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ORIGINAL RESEARCH

Bisphenol A Increases Atherosclerosis in Pregnane X Receptor-Humanized ApoE Deficient Mice

Yipeng Sui, PhD; Se-Hyung Park, PhD; Robert N. Helsley, BS; Manjula Sunkara, MS; Frank J. Gonzalez, PhD; Andrew J. Morris, PhD; Changcheng Zhou, PhD

***Background*—**Bisphenol A (BPA) is a base chemical used extensively in many consumer products. BPA has recently been associated with increased risk of cardiovascular disease (CVD) in multiple large-scale human population studies, but the underlying mechanisms remain elusive. We previously reported that BPA activates the pregnane X receptor (PXR), which acts as a xenobiotic sensor to regulate xenobiotic metabolism and has pro-atherogenic effects in animal models upon activation. Interestingly, BPA is a potent agonist of human PXR but does not activate mouse or rat PXR signaling, which confounds the use of rodent models to evaluate mechanisms of BPA-mediated CVD risk. This study aimed to investigate the atherogenic mechanism of BPA using a PXR-humanized mouse model.

***Methods and Results*—**A PXR-humanized ApoE deficient (huPXR•ApoE$^{-/-}$) mouse line was generated that respond to human PXR ligands and feeding studies were performed to determine the effects of BPA exposure on atherosclerosis development. Exposure to BPA significantly increased atherosclerotic lesion area in the aortic root and brachiocephalic artery of huPXR•ApoE$^{-/-}$ mice by 104% ($P<0.001$) and 120% ($P<0.05$), respectively. By contrast, BPA did not affect atherosclerosis development in the control littermates without human PXR. BPA exposure did not affect plasma lipid levels but increased CD36 expression and lipid accumulation in macrophages of huPXR•ApoE$^{-/-}$ mice.

***Conclusion*—**These findings identify a molecular mechanism that could link BPA exposure to increased risk of CVD in exposed individuals. PXR is therefore a relevant target for future risk assessment of BPA and related environmental chemicals in humans.
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Key Words: atherosclerosis • cells • receptors • risk factors

Bisphenol A (BPA), a base chemical used extensively in polycarbonate plastics in many consumer products, is among the world's highest production-volume chemicals, with more than 8 billion pounds produced each year.[1] More than 80 biomonitoring studies indicate that human exposure to BPA is ubiquitous, and over 95% of the U.S. population is exposed to BPA.[1,2] BPA has been detected in human blood, urine, tissues and other fluids,[1] and numerous animal studies show that exposure to BPA causes diverse adverse effects.[3,4] Despite strong evidence for BPA's adverse effects in animals and, by extrapolation, in humans, recent evaluations of BPA safety by multiple panels have arrived at disparate conclusions and thus controversy remains about the specificity and mechanisms of the potential adverse effects of BPA.[5,6]

Recent large and well-conducted cross-sectional and longitudinal studies have found that higher BPA exposure is consistently associated with increased risk of cardiovascular disease (CVD).[7–9] Lang et al[7] first reported positive associations between urinary BPA concentrations and the CVD, type 2 diabetes, and liver enzyme abnormalities using data from the National Health and Nutrition Examination Survey (NHANES) 2003-2004. Higher urinary BPA levels were significantly associated with increased diagnosis of CVD including coronary heart disease, myocardial infarction, and angina.[7] Melzer et al[8] replicated the early association between urinary BPA concentrations and coronary heart disease using a separate NHANES 2005-2006 database. A separate large-scale longitudinal study has confirmed associations between

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higher BPA exposure levels and incident coronary artery disease during >10 years of follow-up of a group of healthy people who took part in the European Prospective Investigation of Cancer-Norfolk UK in the 1990s.[9] Interestingly, exclusion of subjects with obesity and adjustment for blood lipid concentrations or levels of physical activity had little effect on the associations,[7,9] suggesting that the associations are independent of traditional CVD risk factors. More recently, independent studies have associated BPA exposure with coronary atherosclerosis,[10] carotid atherosclerosis,[11] and peripheral arterial disease,[12] indicating potential effects of BPA exposure on atherosclerosis, the most common cause of CVD. However, the underlying mechanisms responsible for these associations remain unclear, which continues to hamper rational assessment of the health risks of BPA exposure.

BPA, regarded as a xenoestrogen, is a weak agonist of the estrogen receptor (ER), and most health studies of BPA have focused on its estrogenic effects.[4,13] However, the estrogenic effects of BPA probably do not explain the link between BPA exposure and CVD, as animal and human studies identify protective effects of estrogen against atherosclerosis or CVD.[14–18] To date, BPA has not been reported to have atherogenic effects in any animal models. Further, despite compelling evidence about BPA's estrogenic activity, doubts remain whether BPA exerts adverse estrogenic effects in animals and humans.[19–21] Thus, the endocrine-disrupting effects of BPA cannot be entirely attributed to its estrogenic activity and more mechanistic studies are urgently needed to explore the effect of BPA on other signaling pathways.

We previously reported that BPA and its analogs activate another nuclear receptor, the pregnane X receptor (PXR; also known as steroid and xenobiotic receptor, or SXR).[22] PXR functions as a xenobiotic sensor that regulates genes involved in drug and xenobiotic transport and metabolism, including cytochromes P450 (CYP), conjugating enzymes (eg, glutathione transferase (GST)), and ABC family transporters (eg, multidrug resistance 1 (MDR1)).[23,24] PXR is activated by endogenous hormones, dietary steroids, pharmaceutical agents, and other xenobiotic chemicals.[24–26] In mammals, PXR also exhibits considerable differences in its pharmacology and its ligand-binding domain (LBD) is remarkably divergent across species.[24,25] Interestingly, we found that BPA is a potent agonist for human PXR (hPXR) but not for mouse or rat PXR (mPXR and rPXR, respectively),[22] underscoring the importance of species choice in predicting the human risk assessment of BPA.

