases at 16 weeks. Although MMPs levels were not examined here, others have recently described in a model of lifelong exposure to BPA a decreased recovery after myocardial infarction associated with adverse cardiac remodeling due to increased MMP2 and MMP9 expression[12]. Besides, Belcher *et al.* reported that cardiac transcriptome after BPA exposure caused sex-specific dysregulation of the collagen extracellular matrix (ECM)[7]. Since BPA impairs NO production due to eNOS uncoupling, BPA could likely affect ECM turnover via NO signaling leading to a weakening of coronary vascular wall, which in addition to ischemia, could be the culprit behind microhemorrhages. A detailed characterization of the molecular mechanism responsible for early microvascular hemorrhage is critical to

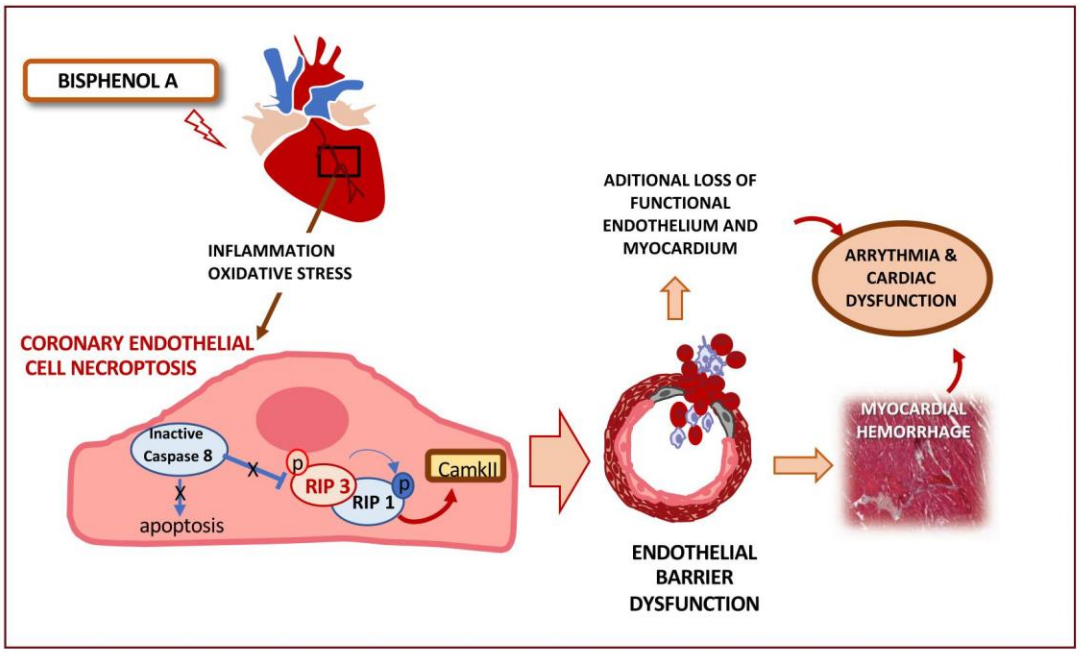

Figure 7. Mechanism of the cardiotoxic effects of BPA. BPA induces early inflammation and oxidative stress leading to endothelial cell death. BPA activates the RIP 3-CamKII necroptotic pathway at endothelial cells. Necroptosis could be contributing to exacerbate the inflammatory conditions induced by BPA although direct effects on myocardium can not be excluded. Decreased endothelial barrier function and weakening of the coronary vascular wall in the setting of hypertension may cause ventricular hemorrhages, cardiac and lung congestion, ultimately leading to heart failure.

understand the cardiac effects of BPA, especially during cardiac ischemia, since myocardial hemorrhage has been associated with human adverse remodeling and adverse health outcome in the longer term[71].

Here, we provide a potential mechanism for detrimental cardiac effects of BPA *in vivo*, finding the activation of necroptosis cascade as the cause of endothelial cell death leading to hypoperfusion and ischemia. Our data showed increased cell death in hearts from BPA treated mice, especially in the areas containing blood vessels, in the absence of increased apoptotic markers. Necroptosis is a newly discovered pathway of programmed cell death with an essential role in human ischemic injury[72]. RIP 3 is a critical determinant in the necroptotic pathway and is expressed in the myocardium[73]. The process of necroptosis is dependent on the activation of the RIP 1–RIP 3–MLKL axis initiated by inflammatory TNF-α and other pro-apoptotic stimuli such as oxidative stress[74]. Our results establish a role for RIP 3 in BPA-induced endothelial cell injury since, since RIP 3 levels increased only in endothelial cells and perivascular areas while this protein was not found increased in cardiomyocytes. Nonetheless, we can not exclude a BPA role in RIP 3 mediated necroptosis in fibroblast or macrophages. The necroptotic pathway is also proven to relate to vascular disease as elevated RIP 3 levels are detected in ischemic stroke, atherosclerosis, and aortic aneurisms[74]. Interestingly, BPA decreased endothelial cell viability in a dose-dependent manner but failed to induce apoptotic cell death. In our study, gene silencing of RIP 3 reversed the effect of BPA on endothelial cell necroptosis. It has been reported that RIP 3 knockout decreases myocardial necroptosis in myocardial infarction[73]. Accordingly, the treatment of mice with BPA and the necroptosis inhibitor NEC-1 reversed cardiotoxic effects of BPA producing fewer hemorrhages and less inflammation. In support of our results, Chen J. *et al.* showed that NEC-1 prevented the disruption of the brain-blood barrier reducing brain hemorrhages[75]. However, NEC-1 only induced a moderate improvement of cardiac function so the possibility of a direct effect of BPA on myocardium by a different pathway cannot be completely ruled out.

Two mechanisms can contribute to BPA triggering the necroptotic pathway: early inflammation, which increased TNF-α, a known initiator of RIP 1/3 necroptosis, and, secondly, inhibition of caspase-8 activity which allowed RIP 3 phosphorylation and activation. Recent reports documented that RIP3 can also facilitate inflammation independent of myocardial necrosis by releasing damage-associated molecular patterns (DAMPS)[76]. In our study, inflammation appears as early as 4 weeks and remains elevated after 16 weeks. Thus, RIP 3-induced necroptosis could be initiated by TNF-α and oxidative stress, and later could exacerbate the inflammatory conditions induced by BPA in later stages. The necroptosis-inflammatory response might explain the lack of M2 phenotype macrophages found at 16 weeks. This hypothesis is supported by the decreased inflammation found in BPA + NEC-1-treated mouse.

BPA-dependent cardiac damage could be initiated by inflammation and oxidative stress increasing CamKII activation. Moreover, oxidation of CamKII might be the primary mechanism for myocardial rupture following myocardial infarction[77]. Here we have shown increased activation of CamKII in hearts from 4 and 8 weeks BPA treated mice. CamKII activation by autophosphorylation and oxidation has been proposed as a potential mechanism to activate necroptosis and conversely CamKII has been proposed as an alternative substrate of RIP 3,

leading to myocardial necroptosis during ischemia-reperfusion injury[28]. Our data demonstrate that inhibition of CamKII activity with KN-93 decreases necroptosis but does not affect BPA increased-RIP 3. However, RIP 3 silencing prevented CamKII phosphorylation and necroptosis induction. Therefore our results point to RIP 3 acting through CamKII to induce necroptosis in response to BPA.

In summary, our study provides a mechanism to explain the cardiotoxic effects of BPA. BPA activates the RIP 3-CamKII necroptotic pathway leading to endothelial cell death. Decreased endothelial barrier function and weakening of the coronary vascular wall in the setting of hypertension may cause ventricular hemorrhages, cardiac, and lung congestion, which ultimately led to heart failure (summarized in Fig. 7). Targeting the RIP 3 pathway may have beneficial effects in BPA induced cardiac damage, mainly when associated with ischemia.

Our results are particularly interesting because exposure to this common environmental chemical could pose an additional risk for individuals with preexisting cardiac conduction abnormalities, cardiac disease, and other cardiovascular risk factors.

Data availability

All data generated or analyzed during this study are included in this published article and the Supplementary Information files

Received: 29 May 2019; Accepted: 20 February 2020;
Published online: 06 March 2020

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Acknowledgements
This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02139) - Cofinanced by the European Development Regional Fund A way to achieve Europe (FEDER)-. Moreno-Gomez-Toledano R is a recipient of a research contract from Comunidad Autónoma de Madrid-Programa de Actividades I + D en Biomedicina 2017- (B2017-BMD-3686). P. Reventún is a fellow of the program "Ayuda para contratos predoctorales para personal investigador en formación" from UAH. A. Cook is a recipient of an " Ayuda Iniciación a la Investigación" from UAH.

Author contributions
P.R. and S.S. contributed equally to this work. P.R., S.S, I.C., A.C. and R.M. conducted experiments, performed data analysis and interpreation and prepared the Figures. C.R. and C.M. conducted experiments and performed data analysis. R.J.B. Contributed in the conception and design of the study, performed a critical revision of the manuscript and provided grant funding. C.Z. Conducted experiments and performed data analysis. Conception and design of the experiments and critical revision of the manuscript. M.S. Conception design and supervision of the experiments, drafting and critical revision of the manuscript and participated in the experiments, analysis and interpretation of data. Was involved in project administration, supervision of the study and provided research resources and grant funding. All authos read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-61014-1.

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XIV. ANEXO V

BISPHENOL A INDUCES ACCELERATED CELL AGING IN MURINE ENDOTHELIUM. **BIOMOLECULES** (4.879, Q2 BIOCHEMISTRY & MOLECULAR BIOLOGY [2020]).

DOI: 10.3390/BIOM11101429

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Bisphenol A Induces Accelerated Cell Aging in Murine Endothelium

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Abstract: Bisphenol A (BPA) is a widespread endocrine disruptor affecting many organs and systems. Previous work in our laboratory demonstrated that BPA could induce death due to necroptosis in murine aortic endothelial cells (MAECs). This work aims to evaluate the possible involvement of BPA-induced senescence mechanisms in endothelial cells. The β-Gal assays showed interesting differences in cell senescence at relatively low doses (100 nM and 5 μM). Western blots confirmed that proteins involved in senescence mechanisms, p16 and p21, were overexpressed in the presence of BPA. In addition, the UPR (unfolding protein response) system, which is part of the senescent phenotype, was also explored by Western blot and qPCR, confirming the involvement of the PERK-ATF4-CHOP pathway (related to pathological processes). The endothelium of mice treated with BPA showed an evident increase in the expression of the proteins p16, p21, and CHOP, confirming the results observed in cells. Our results demonstrate that oxidative stress induced by BPA leads to UPR activation and senescence since pretreatment with N-acetylcysteine (NAC) in BPA-treated cells reduced the percentage of senescent cells prevented the overexpression of proteins related to BPA-induced senescence and reduced the activation of the UPR system. The results suggest that BPA participates actively in accelerated cell aging mechanisms, affecting the vascular endothelium and promoting cardiovascular diseases.

