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The genomic response of Ishikawa cells to bisphenol A exposure is dose- and time-dependent

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ABSTRACT

A reliable *in vitro* model to determine the potential estrogenic activity of chemicals of interest is still unavailable. To further investigate the usefulness of a human-derived cell line, we determined the transcriptional changes induced by bisphenol A (BPA) in Ishikawa cells at various doses (1 nM, 100 nM, 10 μM, and 100 μM) and time points (8, 24 and 48 h) by comparing the response of approximately 38,500 human genes and ESTs between treatment groups and controls (vehicle-treated). By trend analysis, we determined that the expression of 2794 genes was modified by BPA in a dose- and time-dependent manner ($p \leq 0.0001$). However, the majority of gene expression changes induced in Ishikawa cells were elicited by the highest doses of BPA evaluated (10–100 μM), while the genomic response of the cells exposed to low doses of BPA was essentially negligible. By comparing the Ishikawa cells' response to BPA vs.17α-ethynyl estradiol we determined that the change in the expression of 307 genes was identical in the direction of the change, although the magnitude of the change for some genes was different. Further, the response of Ishikawa cells to high doses of BPA shared similarities to the estrogenic response of the rat uterus, specifically, 362 genes were regulated in a similar manner *in vivo* as well as *in vitro*. Gene ontology analysis indicated that BPA results in changes to multiple molecular pathways affecting various biological processes particularly associated with cell organization and biogenesis, regulation of translation, cell proliferation, and intracellular transport; processes also affected by estrogen exposure in the uterus of the rat. These results indicate that Ishikawa cells are capable of generating a biologically relevant estrogenic response after exposure to chemicals with varied estrogenic activity, and offer an *in vitro* model to assess this mode of action.

1. Introduction

Bisphenol A [BPA, 2,2-bis(4-hydroxyphenyl)propane] is a high production volume chemical, used primarily in the production of polycarbonate plastics and epoxy resins. Polycarbonate plastics are used to make some food and drink containers; the epoxy resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. Some polymers used in dental sealants and tooth coatings contain BPA. Given the extensive use of BPA in the manufacture of consumer goods and products, there is a widespread potential for human exposure to this chemical. In fact, there is evidence indicating that most humans are exposed to BPA, and it has been estimated that the level of human exposure ranges from under 1 μg/kg/day to almost 5 μg/kg/day (Vandenberg et al., 2007). Acute studies with relatively high doses of BPA, in both animals and humans, indicate that this chemical is rapidly metabolized (mostly by conjugation with glucuronic acid) and excreted in the urine (Yokota et al., 1999; Volkel et al., 2002). Free BPA (active form) has been measured in human blood (serum and plasma), breast milk, amniotic fluid, and placental tissue. Its concentration is relatively low, in the low ng/ml (low nM range) or ng/g range using various analytical techniques (Ikezuki et al., 2002; Kang et al., 2006; Vandenberg et al., 2007), while glucuronide-conjugated BPA (most abundant and inactive form) has been found in the low ng/ml range in the urine of over 90% of individuals tested in several countries and continents (Vandenberg et al., 2007). Although only a few small studies have explored the associations between BPA levels and human health issues, thus far no solid evidence of any association has been found (see for example: Lang et al., 2008; vom Saal and Myers, 2008; Young and Yu, 2009; Ye et al., 2009).

BPA has been considered to be a very weak environmental estrogen because of its low estrogen receptor (ER) affinity and since in many bioassays (e.g., the rodent uterotrophic assay and some

