Journal of Cellular Physiology

Bisphenol-A Induces Podocytopathy With Proteinuria in Mice

NURIA OLEA-HERRERO,[1] MARÍA ISABEL ARENAS,[2] CARMEN MUÑÓZ-MORENO,[1] RAFAEL MORENO-GÓMEZ-TOLEDANO,[1] MARTA GONZÁLEZ-SANTANDER,[3] IGNACIO ARRIBAS,[4] AND RICARDO J. BOSCH[1]*

[1]*Laboratory of Renal Physiology and Experimental Nephrology, Department of System Biology/Physiology Unit, University of Alcalá, Alcalá de Henares (28871), Spain*

[2]*Department of Biomedicine and Biotechnology/Cell Biology Unit, University of Alcalá, Alcalá de Henares (28871), Spain*

[3]*Department of Medicine and Medical Specialities, University of Alcalá, Alcalá de Henares (28871), Spain*

[4]*Department of Clinical Chemistry University Hospital "Príncipe de Asturias", University of Alcalá, Alcalá de Henares (28871), Spain*

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. © 2014 The Authors. *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).

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

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.

*Correspondence to: Ricardo J. Bosch, Departamento de Biología de Sistemas/ Unidad de Fisiología, Facultad de Medicina, Campus Universitario, Universidad de Alcalá, 28871 Alcalá de Henares, Spain
E-mail: ricardoj.bosch@uah.es

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

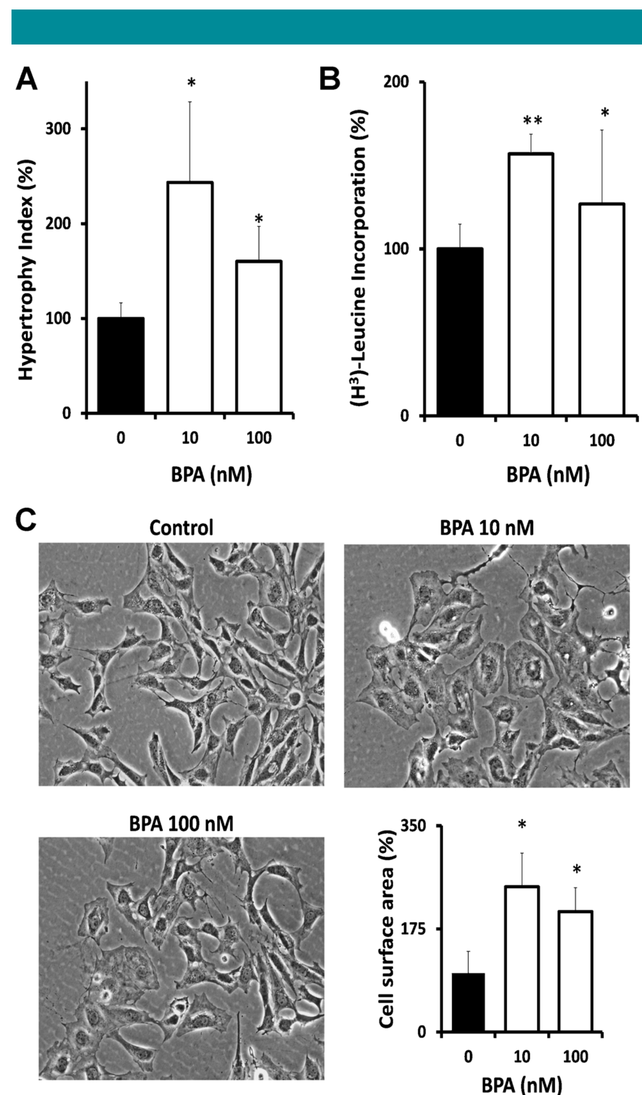

Fig. 1. BPA induces podocyte hypertrophy. (A) hypertrophy index, (B) [H^3]-leucine incorporation, (C) microphotographs of podocytes incubated with low and high BPA concentration as well as planar podocyte surface area analysis (X200). Data are the means ± SD of three different experiments, each performed 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.

