Bisphenol A Disrupts Steroidogenesis in Human H295R Cells

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There is increasing concern over the risk of environmentally relevant doses of bisphenol A (BPA) on human endocrine systems. Effects of BPA on steroidogenesis and the related molecular mechanisms were investigated in H295R human adenocarcinoma cells. This immortal cell line is unique in expressing all the enzymes of the steroidogenic pathways. The effects of BPA on steroidogenesis, 17β-estradiol (E2) metabolism, and aromatase activity were examined in H295R cells exposed to BPA from 3.0×10^{-1} to 3.0×10^{3} ng/ml. Concentrations of BPA in basic cell culture materials were verified. Stable CYP17A-knockdown H295R cells were developed to verify the mechanism of inhibited steroidogenesis by BPA. Background concentrations of BPA in control cell culture media ranged from 0.03 to 0.38 ng/ml. Significantly lesser concentrations of androstenedione, testosterone, cortisol, and cortisone were caused by exposure to 30–3000 ng BPA/ml. In contrast, sconcentrations of estrone (E1) and E2 were significantly greater in BPA-exposed H295R cells. Lesser production of androstenedione and testosterone by H295R cells exposed to BPA was the most sensitive endpoint (no observable effect concentrations < 30 ng BPA/ml). CYP17A knockdown in H295R cells resulted in less production of both 17α hydroxyprogesterone and androstenedione. The results are consistent with the hypothesis that in H295R cells, BPA selectively inhibits 17,20-lyase but not 17α-hydroxylase. The primary mechanism causing increased E2 in the medium was inhibition of E2 metabolism rather than greater aromatase (CYP19) activity. These results suggest that BPA has the potential to interfere with cellular steroidogenesis in humans through multiple molecular mechanisms.

Key Words: CYP17A; 17,20-lyase; estradiol; metabolism; cellular uptake; endocrine disruption; aromatase; CYP19.

The synthetic chemical bisphenol A [2, 2-bis (4-hydroxyphenyl) propane], abbreviated as BPA, is used in manufacture of industrial and consumer products. Currently, over 2.7 million metric tons of BPA are produced annually, primarily for use in manufacturing of epoxy resins and polycarbonate plastics. These products are constituents of a wide variety of products, including plastic food containers and water/milk carboys/bottles, food wrapping, food cans, and dental fillings (Shin *et al.*, 2004; Vandenberg *et al.*, 2007). Over 100 tons of BPA are released into the atmosphere annually (Halden, 2010; Thompson *et al.*, 2009). BPA can leach into food and beverages from plastic containers and has been found in various human food samples at concentrations from < 1 to 300 ng BPA/l. Humans and infants in particular are exposed continuously to BPA, primarily through the diet. Concentrations of BPA in human urine ranged from 3 to 200 ng BPA/l, and those in human serum were in the range of 0.1–10 ng BPA/ml (Vandenberg *et al.*, 2007, 2010).

Increasing concern has been focused on the risk of environmentally relevant doses of BPA to human development and reproduction (Goodman *et al.*, 2009; Vandenberg *et al.*, 2007; vom Saal and Hughes, 2005). Recently, the Canadian government placed a ban on the sale of polycarbonate baby bottles containing BPA and became the first country to designate BPA as being "toxic" at environmentally relevant concentrations. Such actions have initiated ongoing debate among scientists and risk assessors about the safety of current concentrations of BPA to human health. Several studies using laboratory rodent models have found small doses of BPA to be associated with defects in the reproductive tract, meiotic abnormalities in fetal oocytes, complications of pregnancy, and morphological changes in mammary and prostate glands (Hunt *et al.*, 2009). The principal hypothesis invoked to explain these effects is that BPA acts as an estrogen agonist by binding to the estrogen receptor (ER) (Steinmetz *et al.*, 1997; Washington *et al.*, 2001). However, the validity of these findings has been compromised by a lack of reproducibility and discrepancies with the results of standard *in vivo* multigeneration studies