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responses in human breast cancer cells) it elicits weak estrogenic responses. Multiple researchers have used biochemical assays to examine the kinetics of BPA binding to ERs and have determined that BPA binds both ERα and ERβ, with approximately 10-fold higher affinity to ERβ (Gould et al., 1998; Kuiper et al., 1998; Pennie et al., 1998; Gutendorf and Westendorf, 2001; Han et al., 2002). However, the affinity of BPA for human ERs is 10,000–100,000-fold weaker than that of 17 β-estradiol, the natural endogenous estrogen. BPA exerts estrogenic effects through the classical nuclear estrogen receptors, acting as an agonist as well as a selective estrogen receptor modulator [SERM] (Chapin et al., 2008). Further, Takayanagi et al. (2006) determined that BPA is capable of binding the human estrogen-related receptor gamma (ESRRG), an orphan nuclear receptor whose natural ligand and cellular functions are still unknown. In a binding assay using [^{3}H]4-hydroxytamoxifen as a tracer, BPA showed a dose-dependent receptor binding curve with an IC_{50} value of 13.1 nM, which indicates a relatively strong affinity for this receptor. 4-hydroxytamoxifen has a higher affinity (~2-fold) for both ERα and ERβ than estradiol (Kuiper et al., 1998). In a reporter gene assay for ESRRG using HeLa cells, Takayanagi et al. (2006) determined that BPA (1 μM) was capable of activating this receptor, or rather it preserved its basal constitutive activity. It has to be emphasized that ESRRG was identified on the basis of its sequence similarity to the estrogen receptors, however this nuclear receptor does not bind 17 β-estradiol (Takayanagi et al., 2006; Okada et al., 2008). It has also been proposed that BPA can elicit an immediate response that is not mediated through changes in gene expression, as estrogen does (Nadal et al., 2000; Watson et al., 2007). Rapid non-genomic effects of BPA could be mediated by interacting with cytoplasmic or cell membrane-bound regulatory proteins, which after interacting with BPA modulate the activity of membrane and/or cytoplasmic signaling molecules such as adapter proteins, G proteins, ion channels, and protein kinases (Boonyaratanakornkit and Edwards, 2007). However, these rapid non-genomic effects of BPA can also result indirectly (minutes to hours after BPA exposure) in changes in the expression of specific genes.

The establishment of reliable *in vitro* systems to determine the potential estrogenic activity of chemicals of interest is indispensable in order to develop dependable high-throughput screening assays and to minimize animal testing studies for this mode of action. Using a transcriptomics approach to determine the response to a potent estrogen receptor agonist, 17α-ethynyl estradiol (EE), we identified a cultured cell line, Ishikawa cells, capable of generating a robust genomic response to estrogen exposure which has a high degree of concordance with the response determined *in vivo* (Naciff et al., 2009). Ishikawa cells are one of the best characterized human endometrial cell lines currently available. These are cells derived from a well-differentiated adenocarcinoma of the human endometrial epithelium, which express functional steroid receptors for estrogen, progesterone and androgen (Nishida et al., 1985; Croxtall et al., 1990; Lessey et al., 1996; Lovely et al., 2000; Nishida, 2002). Specifically, Ishikawa cells constitutively express both ERα and ERβ, as well as ESRRG, thus offering the opportunity to evaluate the effects of chemicals with estrogenic activity on the cellular responses mediated by of these receptors. These characteristics make these cells an ideal model to study the response of the endometrial epithelium to estrogen exposure *in vitro*.

In order to better understand the estrogenic response of Ishikawa cells, define their value as an *in vitro* alternative to evaluate estrogenicity, and at the same time obtain an insight into the molecular mechanism involved in the response of the human-derived cells to BPA, we have determined the gene expression profiles induced by BPA at various doses and time points.

2. Materials and methods

2.1. Chemicals

Bisphenol A (99+%) was obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Cell culture