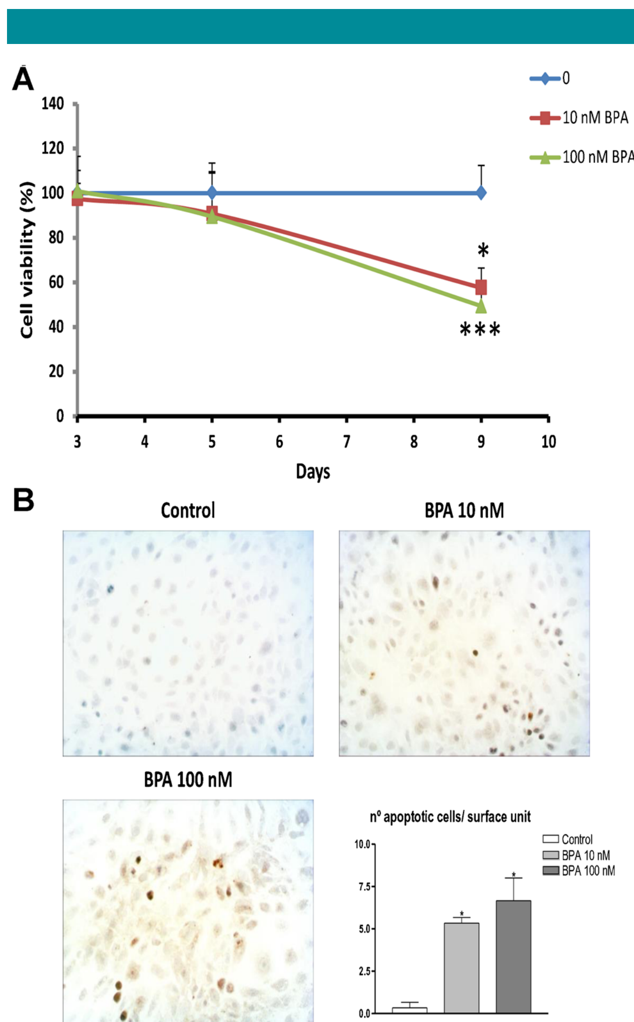

Fig. 2. **Effect of BPA on podocyte viability. (A) Mouse podocytes were incubated with different doses of BPA during 3, 6, and 9 days, and podocyte viability was assayed by MTT. Data are the means ± SD of three different experiments, each performed in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ using 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

