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Effect of Bisphenol A on Drug Efflux in BeWo, a Human Trophoblast-like Cell Line

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Bisphenol A (BPA) is a monomer of polycarbonate plastics that has estrogenic activities and has been shown to be a substrate for multidrug resistant efflux mechanisms, specifically, P-glycoprotein. Since the natural hormone estrogen reverses multidrug resistance in some cell types, we hypothesized that BPA might have a similar activity in trophoblasts. We have used BeWo cells as an in vitro model for human trophoblasts and calcein AM as a substrate for drug efflux mechanism to characterize BPA interactions with placental P-glycoprotein. We found that chronic exposure of BeWo cells to BPA did not alter intracellular calcein accumulation in a fashion that would be reflective of changes in P-glycoprotein expression. Immunoblots affirmed that BPA had small effects on P-glycoprotein expression. However, BeWo cells acutely exposed to BPA pretreatment were observed to have a significantly decreased calcein accumulation. Addition of cyclosporin A, a P-glycoprotein inhibitor and substrate, completely reversed BPA's effects on calcein accumulation and resulted in a net increase, relative to controls, in calcein accumulation by the BeWo cells. BPA was found not to stimulate P-gp ATPase or alter intracellular esterases mediating calcein release from calcein AM. Therefore, our results suggested that BPA stimulated drug efflux by BeWo cells probably by direct effects on P-glycoprotein.
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INTRODUCTION

Bisphenol A (BPA) is a monomer of epoxy resins and polycarbonate plastics. It is widely used for the production of plastics, resins and coatings [1], including the inner coating of food cans and in dental sealant. BPA can enter the body by adsorption or ingestion since a significant amount of BPA has been detected in liquids from the plastic lining of food cans [2] and in the saliva of patients from dental composites or sealants [3]. Additionally, many aquatic ecosystems appear to be polluted with BPA [1,4,5]. BPA has been detected in early gestation of human pregnancy in serum at ng/ml levels and is significantly higher (up to 5-fold and greater) in amniotic fluid suggesting accumulation early in fetuses. Moreover, accumulation appears highest in the placenta and to favor male fetuses [6,7].

BPA is thought to mimic the action of estrogen in vitro and in vivo through binding to the estrogen receptor α (ERα) and β (ERβ) and regulating target gene expression. For example, BPA has been shown to activate ERα and ERβ [8,9], induce the expression of estrogen responsive genes and stimulate cell proliferation in MCF-7 breast cancer cells [10], and in vivo, disrupts growth and differentiation of the rodent reproductive tract and alters the release of prolactin from rat pituitary [11–14]. Thus, BPA is generally considered to be an environmental estrogen and is an endocrine disrupter.

The human placenta serves as an interface for regulating xenobiotic distribution between fetal and maternal blood supplies. The rate limiting barrier in the placenta is a single layer of syncytial trophoblasts which appear to regulate drug and chemical exposure of the fetus through a group of transporters [15], a number of which are functionally expressed proteins and are localized to the maternal plasma membrane of the trophoblast [16–18]. The prototype multidrug resistant mechanism in the placenta is the multidrug resistant gene product 1 (MDR1) also known as P-glycoprotein (P-gp). In addition, known to be present in the placenta are the multidrug resistance associated proteins (MRPs) and breast cancer resistance protein (BCRP) [19]. Steroid hormones are transported and are modulators of these multidrug resistance mechanisms. For example, progesterone, one of the primary hormones of pregnancy, depending on concentration, has been reported to show inhibitory effects (above 10 μM) and stimulatory effects (below 1 μM) on P-gp activity. Progesterone, however, is not transported and influences P-gp transport through an allosteric binding site [20,21]. 17β-estradiol (E2), another primary hormone of pregnancy,

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is found to reverse BCRP mediated drug resistance [22] and the estrogen receptor antagonist, tamoxifen, reverses P-gp-mediated drug resistance in vitro. The nature of estrogen interactions with P-gp is less clear than that observed for progesterone [23,24]. BPA has also been shown to be a substrate for P-gp. Accordingly, tissues expressing P-gp may therefore form a barrier to absorption of both natural and environmental estrogens and related structures [25].