(Hunt *et al.*, 2009; Vandenberg *et al.*, 2009). If BPA is causing adverse effects, it is unlikely that these effects are through direct ER-mediated processes. BPA is weakly estrogenic, with a relative potency between 1000- and ~10,000-fold less than that of the endogenous estrogen, 17β-estradiol (E2) (Steinmetz *et al.*, 1997). However, the results of some "low-dose" studies have suggested that BPA has a greater *in vivo* potency than would be predicted based on binding to the ER. The lack of concordance in potency estimates based on ER binding and *in vivo* biological activity indicates that current understanding of the endocrine disrupting mechanisms of BPA is incomplete. There are mechanisms other than ER-mediated effects through which BPA could affect physiological functioning, including modulation of steroidogenesis and interference with metabolic breakdown of estrogens and effects on signaling cascades (Hilscherova *et al.*, 2004; Sanderson *et al.*, 2000; Zhang *et al.*, 2005). Chemicals can alter production or metabolism of steroids at the cellular level and have the potential to disrupt the endocrine system in living organisms. Examination of the expression of different steroidogenic enzymes provides mechanistic information on the molecular basis for alterations in hormone biosynthesis caused by exposure to chemicals. Biological effects can then be verified at higher organizational levels, such as protein expression, enzyme activities, or hormone production as desired (He *et al.*, 2010; Higley *et al.*, 2010). Among the issues contributing to the controversy surrounding BPA are validity of low-dose exposure results and cross-species prediction of human toxicity.

The use of *in vitro* models has been critical in evaluating toxic effects and determining mechanisms of action of chemicals. Several studies using cell culture have suggested that BPA induces significant estrogenic effects at concentrations as little as 10^{-12}M or 0.23 pg BPA/ml (Bouskine *et al.*, 2009; Wozniak *et al.*, 2005). These results suggest that certain human cell types might be susceptible to BPA at concentrations 1000-fold less than magnitudes of estimated current human exposures. Consequently, accurate quantification of BPA is essential for proper interpretation of results, especially in studies where low doses of BPA are used. Some of the reported effects have occurred at calculated concentrations that are less than what can be confirmed by instrumental analyses. There are characteristics of BPA that could result in either the overestimation or underestimation of the actual exposure. The actual exposure could be underestimated due to the presence of BPA as a contaminant in medium or plastics. Alternatively, the dose could be overestimated due to biotransformation of BPA. However, none of the above *in vitro* studies evaluated the background BPA concentrations in their testing system and verified the actual BPA concentrations in cell culture medium. Furthermore, current understanding of the toxic effects of BPA is based primarily on the results of studies in which rats or mice were exposed to BPA (Hunt *et al.*, 2009; Vandenberg *et al.*, 2009).

Using human H295R cells, the present study investigated the effect of BPA on steroidogenesis and E2 metabolism. To control for potential contamination with BPA through the materials used in this studies, concentrations of BPA in the basic cell culture materials were verified.

MATERIALS AND METHODS

Chemicals and materials. The following materials were obtained from Sigma (St Louis, MO): BPA, estrone (E1), 17β-estradiol (E2), and dansyl chloride. Deuterated standards, including bisphenol A-d_{16} (d_{16}-BPA), estronel-2,4,16,16-d_4 (d_4-E1), 17β-estradiol-2,4,16,16-d_4 (d_4-βE2), progesterone-d_9, 17α-OH-progesterone-d_8, androstenedione-d_7, testosterone-d_5, and deoxycorticosterone-d_8, were obtained from C/D/N Isotope (Pointe-Claire, Quebec, Canada). Cortisol-d_4 was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). Solvents including dichloromethane (DCM), *n*-hexane, acetone, acetonitrile, and methanol were pesticide residue grade obtained from OmniSolv (EM Science, Lawrence, KS). Costar 24-well flat bottom cell culture plate was purchased from Corning Inc. (Corning, NY). Other plastic materials used in medium preparation and extraction included disposable medium filtration set (Corning Inc.) and disposable polystyrene pipettes of various sizes (DOT Scientific Inc., Burton, MI)

Cell culture and exposure. Culture and maintenance of the H295R human adrenocortical carcinoma cells (ATCC, Beltsville, MD) followed the protocol previously described (Gracia *et al.*, 2007; Zhang *et al.*, 2005). Briefly, H295R cells were cultured in Dulbecco's modified Eagle's medium supplemented with Ham's nutrient mixture F-12 (Sigma) with 1 ml/100 ml ITS + Premix (BD Bioscience, San Jose, CA) and 2.5% BD Nu-Serum (BD Bioscience) at 37°C in a 5% CO_2 atmosphere. Exposure was conducted in Costar 24-well cell culture flat bottom plate (Corning Inc.). One microliter of cell suspension was added to each well at a density of 1×10^6 cells/ml. Twenty-four hours after plating, H295R cells were exposed to various concentrations of BPA dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Nominal BPA doses were 0.0098, 0.039, 0.156, 0.625, 2.50, and 10.0μM with three replicates in each experiment. The final concentration of DMSO in culture medium was 0.01%. The rate of BPA metabolism by the H295R cells was examined by exposing the cells to nominal concentrations of 0.039, 0.156, 0.625, 2.50, or 10.0μM BPA, and the medium was sampled at 0, 3, 10, 24, and 48 h after the initiation of the exposure. The culture medium from each well was collected after 24 h and stored at −80°C until analysis. Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as previously described (Mosmann, 1983), but no significant inhibition on cell viability was observed in any of the tested BPA concentrations.