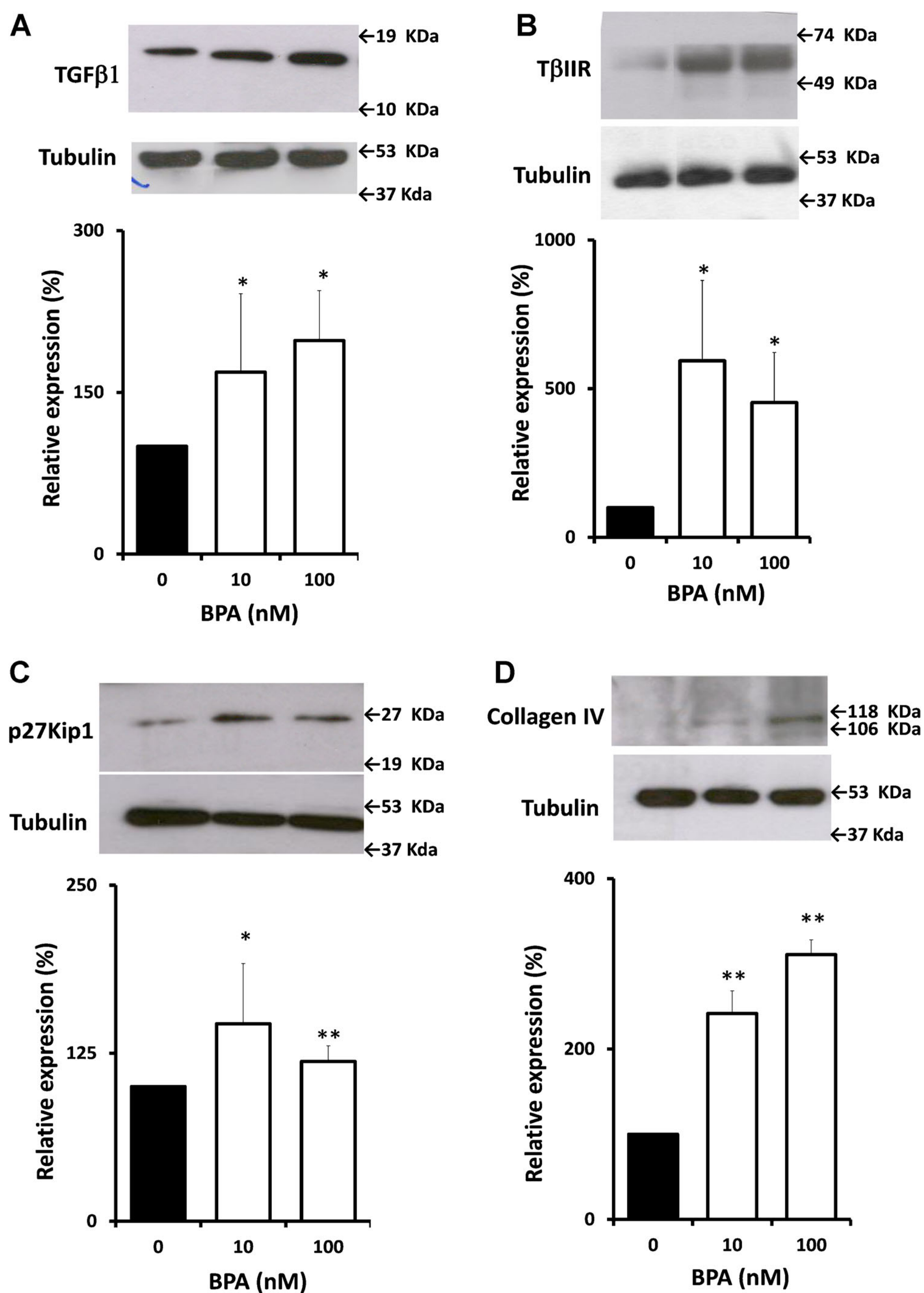

Fig. 3. Effects of BPA on the expression of TGFβ1 system, p27Kip1 and collagen IV on cultured podocytes. Protein TGFβ1 expression (A), TβIIR expression (B), p27Kip1 expression (C), and collagen IV expression (D) were analized by Western blot. Data are the means ± SD of three different experiments, each 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