We recently revealed the pro-atherogenic effects of PXR in animal models and found that chronic activation of PXR increases atherosclerosis in ApoE-deficient (ApoE$^{-/-}$) mice.[27] These observations suggest that BPA-mediated PXR activation could potentially accelerate atherosclerosis development and increase CVD risk in humans. Because BPA is a potent agonist of human but not mouse or rat PXR,[22] the choice of animal model is a paramount issue in conducting preclinical studies to evaluate the contribution of BPA exposure to CVD risk. To investigate the effects of BPA exposure on atherosclerosis development, a PXR-humanized ApoE-deficient (huPXR•ApoE$^{-/-}$) mouse line was generated. Here we report that BPA increases atherosclerosis in ApoE$^{-/-}$ mice in a human PXR-dependent manner. BPA exposure does not affect plasma lipid levels but increases lipid accumulation and foam cell formation in macrophages of huPXR•ApoE$^{-/-}$ mice.

Methods

Animals and Diets

ApoE$^{-/-}$ mice on the C57BL/6 background were purchased from The Jackson Laboratory. PXR-humanized mice (huPXR, mouse PXR knockout/human PXR transgenic) were generated by transgenesis on a *Pxr*-null mice using a BAC clone containing the complete human PXR gene and including 5′- and 3′-flanking sequences as previously described.[28] huPXR mice have similar tissue distribution of PXR expression in liver and intestine as native PXR in humans and mice.[28] huPXR mice were backcrossed with C57BL/6 wild-type (WT) mice for at least 4 generations at NCI,[28] and backcrossed for 4 additional generations onto the C57BL/6 background and then bred with ApoE$^{-/-}$ mice to generate huPXR•ApoE$^{-/-}$ (hPXRtg-PXR$^{-/-}$ApoE$^{-/-}$) and PXR$^{-/-}$ApoE$^{-/-}$ mice. All the mice used in this study have the same background (PXR and ApoE null alleles) except for one allele of huPXR•ApoE$^{-/-}$ mice carrying the human PXR gene. All the animals were housed in a specific pathogen-free room with a 12-hour light-dark cycle in the University of Kentucky Division of Laboratory Animal Resources under a protocol approved by the Institutional Animal Care and Use Committee. BPA, rifampicin (RIF), and pregnenolone 16α-carbonitrile (PCN) were purchased from Sigma-Aldrich. BPA was incorporated into a modified semisynthetic diet containing 4.2% fat and 0.02% cholesterol[29,30] at a dose of 50 mg/kg by Harland Laboratories, Inc.[31–33] Four-week-old experimental male huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ littermates were weaned and fed with a control diet or a diet supplemented with BPA for 12 weeks until euthanization at 16 weeks of age (15 to 20 mice per group). Five to 10 mice were used for primary cell isolation and tissue analysis, and the rest were used for atherosclerosis analysis. The number of mice used in each study is listed in figure legends or shown by scatter points.

Blood Analysis and Atherosclerotic Lesion Quantification

Plasma total cholesterol and triglyceride concentrations were determined enzymatically by a colorimetric method.[29] Plasma

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from multiple mice (n=6) was pooled and plasma lipoprotein cholesterol distribution was determined by fast-performance liquid chromatography (FPLC).[34] OCT-embedded hearts or brachiocephalic arteries were sectioned and stained with Oil-red-O, and atherosclerotic lesions were quantified as previously described.[29,34] Immunohistochemistry were performed on sections of aortic roots with specific antibodies against PXR, monocyte/macrophage marker MOMA-2, or CD36 as previously described.[29,34]

Analysis of Urinary BPA by LC-ESI-MS/MS

Analysis of urinary BPA was performed using a modified chemical derivatization liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method.[35] Labeled BPA-d_{16} internal standard (Sigma-Aldrich) was added to each urine sample (50 μL) prior to extraction. An amount of 500 μL of cold acetonitrile was added to the samples and protein precipitation was collected by centrifugations. Supernatants were dried in 4 mL vials under N_2. An amount of 100 μL of sodium bicarbonate buffer (0.1 mol/L, pH 10) was added to the vials, followed by 100 μL of 1 mg/mL solution of pyridine-3-sulfonly chloride (PSC) in acetone. Vials were vortexed and placed in a heater block at 70°C for 5 to 10 minutes for preparation of PSC derivatives. Reaction mixtures were then cooled on ice for 10 minutes and dried under N_2. The samples were then reconstituted with 100 μL of methanol and transferred to autosampler vials for LC-ESI-MS/MS analysis. PSC derivatives of BPA and BPA-glucuronide samples were detected and quantitated by reverse phase HPLC using a Waters XTerra MS C8 column. The mobile phase consisted of 20% methanol with 1 mmol/L ammonium formate as solvent A and 100% methanol as solvent B. Analysis of BPA was achieved from 0% to 70% solvent B for 1 minute, which was gradually increased to 80% over 3 minutes and then to 90% over the next 4 minutes and maintained at 90% for the last 2 minutes. The column was equilibrated to initial conditions in 3 minutes. The flow rate was 0.5 mL/min with a column temperature of 30°C. The sample injection volume was 10 μL. The mass spectrometer was operated in positive electrospray ionization mode with a declustering potential of 51 V, entrance potential of 10 V, collision energy of 37 V, collision cell exit potential of 12 V, curtain gas of 10 psi, ion spray voltage of 5500 V, ion source gas1/gas2 of 40 psi and temperature of 550°C. The instrument was operated in selected ion monitoring mode with the following precursor product ion pairs monitored for the indicated analytes: m/z 511.1/354, m/z 511.1/212, m/z 511.1/79 for PSC-BPA and m/z 527.2/223.2, m/z 527.2/367.1, m/z 527.2/79 for PSC-BPA-d_{16} and m/z 546.2/213.2, m/z 546.2/276.1, m/z 546.2/79.2 for PSC-BPA-glucuronide.

Peritoneal Macrophage Isolation and Staining

Mice were injected intraperitoneally with 1 mL of 3% thioglycollate. Peritoneal macrophages were collected 4 days later and Oil red O/hematoxylin staining was performed as described before.[27,34] Cells containing lipid droplets (>10) were counted as foam cells and at least 10 fields per condition were counted.[36]

RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was isolated from mouse tissues or cells using TRIzol Reagent (Life Technologies) and quantitative real-time PCR (QPCR) was performed using gene-specific primers and the SYBR green PCR kit (Life Technologies) as described previously.[22] The primer sets used in this study are listed in Table 1.

Statistical Analysis

Statistical analysis was performed using a 2-sample, 2-tailed Student's *t* test for comparisons between 2 groups, in which $P<0.05$ was regarded as significant. One-way ANOVA analysis of variance was used when multiple comparisons were made followed by post hoc Bonferroni *t* test. All data were presented by mean±SD.