Keywords: bisphenol A; murine aortic endothelial cell; senescence; unfolding protein response

Citation: Moreno-Gómez-Toledano, R.; Sánchez-Esteban, S.; Cook, A.; Mínguez-Moratinos, M.; Ramírez-Carracedo, R.; Reventún, P.; Delgado-Marín, M.; Bosch, R.J.; Saura, M. Bisphenol A Induces Accelerated Cell Aging in Murine Endothelium. *Biomolecules* **2021**, *11*, 1429. https://doi.org/10.3390/biom11101429

Academic Editor: Ryan Moseley

Received: 8 August 2021
Accepted: 26 September 2021
Published: 29 September 2021

1. Introduction

Bisphenol A (BPA) is a phenolic-type molecule widely used in the manufacture of plastics. Its ability to improve the properties of plastics and its role as a monomer in synthesizing polycarbonates and epoxy resins have made it an element present in many everyday objects [1,2]. BPA has become an essential element for many global industries, from food containers, toys, and clothing to electronic devices and medical-surgical supplies [1,3–7]. Its production and demand increase every year, and the trend is expected to continue upward in the coming years [8].

BPA is classified as an endocrine disruptor or xenoestrogen due to its ability to bind to estrogen receptors [9]. For this reason, numerous studies are exploring its possible effects on both the female and male genitourinary systems [10–13]. However, in the last two decades, evidence correlates BPA with other types of pathologies such as diabetes, obesity, and even cognitive and behavioral disorders [14–17]. Our group has recently found new evidence that positions BPA as a possible environmental factor promoting nephro-vascular pathologies [18–21]. Currently, few studies explore the cardiovascular system, but there are population studies that correlate high concentrations of urinary BPA with an increased risk of developing arterial hypertension [22,23].

A process highly implicated in the development of cardiovascular pathologies is aging and cellular senescence [24]. Cellular senescence is a process that can be considered a hallmark of aging [25] and is characterized by the irreversible arrest of the cell cycle in the presence of different inducing factors, such as telomere dysfunctions, oxidative stress, activation of oncogenes, or cell damage [26]. Senescence is frequently called quiescence, although there are notable differences between the two processes at the morphological and molecular levels [27]. The p53/p21 and p16/pRB pathways are classical pathways regulating cellular senescence [26]. There is substantial evidence that supports the close relationship between cellular senescence and cardiovascular disorders. In humans, significant increases in the expression of senescence proteins have been observed in endothelial cells of aged sedentary individuals, compared with aged exercising adults (57–60 years old) [24]. Furthermore, there is evidence that BPA could be involved in the acceleration of cellular aging in different cell lines [28,29], species [30], as well as in human studies [31]. We previously reported that BPA can induce arterial hypertension through angiotensin II (AngII)-mediated eNOS uncoupling [18]. Importantly, AngII stimulation recapitulates the senescent characteristics in vascular cells, which may contribute to the acceleration of atherosclerosis [32,33]. Aged human [34] and murine [35,36] arterial endothelial cells as well as EPCs [37,38] also show heightened levels of ROS partially due to activation of NADPH oxidase, which further uncouples eNOS [34,39,40]. All of the above led us to formulate the hypothesis that BPA could induce endothelial cell senescence.

A fundamental component activated in response to stress within the senescent phenotype is the unfolding protein response (UPR). The activation of proteins of the UPR system in response to senescence-inducing stimuli has been observed in different cell lines, and the inhibition of some of these proteins can modify the percentage of SA-β-galactosidase-positive cells [41]. The UPR system has three main signaling pathways, initiated by the ATF6, IRE1, and PERK stress sensors. Activation of each sensor activates the transcription factors ATF6 (N), XBP1, and ATF4, respectively. These factors are capable of increasing the folding capacity of the endoplasmic reticulum [42]. An important difference between the signaling pathways is that the PERK-ATF4-CHOP pathway is related to myocardial ischemia, hypertension, cardiac atrophy, hypertrophy, or even vascular calcification [43–47], while the IRE1 and ATF6 pathways may have cardioprotective effects [43,48].

Classically, the PERK-ATF4-CHOP signaling pathway has been associated with cell apoptosis [49–52]. Other authors have observed that the treatment of BPA at cytotoxic concentrations in non-parenchymal hepatocytes mice (100 μM) [51] or mouse spermatocytes GC-2 cells (20–80 μM) [52] induced a significant increase in apoptotic processes related to ER stress. However, there is evidence in the literature that the reduction in the PERK-ATF4-CHOP pathway can reduce the number of senescent cells in certain cell lines, such as melanocytes [41]. It has even been observed that the overexpression of CHOP in senescent cells does not lead to cell death [53].

The present work aims to explore the role of BPA in endothelial cell senescence, exploring the involvement of the unfolding protein response. Thus, BPA may contribute to cardiovascular diseases by activating this mechanism of accelerated cellular aging.

2. Materials and Methods

2.1. Cell Culture

Murine aortic endothelial cells (MAECs) were isolated from mouse aorta, as previously reported [19,54]. Briefly, the aortas were sectioned a deposited in Matrigel solution and fed with fresh growth medium for seven days (DMEM/HAM's medium, 20% FBS, 0.05 mg/mL penicillin/streptomycin, and 2.5 μg/mL amphotericin). The tissue was removed, and 500 μL of cell recovery solution was added to each culture. The solution was centrifuged at 4 °C, resuspended in 4 mL of growing medium, and plated. MAECs were selected by their ability to express the intercellular adhesion molecule-2 (ICAM-2) protein and purified with a flow cytometry cell sorter (DAKO, Carpinteria, CA, USA). Purification was verified by confocal microscopy of MAECs double-stained with Von Willebrand factor antibodies.

MAEC were cultured with Dulbecco's Modified Eagle's Medium (DMEM/F12), supplemented with 0.05 mg/mL penicillin/streptomycin, 2.5 µg/mL amphotericin, and 10% fetal bovine serum (Gibco, Waltham, MA, USA) in a humidified CO_2 incubator with 5% CO_2 at 37 °C. MAEC were used between passage 4 and 9. BPA concentrations between 1 nM and 100 µM were used to delimit the cytotoxicity on the MTT assay. Subsequently, in the senescence test, doses lower than cytotoxic in the range of 1 nM–5 µM were used (5 µM is half of the maximum concentration at which no cytotoxic effects were observed). The two concentrations at which the highest effects on cell senescence were observed (100 nM and 5 µM) were used for Western blot and qPCR. Finally, in the approach with the antioxidant N-Acetyl-L-cysteine (NAC) (Sigma, San Luis, MO, USA), a concentration commonly used in the academic literature was used, 5 mM two hours before treatment with BPA [55,56].

2.2. Animal Model

Wild-type CD1 mice were purchased from Charles River (Wilmington, MA, USA) and housed in our animal facilities with four mice/cage located in isolated rooms. All animal procedures were approved by the University of Alcala Animal Care Committee and Autonomous Community of Madrid) and conformed to the EU directive regarding protecting animals used for experimental and other scientific purposes (enacted under Spanish law 1201/2005).Three-month-old CD1 male mice (~40 g weight) were distributed into two groups: control and BPA. Control treatment consisted of an equivalent volume of ethanol (final concentration 0.01%) in drinking water. Ethanol dissolved-BPA was added to the drinking water at a final concentration of 150 µg/mL. This dose of BPA is equivalent to 25 mg/kg; that is, half of the concentration used by the European Food Safety Authority as a reference in the calculation of the tolerable daily intake [1] and also the U.S. National Toxicology Program defines doses less than 50 mg/kg/day as "low dose" [57]. Furthermore, in the CLARITY-BPA study (Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity), one of the largest animal studies conducted in the context of BPA, the authors stated, "BPA did not elicit adverse effects in the in-life or terminal endpoints monitored in either sex below 25,000 µg/kg bw/day" [58]. However, other authors suggest that some results described in this study show non-monotonic effects, reaffirming the need to study the effects of BPA at low doses [59].

Mice received BPA or vehicle in the drinking water for 8 weeks. Mice were given free access to water and drank approximately 5 mL/day/mouse [18]. At the end of the experiment, animals were sacrificed, and aortas were collected and stored for posterior Western blot and immunofluorescence.

2.3. MTT Cell Viability Assay

MAEC were then seeded into 24 well plates (1500 cells/well) in complete medium. After overnight incubation, the medium was removed, and 1 mL of growth culture containing a series of different concentrations of BPA ranging from 0 (control) to 100 µM was added during 5 days. After BPA treatment, 100 µL of MTT (5 mg/mL) were added to each well in 900 µL of the medium, and the plates were incubated for 3 h at 37 °C. Then, DMSO (Sigma, San Luis, MO, USA) was added to solubilize the formazan crystals. The absorbance was measured at a test wavelength of 570 nm [60].

2.4. Senescence-Associated β-Galactosidase Assay

Senescence-associated β-Galactosidase (β-Gal) staining was performed on mouse aortic endothelial cells (MAEC) using a senescence detection kit (Abcam) according to the manufacturer's protocol. Briefly, cells were fixed and incubated with staining solution mix for 12 h at 37 °C. After that time, the cells were photographed under the microscope (Nikon DIAPHOT 300, Tokyo, Japan). Eight photographs were taken per well in different areas using the 10× objective. Afterward, a count of senescent and total cells was performed, and the data were analyzed.

2.5. Western Blot

Protein lysates were immunoblotted as previously described [61]. Total protein was separated in SDS-polyacrylamide gel electrophoresis and transferred to a PDVF membrane. For protein detection, blocked membranes were incubated with specific antibodies, washed, and incubated with a secondary antibody. Immunoreactive bands were visualized with the SuperSignal detection system Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Primary antibodies used: p16 from Abcam (reference ab51243; 1:1000), p21 from eBioScience (reference 14-6715-81; 1:1000), CHOP from Cell Signaling (reference 5554S; 1:500), XPB1 from Sigma (reference SAB2102720; 1:500) and β-Actin from Sigma (reference A2066; 1:1000).

