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Research Article

Comparing the Effects of Bisphenol A and Bisphenol S on Human Podocyte Biology

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Abstract

Human podocytes (hPC) are crucial for renal structural integrity and function. Loss of hPC plays an important role in pathogenesis of various renal disorders. Bisphenol A (BPA) is widely used in various everyday goods. Due to its potential cytotoxic activities, especially in the cardio-renal system, alternatives were used for "BPA-free" manufacturing, such as bisphenol S (BPS).Similar to BPA, BPS exhibits estrogenic activities and was suggested to induce cardiotoxic effects. In contrast to BPA, the renal impact of BPS is unknown so far. Here, we performed a comparative analysis of the effects of BPS and BPA on hPC viability and protein expression.

Functional assays were used for cell viability measurement and expression was characterized via real-time PCR and Western blotting in hPC. We found that BPA and BPS (0.01 µM - 1000 µM) differentially affect hPC viability as well as expression of nephrin, protein kinase B, podoplanin and nuclear factor 'kappa-light-chain-enhancer' of activated B - cells p65 subunit RelA in renal cells in a dose-dependent manner. Inhibition of protein kinase B, RelA, or podoplanin, respectively, were also shown to reduce hPC viability. Moreover, 10 µM BPA but not BPS, induced interleukin-1 beta and Bcl-2-associated death promoter expression in hPC. Here, we provide first novel findings about potential cytotoxic effects of BPS on renal cells. Moreover, we show that BPA's nephrotoxic impact on hPC viability and protein expressions were much stronger than that of BPS in this study.

Introduction

Bisphenol A (BPA) is one of the most important man-made synthetic polymers used for the production of various everyday articles, such as polycarbonate water bottles, plastic food container, baby bottles and the inner coating of tin cans [1]. This consequently leads to a high risk of customer exposure to BPA, e.g. by food contaminations [1]. BPA is metabolized in the liver and predominantly excreted renally [2]. Several studies indicated that BPA may mediate cytotoxic, genotoxic and carcinogenic activities in the context of different human pathologies, such as cardiovascular disease, reproductive disorders and in renal diseases [1,3,4]. However, these data are still controversially debated in the context BPA's safety and its high industrial importance [5]. Due to BPA's potential toxic effects, the European Food Safety Authority (EFSA) prohibited in 2011 its use in the manufacture of polycarbonate infant feeding bottles and limited BPA's tolerable daily intake in the European Union (EU) to 4 µg/kg body weight per day in 2015 [6]. In consequence to these restrictions, manufacturers are increasingly tending to replace BPA by alternatives, such as bisphenol S (BPS) to manufacture "BPA-free" products [7,8]. BPS can be found in higher levels - amongst others - in thermal paper and food, which consequently leads to an increased risk for customer exposure to this compound, too [9]. BPS was shown to mediate estrogenic activities, comparable to that of BPA [10]. Furthermore, BPS was recently demonstrated to mediate arrhythmogenic effects by over-activating ventricular myocytes and increasing electrical activities in female rat hearts [11]. These data indicated that BPS induces cardiotoxic effects in the context of female-specific heart arrhythmias, similar to those reported for BPA [11]. However, renal activities of BPS, especially regarding its potential nephrotoxicity are largely unexplored.

Human podocytes (hPC) are crucial for renal structural integrity and function [12,13]. In this context, hPC play an essential role for maintenance of the glomerular filtration barrier via generating and providing essential compounds of the slit diaphragm, such as nephrin [14,15]. Beside nephrin as structural factor, biological function of hPC were also regulated via various other proteins, including factors involved in cell survival and viability, such as protein kinase B (Akt) or podoplanin (PDPN) [12,16,17] and proteins involved in gene expression control, e.g. the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) p65 subunit RelA [15,18]. Damage or loss of hPC is of major importance for the pathogenesis of various renal disorders, including chronic kidney disease or diabetic nephropathy [14,19]. In this context, reduced hPC viability as consequence, e.g. of increased cell apoptosis or reduced survival, plays a crucial role in the development of renal insufficiency [17-19].