Results

Generation and Characterization of PXR-Humanized ApoE$^{-/-}$ Mice for BPA Risk Assessment

Since BPA is a human PXR-selective ligand, one of the key challenges to study the effects of BPA-mediated PXR activation on atherosclerosis is development of a mouse model that recapitulates the human response to PXR ligands. To address this issue, PXR-humanized ApoE knockout mice (huPXR•ApoE$^{-/-}$) were generated. The huPXR mice, expressing the human *PXR* gene in place of mouse *Pxr* gene,[28,37] were crossed with atherosclerosis-prone ApoE$^{-/-}$ mice to generate huPXR•ApoE$^{-/-}$ mice and PXR$^{-/-}$ApoE$^{-/-}$ mice (Figure 1A). The huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice generated by this strategy have the same genetic background (mPXR and ApoE null alleles) except for one allele of huPXR•ApoE$^{-/-}$ mice carrying the human PXR transgene (Figure 1A). The huPXR•ApoE$^{-/-}$ mice were then treated with the hPXR-specific ligand rifampicin (RIF) or the mPXR-specific ligand pregnenolone 16α-carbonitrile (PCN).[22] As expected, huPXR•ApoE$^{-/-}$mice can respond to the human-specific PXR agonist RIF but not to the mouse-specific activator PCN

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Table 1. Primer Sequences for Genomic PCR and QPCR

Gene	Primer Sequence	Gene	Primer Sequence
ApoE	5′-GCCTAGCCGAGGGAGAGCCG-3′	CD36	5′-CAGTCGGAGACATGCT-3′
	5′-TGTGACTTGGGAGCTCTGCAGC-3′		5′-CTCGGGGTCCTGAGTT-3′
	5′-GCCGCCCCGACTGCATCT-3′	SR-A	5′-GGAGTGTAGGCGGATC-3′
mPXR	5′-CTGGTCATCACTGTTGCTGTACCA-3′		5′-GTCAATGGAGGCCCCA-3′
	5′-GCAGCATAGGACAAGTTATTCTAGAG-3′	SR-BI	5′-CTCATCAAGCAGCAGGTGCTCA-3′
	5′-CTAAAGCGCATGCTCCAGACTGC-3′		5′-GAGGATTCGGGTGTCATGAA-3′
hPXR	5′-GCACCTGCTGCTAGGGAATA-3′	ABCA1	5′-CCGAGGAAGACGTGGACACCTTC-3′
	5′-CTCCATTGCCCCTCCTAAGT-3′		5′-CCTCAGCCATGACCTGCCTTGTAG-3′
CYP3A11	5′-CAGCTTGGTGCTCCTCTACC-3′	ABCG1	5′-AGGTCTCAGCCTTCTAAAGTTCCTC-3′
	5′-TCAAACAACCCCCATGTTTT-3′		5′-TCTCTCGAATGAAATTTATCG-3′
MDR1a	5′-CCCCCGAGATTGACAGCTAC-3′	GAPDH	5′-AACTTTGGCATTGTGGAAGG-3′
	5′-ACTCCACTAAATTGCACATTTCCTTC-3′		5′-GGATGCAGGGATGATGTTCT-3′

PCR indicates polymerase chain reaction; QPCR, quantitative real-time polymerase chain reaction.

(Figure 1B). RIF-mediated PXR target gene *Cyp3a11* upregulation in the liver was abolished in PXR$^{-/-}$ApoE$^{-/-}$ mice (Figure 1B). These results confirm the presence of the functional hPXR in huPXR•ApoE$^{-/-}$ mice. Thus, huPXR•ApoE$^{-/-}$ mice provides an in vivo system to assess atherogenic responses to relevant environment chemicals such as BPA, while allowing the use of a murine model to evaluate mechanisms of deleterious effects of BPA arising from human exposure.

BPA Activates hPXR and Stimulates PXR Target Gene Expression in huPXR•ApoE$^{-/-}$ Mice

The main route of human exposure to BPA is oral and pharmacokinetic studies have demonstrated that exposure via diet is a more natural continuous exposure route than other methods commonly used in chronically exposed animals.[38] To determine the impact of chronic exposure to BPA on atherosclerosis development, 4-week-old male huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ littermates were fed a control diet or a diet supplemented with BPA at a dose of 50 mg/kg. The choice of 50 mg BPA/kg feed weight was based on previous studies demonstrating that 50 mg BPA/kg feed weight represents a moderate or low dose exposure to BPA in experimental animal models.[31–33,38]

To determine whether 50 mg/kg feed weight provides urinary BPA concentrations similar to that observed in humans, a chemical derivatization LC-ESI-MS/MS method was developed to measure urinary BPA concentrations (Figure 2). Consistent with previous reports that BPA undergoes metabolism (conjugation) and clearance from the body, we were able to detect both conjugated BPA-glucuronide and

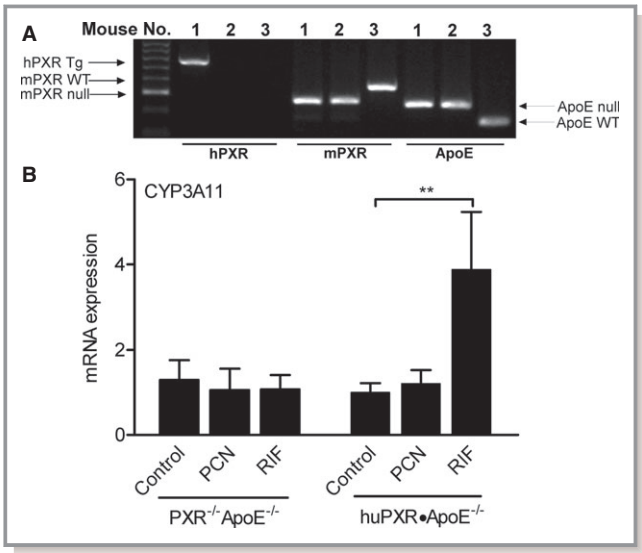

Figure 1. Generation of huPXR•ApoE$^{-/-}$ mice. A, Genotype analysis of huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice by 3 different PCR assays. The presence of the human (h) PXR transgene was determined by hPXR primers (576 bp). Mouse (m) PXR primers were used to identify WT allele (348 bp) and PXR null allele (265 bp). Mouse ApoE primers were used to identify WT allele (155 bp) and ApoE null allele (245 bp). Mouse no. 1 is huPXR•ApoE$^{-/-}$, no. 2 is PXR$^{-/-}$ApoE$^{-/-}$, and no. 3 is WT control. B, Six-week-old male huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice were treated with DMSO vehicle control, mPXR-specific ligand pregnenolone 16α-carbonitrile (PCN), or hPXR-specific ligand rifampicin (RIF) by intraperitoneal injection at the dose of 10 mg/kg per day for 3 days. Total RNA was extracted from the liver, and the mRNA levels of prototypic PXR activated gene *CYP3A11* were measured by QPCR (n=5 per group, $^{**}P<0.01$). PCR indicates polymerase chain reaction; PXR, pregnane X receptor; QPCR, quantitative real-time polymerase chain reaction; WT, wild-type.