2.6. Protein Oxidation

Protein oxidation detected by reaction with 2,4-dinitrophenyl hydrazine (DNP) using an OxyBlot™ Protein Oxidation Detection Kit (Sigma, San Luis, MO, USA). Briefly, samples were denatured by SDS, and the carbonyl groups in the protein side chains were derivatized to DNP-hydrazone by reaction with DNPH. The proteins were electrophoresed on an SDS-PAGE gel and followed by immunoblotting of the anti-DNP antibody (1:150). The same membrane was incubated with the anti-b-actin antibody for loading control. For densitometric analysis, the proteins migrating between 97 and 68 KDa were analyzed.

2.7. qPCR

Total RNA was extracted with TRIzol reagent from Invitrogen Corporation (Carlsbad, CA, USA) following the manufacturer´s instructions. First-strand cDNA was synthesized from 2 μg of total RNA in a 20 μL reaction mixture using the High-Capacity cDNA reverse transcription kit, and the qPCR reaction was performed with SYBR select master mix both of Life technologies (Carlsbad, CA, USA). The qPCR conditions were standard cycling mode (primer Tm $\geq$ 60 °C) first UDG activation 50 °C 2 min, then AmpliTaq® DNA polymerase, UP activation 95 °C 2 min, denature 95 °C 15 s and anneal/Extend 60 °C 1 min. The following primers were used: CHOP forward 5′ CAG GAG AAC GAG CGG AAA GTG G 3′, CHOP reverse 5′ TGC TGG GTA CAC TTC CGG AGA G 3′, ATF-6 forward 5′ TTC GAG GCT GGG TTC ATA G 3′, ATF-6 reverse 5′ GGG AGG CGT AAT ACA CTT 3′, PERK forward 5′ ATG CAC AGG GAC CTC AAG 3′, PERK reverse 5′ CTG CTC TGG GCT CAT GTA TAG 3′, IRE-1α forward 5′ AAG ATG GAC TGG CGG GAG AAC IRE-1α reverse 5′ GGG AAG CGG GAA GTG AAG TAG 3′, Actin forward 5′ CGA TGC CCT GAG GCT CTT T 3′, Actin reverse 5′ TGG ATG CCA CAG GAT TCC A 3′.

2.8. Immunohistochemistry

Aorta arteries were fixed in a 10% formalin solution, dehydrated in ethanol, and then embedded in paraffin as previously described [62]. Tissue sections (5 μm) were obtained in a microtome, were deparaffinized, rehydrated, and stained. Samples were boiled in retrieval buffer for 20 min after xylene deparaffinization. A Mouse- and Rabbit-Specific HRP/DAB (ABC) Detection IHC kit (Abcam, Cambrige, UK) was used according to the manufacturer's protocol, and antibody incubation was overnight at 4 °C. Sections were counterstained with Carazzi hematoxylin, dehydrated, and mounted with DPX (Casa Alvarez, Madrid, Spain). Images obtained of at least five different animals per condition were taken for data quantification using a bright-field microscope (Eclipse 50i; Nikon, Tokyo, Japan). Primary antibodies used in immunohistochemistry: p16 from Abcam (reference ab51243; 1:50), p21 from eBioScience (reference 14-6715-81; 1:50) and CHOP from Cell Signaling (reference 5554S; 1:50).

2.9. Confocal Microscopy

Slides containing tissue sections were incubated with the primary antibodies overnight 4 °C. After washing with PBS, the slides were incubated with FITC, Alexa-488, or Alexa-647-conjugated secondary antibodies for 1 hour at room temperature. Isolectin IB4 labeled

with FITC was used as a specific marker of vascular endothelium. Nuclei were stained with Hoechst. Images were taken for data quantification using a Leica TCS SP5 confocal microscope (UAH-NANBIOSIS-CIBER-BNN). At least five different fields per condition were obtained. Quantification of signal intensity was performed using Image J software (NIH Bethesda, MD, USA).

2.10. Superoxide Anion Production

MAECs were incubated with PBS (control) or BPA (5 μM) for 24 h. On the day of the experiment, cells were treated with 10^{-5} M apocynin for 30 min to inhibit NADPH oxidase and then stimulated with eNOS agonists. After exposure to different experimental conditions, cells were trypsin dispersed and labeled with the DHE at 37 °C. Cells were analyzed by FACS Calibur (Becton Dickinson Company, Franklin Lakes, NJ, USA). A total of 10,000 events were analyzed for each condition.

2.11. Statistical Analysis

All results were expressed as mean ± standard deviation (SD). GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. First, the data distribution was analyzed using the D'Agostino-Pearson and Shapiro–Wilk normality tests. Subsequently, one-way ANOVA or Kruskal–Wallis followed by a Bonferroni or Dunns' test, respectively, were carried out. The *p*-values presented in figures and tables correspond to the post hoc test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. BPA Increases Cellular Senescence at Low Concentrations

Previously, we have reported that BPA can induce a substantial reduction in cell viability at high concentrations in different cell lines, including endothelial cells at 24 h [18,20]. To test the effects on cell viability of longer BPA administration, we extended our study to 5 days. As is shown in Figure 1A, MAEC treatment with BPA did not induce any change in cell viability at BPA concentrations below 100 μM. However, it induces a significant reduction in cell viability at concentrations of 100 μM at 5 days. To test whether BPA may induce cell senescence, we performed a β-Gal assay. Five days BPA treatment, at concentrations below the cytotoxic threshold (1 nM–5 μM), induced a concentration-dependent increase in the percentage of β-Gal-positive cells. As shown in Figure 1B, the concentrations that induced significant changes were 100 nM and 5 μM, half of the highest concentration at which no effects on cell viability were observed.

To confirm the results observed in the β-Gal functional assay, relative expression of the senescence marker proteins p21 and p16 were analyzed. We used 100 nM and 5 μM BPA concentrations, which induced maximal changes in the previous β-gal assay. After five days of BPA treatment, both p21 and p16 proteins expression increased compared with the control group only at 5 μM BPA. No detectable differences in protein expression were found at 100 nM BPA (Figure 2).

Since oxidative stress is one of the stressors capable of inducing the senescence program, we studied if oxidative stress was preceding the effects observed. BPA treatment for 24 h induced an increase in carbonylated proteins, which are surrogate markers of protein oxidation in a dose-dependent manner. Accordingly, superoxide generation as detected by DHE staining of DNA in MAEC was increased at 5 μM BPA as early as 4 h, as demonstrated by orange nuclear staining. Superoxide production in MAEC treated with BPA 5 μM for 24 h and stimulated with eNOS agonists VEGF (50 ng/mL, 3 min) and A23187 (10^{-6} M, 5 min) was quantified in the presence of an inhibitor of NADPH oxidase (apocynin, 10^{-5} M). We observed an increased production of superoxide in the presence of BPA, which was enhanced with VEGF and calcium ionophore A23187. This result suggests that eNOS uncoupling could be contributing to the increased superoxide. In summary, BPA induces oxidative stress at early time points and stimulates cellular senescence in endothelial cells.

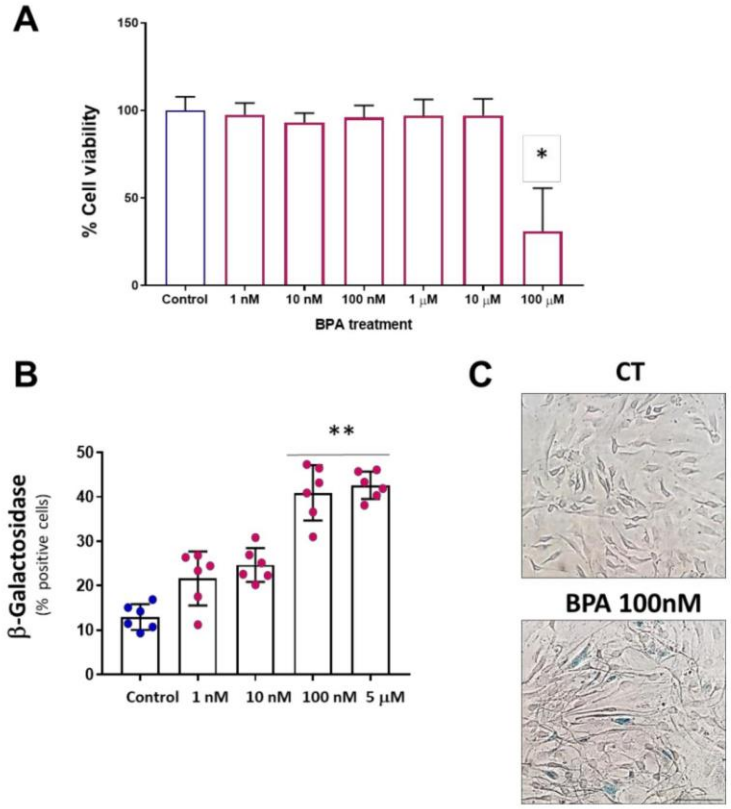

Figure 1. BPA induces cellular senescence at low concentrations. (**A**) MTT viability assay in MAEC treated with BPA 5 days at concentrations ranging from 1nM to 100 μM. A significant reduction in cell viability is observed at 100 μM. Data are the means ± SD (n = 3 with a triplicate per experimental condition). *p* was determined by a Kruskal–Wallis test with Dunn's multiple comparisons test. * $p < 0.0001$. (**B**) Senescence-associated β-Gal assay in MAEC treated with BPA 5 days at the indicated concentrations. Note that the maximum effect on cell senescence was observed at concentrations of 100 nM and 5 μM. Data points represent the mean ± SD (n = 6 in triplicate). *p* was determined by a Kruskal–Wallis test with Dunn's multiple comparisons test. ** $p < 0.001$ (**C**). Representative microphotographs of the senescence assay (scale bar = 50 μm). Senescent cells show the characteristic staining of β-Gal in blue color, CT means control group.

3.2. BPA Modulates the PERK-ATF4-CHOP Pathway after Five Days Treatment

Since the UPR is known to be part of the senescent phenotype, we next explored whether UPR is involved in BPA responses in MAEC. As shown in Figure 3A, BPA treatment induced an increase in the PERK-ATF4-CHOP pathway. Slight increases in BIP and ATF6 mRNAs expression are observed at 5 μM, although they were not statistically significant. In addition, the relative expression of two essential proteins of the UPR system, XBP1 and CHOP, was also analyzed (Figure 3B). On the one hand, the IRE-XBP1 signaling pathway did not show significant changes. On the other hand, the CHOP pathway only showed significant changes at 100 nM.