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Literature regarding renal effects of BPS as well as data showing mechanistic insights into its mode of action in the renal context, especially in hPC are sparse. Therefore, we performed a comparative analysis of the impact of BPS and BPA on hPC viability. Moreover, we characterized the differential effects of both compounds on the expression of nephrin, Akt, PDPN and RelA inrenal cells. Finally, we studied the functional impact of Akt, RelA and PDPN on cell viability in hPC incubated with BPS or BPA, respectively. In sum, our data show for the first time that the nephrotoxic potential of BPS, regarding its impact on hPC viability and gene expression, is much lower than that of BPA.

Methods

Cell culture

Immortalized hPC were obtained from Dr. M. Saleem (University of Bristol, Bristol, UK) and were cultured in RPMI 1640 supplemented with fetal bovine serum (FBS; 10%), insulin-transferrin-selenium (1x) and penicillin/streptomycin (1%), all purchased from BIOCHROM GmbH, Berlin, Germany. These cells proliferate at 33°C and differentiate at 37°C for 14 d to mature hPC as described earlier [13]. Cells were incubated with different concentrations ranging from 0.01 µM to 1000 µM of BPA (BIOZOL GmbH, Eching, Germany) or BPS (BIOZOL GmbH, Eching, Germany) in FBS-free RPMI 1640 Medium (BIOCHROM GmbH, Berlin, Germany) for 24 h or 48 h, respectively. Controls were treated with corresponding amounts of ethanol (99.9 %, solvent; Fisher Scientific GmbH, Schwerte, Germany). BAY 11-7082 (10 µM; Adipogen AG, Liestal, Switzerland) was used for pharmacological inhibition of NFκB and Akt was blocked via triciribine (10 µM; Selleck Chemicals, Houston, TX, USA) as described before [15,20]. Transfection of hPC was performed using LipofectamineTM 2000 (Life Technologies GmbH, Darmstadt, Germany) and 200 nM specific small interfering ribonucleic acid (siRNA) directed against PDPN (siPDPN, Sigma-Aldrich Chemie GmbH, Munich, Germany) or a non-sense control siRNA (siControl; Sigma-Aldrich Chemie GmbH, Munich, Germany), respectively. Transfection efficiency was 25 % [12].

Cell viability assay

Calcein AM (acetoxymethyl) cell viability kit (Trevigen Inc. Gaithersburg, MD, USA) was used for measurement of hPC viability as described earlier. In brief, hPC (1 x 104 / well) were cultured in 96-well plates and treated - as mentioned above - with different stimuli, inhibitors, or siRNAs, respectively, for 24 h. Following the manufacturer's protocol, hPC were washed with phosphate-buffered saline (Life Technologies GmbH, Darmstadt, Germany) and then incubated for 30 min with calcein AM working solution. Finally, fluorescence was determined (490 nm excitation, 520 nm emission).

Western blot

Protein expression was determined in hPC cell lysates via sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blot analyses as previously described [12,15]. Protein detection was done using specific antibodies: anti-nephrin (1:500; Thermo Fisher Scientific, Waltham, MA, USA), anti-PDPN (1:500; Sigma-Aldrich Chemie GmbH, Munich, Germany), anti-Akt (1:500, Merck Chemicals GmbH, Schwalbach, Germany), anti-GAPDH (1:20000, Calbiochem, Darmstadt, Germany), anti-RelA (1:500, Aviva Systems Biology, Corp., San Diego, CA, USA), rabbit anti-mouse and goat anti-rabbit (both 1:5000, DAKO, Glostrup, Denmark). Final quantification of protein expression was done via Gel-Pro AnalyzerTM software (version 4.0.00.001; Media Cybernetics, Bethesda, MD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Universal RNA purification kit (Roboklon GmbH, Berlin, Germany) was used for isolation of total RNA which was then reverse transcribed into cDNA templates via the High-Capacity cDNA Reverse Transcription Kit (Life Technologies GmbH, Darmstadt, Germany). For qRT-PCR a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used under the following conditions: 50°C, 2 min; 95°C, 20 s; 45 cycles 95°C, 3 s; 60°C, 30 s. For analyses of the gene expression of Bcl-2-associated death promoter (BAD), interleukin-1 beta (IL-1β) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan® Gene Expression Assays (Life Technologies GmbH, Darmstadt, Germany) were used following the manufacturer's instructions.