CYP17A knockdown in H295R cells. For transfection, cells were seeded into six-well plates and were transfected with either a human CYP17A pLKO.1 lentiviral short hairpin RNA (shRNA) vector or a nonsilencing vector (no hairpin insert, as control) (Thermo Scientific Open Biosystems, Huntsville, AL) using Arrest-In transfection reagent (Thermo Scientific Open Biosystems) for 5 h before the addition of serum-containing culture medium. After 2 days of culture, transfected cells were selected using culture medium containing 0.4 μg/ml puromycin (Sigma) for an extended selection period (48–96 h). Surviving cells were further cultured and tested for the ability to produce steroids in the culture medium.

Instrumental analysis. Quantification of BPA and steroid hormone concentrations in water and culture media were performed as described previously (Chang *et al.*, 2010; Liu *et al.*, 2010). Briefly, surrogate deuterium-labeled standards were spiked into samples of media before extraction with ethyl acetate/hexane (vol/vol, 50/50). The water phase was discarded and the solvent phase was evaporated under nitrogen. The dried residue was dissolved in 200 μl methanol. For steroids except estrogens, 100 μl of methanol solution

was separated into a vial for liquid chromatography-quadrupole mass spectrometer analysis. For estrogens and BPA, the remaining 100 µl aliquot of methanol solution was evaporated and redissolved in 100 µl of NaHCO$_3$ buffer (pH 10.5). An aliquot of 100 µl of dansyl chloride (1.0 mg/ml in acetone) was added as a derivatizing agent. The derivation reaction of estrogens and BPA was conducted for 5 min at 60°C and was stopped by cooling to room temperature. The reaction mixture was then extracted with ethyl acetate/hexane (vol/vol, 50/50), dried, and reconstituted with 100 µl of acetonitrile for analysis.

Instrumental analyses were conducted using an Agilent 1200 series HPLC system (Santa Clara, CA) connected to an API 3000 triple-quadrupole MS/MS system (PE Sciex, Concord, ON, Canada). For estrogens and BPA, the mobile phase was acetonitrile and 0.1% formic acid; for other steroid hormones, chromatography was performed using nanopure water and methanol. Sample extracts were separated on a Betasil C18 column (100 × 2.1 mm, 5-µm particle size) purchased from Thermo (Waltham, MA) before MS/MS analysis. All data were acquired and processed with ABI Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA). Triplicate analyses were performed on each sample.

Overall, the mean ($n = 4$) absolute recoveries at two concentration levels of 1000 and 5000 pg/ml for each of the target steroids ranged from 88 to 101% (Table 1). The method accuracy for the two spiked levels was 8–101 and 91–102%, respectively. The intrabatch precision was 5.4–10 and 4.8–7.4% for the two concentrations, respectively. The interbatch precision was 9.1–13 and 6.6–8.7% for the two concentrations, respectively. The method limit of quantification, with a signal-to-noise ratio of ~10:1, was 3–42 pg/g.

Messenger RNA quantification by RT-PCR. Messenger RNA (mRNA) expression of steroidogenic genes in H295R cells was measured as previously described (Hilscherova et al., 2004; Zhang et al., 2005). Briefly, after exposure, the medium was removed and the cells were used for total RNA isolation (Agilent Technologies Inc., Wilmington, DE), first-strand complementary DNA (cDNA) synthesis (BioRad, Mississauga, ON, Canada) and quantitative real-time PCR (qRT-PCR) (Applied Biosystems) following the manufacturer's instructions. The target genes included HMGR, *StAR*, *CYP17*, *CYP11A*, *CYP21*, *CYP19*, *CYP11B1*, *CYP11B2*, *17βHSD*, *3βHSD*, and *PBGD* were used as the reference gene. The primer sequences have been described previously (Zhang et al., 2005). A final reaction volume of 20 µl was made up with 10 µl of diluted cDNA and nuclease-free distilled water (Invitrogen). The PCR reaction mix was first denatured at 95°C for 10 min and then followed with 50 PCR cycles. The PCR thermal cycle profile was (1) denaturation for 15 s at 95°C, (2) annealing for 30 s at 60°C, and (3) extension for 30 s at 72°C. Gene expression was calculated by Delta *Ct* method and was repeated three times. None of the examined steroidogenic genes displayed a significant over 1.5-fold changes at the transcriptional level.