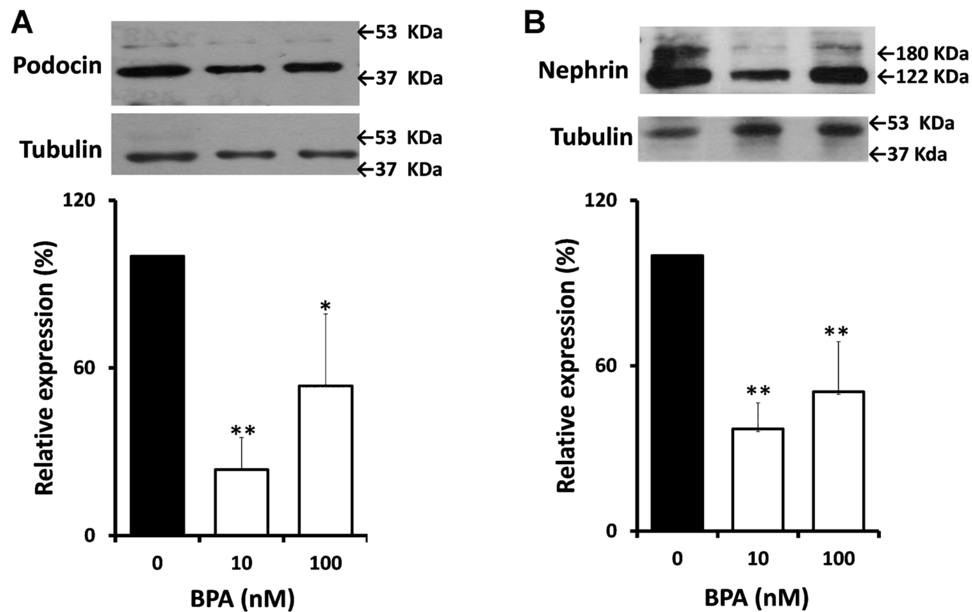

Fig. 4. Podocin and nephrine expression induced by BPA. Protein podocin expression (A) and nephrine expression (B) were measured by Western blot. Data are the means ± SD of three different experiments, each performed in duplicate. $^*P < 0.05$ and $^{**}P < 0.01$ using ANOVA test for the comparison between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

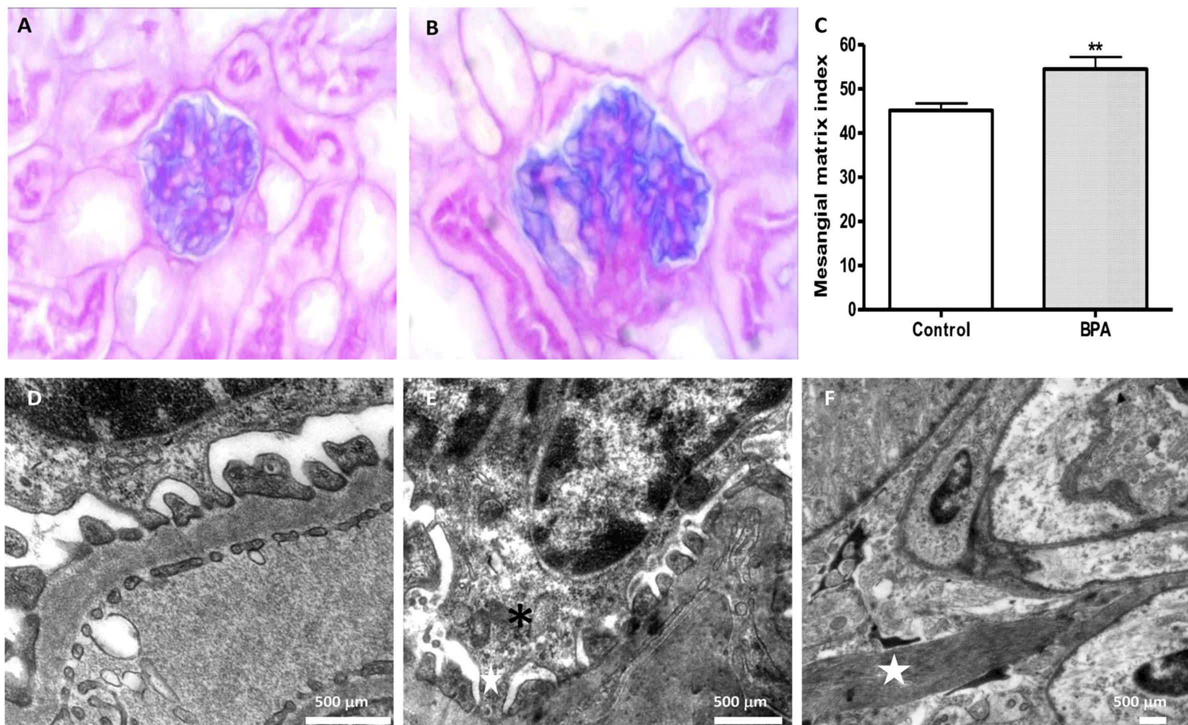

Fig. 5. Renal histological changes in BPA-injected mice. (A,B) Sections from kidney of control and BPA-injected mice stained with PAS-Alcian blue. The renal corpuscles from BPA mice showed higher staining with PAS and alcian blue than controls (pink and blue colors) (X300). (C) Mesangial matrix index. 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.