Considering that the human placenta expresses P-gp [15–18], reports of BPA accumulation in the placenta and significant fetal exposure [6,7] are seemingly inconsistent with the presence of P-gp in the trophoblast. To consider this discrepancy, we have investigated how BPA might interact with P-gp in a human trophoblast model. Specifically, we are hypothesizing that BPA, similar to the natural steroid hormones, may alter P-gp efflux of drugs by the trophoblast. The availability of a human trophoblast-like cell line, BeWo, which forms an asymmetric monolayer to facilitate *trans*-cellular transport [26] provides the opportunity to examine the function and regulation of P-gp and other transporters associated with the trophoblast. Importantly, P-gp which is expressed by human trophoblasts has been shown to be expressed and functional in BeWo cell line [27]. We therefore have utilized the BeWo cell line to determine whether BPA alters P-gp protein expression and the degree to which the agent may interfere with the efflux mechanism directly.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Penicillin/streptomycin as a mixture (10,000 U/ml), non-essential amino acids (MEM) and Novex® 8% Tris–glycine gels were from Invitrogen Life Tech (Carlsbad, CA). Tissue culture flasks, 12-well and 24-well tissue culture plates were purchased from Corning Costar (Corning, NY). Dulbecco's modified Eagle's medium (DMEM), sodium bicarbonate, trypsin–EDTA (10×) solution, cyclosporin A, 4,4′-isopropylidenediphenol (bisphenol A), and Hanks' Balanced Salt Solution (HBSS) were from Sigma (St. Louis, MO). Calcein AM was obtained from Molecular Probes (Eugene, OR). Anti-human P-glycoprotein (C219) mouse monoclonal antibody was from Signet Laboratories (Dedham, MA). Peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA). ECL Plus Western Blotting Detection System was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Human P-glycoprotein membranes were purchased from Gentest (Woburn, MA). Microplate fluorescence reader was purchased from Bio-TEK Instruments, Inc (Winooski, VT). All other chemicals were also commercially available.

BeWo cell culture

The BeWo clone (b30) was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO, USA). The cells were cultured as previously described [28]. Briefly, cells were grown in DMEM with 10% heat-inactivated FBS complemented with 0.37% sodium bicarbonate, 100 U/ml of penicillin/streptomycin, 2 mM glutamine and 0.1 mM MEM. The cells were maintained in 75-cm^2 surface area flasks at pH 7.4 under 5% CO_2 and 95% humidity at 37 °C. To pass the cells, the cells were exposed to a trypsin–EDTA solution (0.25% trypsin and 0.02% EDTA in PBS). For uptake assays, cells were plated onto 12-well standard tissue culture plates with a density of 10,000 cells/cm^2. Cells were used upon forming a confluent monolayer (approximately 5–6 days in culture).

Chronic treatment of BeWo cells with bisphenol A

Selected concentrations of BPA were prepared as 1000× stocks in ethanol. For 48 h pretreatment, cells at about 50% confluence were exposed to selected concentrations of BPA. The medium was changed once before use and BPA at the same concentration was included in the change medium. The control cells were cultured in the media containing vehicle (0.1% ethanol) under the same conditions.

Calcein accumulation studies

The accumulation studies were performed according to the methods described elsewhere with some modifications [29]. Calcein AM is a substrate for P-gp and calcein, the fluorescent product, has been shown to be a substrate for both P-gp and MRP [30]. The experimental procedures were conducted at 37 °C unless otherwise indicated. Briefly, the BeWo cells at confluence were washed twice with HBSS supplemented with 25 mM D-glucose (HBSS-Glc) followed by the pre-incubation with HBSS-Glc buffer with or without potential P-gp inhibitors for 30–45 min. The accumulation of calcein was performed by incubating cells with calcein AM (1 μg/ml final) in the presence of selected concentrations of BPA or the vehicle (0.1% ethanol) for 45–60 min. The accumulation was stopped by rapidly rinsing the cells three times with 1 ml/well of ice-cold HBSS-Glc. Following the rinse, each well received 1 ml of lysis buffer (2% Triton X-100). After incubation for 2 h, the cell lysate from each well was mixed by repeated pipetting. The lysate was assayed to determine the intracellular level of calcein using a microplate fluorescence reader. Cellular protein in each lysate was also assayed with a BCA protein kit (Pierce). The accumulation of calcein was normalized as pmol calcein/mg cell protein.

Protein extraction and Western blot analysis

The method for membrane isolation was as described previously with some modification [31]. Briefly, the cells were harvested in Tris–mannitol buffer [(50 mM Tris–HCl, pH 7.0 containing 300 mM mannitol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] followed by centrifugation at 200 × *g* for 6 min. The cell pellet was lysed by homogenization in TMEP buffer

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