Estrogen biosynthesis and metabolism. Synthesis of E2 catalyzed by aromatase (CYP19A) was determined by previously described methods (Higley et al., 2010; Sanderson et al., 2002). Briefly, 1.0 ml of H295R cell suspension was plated in each well of a 24-well plate at a density of 1×10^6 cells/ml and incubated at 37°C for 1 h. After treatment, the culture medium was replaced with 250 µl of serum-free medium spiked with 54nM^{3}H-[1β-] androstenedione (PerkinElmer, Boston, MA) and cells were further incubated at 37°C for 1 h. Aromatase activity was quantified by the rate of release of tritiated water due to the conversion of ^{3}H-1β-androstenedione to estrone by aromatase. The rate of E2 metabolism in H295R cells was measured by a previously described protocol (He et al., 2010). To assess concentration-dependent effects of BPA on transformation of E2, cells were first cultured in 24-well plates for 24 h. Culture medium was then replaced with 250 µl of fresh medium spiked with 1.0nM 6, 7 [^{3}H]-E2 (PerkinElmer) and different nominal concentrations of BPA. Cells were further incubated at 37°C for 2 h. The amount of E2 in the aqueous phase was determined by measurement of radioactivity by liquid scintillation counting by use of a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Brea, CA). The [3H] measured in the aqueous fraction were sulfonated or glucoronidated metabolites of E2, whereas the parent partitioned primarily to the DCM fraction. The rate of E2 metabolism was calculated as the change in E2 in the spiked media compared with controls. To compare the direct and indirect effects of BPA, cells were preincubated with medium without and with 5nM BPA, respectively, in 24-well plates for 24 h. Direct effects of BPA were assessed by replacing the culture medium with freshly prepared medium spiked with 1.0nM 6,7 [^{3}H]-E2 and 5nM BPA. Indirect effects of BPA were assessed by replacing the culture medium with freshly prepared medium spiked with 1.0nM 6,7 [^{3}H]-E2. After 2 h further incubation, cell culture medium was collected and extracted to measure the rate of E2 metabolism. The cells were rapidly washed twice with ice-cold PBS and then were lysed by 250 µl lysis buffer containing 0.1M NaOH, 0.1% SDS, and 0.1% Na$_2$CO$_3$ for 15 min at room temperature. Cell homogenates were collected and cellular uptake of estrogen was determined by measurement of radioactivity of cell homogenates.

TABLE 1
Accuracy, Intrabatch Precision and Interbatch Precision, and Method Limit of Quantification (LOQs, picograms per milliliter) of LC-MS/MS Measurement of Steroids

Steroids	1000 pg/ml spiked			5000 pg/ml spiked			
	Accuracy (%)[a]	Intrabatch precision (%)[b]	Interbatch precision (%)[c]	Accuracy (%)[a]	Intrabatch precision (%)[b]	Interbatch precision (%)[c]	LOQ
BPA	97	5.4	10	100	4.8	8.1	5
Estrone	95	8.5	9.4	92	5.6	8.7	4
Estradiol	101	9.6	12	102	5.3	6.6	3
Androstenedione	93	8.2	11	102	6.9	7.8	20
Testosterone	90	9.2	13	98	3.8	7.5	24
Cortisol	95	7.4	14	92	5.9	6.8	42
Corticosterone	96	10	10	95	7.4	8.0	40
17α-Hydroxylprogesterone	91	8.1	9.1	91	4.6	6.5	35
21α-Hydroxyprogesterone	87	10	12	89	5.9	6.7	32
Progesterone	93	7.9	10	95	7.2	7.7	18

[a]Accuracy was measured as the percent of the spiked amount of steroids that was recovered.
[b]Intrabatch precision (coefficient of variation) was measured by the percent relative standard deviation (RSD).
[c]Interbatch precision (coefficient of variation) was measured by the percent RSD.