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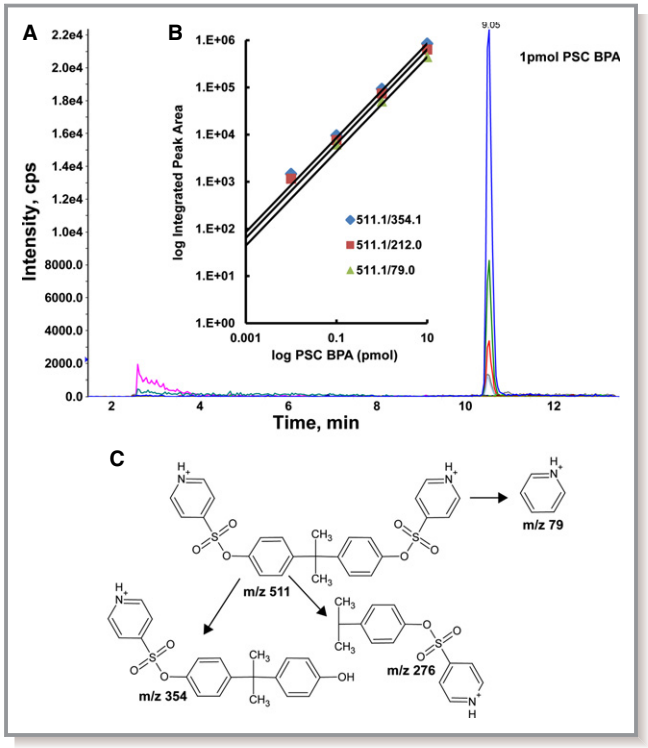

Figure 2. A novel LC-ESI-MS/MS method to quantitate BPA. A, BPA was derivatized by reaction with pyridine-3-sulfonyl chloride (PSC) in acetone and 1 pmol of the derivatized material was analyzed by reverse phase HPLC using a Waters XTerra MS C8 column. PSC-BPA was detected by positive mode electrospray ionization (ESI) selective reaction monitoring mode tandem MS using an ABSciex 4000 Q-Trap instrument as described in the methods monitoring the precursor product ion pairs shown in the calibration (B). The structure of the PSC derivative of BPA is shown (C) with a fragmentation scheme generating the product ions monitored in (A) and (B). BPA indicates bisphenol A; LC-ESI-MS/MS liquid chromatography-electrospray ionization-tandem mass spectrometry.

unconjugated BPA in the urine of BPA-exposed mice. While the concentrations of BPA-glucuronide were higher than unconjugated BPA, the urinary concentrations of unconjugated BPA were 1.19 and 2.33 ng/mL in PXR$^{-/-}$ApoE$^{-/-}$ and huPXR•ApoE$^{-/-}$ mice, respectively (Table 2). These values are similar to those detected in human urine samples with the unconjugated BPA concentrations that ranged from undetectable to 2.5 ng/mL.[1,39,40] BPA feeding stimulated expression of the prototypic PXR target genes, *Cyp3a11*, *Mdr1a*, and *Cd36* in the liver of huPXR•ApoE$^{-/-}$ mice, but not in PXR$^{-/-}$ApoE$^{-/-}$ mice (Figure 3), indicating that feeding huPXR•ApoE$^{-/-}$ mice 50 mg BPA/kg feed weight can efficiently activate human PXR in vivo.

Exposure to BPA Does Not Affect Plasma Lipid Levels but Increases Atherosclerosis in huPXR•ApoE$^{-/-}$ Mice

Exposure to BPA for 12 weeks did not affect the body weight of huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice (Figure 4). The effect of BPA exposure on plasma lipid and lipoprotein levels revealed no changes in plasma triglyceride and cholesterol levels (Figure 5). In addition, FPLC analysis showed that huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice had similar plasma cholesterol distribution patterns, which were not affected by BPA treatment (Figures 5C and 5F).

Atherosclerotic lesion areas were determined in the aortic root and brachiocephalic artery (BCA) as shown in Figure 6. Feeding huPXR•ApoE$^{-/-}$ mice BPA for 12 weeks significantly increased lesion areas in the aortic root by 104% (P<0.001; Student's t test) (Figure 6A). BPA feeding also accelerated atherosclerosis development in the BCA, an artery prone to developing advanced lesions (Figure 6B). Compared to mice fed control diet, BCA cross-section lesion areas were increased by 120% (P<0.05; Student's t test) in BPA-fed huPXR•ApoE$^{-/-}$ mice (Figure 6B). By contrast, exposure to BPA did not affect atherosclerotic lesion development in either the aortic root or BCA of PXR$^{-/-}$ApoE$^{-/-}$ mice (Figures 6C and 6D). Thus, BPA increases atherosclerosis in these models in a human PXR-dependent manner.

BPA Increases Lipid Accumulation and Foam Cell Formation in Macrophages of huPXR•ApoE$^{-/-}$ Mice

Macrophages play a critical role in atherogenesis and accumulation of lipid-loaded macrophages is a hallmark of atherosclerosis.[41,42] We previously reported that activation of

Table 2. Urinary BPA and BPA-Glucuronide Concentrations in Mice Fed the Control or BPA Diet for 12 Weeks

Genotype	Diet	BPA (ng/mL)	BPA-Glucuronide (ng/mL)
PXR$^{-/-}$ApoE$^{-/-}$	Control	N.D.	N.D.
huPXR•ApoE$^{-/-}$	Control	N.D.	N.D.
PXR$^{-/-}$ApoE$^{-/-}$	BPA	1.19±0.98	19.97±15.62
huPXR•ApoE$^{-/-}$	BPA	2.33±1.93	11.60±6.48

All values shown are mean±SD (n=9). BPA indicates bisphenol A; PXR, pregnane X receptor; N.D., not detectable.