Thus, only the PERK-ATF4-CHOP branch of UPR is activated in the vascular endothelial cells after BPA treatment and may contribute to the senescent phenotype observed.

3.3. BPA Induces Senescence and Activation of the PERK-ATF4-CHOP Pathway in Mice

To test the potential of BPA to trigger senescence in vivo, 3-month-old CD1 mice were administered BPA in the drinking water for eight weeks. p16 and p21, the main proteins involved in stress-associated cell senescence, were overexpressed in the endothelium of

animals treated with BPA compared to the control mice (Figure 4A). In addition, there was also an increase in CHOP expression, confirming the participation of this pathway in the senescent response to BPA. Next, we examined the expression of senescence proteins p16 and p21 in the aorta by Western blot. Quantitative analysis showed a significant increase in p16 and p21 levels in BPA-treated mice compared to control (Figure 4B).

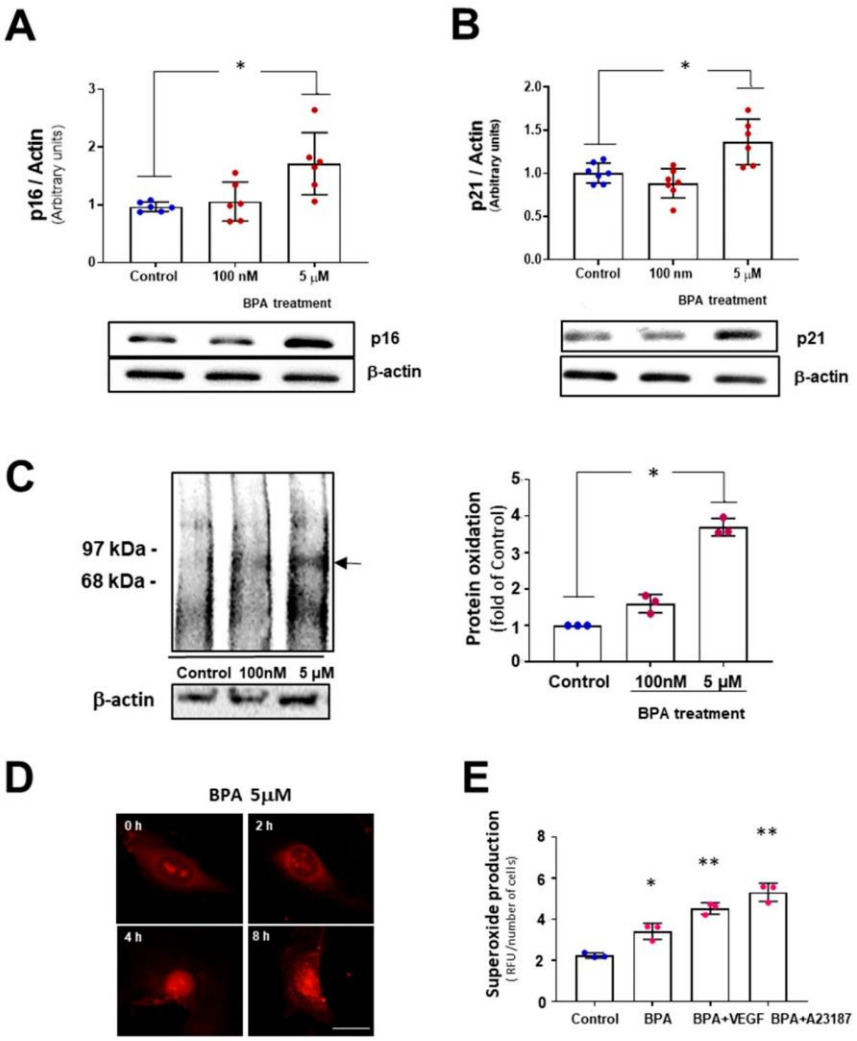

Figure 2. BPA induces senescent protein markers p21 and p16 and increases oxidative stress. Western blotting analysis of MAEC treated with 100 nM and 5 μM BPA for five days using antibodies against (**A**) p21 and (**B**) p16. β-actin was used as a loading control. (n = 6). Results are given as mean ± SD, *p* was determined by a Kruskal–Wallis test for the comparison between control and BPA-treated cells.* $p < 0.05$); (**C**) The levels of reactive oxygen species induced in MAEC by BPA were analyzed by oxyblot assay in 100 nM and 5 μM BPA-treated MAEC for 24 h to detect carbonyl groups in proteins as a marker of protein oxidation. The densities of the proteins between 97 and 68 KDa were normalized using the expression of β-actin. (n = 3 with duplicate for each condition).* $p < 0.001$ using Kruskal–Wallis test for the comparison between control and BPA-treated cells; (**D**) Immunofluorescence (IF) of superoxide production in MAEC in response to 5 μM BPA for 0–8 h, using the fluorescence probe dihydroetidium (DHE). After 4 h, fluorescence can be observed at the cell nucleus. Scale bar: 10 μm; (**E**) Quantification of superoxide in MAEC upon 5 μM BPA treatment for 24 h, BPA+VEGF and BPA+ A23187 and vehicle (control). (n = 3, per duplicate). Results are given as mean ± SD, *p* was determined by a Kruskal–Wallis test, * $p < 0.001$ vs. Control and ** $p < 0.001$ vs. BPA.

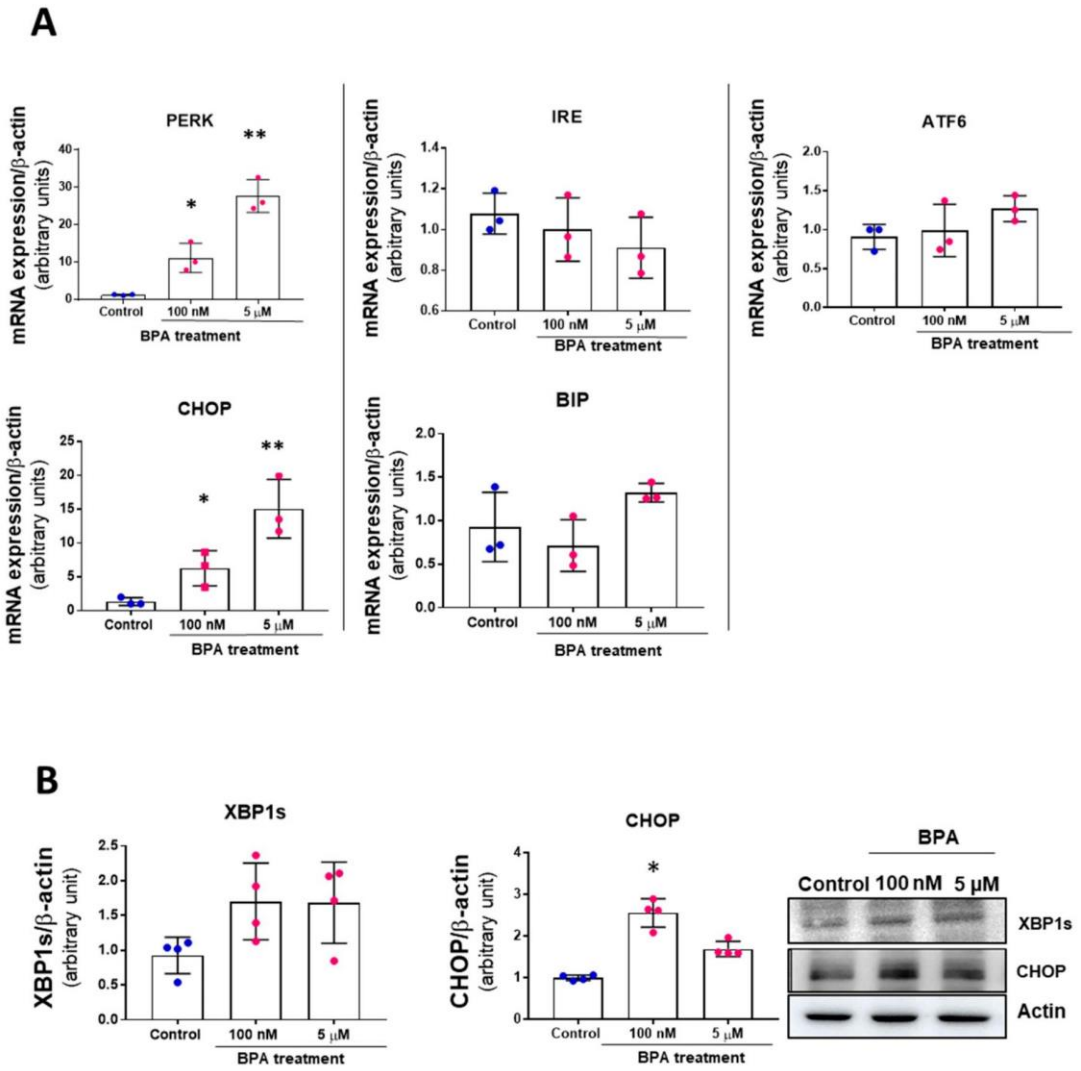

Figure 3. BPA induces the PERK/ATF4/CHOP branch of the UPR. (**A**) RT qPCR of control and 5 days BPA MAEC showing endothelial mRNA expression of PERK, IRE, ATF6, CHOP, and BIP. mRNA expression was normalized by β-actin. (**B**) MAEC were treated with 100 nM and 5 μM BPA for five days, and XBP-1 spliced form (XBP-1s) indicative of IRE1α activation, and CHOP protein expressions were measured by Western blot. β-actin was used as a loading control. Data represent media + SD of 4 independent experiments, each performed in duplicate. * $p < 0.05$ and ** $p < 0.01$ using Kruskal–Wallis test for the comparison between control and BPA-treated cells.

3.4. NAC Reduces Cell Senescence and Attenuates the BPA-Induced Response on the UPR System

Since BPA can lead to cell senescence in vivo and cultured endothelial cells and also induce oxidative stress, a known inductor of cell senescence, we wonder whether an antioxidant could revert its effects. Thus, we tested whether a general antioxidant drug, N-acetylcysteine (NAC), could ameliorate BPA effects on MAEC. We found that NAC co-treatment reversed BPA effects on endothelial cell senescence. Indeed, β-Gal assays in MAEC treated 5 days with BPA or BPA+ NAC showed that NAC not only prevented the senescence triggered by BPA but reduced the number of senescence cells below those in the control group (Figure 5A). Accordingly, p16 and p21 protein expression was also reduced in the presence of NAC (Figure 5B). These results suggest a causative role of BPA-induced oxidative stress in cellular senescence.