Statistical analyses. Prior to conducting statistical comparisons, data were tested for normality using the Shapiro-Wilk's test and probability plots. If necessary, values were log transformed to approximate normality. Two-sample comparisons were made by means of the Student's t-test. A linear regression model was used to compare hormone production between the two cell lines (silenced and nonsilenced) in response to chemical exposure. Effects of BPA on steroid production were fitted to an ANOVA model and the comparison between different concentrations and control was carried out using Tukey's 'Honest Significant Difference' method. Statistical analyses were conducted using the R project language (http://www.r-project.org/). Differences with $p < 0.05$ were considered to be statistically significant.

RESULTS

BPA was measurable in the basic laboratory materials used during the culture of H295R cells (Table 2). The mean background BPA concentrations in nanopure water collected directly from the system, freshly prepared unsupplemented Dulbecco's modified Eagle's medium, and supplemented medium after a 24-h incubation at 37°C in a 24-well plate were 3.0×10^{-2}, 1.4×10^{-1}, and 3.8×10^{-1} ng/ml, respectively. In addition, because BPA is metabolized by H295R cells, the actual exposure concentration decreased as a function of time. The rate of transformation of BPA followed pseudo first-order kinetics ($Ct = Co \ Exp \ (-0.0064 \ t)$) with a half-life of 47 h ($r^2 = 0.94$, $n = 11$).

BPA modulated production of multiple steroids in H295R cells. Production of both androstenedione and testosterone was inhibited by BPA in a concentration-dependent manner over the course of the 24-h incubation (Fig. 1). Among the nine steroids investigated, four, androstenedione, testosterone, cortisol and corticosterone, exhibited concentration-dependent lesser concentrations relative to controls. 17α-Hydroxylprogesterone and 21α-hydroxyprogesterone were the only two steroids whose synthesis was not affected by BPA. However, concentrations of three hormones, progesterone, E1, and E2, were greater in the presence of BPA, especially at greater exposure concentrations. Among the six steroids modulated by BPA exposure, testosterone and androstenedione were the two most sensitive endpoints with no observable effect concentrations (NOECs) of 8.76 ± 2.25 and 26.3 ± 2.5 ng BPA/ml, respectively.

To further investigate the mechanistic basis of the effects of BPA on steroidogenesis in H295R cells, stable CYP17A-

TABLE 2
Background BPA Concentrations (Mean ± SD) in the H295R Cell Culture System

Sample description	BPA concentration (ng/ml)
Nanopure water	0.03 ± 0.02
Unsupplemented medium	0.14 ± 0.07
Supplemented medium after 24-h incubation at 37°C	0.38 ± 0.04

knockdown H295R cells were developed using a human CYP17A lentiviral shRNA plasmid construct carrying a puromycin resistance gene as a selection trait (Table 3). The puromycin-resistant stable H295R/CYP17A-knockdown cells displayed less production of 17α hydroxyprogesterone and androstenedione compared with puromycin-resistant nonsilenced H295R cells, which confirmed that the activities of both 17α-hydroxylase and 17,20-lyase were inhibited (Table 2). Reduced activity of the enzyme 17α-hydroxylase resulted in less production of its direct product 17α-hydroxyprogesterone to 32% of control levels and caused a 3.2-fold increase in production of the alternative product, 21α-hydroxyprogesterone. The decreased activity of 17,20-lyase in H295R/CYP17A-knockdown cells further decreased the conversion rate of 17α-hydroxyprogesterone to androstenedione, which resulted in a 7.7-fold decrease in production of androstenedione and a 2.4-fold decrease in production of testosterone.

Exposure to BPA resulted in greater concentrations of both E1 and E2 in the medium. The activity of aromatase (CYP19A) in H295R cells was not altered by BPA (data not shown). However, BPA did modulate the concentration of E2 by inhibition of E2 metabolism (Fig. 2). No alteration in E2 metabolism or cellular intake of E2 was observed in H295R cells preincubated with BPA. Direct exposure to BPA inhibited metabolism of E2 in a concentration-dependent manner within 30 min. Within 2 h of direct incubation with 5μM BPA, H295R cells displayed less cellular incorporation of 6,7,[3H]-E2 during the first 60 min but a greater level of cellular 6,7,[3H]-E2 after 80 min relative to the control (Fig. 3).