Statistical analysis

Statistics were done via GraphPad Prism (version 6.00; GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed via Student's t-test or one-way analysis of variance (ANOVA), as appropriate and were expressed as mean ± SEM. A probability value (p) ≤0.05 was regarded as significant.

Results

First of all, we compared the effects of BPA *vs* BPS on hPC viability post 24 h. Treatment of cells with 0.01 - 1 µM BPA had no significant influence on cell viability (Figure 1). Compared to controls, incubation of hPC with 10 µM and 100 µM BPA led to a significant reduction of cell viability. Moreover, treatment of renal cells with 1000 µM led to a total loss of hPC at this time point. Compared to BPA, application of BPS ranging from 0.01 µM to 100 µM had no significant inhibitory effect on renal cell viability. In contrast to low BPS concentration, incubation of hPC with 1000 µM BPS significantly reduced cell viability. Compared to BPA, the impact of BPS on cell viability was significantly lower in cells treated with the same concentrations of BPA *vs* BPS (Figure 1).

Treatment of hPC with BPA and BPS for 24 h differentially modulated the protein expression of Akt, RelA, nephrin and PDPN (Figure 2). Compared to controls, incubation of renal cells with 0.1 µM and 10 µM BPA and BPS had no significant impact on Akt and RelA expression (Figure 2A,B). In contrast, treatment of hPC with 1000 µM BPA completely inhibited protein expression of both proteins in hPC. Whereas, treatment of cells with 1000 µM BPS led to a significant but only slight reduction of Akt and RelA (Figure 2A,B). Comparable results were also found for protein expression of nephrin and PDPN (Figure 2C,D). Low concentrations (0.1 µM and 10 µM) of BPA and BPS had no effect on the expression of nephrin and PDPN in hPC. Compared to controls, application of 1000 µM BPA significantly reduced nephrin and PDPN protein, whereas, 1000 µM BPS had no significant influence on protein expression of both factors in hPC (Figure 2C,D).

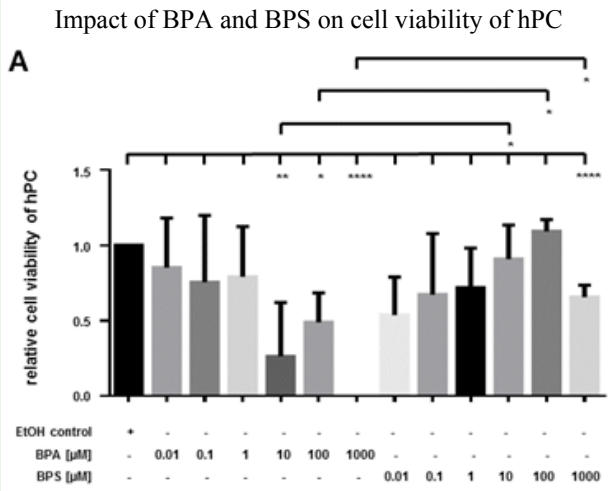

Figure 1: BPA and BPS differentially affect cell viability of hPC. Shown is the cell viability of hPC 24 h after incubated with different concentrations (0 µM - 1000 µM) of BPA or BPS, respectively? Controls were treated with an appropriate volume of ethanol (solvent; EtOH control). (*) P ≤ 0.05, (**) P ≤ 0.01, (****) P ≤ 0.0001; n = 5.