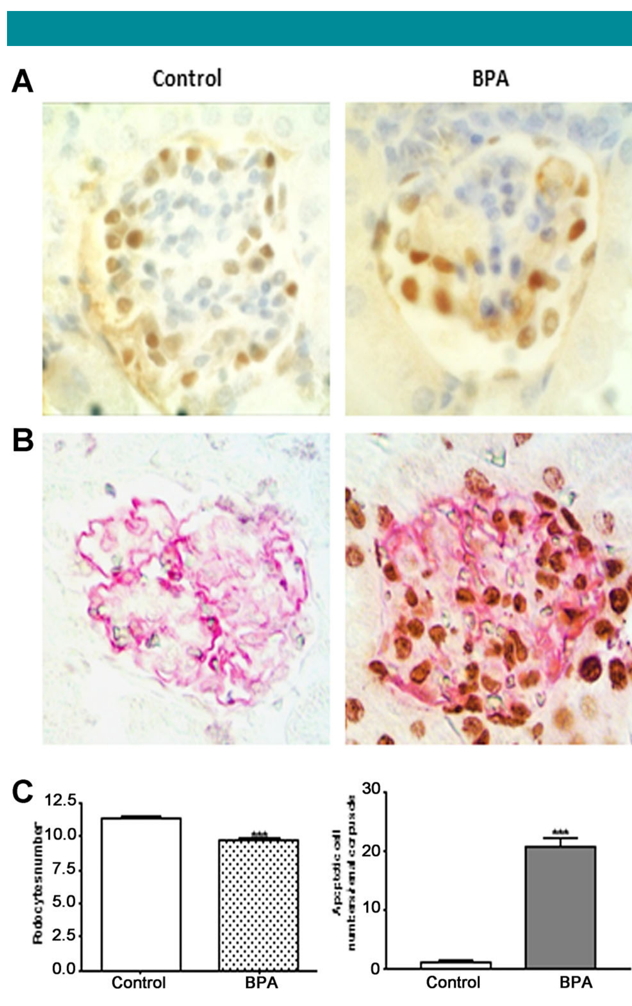

Fig. 6. Podocytopenia in BPA-injected mice. (A) WT-1 labelling. In BPA-injected mice the podocyte cell number (brown nuclei) was decreased with respect to control mice. X300. (B) TUNEL assay (brown nuclei) combined with podocin immunohistochemistry, a marker of podocyte foot processes (red). The renal 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