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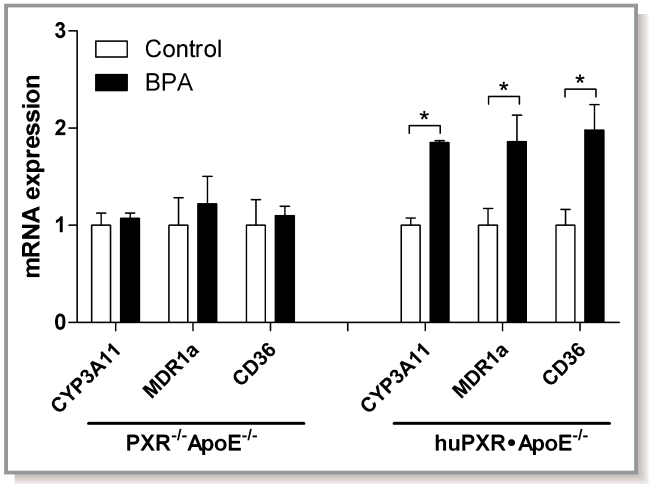

Figure 3. BPA increases PXR target gene expression in huPXR•ApoE$^{-/-}$ mice. Four-week-old male PXR$^{-/-}$ApoE$^{-/-}$ and huPXR•ApoE$^{-/-}$ littermates were fed a control diet or supplemented with 50 mg/kg BPA (BPA) for 12 weeks. The expression of hepatic PXR target gene mRNAs was measured by QPCR (n=4 per group, *P<0.05). BPA indicates bisphenol A; PXR, pregnane X receptor.

PXR increases lipid accumulation in macrophages of ApoE$^{-/-}$ mice, which contributes to PXR's pro-atherogenic effects.[27] To elucidate possible molecular mechanisms through which BPA increases atherosclerosis, exposure to BPA-affected macrophage functions was investigated. Peritoneal macrophages were isolated from huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice fed control diet or supplemented with BPA for 12 weeks and neutral lipid levels and foam cell formation in peritoneal macrophages were determined by oil-red-O staining. BPA feeding promoted lipid accumulation and foam cell formation in peritoneal macrophages of huPXR•ApoE$^{-/-}$ mice but not in that of PXR$^{-/-}$ApoE$^{-/-}$ mice (Figures 7A and 7B). Gene expression analysis showed that BPA exposure stimulated mRNA levels of the prototypic PXR activated genes, *Mdr1a* (P<0.05; Student's *t* test) and *Cd36* (P<0.01; Student's *t* test), in the macrophages of huPXR•ApoE$^{-/-}$ but not PXR$^{-/-}$ApoE$^{-/-}$ mice (Figure 7C). CD36 is a member of the scavenger receptor class B family and plays an important role in mediating macrophage lipid uptake and foam cell formation.[43] In contrast, the expression levels of mRNA encoding scavenger receptors, SR-A and SR-BI, and ABC transporters, ABCA1, and ABCG1, were not affected by BPA exposure in the macrophages derived from either huPXR•ApoE$^{-/-}$ or PXR$^{-/-}$ApoE$^{-/-}$ mice (Figure 7C).

The significantly increased *Cd36* mRNA levels and elevated lipid accumulation in the macrophages of BPA-treated huPXR•ApoE$^{-/-}$ mice promoted us to investigate the protein content of CD36 in the atherosclerotic lesions. Immunofluorescence staining showed that PXR is present in the lesions of huPXR•ApoE$^{-/-}$ mice and expressed by lesional macrophages (Figure 7D). Consistent with macrophage gene expression analysis, BPA exposure substantially increased CD36 protein levels in atherosclerotic lesions of huPXR•ApoE$^{-/-}$ mice (Figure 7D). Analysis of atherosclerotic lesions further confirmed that BPA significantly increased macrophage (P<0.05; Student's *t* test) and CD36 content (P<0.05; Student's *t* test) in plaques of huPXR•ApoE$^{-/-}$ mice but not PXR$^{-/-}$ApoE$^{-/-}$ littermates (Figure 7E). Thus, the increase in atherosclerotic lesions in BPA-fed huPXR•ApoE$^{-/-}$ mice is associated with increased CD36 expression and foam cell formation in macrophages.

Discussion

Risk assessment of BPA is still hampered by large scientific uncertainties and the impact of BPA exposure on human health is not clearly understood. While >95% of the US population is exposed to BPA, there is an urgent need to understand the molecular mechanisms underlying the associations between BPA exposure and CVD. We recently

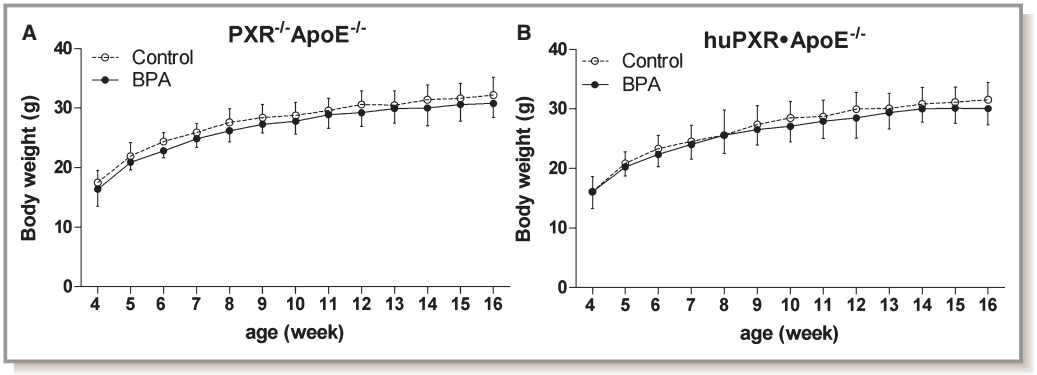

Figure 4. BPA exposure does not affect body weight. Growth curves of 4-week-old male PXR$^{-/-}$ApoE$^{-/-}$ (A) and huPXR•ApoE$^{-/-}$ (B) littermates fed a control diet or supplemented with 50 mg/kg BPA (BPA) for 12 weeks (n=11 to 17 per group). BPA indicates bisphenol A; PXR, pregnane X receptor.