DISCUSSION

Background Contamination and Metabolism of BPA

The actual concentrations of BPA in H295R cell culture media were affected by background contamination and metabolism of BPA. BPA was present in the basic laboratory materials used during the culture of H295R cells in a concentration range from 3.0×10^{-2} to 3.8×10^{-1} ng/ml (Table 1). There were several standard laboratory procedures involved in preparation of sterile serum-supplemented medium from nanopure water, such as mixing, filtration, and liquid transferring. During the processes used to prepare media, the background concentration of BPA increased approximately 13-fold relative to the original nanopure water. The detectable BPA background concentration in cell culture medium is likely due to unavoidable contamination of BPA from various plastic materials used in standard laboratory practice. In standard cell culture practice, much disposable plastic ware is used, including the media filtration vessel, cell culture plates (e.g., six-well plate), pipette tips, and other containers. It has been reported that chemicals leaching from disposable plastic ware can affect assays conducted in academic, medical, and commercial labs worldwide (McDonald *et al.*, 2008; Soto *et al.*, 1996).

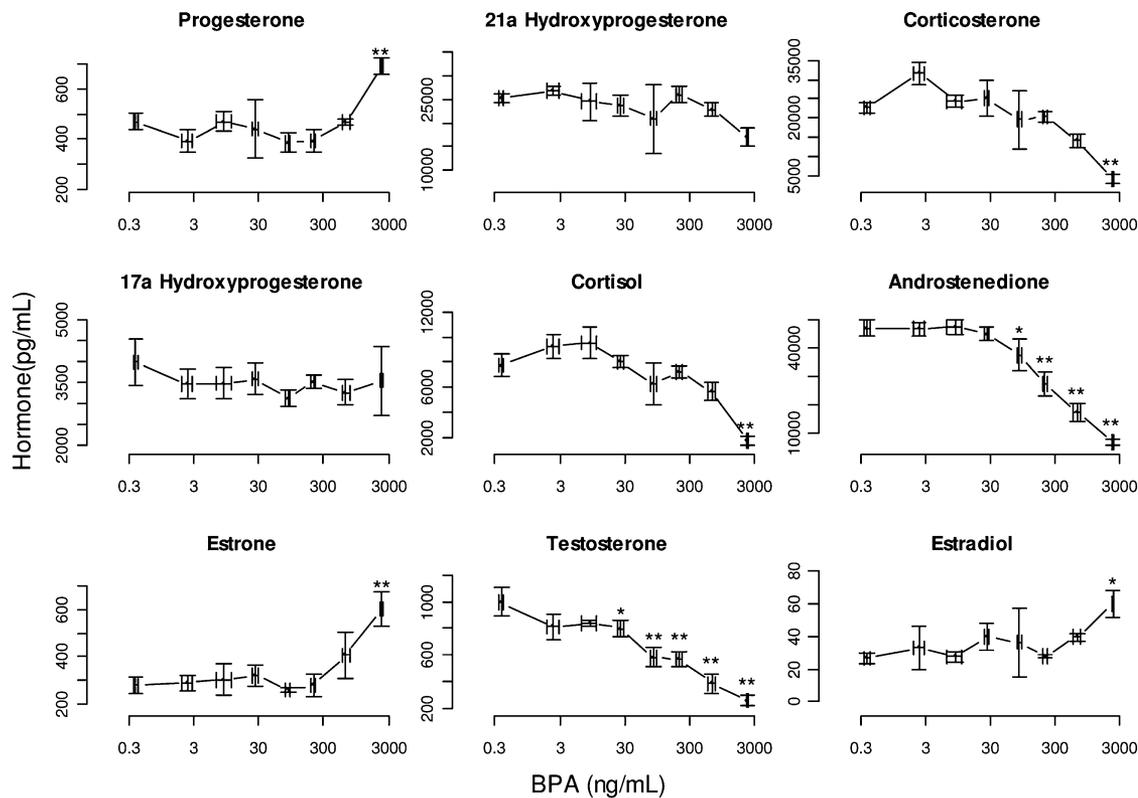FIG. 1. Concentrations of steroid hormones in BPA-exposed H295R cell culture medium after 24 h. Results are expressed as mean ± SD. This data set was from one experiment and is representative of three replicated experiments. The background BPA concentration in the control group was 0.38 ± 0.04 ng/ml. Significant difference between control and other concentrations is indicated by *$p < 0.05$.

BPA is metabolized by H295R cells with a half-life of 47 h ($r^2 = 0.94$, $n = 11$). *In vitro* cell cultures, especially for nonhepatic cells, are believed to normally have little capacity to metabolize hormones. However, the fact that greater than 50% of BPA could be metabolized within 48 h by H295R cells

FIG. 2. Effect of BPA on estrogen metabolism. Estrogen metabolism was analyzed by measuring the formation of water-soluble [^{3}H] E2 species in cell culture medium after 30-min coexposure. This data set was from one experiment and is representative of three replicated experiments. Significant difference between control and other concentrations is indicated by *$p < 0.05$.

warrants the measurement of BPA concentrations in cell-based *in vitro* investigations.