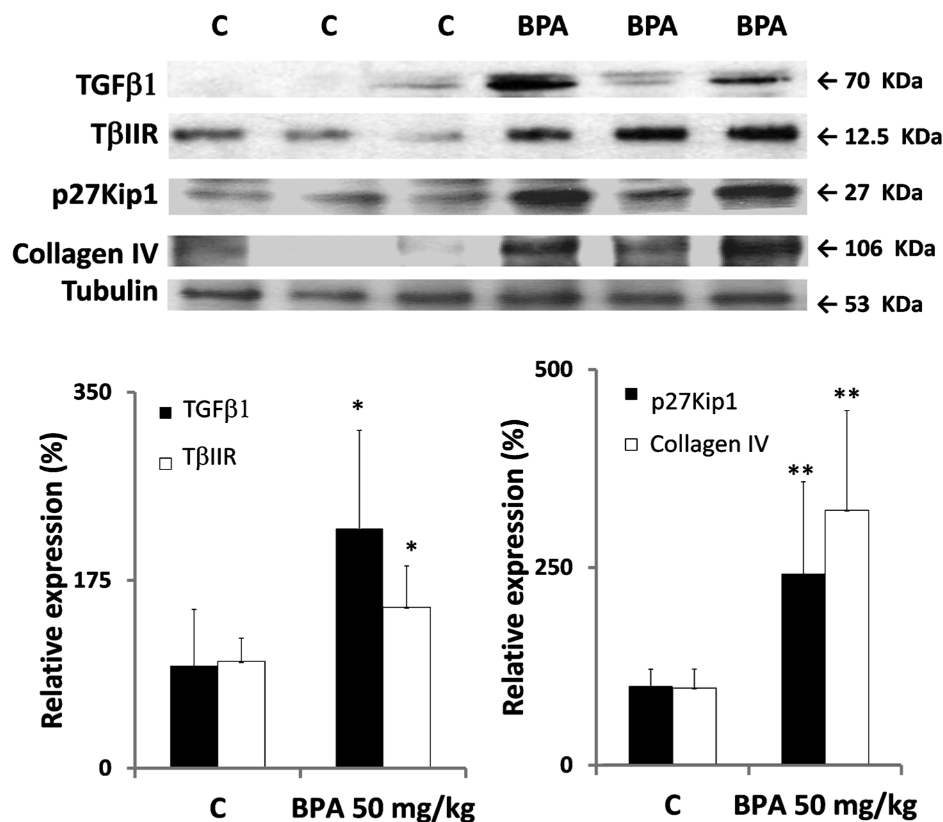

Fig. 7. **Renal protein expression in BPA-injected mice. The TGFβ1 system, p27Kip1 as well as collagen IV were analyzed by Western blot. Data are the means ± SD of three different experiments, each performed in duplicate. *$P < 0.05$ and **$P < 0.01$ using ANOVA test for the comparison between control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