Next, we characterized the impact of BPA *vs* BPS on Akt, RelA, nephrin and PDPN protein expression in hPC after 48 h (Figure 3). Compared to control cells, treatment of hPC with low concentrations of BPA ranging from 0.01 µM - 10 µM had no impact on the expression of Akt, RelA, nephrin and PDPN (Figure 3A-D). Whereas, incubation of cells with 100 µM BPA led to a significant reduction of Akt, RelA, nephrin and PDPN. Compared to controls, treatment of renal cells with BPS did not affect Akt, RelA, nephrin, and PDPN expression at any tested concentration (0.01 µM - 100 µM) (Figure 3A-D). Moreover, there was a significant difference regarding

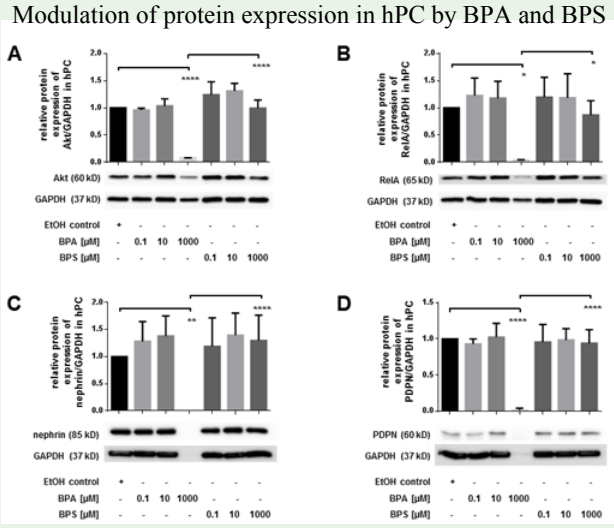

Figure 2: Modulation of protein expression by BPA vs BPS in hPC. (A-D) Protein expression of (A) Akt, (B) RelA, (C) nephrin and (D) PDPN normalized against GAPDH expression in hPC post 24 h. Compared were hPC incubated with 0 µM, 0.1 µM, 10 µM and 1000 µMBPA or BPS, respectively. Control cells were treated with an appropriate volume of ethanol (EtOH control).Shown is the mean ± SEM of at least 3 independent experiments. (*) P ≤ 0.05, (**) P ≤ 0.01,(****) P ≤ 0.0001.

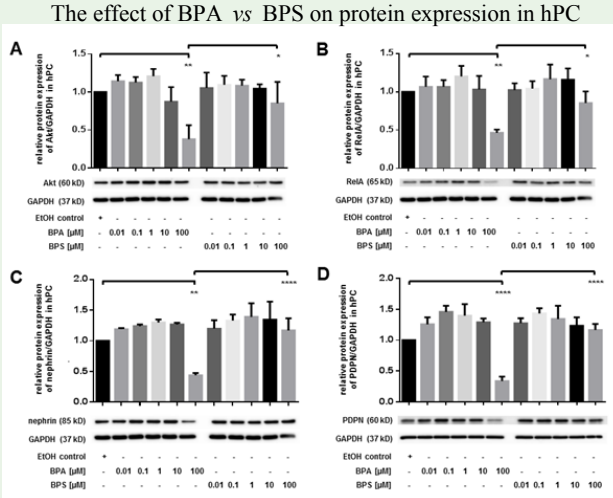

Figure 3: BPA *vs* BPS differentially affect Akt, RelA, nephrin, and PDPN in hPC. Depicted is the ratio of (A) Akt/GAPDH, (B) RelA/GAPDH,(C) nephrin/GAPDH and (D)PDPN/GAPDH protein expression 48 h post incubation of hPC with different concentrations (0 µM - 100 µM) of BPA or BPS, respectively. Application of an equal amount of the used solvent ethanol to hPC was used as control (EtOH control).Shown is the mean ± SEM of at least 3 independent experiments. (*) P ≤ 0.05, (**) P ≤ 0.01,(****) P ≤ 0.0001.

the impact of 100 µM BPA compared to the influence of 100 µM BPS on the expression of all four analyzed proteins.