Next, we tested if NAC is able to prevent also the UPR response. Western blots were performed to analyze the relative expression of the UPR proteins CHOP and XBP1 at 24 h. A significant increase in the expression of the XBP1 and CHOP proteins was observed in response to 24 h treatment with BPA, and NAC reversed the BPA effect. Thus, increased oxidative stress and oxidation of proteins lead to early UPR response, which can be inhibited by NAC (Figure 6).

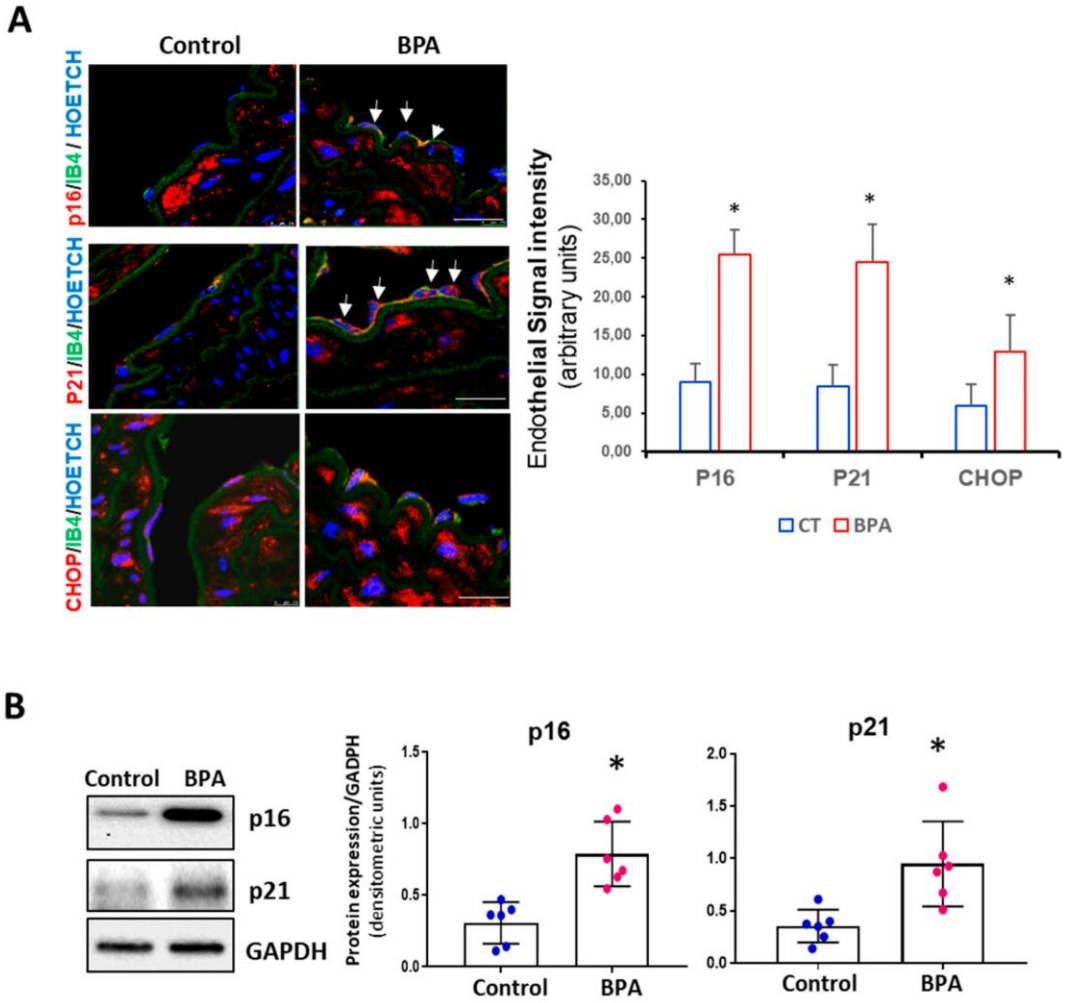

Figure 4. Senescence proteins (p16 and p21) and CHOP expression in BPA-treated-mice aort. Treatment with BPA in drinking water induced notable increases in expression of both the p16, p21, and CHOP proteins. (**A**) Representative confocal images from aorta sections of control and 8-weeks-treated BPA mice followed by immunostaining for p16 (upper panel), p21 (middle panel), and CHOP (lower panel) (red). Endothelium was marked in green with isolectin B4-FITC. Nuclei were labeled with hoechst in blue (n = 8 mice per condition). Scale bar = 60 μM. (**B**) Immunoblot detection of p16 and p21, in total aorta lysates from CT and 8 weeks BPA-treated mice. GAPDH was used as a loading control. A representative immunoblot is shown. The densitometric analysis is shown below (data are mean ± SD, n = 6 mice per condition) * $p < 0.001$ vs. CT.

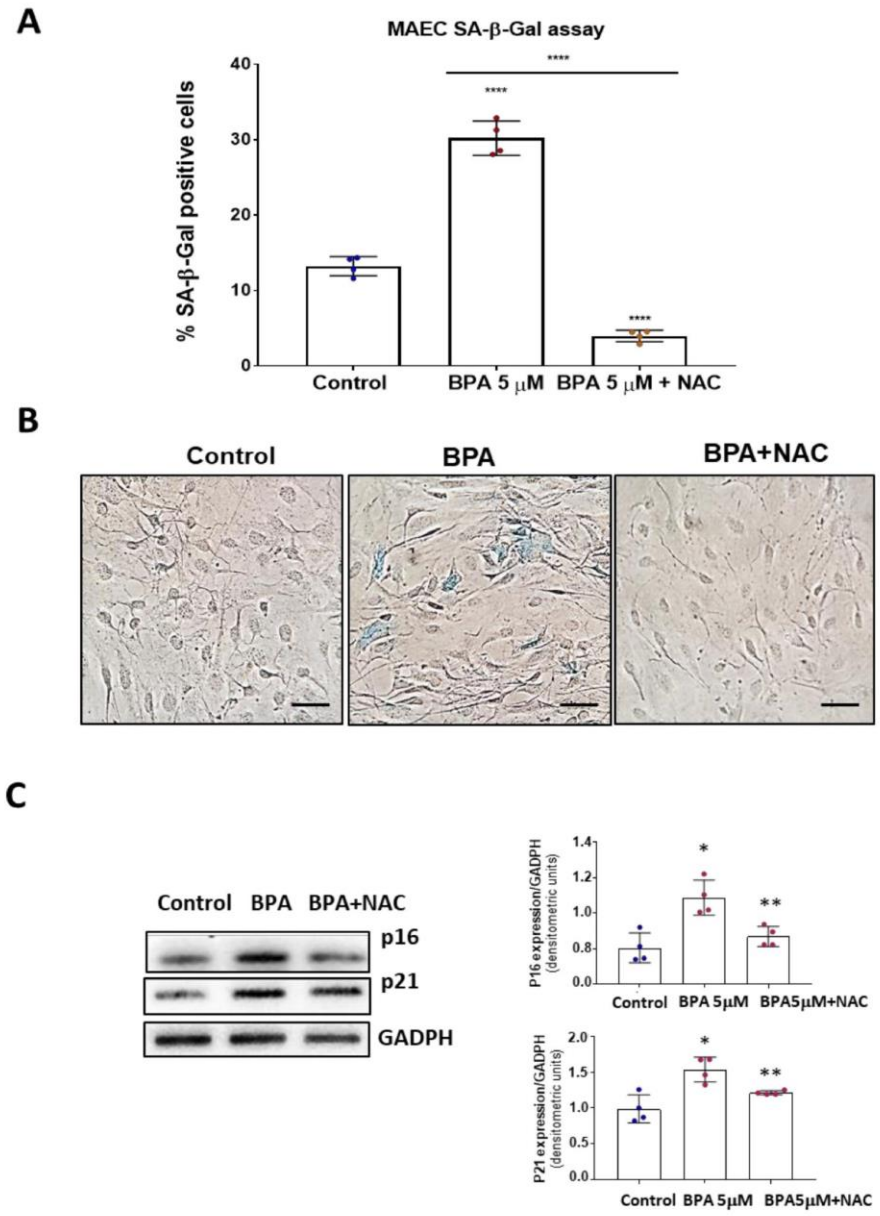

Figure 5. N-Acetylcistein decreases senescence-associated β-Gal assay in MAEC treated with BPA 5 days. (**A**) Senescence-associated β-Gal assay in MAEC treated with BPA 5 days at 5 μM and BPA 5 μM + NAC 5 mM. Note that pretreatment with NAC in BPA-treated cells reduced the percentage of senescent cells even below the control group. Results represented are means ± SD. (n = 4, each performed in triplicate). **** $p < 0.0001$ using Kruskal–Wallis test. (**B**) Representative microphotographs of the senescence assay made at x40 magnification. (**C**) Western blot analysis of MAEC treated as in **A**, using antibodies to p21 and p16. Data are the means ± SD of four different experiments, each performed in duplicate. * $p < 0.05$ using Kruskal–Wallis test for the comparison between control and BPA-treated cells; ** $p < 0.001$ using Kruskal–Wallis test for the comparison between BPA 5 μM and BPA 5 μM + NAC 5 mM.