Literature Cited

Barisoni L, Schnaper HW, Kopp JB. 2007. A proposed taxonomy for the podocytopathies: A reassessment of the primary nephrotic diseases. Clin J Am Soc Nephrol 2:529–542.
Benachour N, Aris A. 2009. Toxic effects of low doses of Bisphenol-A on human placental cells. Toxicol Appl Pharmacol 241:322–328.
Breyer MD, Bottinger E, Brosius FC, 3rd, Coffman TM, Harris RC, Heilig CW, Sharma K. 2005. Mouse models of diabetic nephropathy. J Am Soc Nephrol 16:27–45.
Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL. 2005. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect 113:391–395.
Carwile JL, Ye X, Zhou X, Calafat AM, Michels KB. 2011. Canned soup consumption and urinary bisphenol A: A randomized crossover trial. JAMA 306:2218–2220.
Chou WC, Chen JL, Lin CF, Chen YC, Shih FC, Chuang CY. 2011. Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: A birth cohort study in Taiwan. Environ Health 10:94.
Coward RJ, Foster RR, Patton D, Ni L, Lennon R, Bates DO, Harper SJ, Mathieson PW, Saleem MA. 2005. Nephrotic plasma alters slit diaphragm-dependent signaling and translocates nephrin, Podocin, and CD2 associated protein in cultured human podocytes. J Am Soc Nephrol 16:629–637.
D'Agati VD. 2008. Podocyte injury in focal segmental glomerulosclerosis: Lessons from animal models (a play in five acts). Kidney Int 73:399–406.
D'Amico G, Bazzi C. 2003. Pathophysiology of proteinuria. Kidney Int 63:809–825.
Diez-Sampedro A, Lenz O, Fornoni A. 2011. Podocytopathy in diabetes: A metabolic and endocrine disorder. Am J Kidney Dis 58:637–646.
Doerge DR, Twaddle NC, Vanlandingham M, Fisher JW. 2012. Pharmacokinetics of bisphenol A in serum and adipose tissue following intravenous administration to adult female CD-1 mice. Toxicol Lett 211:114–119.
Gonzalez-Parra E, Herrero JA, Elewa U, Bosch RJ, Arduan AO, Egido J. 2013. Bisphenol A in chronic kidney disease. Int J Nephrol 2013:437857.
Griffin S, Shankland S. 2008. Renal hyperplasia and hypertrophy: role of cell cylce regulatory proteins. In: Alpern R Hebert S editors. The Kidney Physiology and Pathophysiology. New York, NY, USA: pp 723–742.
Howdeshell KL, Peterman PH, Judy BM, Taylor JA, Orazio CE, Ruhlen RL, Vom Saal FS, Welshons WV. 2003. Bisphenol A is released from used polycarbonate animal cages into water at room temperature. Environ Health Perspect 111:1180–1187.
Huang YQ, Wong CK, Zheng JS, Bouwman H, Barra R, Wahlstrom B, Neretin L, Wong MH. 2012. Bisphenol A (BPA) in China: A review of sources, environmental levels, and potential human health impacts. Environ Int 42:91–99.
Iglesias-de la Cruz MC, Ziyadeh FN, Isono M, Kouahou M, Han DC, Kalluri R, Mundel P, Chen S. 2002. Effects of high glucose and TGF-beta1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes. Kidney Int 62:901–913.
Izquierdo A, Lopez-Luna P, Ortega A, Romero M, Guitierrez-Tarres MA, Arribas I, Alvarez MJ, Esbrit P, Bosch RJ. 2006. The parathyroid hormone-related protein system and diabetic nephropathy outcome in streptozotocin-induced diabetes. Kidney Int 69:2171–2177.
Jefferson JA, Shankland SJ, Pichler RH. 2008. Proteinuria in diabetic kidney disease: A mechanistic viewpoint. Kidney Int 74(1):22–36.
Kabuto H, Hasuike S, Minagawa N, Shishibori T. 2003. Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ Res 93:31–35.
Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA. 2013. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proc Natl Acad Sci USA 110:9956–9961.
Kurosawa T, Hiroi H, Tsutsumi O, Ishikawa T, Osuga Y, Fujiwara T, Inoue S, Muramatsu M, Momoeda M, Taketani Y. 2002. The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. Endocr J 49:465–471.
Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, Melzer D. 2008. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. JAMA 300:1303–1310.
Li M, Bi Y, Qi L, Wang T, Xu M, Huang Y, Xu Y, Chen Y, Lu J, Wang W, Ning G. 2012. Exposure to bisphenol A is associated with low-grade albuminuria in Chinese adults. Kidney Int 81:1131–1139.
Li YJ, Song TB, Cai YY, Zhou JS, Song X, Zhao X, Wu XL. 2009. Bisphenol A exposure induces apoptosis and upregulation of Fas/FasL and caspase-3 expression in the testes of mice. Toxicol Sci 108:427–436.
Naciff JM, Jump ML, Torontali SM, Carr GJ, Tiesman JP, Overmann GJ, Daston GP. 2002. Gene expression profile induced by 17alpha-ethynyl estradiol, bisphenol A, and genistein in the developing female reproductive system of the rat. Toxicol Sci 68:184–199.
Ortega A, Romero M, Izquierdo A, Troyano N, Arce Y, Ardura JA, Arenas MI, Bover J, Esbrit P, Bosch RJ. 2012. Parathyroid hormone-related protein is a hypertrophy factor for human mesangial cells: Implications for diabetic nephropathy. J Cell Physiol 227:1980–1987.
Pottenger LH, Domoradzki JY, Markham DA, Hansen SC, Cagen SZ, Waechter JM, Jr. 2000. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicol Sci 54:3–18.
Qin XY, Kojima Y, Mizuno K, Ueoka K, Muroya K, Miyado M, Zaha H, Akanuma H, Zeng Q, Fukuda T, Yoshinaga J, Yonemoto J, Kohri K, Hayashi Y, Fukami M, Ogata T, Sone H. 2012. Identification of novel low-dose bisphenol a targets in human foreskin fibroblast cells derived from hypospadias patients. PLoS One 7:e36711.
Richter CA, Taylor JA, Ruhlen RL, Welshons WV, Vom Saal FS. 2007. Estradiol and Bisphenol A stimulate androgen receptor and estrogen receptor gene expression in fetal mouse prostate mesenchyme cells. Environ Health Perspect 115:902–908.
Ritz E, Rychlík I, Locatelli F, Halimi S. 1999. End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions. Am J Kidney Dis 34:795–808.
Romero M, Ortega A, Izquierdo A, Lopez-Luna P, Bosch RJ. 2010. Parathyroid hormone-related protein induces hypertrophy in podocytes via TGF-beta(1) and p27(Kip1): Implications for diabetic nephropathy. Nephrol Dial Transplant 25:2447–2457.
Schiffer M, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel P, Bottinger EP. 2001. Apoptosis in podocytes induced by TGF-beta and Smad7. J Clin Invest 108:807–816.
Shankland SJ. 2006. The podocyte's response to injury: Role in proteinuria and glomerulosclerosis. Kidney Int 69:2131–2147.
Shono A, Tsukaguchi H, Yaoita E, Nameta M, Kurihara H, Qin XS, Yamamoto T, Doi T. 2007. Podocin participates in the assembly of tight junctions between foot processes in nephrotic podocytes. J Am Soc Nephrol 18:2525–2533.
Sriphrapradang C, Chailurkit LO, Aekplakorn W, Ongphiphadhanakul B. 2013. Association between bisphenol A and abnormal free thyroxine level in men. Endocrine 44:441–447.
Takeuchi T, Tsutsumi O, Ikezuki Y, Kamei Y, Osuga Y, Fujiwara T, Takai Y, Momoeda M, Yano T, Taketani Y. 2006. Elevated serum bisphenol A levels under hyperandrogenic conditions may be caused by decreased UDP-glucuronosyltransferase activity. Endocr J 53:485–491.
Taylor JA, Vom Saal FS, Welshons WV, Drury B, Rottinghaus G, Hunt PA, Toutain PL, Laffont CM, VandeVoort CA. 2011. Similarity of bisphenol A pharmacokinetics in rhesus monkeys and mice: relevance for human exposure. Environ Health Perspect 119:422–430.