Since, Akt, RelA and PDPN were shown to modulate cell survival and viability of renal cells [12,17,18] we studiedthe role of these factors in modulating cell viability of hPC treated with BPA or BPS, respectively (Figure 4). Compared to non-treated control cells, inhibition of Aktby application of 10 µM triciribine alone led to a significant decrease of hPC viability post 24 h (Figure 4A). Co-treatment of renal cells with triciribine and BPA or BPS, respectively, exhibited no further reductive effects on cell viability. Blocking of

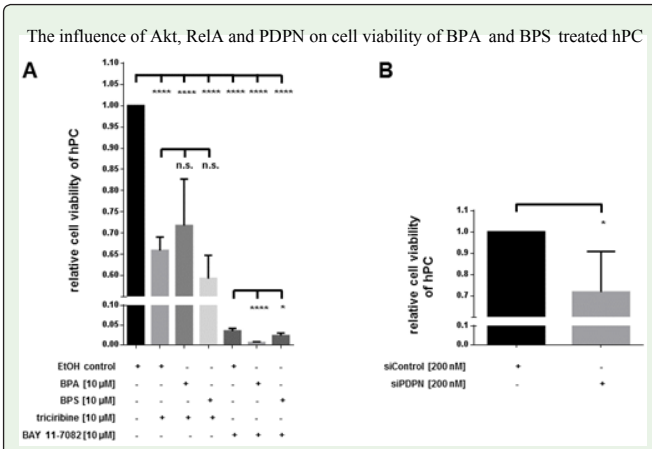

Figure 4: The influence of Akt, RelA, and PDPN on hPC viability. (A) Cell viability of hPC 24 h after inhibition of RelA-associated NFκB activity by 10 µM BAY 11-7082 or Akt via incubation with 10 µM triciribine, respectively, in cells activated with 10 µM BPA and BPS. Control cells were treated with an appropriatevolume of ethanol (EtOH control). (B) Shows the reduction of hPC viability by siRNA-mediated inhibition of PDPN. (siPDPN, 200 nM). A non-functional siRNA (siControl, 200 nM) was used as control. (*) P ≤ 0.05, (****) P ≤ 0.0001,(n.s.) no significant difference; n = 5.

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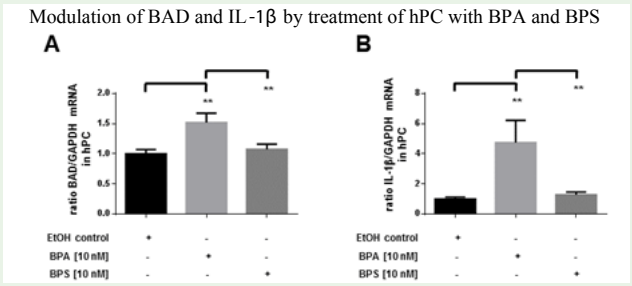

Figure 5: The impact BPA and BPS of BAD and IL-1β mRNA expression. Shown is the mRNA expression of (A) BAD and (B) IL-1β normalized against GAPDH in hPC. Compared were cells treated for 24 h with 10 μM BPA or 10 μM BPS, respectively. Application of an appropriate volume of ethanol (solvent) was used as control (EtOH control). (**) P ≤ 0.01; n=3.

RelA-associated NFκB activity by the pharmacological inhibitor BAY 11-7082 (10 μM) also decreased hPC viability. Compared to cells only treated with BAY 11-7082, additional incubation of cells with BPA or BPS, respectively, led to a further significant reduction of cell viability in those treated cells (Figure 4A). Moreover, siRNA-mediated knockdown of PDPN also reduced hPC viability (Figure 4B).

Compared to controls, application of 10 nM BPA induced the expression of pro-apoptotic BAD in renal cells (Figure 5A). In contrast to BPA, treatment of hPC with BPS had no significant effect on BAD generation. Moreover, BPA incubation also increased the expression of pro-inflammatory IL-1β (Figure 5B). Compared to control cells, treatment of hPC with 10 nM BPS did not affect the generation of IL-1β.

Discussion

BPAwas indicated to mediate cytotoxic effects in the context of different human pathologies, such in renal diseases [1,3,4]. In order to manufacture "BPA-free" products, BPS was used as alternative to BPA [7,8]. Similar to BPA, BPS was found to mediate estrogenic activities and to induce cardiotoxic effects in the context of female-specific heart arrhythmias [10,11]. However, in contrast to BPA [4,5], little is known about potential nephrotoxic effects of its substitute BPS.