Tissue culture flasks (catalogue # 430725 and 3506) and plastic disposables were obtained from Corning (Corning Inc.; Corning, NY). Dulbecco's modified Eagle medium (DMEM)/F12 was purchased from Invitrogen Corporation (Carlsbad, CA), and the fetal calf serum (FCS) came from Hyclone (Logan, UT). The Ishikawa cell line was a generous gift from Dr. Masato Nishida (Kasumigaura National Hospital, Tsuchiura-shi, Ibaraki-ken, Japan). The cells were routinely maintained in DMEM/F12 supplemented with 10% FBS and antibiotic/antimycotic solution containing 100 units/ml penicillin-G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Invitrogen Corporation/Gibco Life Technologies, Grand Island, NY) in a 37 °C incubator at 5% CO_2. As we have done previously with these cells (Naciff et al., 2009), we used serum starvation to synchronize the cells at G0/G1 stage of the cell's cycle, in order to optimize the detection of the genomic response of cells after exposure to graded doses of BPA. However, since the serum provides essential components to maintain cellular viability and adequate responsiveness of most mammalian cell culture systems, the exposure of Ishikawa cells to BPA was done in media supplemented with dextran-coated charcoal (DCC) stripped serum, which contains minimal quantities of steroids. Using this approach we also ensure that the steroids present in the regular serum would not interfere with the BPA response, particularly at low doses. Cells were stripped of endogenous steroids by 48 h culture in phenol red–free DMEM/F12 + 10% DCC-treated fetal bovine serum (Invitrogen Corporation/Biosource Biofluids Cell Culture Products; Rockville, MD), before the experiments started. After steroid starvation, the cells were exposed to vehicle-control (0.1% ethanol in DCC culture media), or to BPA at doses of 10^{-9} M (1 nM, vL), 10^{-7} M (100 nM, L), 10^{-5} M (10 μM, H), and 10^{-4} M (100 μM, vH) for 8, 24 or 48 h. The doses tested include the BPA concentrations that correspond to the IC_{50} determined for the recombinant human ERα (~15 μM) and ERβ (~25 μM) (Gutendorf and Westendorf, 2001) (for the hERβ, Han et al., 2002, determined an IC_{50} of 5×10^{-5} M) and should result in significant activation of these receptors. The indicated concentrations of BPA were prepared in fresh phenol red—free DMEM/F12 + 5% DCC fetal bovine serum. Five independent cell-cultures per time point and dose were used as "biological replicates". Cell viability was evaluated by dye exclusion assay, using 10% trypan blue, and quantified by counting viable and dead cells in a hemacytometer.

2.3. Expression profiling

Total RNA was extracted from each individual cell-culture "biological replica" using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH), 8, 24 and 48 h after exposure to vehicle (controls) or BPA (at the different doses tested). Total RNA was further purified by RNeasy kit (QIAGEN, Valencia CA). After determining the total RNA quality, using the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA), 10 μg of total RNA from each sample were converted into double-stranded cDNA using SuperScript Choice system (Invitrogen Corporation/GIBCO BRL, Rockville, MD) with an oligo-dT primer containing a T7 RNA polymerase promoter. The double-stranded cDNA was purified by phenol/chloroform extraction, and then used for *in vitro* transcription using ENZO BioArray RNA transcript labeling kit (Enzo Life Sciences, Inc., Farmingdale, NY). Biotin-labeled cRNA was purified by RNeasy kit (QIAGEN), and a total of 20 μg of cRNA were fragmented randomly to ~200 bp at 94 °C for 35 min (200 mM Tris-acetate, pH 8.2, 500 mM potassium acetate, 150 mM magnesium acetate). Samples from four individual replicates from each treatment group with high quality cRNA, determined using the Agilent 2100 bioanalyzer, were selected and hybridized to Affymetrix Human Genome U133 Plus 2.0 high-density oligonucleotide microarrays (Affymetrix Inc., Santa Clara, CA) for 16 h. The microarrays were washed and stained by streptavidin-phycoerythrin (SAPE) to detect bound cRNA. The signal intensity was amplified by second staining with biotin-labeled anti-streptavidin antibody and followed by streptavidin-phycoerythrin staining. Fluorescent images were read using the Affymetrix GeneChip Scanner 3000 with Autoloader (Affymetrix Inc., Santa Clara, CA).

2.4. Real-time QRT-PCR

In order to corroborate the relative changes in gene expression in selected genes identified by the oligonucleotide microarrays, we used a real-time (kinetic) quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) approach, as described (Naciff et al., 2002, 2009). Supplementary Table 1 shows the nucleotide sequences for the primers used to evaluate the expression level of the indicated gene transcripts.

2.5. Data analysis

Potential inter-individual variability was addressed by using independent samples of each dose group ($n = 4$) for analysis. For gene expression analysis, scanned output files of Affymetrix microarrays were visually inspected for hybridization arti-