Teeguarden JG, Calafat AM, Ye X, Doerge DR, Churchwell MI, Gunawan R, Graham MK. 2011. Twenty-four hour human urine and serum profiles of bisphenol a during high-dietary exposure. Toxicol Sci 123:48–57.
Trasande L, Attina TM, Trachtman H. 2013. Bisphenol A exposure is associated with low-grade urinary albumin excretion in children of the United States. Kidney Int 83:741–748.
Tyl RW, Myers CB, Marr MC, Sloan CS, Castillo NP, Veselica MM, Seely JC, Dimond SS, Van Miller JP, Shiotsuka RN, Beyer D, Hentges SG, Waechter JM , Jr. 2008. Two-generation reproductive toxicity study of dietary bisphenol A in CD-1 (Swiss) mice. Toxicol Sci 104:362–384.
Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Environ Health Perspect 118:1055–1070.
Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR , Jr. , Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons WV, Zoeller RT, Myers JP. 2012. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev 33:378–455.
Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. 2009. Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocr Rev 30:75–95.
Volkel W, Bittner N, Dekant W. 2005. Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. Drug Metab Dispos 33:1748–1757.
Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, Watson CS, Zoeller RT, Belcher SM. 2007. In vitro molecular mechanisms of bisphenol A action. Reprod Toxicol 24:178–198.
Wiggins JE, Goyal M, Sanden SK, Wharram BL, Shedden KA, Misek DE, Kuick RD, Wiggins RC. 2005. Podocyte hypertrophy, "adaptation," and "decompensation" associated with glomerular enlargement and glomerulosclerosis in the aging rat: Prevention by calorie restriction. J Am Soc Nephrol 16:2953–2966.
Wolf G, Ziyadeh FN. 1999. Molecular mechanisms of diabetic renal hypertrophy. Kidney Int 56:393–405.
Zalko D, Soto AM, Dolo L, Dorio C, Rathahao E, Debrauwer L, Faure R, Cravedi JP. 2003. Biotransformations of bisphenol A in a mammalian model: Answers and new questions raised by low-dose metabolic fate studies in pregnant CD1 mice. Environ Health Perspect 111:309–319.
Ziyadeh FN, Wolf G. 2008. Pathogenesis of the podocytopathy and proteinuria in diabetic glomerulopathy. Curr Diabetes Rev 4:39–45.