In most studies, only nominal concentrations of BPA have been reported. As a consequence, concentrations of BPA leached into assay systems may have been greater than the BPA added in "low-dose" experiments. The significance of this artifact would be that effects would be reported to occur at lesser concentrations than the actual exposure concentration. The finding of the background contamination and metabolism of BPA in the standard cell culture practice challenges the validity of the previous reports which have suggested that BPA induces significant effects at concentrations as little as 10^{-12}M or 0.23 pg/ml without confirmation of the actual concentrations (Bouskine *et al.*, 2009; Wozniak *et al.*, 2005). Concentrations as small as 0.23 pg BPA/ml triggered ER-mediated Ca^{2+} fluxes and prolactin release in rat GH3/B6 pituitary tumor cells (Ma *et al.*, 1998). It has also been reported that this concentration of BPA promoted proliferation of human seminoma cells (JKT-1) (Bouskine *et al.*, 2009). The underlying mechanism of this effect was via a membrane G-protein-coupled ER that activates protein kinases A and G (PKA and PKG). These results suggest that certain human cell types might be susceptible to BPA at concentrations 1000-fold less than currently estimated background BPA concentrations in cell culture medium (0.38 ± 0.04

FIG. 3. Effect of BPA (5μM) on cellular uptake of estrogen (A) and metabolism (B) in H295R cells. Cell culture medium was replaced with serum-free medium containing 1nM 6,7,[³H]-E2 and BPA if for direct exposure. E2 cellular uptake and metabolism were measured at various times. Preincubation treatment resulted in no significant difference from control. Significant difference between control and direct BPA incubation at different time points is indicated by *$p < 0.05$.

ng/ml). However, linking toxicological effects with BPA exposure, especially at low doses, is complicated by the fact that BPA is prevalent in a range of consumer products, including common laboratory plastics, and has the potential to leach from these products. Consequently, these background concentrations of BPA have the potential to affect the results of studies that detect low concentration effects conducted in academic, medical, and commercial laboratories (McDonald *et al.*, 2008).

BPA Modulates Steroidogenesis at the Enzymatic Level

BPA modulated the production of multiple steroids in H295R cells in a concentration-dependent manner over the course of a 24-h incubation (Fig. 1). Cells used in this study were less than passage 8 after thaw from liquid nitrogen. The lesser concentrations of androstenedione and its direct downstream product, testosterone, after exposure to BPA are consistent with direct inhibition of activities of upstream enzymes, such as 3β-hydroxysteroid dehydrogenase (*3βHSD*), 17,20-lyase (*CYP17A*), and 17α-hydroxylase (*CYP17A*). However, because progesterone and 17α-hydroxyprogesterone are

also direct products of 3β-hydroxysteroid dehydrogenase, the unchanged 17α-hydroxylprogesterone production and slight increase in progesterone at the greatest BPA exposure suggest that 17,20-lyase but not *3βHSD* was inhibited by BPA. Indeed, the 17,20-lyase and 17α-hydroxylase activities are the two functions of *CYP17A* (Van Den Akker *et al.*, 2002). Decreases in the activity of 17, 20 lyase but not 17α-hydroxylase are consistent with BPA inhibiting enzyme function and not at the level of transcription of the protein catalyst. This hypothesis was also supported by the measurement of mRNA expression.

Although CYP17 enzyme activities were not directly evaluated in this study, the profile of steroid production in H295R cells in which CYP17A expression was knocked-down was consistent with the hypothesis that BPA decreased production of androstenedione and testosterone by inhibiting activity of the enzyme 17, 20-lyase. The stable H295R/CYP17A-knockdown cells displayed lesser production of both androstenedione and testosterone, which is consistent with inhibition of activities of 17, 20-lyase. In addition, lesser concentrations of 17α-hydroxyprogesterone and greater

TABLE 3
Effect of Knocking Down CYP17 Expression on Steroid Production by H295R Cells After 24-h Incubation at 37°C. Steroid Concentration (Nanograms Per Milliliter) is Expressed as Mean ± SD

