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Bisphenol-A (BPA) Exposure Alters Endometrial Progesterone Receptor Expression in the Non-human Primate

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Abstract

OBJECTIVE—To evaluate the effect of BPA on endometrial PR expression in non-human primates and human cells. BPA is a xenoestrogen endocrine disruptor. Both BPA exposure and diminished progesterone action have been associated with pregnancy loss, endometriosis and endometrial hyperplasia/cancer.

DESIGN—Controlled trial in primates.

SETTING—University

Animals—African green monkeys

INTERVENTIONS—After oophorectomy, BPA (50µg/kg/day), estradiol, both or vehicle control were administered. . Estradiol and BPA were used in Ishikawa cells.

MAIN OUTCOME MEASURES—PR expression using IHC and qPCR.

RESULTS—PR expression was increased in estradiol treated primates compared to controls. Exposure to the combination of estradiol and BPA resulted in decreased PR expression compared to estradiol exposure alone ($p < 0.01$). In Ishikawa cells treated with estradiol, PR expression increased 5.1 fold, however, when Ishikawa cells were simultaneously treated with estradiol and BPA, PR expression was decreased to 0.6 fold that of cells treated with estradiol alone ($p < 0.05$).

CONCLUSION—BPA alone functions as a weak estrogen. However, when administered with estradiol, BPA diminishes estradiol induced PR expression. The estrogen-like effect of BPA reported in exposed humans may be mediated by PR blockade and a resultant decrease in the estrogen inhibition normally imparted by progesterone. Diminished PR expression may underlie previous reports linking BPA exposure to endometrial dysfunction in humans.

Keywords

Bisphenol A; BPA; estrogen; progesterone receptor (PR); endocrine disruptor; xenoestrogen; endometriosis; endometrial hyperplasia

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Introduction

Humans are widely exposed to chemicals that mimic the actions of endogenous hormones, thereby affecting endocrine function. Low doses of these chemicals can affect endocrine function in many species (1,2). Bisphenol-A (BPA) is used in the manufacture of polycarbonate plastics, epoxy resins, and dental sealants and is an estrogenic endocrine disruptor affecting fertility and reproduction (3). Exposure to BPA occurs as it leaches from polycarbonate bottles, epoxy resin coating the interior of food cans, and dental sealants (4). BPA is virtually ubiquitous in the environment and food supply; ninety five percent of human urine samples contain BPA (5). Perinatal exposure has been associated with female reproductive tract abnormalities (6,7,8,9,10). These reproductive effects are likely mediated by BPA's estrogenic activity. BPA binds both estrogen receptor (ER) alpha and beta, resulting in uterotrophic activity, although only 10^{-4} that of an equivalent dose of estradiol (11).

BPA exposure has been associated with pregnancy loss in humans (12). BPA has adverse effects on oocytes, leading to increased rates of anuploidy in mouse embryos; this may in part explain the observed incidence of pregnancy loss in women exposed to BPA (13). However, BPA, a xenoestrogen, may also affect pregnancy by targeting estrogen responsive organs such as the uterus. BPA exposure has also been associated with other endometrial defects including, endometriosis and endometrial hyperplasia (14,15,16,17). Pregnancy loss, endometriosis and endometrial hyperplasia can each be driven by deficient or absent progesterone action. Here we hypothesized a role for BPA functioning as a selective estrogen receptor modulator and antagonizing estradiol's ability to mediate progesterone action.

Progesterone is produced in the ovaries and placenta and is required for the maintenance of pregnancy, prevention of endometrial hyperplasia and is also useful in treatment of endometriosis. The progesterone receptor (PR) mediates the physiological effects of progesterone and has two isoforms, PRA and PRB, differentiated by the addition of 165 amino acids in the N terminus of PRB (18). An inadequate response to progesterone due to changes in PR gene expression, can lead to infertility, pregnancy loss or endometrial hyperplasia. The excessive endometrial growth in endometriosis is also associated with resistance to the normal effects of progesterone (13,19,20, 21, 22, 23, 24, 25). Estradiol normally increases endometrial PR expression; xenoestrogen endocrine-disrupting compounds such as BPA typically mimic estradiol, acting as weak estrogen receptor agonists. Here we hypothesized that BPA acts as a selective endocrine disruptor with the ability to antagonize estrogen action on the expression of some genes.

We tested the ability of BPA to alter PR expression using a primate model as well as an endometrial adenocarcinoma cell line. We demonstrate that treatment with BPA or estradiol individually each led to increased expression of PR. However, when BPA and estradiol are used in combination, PR expression is decreased compared to treatment with estradiol alone. BPA inhibits PR expression by antagonizing the action of estradiol.

Methods

Primate Treatment

Primate surgery and treatment was preformed as previously described (26). Briefly, we used ten adult female African green monkeys (*Chlorocebus aethiops sabaeus*) of reproductive age (body weight, 4–5 kg). All monkeys were anesthetized (20 mg/kg ketamine, i.m, followed by 20 mg/kg pentobarbital i.v.), intubated, and ovariectomized through a median laparotomy under sterile conditions.

One vehicle-treated control received a cholesterol-filled 4-cm long Silastic capsule (Dow Corning; 3.5 mm i.d., 4.65 mm o.d.) and a vehicle-filled minipump (Alzet 2ML4 osmotic pump, delivering fluid at a rate of 2.5 μ l/h for 4 weeks). Three undergoing estradiol benzoate (EB) treatment received a Silastic capsule containing crystalline EB and an Alzet minipump loaded with vehicle. The three in the BPA-treatment group were given a cholesterol-filled Silastic capsule and a BPA-filled Alzet minipump. The three receiving a combination of EB +BP received a Silastic capsule containing crystalline EB and an Alzet minipump loaded with BPA. The Alzet minipumps supplied BPA at a rate of 50 μ g/kg/day. The Silastic capsules and minipumps were implanted below the skin of the back.