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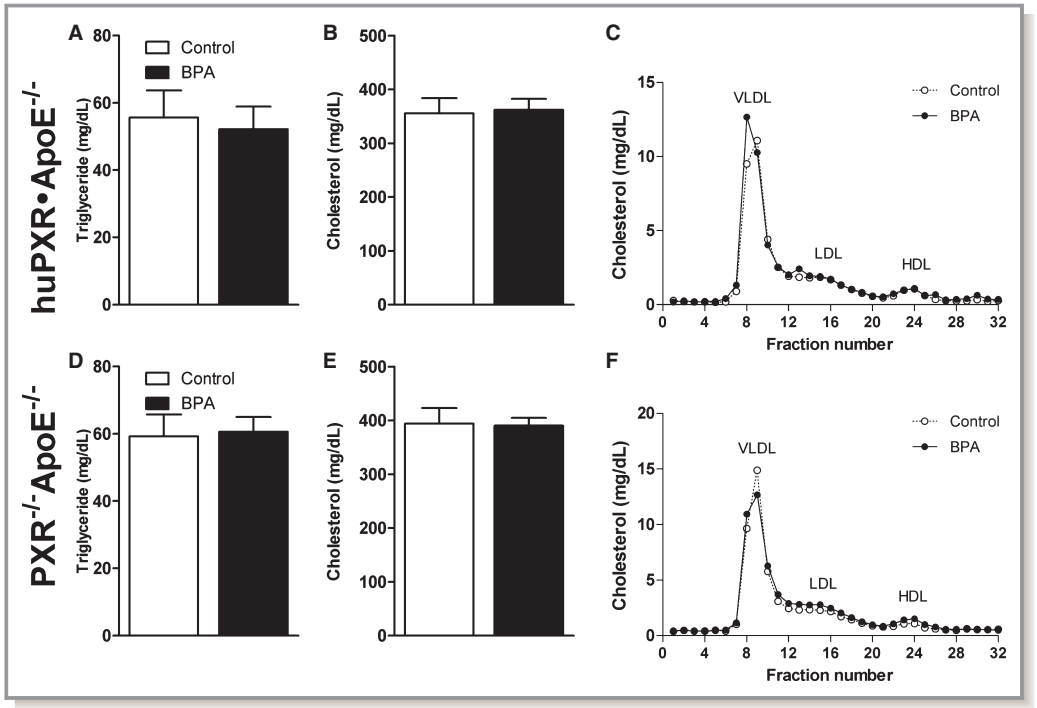

Figure 5. BPA exposure does not affect plasma lipid levels and cholesterol distribution. Four-week-old male huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ littermates were fed a control diet or supplemented with 50 mg/kg BPA (BPA) for 12 weeks. The plasma levels of triglyceride (A and D) and cholesterol (B and E) were measured by standard methods (n=11 to 12 per group) and plasma cholesterol distribution (C and F) was analyzed by FPLC. BPA indicates bisphenol A; FPLC, fast-performance liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PXR, pregnane X receptor.

reported that BPA is a potent agonist of PXR that has pro-atherogenic effects in animal models upon activation.[22,24,27] BPA is an hPXR-selective agonist but does not affect rodent PXR activity; consequently, the choice of an appropriate animal model is paramount in predicting the human risk assessment of BPA. Since the ligand-binding specificity of PXR differs between humans and rodents, PXR-humanized mouse models have been generated to more faithfully predict xenobiotic effects and responses in humans.[24,28,44] Compared with other humanized mice, one of the advantages of the huPXR mouse model is expression in a similar tissue distribution pattern as native PXR gene in humans and mice,[28] which enables the study of hPXR function in multiple tissues/cell types. This model has been successfully used in many studies to investigate human PXR ligand-mediated xenobiotic response in mice, and has been established as a useful tool for the prediction of human drug metabolism and toxicological risk assessment.[37,45–47] Therefore, the huPXR mice were used to generate the huPXR•ApoE$^{-/-}$ mice for studying the effects of BPA on atherogenic effects in vivo.

Since the main source of BPA exposure in humans is through the diet, mice were exposed to 50 mg BPA/kg feed weight in the current study. It was previously assumed that BPA undergoes rapid metabolism and clearance from the body. However, unconjugated BPA has been detected in human urine and tissues, and recent human biomonitoring data demonstrated that the unconjugated BPA concentrations is higher than previously predicted given assumptions about the amount of BPA ingested by humans and its expected rate of clearance.[1,48,49] Using our newly developed LC-ESI-MS/MS method, urinary unconjugated BPA in mice exposed to BPA were readily detected. While most human studies only report the total BPA concentration, several studies have detected unconjugated BPA in human urine with the concentrations ranging from undetectable to 2.5 ng/mL.[1,39,40] Our results confirmed that the dose of 50 mg BPA/kg feed weight is appropriated for long-term exposure studies in mice, which can result in urinary BPA concentrations similar to that observed in human samples. Further, the previously described standard LC-MS/MS methods, although reportedly sensitive, were unable to detect unconjugated BPA in many human samples.[50–52] Therefore, our LC-ESI-MS/MS method, which has significantly improved sensitivity compared to previously described methods may be applied to future biomonitoring studies for the evaluation of BPA exposure levels and safety in humans.