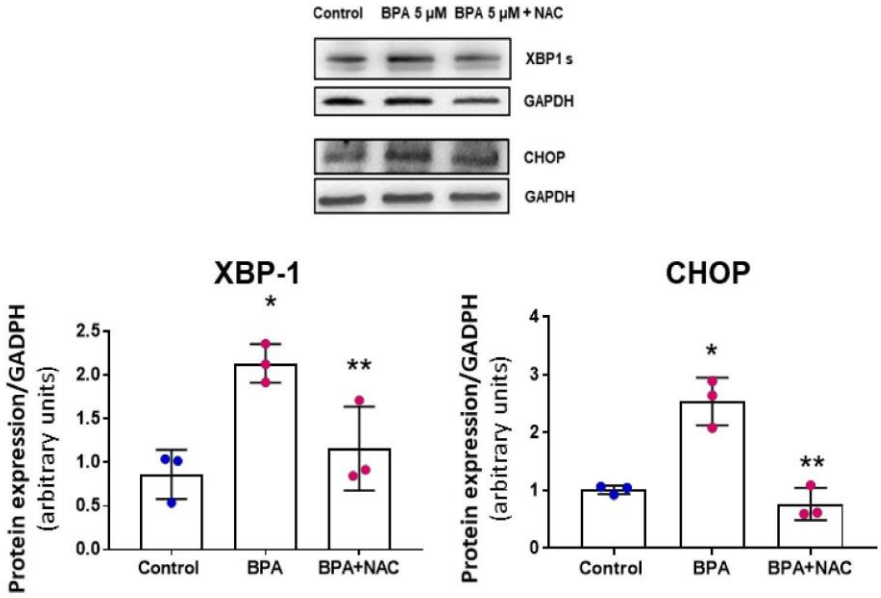

Figure 6. NAC prevents early UPR activation induced by BPA treatment in MAEC. A. Western blotting analysis of MAEC treated with 5 µM BPA and BPA 5 µM + NAC 5 mM for 24 h using antibodies against XBP1 and CHOP. There was a significant increase in XBP-1 and CHOP, which could be avoided by co-treatment with NAC. Results represented are means ± SD. (n = 3, each performed in duplicate). * $p < 0.0001$ using Kruskal–Wallis test for the comparison between control and BPA-treated cells ** $p < 0.0001$ using Kruskal–Wallis test for the comparison between BPA and BPA + NAC.

4. Discussion

Our results show, for the first time, that BPA in doses under the cytotoxic threshold can induce accelerated cell aging in mouse aortic endothelium. Our results suggest a causative role of BPA-induced oxidative stress in cellular senescence and point to UPR as a mediator in this process.

It is widely accepted that during aging exists a poor cellular stress response contributing to age-related diseases [63]. Recently, it has been demonstrated that the accumulating population of senescent cells in the aged human organism indeed experience proteostasis decline, failing to properly activate multiple programs required for stress adaptation at the level of gene transcription, including the UPR. Our results demonstrate that BPA, a ubiquitous contaminant, at low doses can activate an endothelial senescence program in cultured cells and, more importantly, in vivo, inducing a state of accelerated cellular aging.

The viability assays showed cytotoxic effects of BPA at the high micromolar concentration range. This range is consistent with the results described by our group in human podocytes [20], endothelial cells [19], or by other researchers in different cell lines [64–66]. Subsequent experiments were planned, using a range of concentrations that oscillated from 1 nM to 5 µM (half of the highest concentration at which no effects on cell viability were observed).

We observed at the selected concentrations that BPA induces senescence in MAEC. There is strong evidence that BPA is capable of inducing senescence in other cell lines and animal models. Mahmudi et al. [28] observed that BPA could induce senescence in human fetal lung fibroblasts through the ATM-p53 signaling pathway. Ribeiro-Varandas et al. [29] observed differences in p21 gene expression at the vascular level as a function of cell age in HUVEC treated with one µg/mL of BPA. Furthermore, Soundararajan et al. [30] observed that BPA could increase the transcription of senescence markers in zebrafish embryos in a situation of metabolic stress such as hyperglycemia. The same group observed in

patients with type 2 diabetes a positive correlation between systemic levels of BPA and senescence markers such as p16, p21, p53, and GLB1 [31]. The two main effectors activated in response to different senescence-inducing stimuli are p16 and p21 [67]. In our study, five-day treatment with BPA induced significant changes in β-Gal activity at 100 nM and 5 μM, although, in the Western blot analysis, p16 and p21 protein expression was only increased at the higher concentration used. Finally, exposure to BPA has been correlated with obesity-associated breast cancer since it can positively regulate human telomerase reverse transcriptase [68]. In human studies, senescence markers (p21, p16, and p53) have been associated with age and a sedentary lifestyle. Interestingly, physically active old people did not show significant differences with young people [67]. Similarly, significant increases in senescence proteins have been observed in endothelial cells of aged sedentary individuals vs. aged exercising adults [24]. In fact, senescent cells are considered a therapeutic target to treat cardiovascular diseases, and several trials with senolytic drugs are in process [69].

Senescence can be induced by various types of stress, including oxidative stress [70]. Our results demonstrate that BPA exposure even at 100 nM concentrations can increase superoxide production, lipid peroxidation, and protein oxidation in 24 h. Oxidative stress can trigger ER stress, which can induce the UPR pathway. In addition, senescent cells may also contribute to ER stress releasing inflammatory cytokines, growth factors, and enzymes involved in extracellular matrix remodeling. For those reasons, senescence can be considered an adaptive stress response. It has been demonstrated that ER stress/UPR activation occurs at senescence [41]. Evidence suggests that the UPR system can induce the senescent phenotype. However, other authors suggest that the UPR is part of the senescent phenotype because senescence can induce the activation of the UPR system [53]. Our results show that 5 μM BPA treatment increases p16 and p21 proteins levels, which could be related to the significant increase in PERK and CHOP mRNAs. Furthermore, it is noteworthy that after prolonged exposure over time, both in cell cultures and in the animal model, overexpression of the PERK-ATF4-CHOP pathway occurs.

Interestingly, although most studies related the CHOP signaling pathway to death processes in cell cultures [49], others have observed that it regulates or activates cellular aging processes and senescence, independently of cell death [53]. A recent article highlighted that all arms of the UPR were activated and associated with replicative senescence in WI38 cells, and in H2O2-induced senescence, the same cells, only the PERK branch was activated [71]. In addition, this signaling pathway has also been associated with myocardial ischemia, hypertension, cardiac atrophy, hypertrophy, or even vascular calcification [43–47].

Furthermore, our previous studies report that BPA can induce RIP3-necroptosis both in vivo and in endothelial cells [19]. We observed increased necroptosis in MAEC 24 h after BPA treatment at 10 μM, while no increase in apoptotic cell death was detected. However, the percentage of cells undergoing necroptotic cell death was modest. It is accepted that overwhelming stress will cause cell death, while less intense stress will cause senescence [68]. In the present study, BPA needed 5 days to show a significant increase in senescence markers. Thus, it is possible that in the short term, BPA induces necroptosis in endothelial cells, but longer treatments may lead to cellular senescence.

Several experiments show that blocking the PERK pathway can modify the percentage of SA-β-Gal-positive cells in different cell models [53]. However, the mechanisms of interaction between senescence proteins and those of the UPR system remain unclear. Our 24-h protein expression studies showed an increase in protein expression in two different pathways of the UPR system, and co-treatment with NAC prevented the overexpression of XBP-1 and CHOP proteins. NAC treatment reduced cellular senescence at 5 days even below the control group, indicating a temporal pattern in the endothelial responses to low concentrations of BPA. In our experiments, oxidative stress and UPR lead to senescence.

In epidemiological studies, exposure to BPA has been positively correlated with the predisposition to develop cardiovascular diseases. For example, Shankar et al. [22] observed a positive correlation between hypertension and increasing urinary BPA levels, independent of other factors such as age, sex, or ethnicity. Similarly, Aekplakorn et al. [23]

demonstrated a similar relationship in a Thai cohort. Finally, the work of Bae et al. [72] showed that people who drank canned beverages had higher urinary BPA levels than those who drank from glass containers, and those with higher BPA levels had a significant increase in blood pressure.

Today there is still controversy about the degree of exposure to BPA and the doses considered safe. In a recent analysis of our group, it was determined that the mean urinary concentration of BPA in the general population is close to 10 nM, which may be related to ingested concentrations close to the doses used in our study [73]. The doses used in the present work, 100 nM and 5 µM, have been detected in certain groups with high exposure, such as workers in the plastic industry [74–77]. Other population groups in which unusually high concentrations have been detected are patients with stage 5 chronic kidney disease undergoing hemodialysis or patients in intensive care units [78,79]. On the other hand, the dose administered to animals is half the dose used by the European Food Safety Authority as a reference to calculate the human tolerable daily intake [1]. Furthermore, the U.S. National Toxicology Program defines doses less than 50 mg/kg/day as "low dose" [57]. Furthermore, in the CLARITY-BPA study (Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity), one of the largest animal studies conducted in the context of BPA, the authors stated, "BPA did not elicit adverse effects in the in-life or terminal endpoints monitored in either sex below 25,000 µg/kg bw/day" [58]. However, other authors suggest that some results described in this study show non-monotonic effects, reaffirming the need to study the effects of BPA at low doses [59]. In our "low-dose" animal model, BPA visibly affected the vasculature of the exposed animals, promoting the overexpression of the proteins p16, p21, and CHOP. These data confirm the results described in the cell model, showing that BPA can promote accelerated cellular aging.

Thus, in line with the data extracted from the academic literature, BPA is an environmental factor involved in accelerated cell aging mechanisms, affecting the vascular endothelium and promoting cardiovascular diseases.

Author Contributions: Conceptualization, R.J.B. and M.S.; Data curation, R.M.-G.-T., S.S.-E., A.C., M.M.-M., R.R.-C., P.R. and M.D.-M.; Formal analysis, M.S.; Funding acquisition, R.J.B. and M.S.; Investigation, R.M.-G.-T., S.S.-E., A.C., M.M.-M., R.R.-C., P.R. and M.D.-M.; Methodology, R.M.-G.-T. and S.S.-E.; Project administration, M.S.; Writing—original draft, R.M.-G.-T. and S.S.-E.; Writing—review and editing, M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Instituto de Salud Carlos III (PI15/02139) -Fondo Europeo de Desarrollo Regional (FEDER)- (R.J. Bosch and M. Saura). R. Moreno-Gómez-Toledano is recipient of a research contract from CAM (B2017-BMD-3686). A. Cook is a recipient of a FPI fellowship from Universidad de Alcalá.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Universidad de Alcalá (PROEX 007/16, July 2016).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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XV. ANEXO VI

NEW EVIDENCES OF RENAL AND CARDIOVASCULAR ALTERATIONS PROMOTED BY BISPHENOL A. **BIOMOLECULES** (4.879, Q2 BIOCHEMISTRY & MOLECULAR BIOLOGY [2020]).