Loss of hPC and their function plays a central role in the pathogenesis of various renal diseases, such as chronic kidney disease or diabetic nephropathy [14,19]. Data regarding renal effects of BPS, especially thoseshowing mechanistic insights are sparse. Therefore, we set out to perform a comparative analysis of the impact of BPS and BPA on hPC viability and the expression of factors, involved in renal cell function control, such as nephrin, PDPN, Akt and RelA. Here, we showed for the first time that high dose BPS reduced hPC viability. Moreover, BPA had no significant effects on the expression of nephrin, PDPN, Akt and RelA in hPC. And, BPA's renotoxic effectson hPC viability and protein expression were much stronger than that of BPS in this experimental setting.

In our study, we found that BPA reduce hPC viability in a dose-dependent manner. Low BPA doses (up to 1μM) had no significant influence, whereas, higher doses (10 μM and 100 μM) significantly diminished cell viability with a complete hPC loss at 1000 μM BPA. In line with our findings, Michalowicz et al. demonstrated that low level of BPA (<220 μM) had no effect on Human Peripheral Blood Mononuclear Cell (PBMC) viability after 4 h [21]. Treatment of cells with higher BPA doses (220 μM - 440 μM) led to a concentration-dependent reduction of PBMC viability [21]. Comparable data showing dose-dependent effects of BPA on cell viability were also published by others [4,22]. In our experiments, we further found that there was no significant impact of BPS on cell viability at concentrations ranging from 0.01 μM - 100 μM. Solely, treatment of hPC with the highest tested BPS dose (1000 μM) led to a significant but moderate reduction of viability. Substantiating this, Zhang et al. also showed that low BPS doses (0 μM - 100 μM) exhibited no relevant cytotoxic effects on primary isolated murine renal cells whereas, high BPS concentration (300 μM - 1000 μM) led to a significant reduction of renal cell viability. Moreover, we demonstrated in this study for the first time that BPA's cytotoxic and viability-reducing effect on human renal cells was significantly higher than that of BPS. This is in line with the findings of different groups in other experimental settings [21-23]. In this context, Ma and colleagues depicted that application of 300 μM BPA led to a diminished viability of chicken embryonic hepatocytes, whereas, BPS mediated no cytotoxic effects at the same concentration [23]. Substantiating this, Michalowicz et al. also demonstrated 100 μM BPA to reduce PBMC viability, whereas, BPS mediated no cytotoxic activities at this concentration [21].

Modulation of cell viability,as consequence of increased cell apoptosis and/or diminished cell survival, is highly regulated via expression and function of different factors, such as structural proteins (e.g., PDPN and nephrin) [4,12,15], kinases involved in pro-survival signaling (i.e., Akt) [12,16] and transcription-modulating factors, such as NFκB p65 subunit RelA [18,24]. In our study, we found BPS and BPA to differentially affect Akt, RelA, nephrin and PDPN expression in hPC. Treatment of renal cells with low doses (0.1 μM - 10 μM) of BPA or BPS, respectively, had no significant influence on the expression of these factors. In contrast to low concentration, high BPA doses (100 μM - 1000 μM) led to reduced level of Akt, RelA, nephrin and PDPN in hPC, whereas, 100 μM and 1000 μM BPS did not affects protein expression. In line with our data, Benigni and colleagues showed in 2006 that application of with 25 μM of BPA diglycidyl ether to human renal tubulus cells (HK-2) reduced promotor activity and expression of the nephrin gene. Recently, this was further substantiated by findings of Olea-Herrero et al. which demonstrated that activation of primary isolated Murine Podocytes (mPC) with BPA led to reduced expression of nephrin in the context of BPA-induced proteinuria in mice [4].

Regarding modulation of Akt, other groups also showed high but not low BPA doses to reduce Akt expression in other experimental settings, which is in line with our observations in hPC [25-27]. Zhao et al. depicted in 2014 that incubation of primary isolated murine ovaries with low BPA concentrations ranging from 0.1 μM to 10 μM had no significant impact on Akt expression [26]. Comparable results were published by Kim et al., also showing no significant impact of low BPA doses (0.1 nM - 10 nM) on protein level of Akt in testicular rat cells [27]. Recently, Vahdati Hassaniet al. depicted that Akt protein expression was reduced in the liver of Wistar rats exposed to high dose BPA (0.5 mg/kg) for 30 d. Together, these findings are in line with our data.