	Control			
Steroids	(Nonsilenced)	CYP17A-KD	Fold change CYP17-KD	Fold change BPA[a]
21α Hydroxyprogesterone	23900 ± 173	76833 ± 12566	3.21*	0.99
17α Hydroxyprogesterone	7397 ± 1675	2330 ± 485	0.32*	0.91
Corticosterone	23700 ± 625	20567 ± 3075	0.87	0.62
Androstenedione	38267 ± 2084	4890 ± 1092	0.13*	0.37*
Testosterone	3077 ± 256	1273 ± 107	0.41*	0.38*
Estrone	765 ± 26	624 ± 39	0.82*	1.46
Estradiol	163 ± 14	195 ± 58	1.20	1.49

[a]fold change observed at the final concentration of 600 ± 87 ng BPA/ml comparing with the relative control of normal H295R cells.
*$p < 0.05$.

concentrations of 21α-hydroxyprogesterone in the H295R/ CYP17A-knockdown cell culture medium suggested that 17α-hydroxylase was also inhibited (Table 2). This result is consistent with the prediction that less expression of CYP17A would result in lesser activities of both17α-hydroxylase and 17, 20-lyase. Similar inhibitory effects on androstenedione and testosterone were observed in the CYP17A-knockdown cells and those exposed to BPA. However, 17α-hydroxyprogesterone was inhibited in CYP17A-knockdown cells and not in BPA-exposed cells which further confirmed the inhibitory effect of BPA on 17,20-lyase but not 17α-hydroxylase.

Selective inhibition of CYP17A enzyme activity by BPA might be caused by interfering with the normal function of the redox partner interaction site of CYP17A. Both enzymatic functions of CYP17A, 17, 20-lyase activity and 17α-hydroxylase, involve steroid binding followed by electron transfer. However, only 17, 20-lyase activity is dependent on facilitation by interaction of the oxidoreductase with the redox partner-binding site of CYP17A (Van Den Akker et al., 2002). This interaction can be enhanced by cytochrome b5 or phosphorylation of phosphoserine residues on CYP17A. If BPA interfered with either of these two mechanisms, it would lead to decreased 17, 20-lyase activity without any change in 17α-hydroxylase activity. Natural mutations in the redox partner interaction domain (R347C and R347H) of CYP17A result in less severe 17α-hydroxylase deficiency but complete 17,20-lyase deficiency (Van Den Akker et al., 2002). Although this mechanism is consistent with the results of the current study, the actual underlying mechanism of the selective inhibition of 17, 20-lyase by BPA warrants further investigation.

BPA-Modulated Metabolism of Estrogens

BPA increased concentrations of both E1 and E2 in the medium even though their respective direct precursors, androstenedione and testosterone, were both decreased. The activity of aromatase (CYP19A) in H295R cells was not altered by BPA, however, and BPA inhibited E2 metabolism (Fig. 2). H295R cells exhibit endogenous E2 metabolism capability through two endogenous enzymes, E2-sulfotransferase, and E2-glucuronidase (He et al., 2010). H295R cells preincubated with BPA did not alter either E2 metabolism or cellular intake, which is consistent with the conclusion that direct effects of BPA were not through transcriptional mechanisms but by direct inhibition of the enzyme. These findings further indicated that inhibition of E2 metabolism by BPA could prolong the activity of extracellular estrogens. In addition to ER-mediated effects of BPA, indirect estrogenic effects such as inhibition of endogenous E2 metabolism could result in estrogenic effects that were not consistent with BPA being a weak ER agonist (Steinmetz et al., 1997; Washington et al., 2001).

In summary, BPA modulates both synthesis and metabolism of multiple steroids in H295R cells through multiple mechanisms at the enzymatic level. The steroids affected by BPA exposure include progesterone, coticosterone, androstenedione, T, E1 and E2, of which testosterone and androstenedione were the two most sensitive endpoints with NOECs of 8.76 ± 2.25 and 26.3 ± 2.5 ng BPA/ml, respectively. These concentrations were within or close to the range of concentrations observed in human blood (0.2–20 ng/ml)(Vanderberg et al., 2007). BPA exposure caused less production of androstenedione but not 17α-hydroxyprogesterone. However, CYP17A knockdown in H295R cells resulted in lesser production of both 17α-hydroxyprogesterone and androstenedione. These results are consistent with the hypothesis that BPA inhibits activity of 17,20-lyase but not 17α-hydroxylase in human cells. In addition, BPA inhibited E2 metabolism, but no changes were observed in gene expression or aromatase activity, which suggests that the "estrogenic" effects of BPA could be due to non-ER-mediated effects and that further studies to investigate the potential risk of BPA-mediated disruption of steroidogenesis in humans are warranted.

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