DOI: 10.3390/BIOM11111649

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Editorial

New Evidence of Renal and Cardiovascular Alterations Promoted by Bisphenol A

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Bisphenol A (BPA) is a phenolic compound that is widely used to synthesize plastics as a monomer or additive [1]. The unbeatable quality–price relationship has made plastics the central axis of contemporary life, which is why BPA can be found in many articles for food, cosmetic, or even textile use [2–5]. BPA is a well-known molecule that has been widely investigated for its properties as an endocrine disruptor, capable of exerting pro-estrogenic and anti-androgenic effects [6,7]. Numerous contributions have emerged in the last two decades linking BPA to pathologies such as diabetes, hypertension, obesity, kidney disease, cancer, and even behavioral disorders [8–14].

Previous work by our team has observed that BPA could induce apoptosis and dysregulation of structural proteins in cultured mouse podocytes (a highly specialized cell type that forms part of the glomerular filtration barrier, responsible for correct filtering of blood in the renal corpuscle). Furthermore, its intraperitoneal administration in mice induced renal and structural function changes similar to those observed in the early stages of diabetic nephropathy (DN), such as albuminuria, glomerular hyperfiltration, mesangial expansion, and podocytopenia [15]. New evidence suggests that patients with kidney disease release podocytes in their urine, and on numerous occasions, these are viable [16,17]. For this reason, the adhesion mechanisms of human podocytes in culture treated with low doses of BPA were studied, observing a critical detachment and downregulation of numerous structural and adhesion proteins [18]. In parallel, other previous works by our team also demonstrated that BPA administration in drinking water could induce hypertension in a dose-dependent manner, a process related to the uncoupling of the enzyme endothelial nitric oxide synthase [19]. The mechanism of BPA-induced hypertension, regulated by the enzyme calcium-calmodulin dependent protein kinase II, was closely related to necroptosis, a novel mechanism of cell death [20].

Recently, thanks to the Special Issue "Bisphenol A: An Environmental Factor with an Emerging Role in the Pathophysiology of Renal and Cardiovascular Diseases" published in *Biomolecules*, new contributions have been compiled that reinforce the position of BPA as an environmental factor involved in the development or progression of renal and cardiovascular diseases.

Firstly, in Kobroob et al. [21], the authors verified that the kidney damage induced by BPA may be related to oxidative stress, and the administration of an antioxidant such as N-acetyl cysteine (NAC) is capable of restoring mitochondrial integrity and oxidative balance. Interestingly, the authors used a relatively low dose, since the 50 mg/kg dose has been considered no observable effect level (NOEL) in the renal system [22–24]. Despite this, treatment at 16 weeks with BPA was able to induce important changes in kidney function. Body weight and food intake were lower with BPA and NAC + BPA, despite

Citation: Moreno-Gómez-Toledano, R.; Arenas, M.I.; Vélez-Vélez, E.; Saura, M.; Bosch, R.J. New Evidence of Renal and Cardiovascular Alterations Promoted by Bisphenol A. *Biomolecules* **2021**, *11*, 1649. https://doi.org/10.3390/biom11111649

Received: 21 October 2021
Accepted: 5 November 2021
Published: 8 November 2021

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BPA being considered an obesity-related molecule [25,26]. A smaller kidney size was also observed, although no significant changes were observed in the renal hypertrophy index when adjusting for the animal's weight. In the functional aspects of the kidney, treatment with BPA induced a notable and significant increase in urea nitrogen, serum creatinine, 24-hour proteinuria, and the urine protein-to-creatinine ratio, as well as a significant reduction in the clearance of creatinine. Curiously, cotreatment with NAC ameliorated the pathological changes induced by BPA. Similarly, histology showed significant atrophy in the renal corpuscle and many apoptotic cells distributed throughout the renal tubules. Again, cotreatment with NAC significantly alleviated BPA-induced damage. In addition, significant increases in molecules related to oxidative stress were also observed, and considerable reductions in the expression of antioxidant enzymes could be reversed with NAC. Finally, the authors observed that the kidney damage induced by BPA and alleviated by NAC is related to the AMPK-PGC-1α-SIRT3-SOD2 signaling pathway, which is involved in mitochondrial homeostasis.

Interestingly, the oxidative stress induced by low doses of BPA was also studied by Moreno-Gómez-Toledano et al. [27]. The authors used doses of BPA below the cytotoxic threshold and found that BPA was able to promote cell senescence in a primary mouse vascular endothelial cell line. After identifying senescence-inducing concentrations by the β-gal assay, they confirmed that the expression of the senescence markers p16 and p21 only increased to 5 μM. Since oxidative stress is one of the elements responsible for cellular senescence [28], carbamylated proteins and superoxide anion production were analyzed, confirming significant increases at the 5 μM concentration. Next, the relative expression levels of mRNAs related to the unfolding protein response (UPR) system were analyzed using qPCR, since this is related to cell senescence [29]. The results showed that BPA could induce a significant increase in the PERK-ATF4-CHOP pathway, the only one of the three signaling pathways of the UPR system related to pathological processes. In line with the cellular studies, an animal model was carried out with half the NOEL concentration (25 mg/kg) in drinking water. Administration of the compound via this route maintains a relatively constant and natural entry of BPA into the body, which ensures chronic exposure. Analogous to the results observed in endothelial cells in culture, the vascular endothelium of the animals showed a significant increase in the senescence proteins p16 and p21 and the CHOP protein.

Finally, to verify that the acceleration of cellular aging induced by BPA is related to oxidative stress, a new cellular study was carried out, in which NAC was used. In the β-gal assay, it was shown that cotreatment with NAC not only prevented BPA-induced senescence but even reduced it below the level of the control group. Western blot analysis confirmed that the relative expression levels of the p16 and p21 proteins were similar to those of the control group. Finally, the proteins of the UPR system were also expressed similarly to the control group after cotreatment with NAC.

Another work highlighted in this Special Issue is Moreno-Gómez-Toledano et al. [30]. In this case, by conducting complete statistical work, they found new evidence that correlates BPA with human kidney disease. The first part of the work focused on unifying all the studies within the BPA–kidney disease paradigm to perform a combined meta-analysis. The different characteristics of the studies identified by systematic review made it impossible to develop a single meta-analysis. Therefore, several studies were carried out in parallel, unifying studies with similar characteristics. First, the combined analysis of BPA in the blood and risk of chronic kidney disease (CKD) showed a robust and positive result. In the urinary BPA study, the analyses related to albuminuria and low-grade albuminuria did not show conclusive results, although positive trends were shown. However, the combined analysis of the studies related to urinary BPA and renal function (estimated glomerular filtration rate) showed that increases in urinary BPA were related to a decrease in renal function (higher CKD risk).

The second part of the work analyzed the available data from the American National Health and Nutrition Examination Survey (NHANES) cohort, comparing urinary BPA

values with renal function. Thus, it was observed that individuals with a higher concentration of urinary BPA had higher concentrations of albumin in their urine, although the expected relationship with the estimated renal function was not found. Similarly, when analyzing individuals with low-grade albuminuria, a significant increase in urinary BPA was shown. Finally, when studying the urinary BPA value of individuals diagnosed with kidney disease, a significant increase was determined, which was even higher in those undergoing dialysis treatment.

In conclusion, the new evidence supports and reinforces the hypothesis that BPA is an environmental factor related to renal and cardiovascular diseases.

Funding: This work was supported, in part, by grants from Instituto de Salud Carlos III (PI15/02139)-Fondo Europeo de Desarrollo Regional (FEDER). R. Moreno-Gómez-Toledano is the recipient of a research contract from CAM (B2017-BMD-3686).

Conflicts of Interest: The authors declare no conflict of interest.

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XVI. ANEXO VII

NEW EVIDENCE FOR A ROLE OF BISPHENOL A IN CELL INTEGRITY. IMPLICATIONS IN THE HUMAN POPULATION. **BIOCELL** (1.254, Q3 BIOLOGY [2020]).

DOI: 10.32604/BIOCELL.2022.017894

BIOCELL
2022 46(2): 305-308

Tech Science Press

New evidence for a role of Bisphenol A in cell integrity. Implications in the human population

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Key words: Bisphenol A, Podocyte, Cytoskeleton, Cell Adhesion, Chronic Renal Diseases, Dialysis

Abstract: Bisphenol A (BPA) is a xenoestrogen known for its implications for the endocrine systems and several other organs, including the kidneys. Recent renal studies have shown that BPA can induce alterations of the cytoskeleton and cell adhesion mechanisms such as a podocytopathy with proteinuria and hypertension, alterations involved in the progression of renal diseases. These data and the fact that BPA is known to be present in the urine of almost the entire population strongly suggest the critical need to reevaluate BPA exposures considered safe.

Introduction

Bisphenol A (BPA) is a xenobiotic molecule classified within the category of endocrine disruptors, and thanks to its properties as an estrogen modulator, it is also called xenoestrogen (Taylor *et al.*, 2011). BPA is found in countless everyday utensils, such as food containers, bottles, cans, toys, and is even found in clothing (Vandenberg *et al.*, 2007; Dursun *et al.*, 2016; EFSA, 2016; Li and Kannan, 2018; Freire *et al.*, 2019). Its use is not restricted to the domestic sphere, as its ability to improve the qualities of plastics has made it an essential element in other industries, as it is used in the manufacture of cars, LED lights, and even medical-surgical material (Duty *et al.*, 2013; Olabisi and Adewale, 2016; Testai *et al.*, 2016).

Presentation of the Viewpoint

BPA is a molecule whose properties as an estrogen modulator were discovered in the 1930s by Dodds and Lawson (Dodds and Lawson, 1936). For this reason, numerous works study its possible relationship with reproductive or genitourinary disorders (Rochester, 2013; Ziv-Gal and Flaws, 2016; Pergialiotis *et al.*, 2018; Tomza-Marciniak *et al.*, 2018). However, in the last two decades, it has been shown that BPA can exert other types of actions on other organs or systems, favoring the accumulation of fatty tissue, the appearance of diabetes, cancer, and even cognitive and behavioral disorders (Provvisiero *et al.*, 2016; Akash *et al.*, 2020; Wu *et al.*, 2020; Nesan *et al.*, 2021). In recent years, our team has made interesting advances on the possible implications of BPA on the renal and vascular system.

Using experimental animals models we observed that BPA is capable to promote hypertension and renal damage (podocytopathy) as well as to participate in the mechanism of progression of chronic kidney disease (CKD) (Olea-Herrero *et al.*, 2014; Saura *et al.*, 2014; Moreno-Gómez-Toledano *et al.*, 2020; Reventun *et al.*, 2020), reviewed by Bosch *et al.* (2016).