Interestingly, we found that chronic exposure to BPA increased atherosclerosis in huPXR•ApoE$^{-/-}$ mice but not

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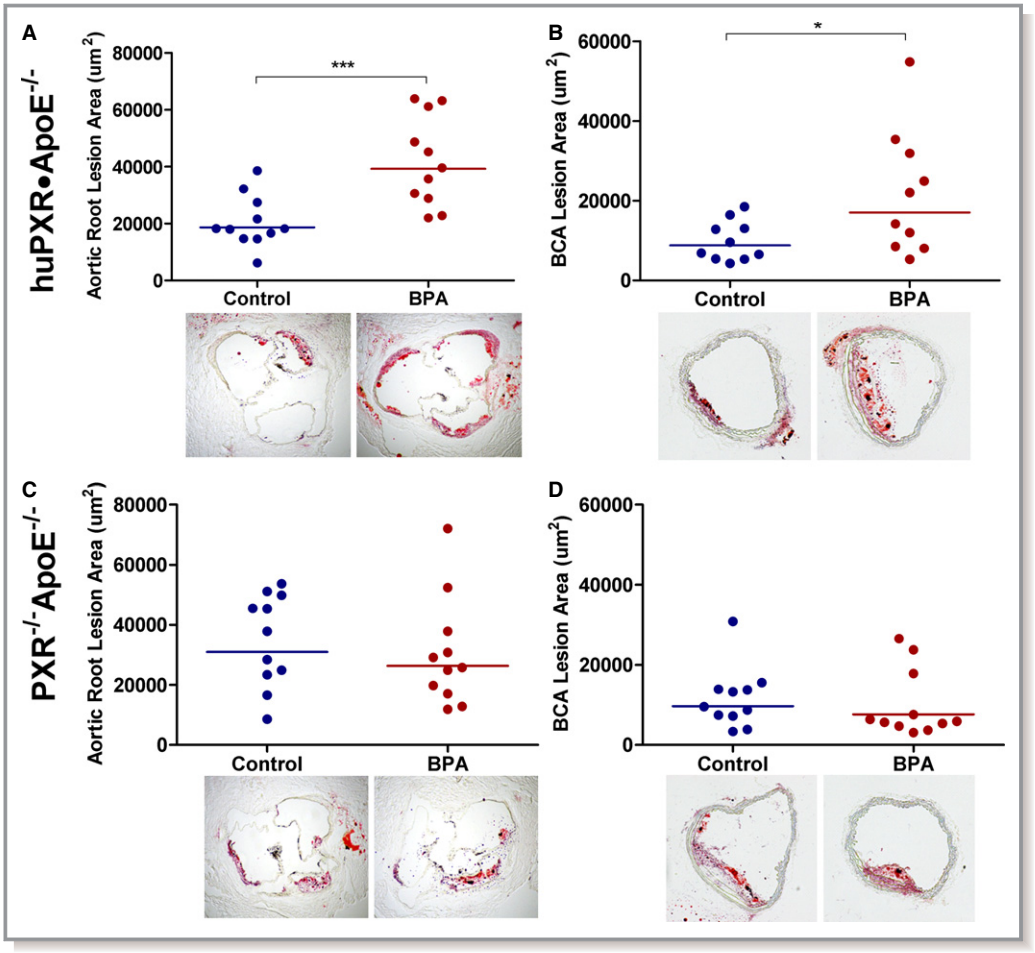

Figure 6. BPA increases atherosclerosis in a human PXR-dependent manner. Four-week-old male huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ littermates were fed a control diet or a diet supplemented with 50 mg/kg BPA for 12 weeks. Quantitative analysis of atherosclerotic lesion size in the aortic root and brachiocephalic artery (BCA) of huPXR•ApoE$^{-/-}$ (A and B) and PXR$^{-/-}$ApoE$^{-/-}$ (C and D) mice (n=10 to 11 per group, *P<0.05 and ***P<0.001). Representative Oil red O-stained sections are shown as indicated. BPA indicates bisphenol A; PXR, pregnane X receptor.

their PXR$^{-/-}$ApoE$^{-/-}$ littermates without altering plasma lipid levels and cholesterol distribution patterns. Therefore, the increased atherosclerosis in huPXR•ApoE$^{-/-}$ mice exposed to BPA could not be explained by the unchanged plasma lipid levels. PXR can directly regulate fatty acid transporter CD36 transcription, and activation of PXR promotes CD36-mediated hepatic lipid accumulation.[53] CD36 plays an important role in atherosclerosis-related processes such as macrophage lipid uptake and foam cell formation.[43,54] We previously demonstrated that activation of PXR increases CD36 levels and lipid accumulation in peritoneal macrophages of ApoE$^{-/-}$ mice.[27] In the current study, the expression levels of CD36 and lipid accumulation were significantly increased in the peritoneal macrophages of huPXR•ApoE$^{-/-}$ mice exposed to BPA. We also observed that PXR is expressed by atherosclerotic lesional macrophages and that BPA exposure increased CD36 and macrophage content in plaques of huPXR•ApoE$^{-/-}$ mice but not that of PXR$^{-/-}$ApoE$^{-/-}$ mice. In addition, the expression levels of several other key receptors and transporters (eg, SR-A, ABCA1, ABCG1) involved in macrophage lipid uptake or efflux were not affected by BPA treatment in macrophages of either huPXR•ApoE$^{-/-}$ or PXR$^{-/-}$ApoE$^{-/-}$ mice. Therefore, a plausible explanation for the increased atherosclerosis observed in huPXR•ApoE$^{-/-}$ mice is the increased CD36 expression and CD36-mediated macrophage lipid uptake and foam cell formation.

BPA is a well-characterized xenoestrogen and the estrogenic effects of BPA have been extensively studied in animals. Many effects of BPA have been found to be similar to effects seen in response to estrogen in laboratory rodent models.[4] Recent studies have also found some adverse effects of exposure to estrogen or BPA on rodent cardiac functions.[55,56] It was reported that both estrogen and BPA can increase