Until now, BPA's impact on RelA and PDPN expression as well as the influence of BPS on Akt, RelA, nephrin, and PDPN was completely uncharted. Here, we found high doses of BPA to reduce expression

of these factors, whereas, BPS had no significant effects on protein expression of all analyzed factors. In 2014 Peyre et al. demonstrated that BPS is less cytotoxic that BPA in the context of metabolic syndromes [28]. They showed that BPA'shepatotoxic potential was mediated viaexpression modulationof different toxicity-, stress-, and transport-, and lipid metabolism-related factors, such as cytochromes P450 (CYP)2B6 and fatty acid synthase in different human hepatocellular cell lines treated with 1 µM - 100 µM of this compound. In contrast to BPA, 1 µM - 100 µM BPS had no influence on these proteins and mediated no toxic effects in hepatocytes [28]. In another experimental setting, Ma et al. also showed 1 µM - 10 µM BPA or BPS, respectively, to differentially affect gene expression of different factors involved in toxicological pathways in chicken embryonic hepatocytes [23]. BPA-mediated modulation of the gene expression pattern was associated with decreased cell viability, whereas, BPS had exhibited no cytotoxic activities in this context [23]. In line with these findings, we also found high doses of BPA to reduce protein generation of factors involved in gene expression control, survival signaling and structural integrity of hPC, such as RelA, Akt, nephrin and PDPN [4,12,16,18]. BPS had no impact on these factors as well as on hPC viability at the same concentrations. Moreover, we found that inhibition of Akt, RelA-dependent NFκB activity and PDPN (Figure 4) led to reduced hPC viability, too. Therefore, reduced expression of these proteins by BPA but not by BPS, may - at least in part - be involved in mediating BPA's more potent viability-reducing effects in hPC, compared to BPS.

Finally, we found 10 µM BPA to induce expression of pro-apoptotic BAD and pro-inflammatory IL-1β in hPC, which was associated with reduced hPC viability. In contrast to BPA, 10 µM BPS had no effect on these factors or renal cell viability. This is in line with results of different other groups [29-31]. In 2012, Kuan et al. depicted incubation of murine RAW267.7 macrophages with BPA-derivates to induce IL-1β expression, which consequently led to increased oxidative stress and pro-inflammatory activation of these cells [29]. In another experimental setting, Bontempo et al. demonstrated that application of 60 µM BPA led to increased BAD activity in human leukemia NB4 cells in vitro [30]. In 2014, Wang and colleagues also showed treatment of rats with 0.25 mg/kg BPA to induce BAD activation in rat mammary glands in vivo [31]. In both studies, BPA-induced BAD activation was associated with increased cell apoptosis in vitro and in vivo [30,31]. In this context, our findings suggest that increased expression of pro-inflammatory IL-1β and pro-apoptotic BAD in BPA-activated hPC may also be involved in mediating BPA's cytotoxic effects in these cells.

Conclusion

In this study, we showed BPA to reduce cell viability of human renal cells which was associated with reduced expression of proteins involved in transcriptional control, cell survival and renal structural integrity (RelA, Akt, nephrin and PDPN) [4,12,16,18] and increased generation of pro-inflammatory IL-1β and pro-apoptotic BAD [29,30]. Furthermore, we found that these effects of BPA were mediated in a dose-dependent manner in hPC. Finally, we demonstrated for the first time that BPS had no significant impact on the expression of the abovementioned factors in human renal cells and exhibited a much lower hPC viability-reducing potential, compared to BPA. In sum, this leads to the conclusion that BPS exhibits less nephrotoxic potential than BPA in hPC. Therefore, BPS may be indicated to be "safer" than BPA in renal settings. However, further experiments, especially in the context of adequate in vivo studies, are needed to substantiate and validate these first experimental findings in human renal cells.

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