Our first work with podocytes (cells that are part of the glomerular filtration barrier) were carried out with immortalized mouse cultures. Podocytes cell lines (mice and human) have a particular condition: at 33°C, they remain undifferentiated, allowing their replication, and at 37°C, they become quiescent, lose their mitotic capacity, and begin to differentiate for 11–15 days. After that time, the podocyte in culture is considered a differentiated, mature, and fully functional podocyte.

The administration of BPA to this cell line demonstrated that low exposure to this molecule (10 and 100 nM) could induce death mechanisms in cultured mouse podocytes (Olea-Herrero *et al.*, 2014) (Fig. 1). Similarly, the intraperitoneal administration of BPA in mice demonstrated that BPA could induce cellular sampling mechanisms in the podocytes of the glomerulus of the animal (Olea-Herrero *et al.*, 2014). These animals developed podocitopathy with

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Received: 15 June 2021; Accepted: 08 July 2021

Doi: 10.32604/biocell.2022.017894

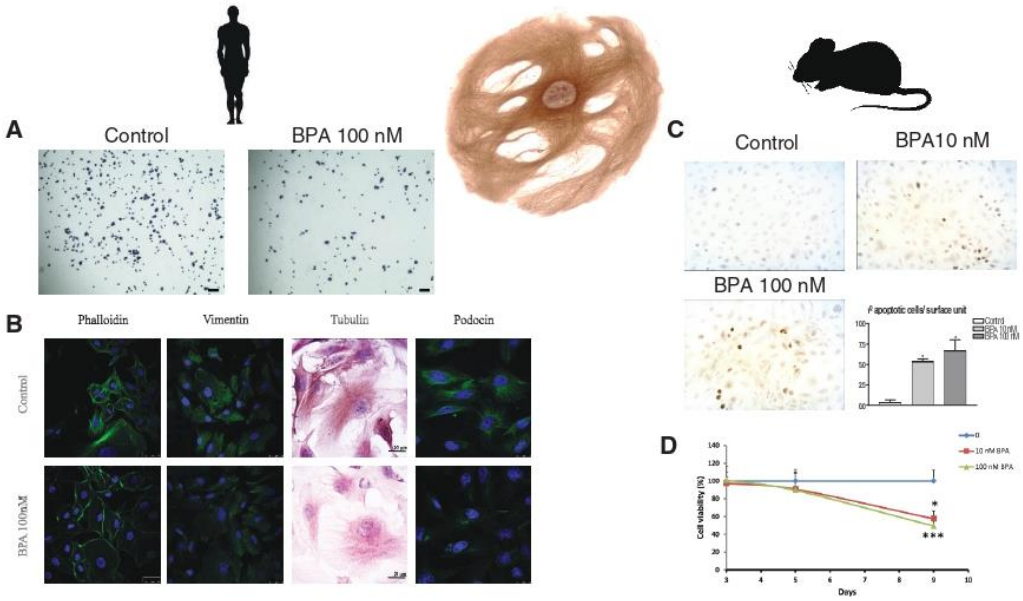

FIGURE 1. Interspecies differences in the effects promoted by BPA in the podocyte. A) Exposure to BPA induces a reduction in the adhesion capacity of the human podocyte (x40) (scale bar: 60 μm). B) Reduction in the relative expression of structural proteins after treatment with BPA, analyzed by immunofluorescence (scale bar: 50 μm) and immunohistochemistry (scale bar: 20 μm). C) In the mouse podocyte, the administration of BPA induces an increase in the mechanisms of cell death (x300). D) Treatment with BPA significantly reduces cell viability (MTT). Figure made with our own results published in the Journal of Cellular Physiology (Olea-Herrero *et al.*, 2014), and Scientific Reports (Moreno-Gómez-Toledano *et al.*, 2020).

proteinuria, similar to those seen in diabetic nephropathy. Although there are limitations when using mouse models for assessing renal failure or long-term histomorphological changes (Breyer *et al.*, 2005), our findings may have pathophysiological implications since the amount of proteinuria and podocytes number are reliable predictors of the progression of renal disease (Meyer *et al.*, 1999; D'Amico and Bazzi, 2003).

Subsequently, we observed that in human podocytes in culture (generously provided by Dr. M Saleem, University of Bristol), 100 nM BPA promotes a novel type of podocytopathy characterized by an impairment of cell adhesion. Transcriptomic and proteomic studies demonstrated that BPA promotes alterations in the expression of structural and adhesion proteins (and messengers). Subsequent western blot and immunofluorescence assays demonstrated alterations in the relative expression of structural proteins such as actin, tubulin, vimentin, and podocin, as well as alterations in proteins related to adhesion mechanisms, such as cofilin-1, vinculin, E-cadherin, nephrin, VCAM-1, tenascin-C, and β-catenin (Moreno-Gómez-Toledano *et al.*, 2020) (Fig. 1). In this way, it was possible to observe solid evidence that the cellular microenvironment and the elements that make it up, including xenobiotic compounds, can substantially affect the cellular structure.

Adamakis *et al.* (2018) observed that BPA in the aquatic environment, at environmentally relevant BPA concentration, was capable of promoting alterations in the cytoskeleton of the seagrass *Cymodocea nodosa*, even stating that the integrity of the actin filament is the most sensitive biomarker to exposure to BPA. Stavropoulou *et al.* (2018) also observed BPA-mediated alterations in the actin filaments of the *Zea mays* (corn) plant. In cell cultures, Yin *et al.* (2020) described BPA-mediated cytoskeletal alterations in a mouse neuroblastoma cell line (neuro-2a cells). When using BPA doses in the micromolar range, they observed a reduction in the number of dendrites and a lower signal intensity when performing phalloidin (F-actin) immunofluorescence. Similarly, Rameshrad *et al.* (2018) observed that BPA is capable of inducing alterations in the expression of VCAM (adhesion protein) in human umbilical vein endothelial cells, also in the micromolar range.

Analysis of the Impact of the Viewpoint

It is evident that BPA can affect cell structure, not only in human cell cultures but also in murine cell cultures and even aquatic and terrestrial plants. This effect in the podocyte is especially interesting since its inability to regenerate makes it a cell of particular relevance in CKD (Glassock and Rule, 2016). Technically, BPA-induced podocyte loss could be as damaging to kidney function as cell death. The loss of podocytes induces an increase in the compensatory mesangial matrix, progressively reducing the renal filtration capacity. However, new studies have observed that although the podocyte does not have replicative capacity *per se*, the surrounding glomerular parietal epithelial cells could play a decisive role in podocyte regeneration, differentiating towards this cell type (Shankland *et al.*, 2017). In any case, when the regenerative capacity cannot compensate for the loss, the kidney will progress to CKD. Since BPA is a possible environmental factor involved in this type of pathology, the degree of exposure to which the susceptible population is exposed,

such as patients in intensive care or patients undergoing dialysis techniques, is of particular importance.

As we previously described, mean urinary BPA values have been observed in patients undergoing conventional dialysis between 52.73-155.84 ng/ml (1.11-3.28 μg/kg BW/day, 230.98-682.64 nM) (Moreno-Gómez-Toledano *et al.*, 2021). These values are lower than those considered safe by the European Food Safety Authority, 4 μg/kg BW/day (TDI, Tolerable Daily Intake) (EFSA, 2016). However, this dose has been calculated using animal models as reference (Tyl *et al.*, 2008), to which several correction factors were applied. As shown in Fig. 1, our cellular models show considerable evidence of the effects produced by BPA at the same doses, which should be a critical element to take into account when extrapolating animal studies to humans. Furthermore, the doses observed in CKD patients in dialysis treatment are lower than the proposed TDI but between 2 and 6 times higher than the concentrations used in the cell models.

However, it must be mentioned that BPA is among other chemicals that can be found circulating in the body. In recent years, various compounds have been identified, such as phthalates (Wang *et al.*, 2019), as well as other phenolic derivatives, such as bisphenol S, F, or AF (BPS, BPF, or BPAF, respectively) (Chen *et al.*, 2016). It has been observed that the combination of these elements could enhance the damage that they can already exert individually. Thus, it has been observed that the combination of BPA and dibutyl phthalate (DBP) increases cytotoxicity, oxidative stress, and genotoxicity in liver cell cultures (Li *et al.*, 2017). In animal models, it has been determined that co-exposure of BPA with Di-(2-Ethylhexyl) -phthalate (DEHP) appears to increase susceptibility to tumor development (Zhang *et al.*, 2021).

Furthermore, computer models that have recently been published showed synergy or antagonism as a function of the combination of phenolic derivatives (Jatkowska *et al.*, 2021). According to Kataria *et al.* (2015), oxidative stress might represent a common pathway that mediates renal injury associated with exposure to environmental chemicals such as BPA, phthalates, polycyclic aromatic hydrocarbon, polychlorinated biphenyl, perfluoroalkyl acid as well as dioxins. This mechanism has biological plausibility and justifies further investigation when examining the adverse effects of these chemicals. Interestingly, these authors also suggest that other functional disturbances contribute to the adverse cardiorenal effects elicited by the described compounds, including effects on modifiable patient-associated factors, such as obesity.

Conclusion

The latest advances in BPA study have determined that it is a molecule with the potential to induce alterations in the cytoskeleton and the capacity for cell adhesion. The concentrations to which the susceptible population is exposed could worsen their pathologies, particularly in patients with CKD. The widespread usage of BPA, especially in the composition of the surgical medical material, should be evaluated and act accordingly, as it could be a crucial factor in the evolution of specific pathologies. Future translational studies need to evaluate the impact of BPA in the human population and reevaluate BPA exposures considered safe.

Copyright of Figures: We want to thank the Journal of Cellular Physiology (Olea-Herrero *et al.*, 2014), and Scientific Reports (Moreno-Gómez-Toledano *et al.*, 2020) for publishing our research results, part of which has been used in Fig. 1.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: R. Moreno-Gómez-Toledano; M. I. Arenas; E. Vélez-Vélez; R. J. Bosch; draft manuscript preparation: R. Moreno-Gómez-Toledano; M. I. Arenas; E. Vélez-Vélez; R. J. Bosch. All authors reviewed the results and approved the final version of the manuscript.

Funding Statement: This work was supported in part by grants from Instituto de Salud Carlos III (PI15/02139) -Fondo Europeo de Desarrollo Regional (FEDER)-. R. Moreno-Gómez-Toledano is recipient of a research contract from CAM (B2017-BMD-3686).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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detrás de los sabios
para poder marchar algún día
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Santiago Ramón y Cajal (1852 – 1934)

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