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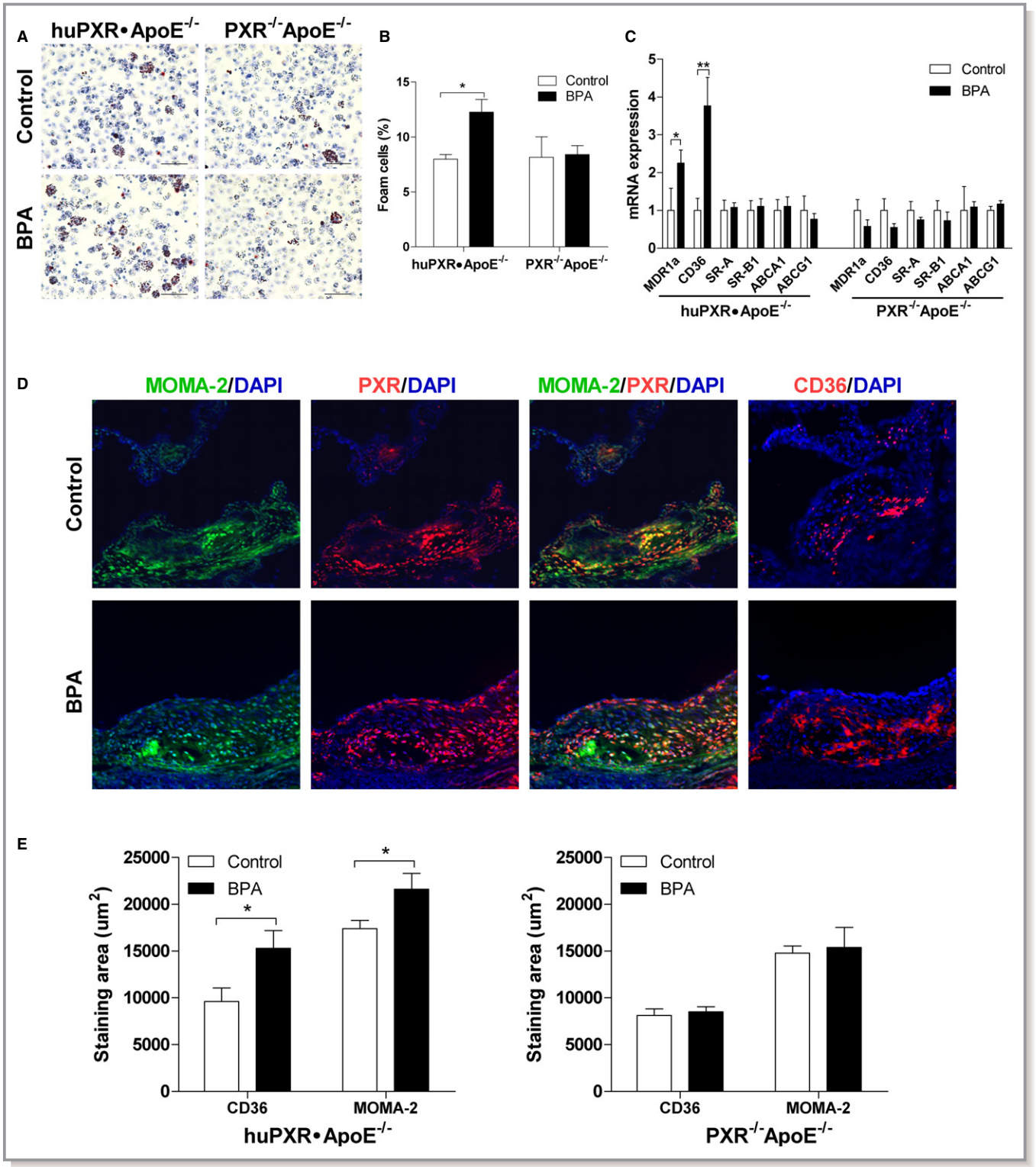

Figure 7. BPA increases foam cell formation and CD36 expression in macrophages and atherosclerotic lesions of huPXR•ApoE$^{-/-}$ mice. A, Freshly isolated peritoneal macrophages from huPXR•ApoE$^{-/-}$ and PXR$^{-/-}$ApoE$^{-/-}$ mice fed a control diet or BPA diet were stained with Oil-red-O and haematoxylin. B, Foam cell quantification from peritoneal macrophages in studies described in panel A (n=4 per group, *P<0.05). C, The expression levels of macrophage genes were measured by QPCR (n=3 per group, *P<0.05 and **P<0.01). D, Sections of atherosclerotic lesion area in the aortic root of huPXR•ApoE$^{-/-}$ mice were stained with anti-monocytes/macrophages (MOMA-2), anti-PXR or anti-CD36 primary antibodies, followed by fluorescein-labeled secondary antibodies. Nuclei were stained with DAPI. E, Quantification of macrophage (MOMA-2) and CD36 staining area in the aortic root of huPXR•ApoE$^{-/-}$ or PXR$^{-/-}$ApoE$^{-/-}$ mice (n=5 to 6 per group, *P<0.05). BPA indicates bisphenol A; PXR, pregnane X receptor; QPCR, quantitative real-time polymerase chain reaction.

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cardiac arrhythmias in isolated hearts from female but not male rodents[56] and alter cardiac calcium homeostasis via ER stimulation in isolated female rodent hearts.[55] However, BPA exposure has been associated with atherosclerosis in humans[10–12] and numerous studies have confirmed that estrogen has atheroprotective effects in animals and humans.[14–17] Therefore, it is unlikely that estrogenic activity of BPA can alone increase atherosclerosis and incidence of CVD in humans. It is still plausible that activation of both PXR and ER by BPA coordinately contribute to increased CVD risk in humans. It would be interesting to study the effects of BPA exposure on atherosclerosis development or cardiac functions in ERα- or ERβ-deficient mice in the presence or absence of hPXR in the future.

In addition to BPA, we have previously identified several environmentally significant BPA analogs including BPB and BPAF as human PXR ligands.[22] Further, we demonstrated that BPA and analogs can synergistically activate human PXR.[22] The synergism between BPA and other environmental chemicals support the need to include mixtures for future in vivo studies, which may have important implications for environmental chemical risk assessment. Combinations of BPA and other environmental chemicals may produce significant effects on PXR activity and atherosclerosis development in humans, even when each chemical is present at low doses that individually do not induce observable effects. In addition, BPA has been implicated to have carcinogenetic potential.[18,57] Activation of PXR has been shown to induce tumor aggressiveness in humans and mice.[58] Future studies are needed to investigate whether BPA-mediated hPXR activation can induce tumorigenesis in PXR-humanized animal models.

In summary, we found that BPA increased atherosclerosis in ApoE$^{-/-}$ mice in a human PXR-dependent manner. BPA exposure did not affect plasma lipid levels but increased CD36 expression and lipid accumulation in macrophages of huPXR•ApoE$^{-/-}$ mice. These findings demonstrate, for the first time, that BPA exposure increases atherosclerosis development in a laboratory animal model, and provide a potential molecular mechanism by which exposure to BPA increases atherosclerosis and CVD risk in humans. Activation of human PXR should be taken into consideration for future risk assessment of BPA and related environmental chemicals.

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Disclosures

None.

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Bisphenol A Increases Atherosclerosis in Pregnane X Receptor-Humanized ApoE Deficient Mice

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