Twenty-eight days later, the animals were deeply anesthetized (ketamine 20 mg/kg i.m., followed by pentobarbital, 100 mg/kg i.v.) and then euthanized by transcardial perfusion of heparinized saline (1.0 liter), followed by a fixative [1.5 liters, containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)] with the descending aorta clamped. The uteri were dissected and postfixed overnight in the same, but glutaraldehyde-free, fixative. The tissues were stored and transported in phosphate buffer containing 0.1% sodium azide. Specimens were embedded in paraffin and serial 5-mm sections were obtained.

Animals and sterile surgical facilities were provided by the Saint Kitts Biomedical Research Foundation (SKBRF). The SKBRF traps or breeds its animals and has a complete 24-h veterinary service. The facility operates in full compliance with all applicable U.S. regulations and has provided an assurance of compliance (#A3005) to the Office of Laboratory Animal Welfare. All animal protocols used in this study were in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and were approved by SKBRF's Institutional Animal Care and Use Committee.

Immunohistochemistry

Slides were deparaffinized and hydrated through three 10 minute xylene washes followed by three 10 minute ethanol washes. The slides then underwent permeabilization in cold 95% ethanol and were rinsed with distilled water. Antigen retrieval was then performed for 20 minutes, by steaming the slides at 90°C in 0.01M sodium citrate. After cooling the slides were rinsed in PBS for 3 minutes and washed in PBS containing 0.1% Tween (PBST) for 5 minutes followed by a 3% hydrogen peroxide rinse for 3 minutes. The slides were then incubated at room temperature with 1.5% normal horse serum diluted in PBST for 1 hour and then with a PR monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by a biotinylated secondary antibody, anti-goat horse IgG (Vector Laboratories, Burlingame, CA) for 1 hour. Then the slides were incubated for 15 minutes in VECTASTAIN® Elite ABC (Vector Laboratories) and then incubated for 8 minutes in 3,3'-diaminobenzidine (DAB) (Vector Laboratories). The tissue was then exposed to hematoxylin for 20 seconds and mounted with Permount.

An analysis of PR expression was performed by two evaluators blinded to the treatment group and quantified using the H-Score. The H-Score represents glandular and stromal cell staining intensity and was calculated using the formula: $H\text{-Score} = \sum \text{Pi} (i + 1)$, where the Pi represents the percentage of stained cells ranging from 0–100% and stain intensity (i) is assigned a value of 1, 2, or 3 indicating weak, moderate, or strong, respectively (27,28). Statistical analysis was performed using ANOVA on ranks and statistical significance was defined as $p < 0.05$.

Cell Culture

Ishikawa cells are a well-differentiated endometrial adenocarcinoma cell line that has been used to model human endometrial epithelial cells (29,30,31,32,33). Ishikawa cells express ER and demonstrate estrogen responsive PR expression. Ishikawa cells were a generous gift from Richard Hochberg (Yale University).

Ishikawa cells were maintained in 25-cm² flasks using Dulbecco's Modified Eagles Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% charcoal-stripped calf serum, 1% penicillin/streptomycin, and 1% sodium pyruvate. Cells were maintained at 37°C in a humidified atmosphere (5% CO₂ in air) and allowed to reach 80% confluency. The cells were treated with BPA, estradiol, or a combination of BPA and estradiol, all at concentrations of 1µM. Control cells were treated with equivalent concentrations of methanol, ethanol, and methanol and ethanol, respectively, as found in the diluents of each active comparator.

RNA isolation, quantitative real-time RT-PCR

Total RNA from all cell cultures was purified using the Qiagen RNeasy Plus Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. For real-time RT-PCR analysis, 200ng of RNA was converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). RT-PCR was performed using the LightCycler SYBR Green RT-PCR kit (Roche, Stockholm, Sweden). PCR for PR was performed for 1 cycle at 50°C for 2 minutes; 1 cycle at 60°C for 30 minutes; 1 cycle at 95°C for 5 minutes; and 40 cycles at 95°C for 20 seconds followed by 62°C for 1 minute. Each experiment was performed in triplicate and repeated three times. Comparisons of PR and PR-B gene expression in all four treatment groups were analyzed by ANOVA on ranks.

Total PR (forward primer 5'-CGCCCTATCTCAACTACCTG -3', reverse primer 5'-CCTGACAGCACTTTCTAAGG - 3');

PR-B (forward primer 5'-CCCAGTTTGAGGAGATGAGG -3', reverse primer 5'-ATCCCTGCCAATATCTTGGG- 3');

B-Actin (forward primer 5'-GTGTTGGCGTACAGGTCTTTG -3', reverse primer 5'-CGTACCACTGGCATCGTGAT- 3');

Results

To determine the effects of Bisphenol-A on endometrial PR gene expression, we treated primates with BPA, estradiol, or a combination of the two. A total of ten African green monkeys (*Cercopithecus aethiops sabaeus*) were ovariectomized. Three were treated with BPA (50 microgram/kg/day), three with estradiol benzoate and three received combined treatment with BPA and estradiol, via Alzet pumps. The control was treated with insertion of an Alzet pump containing vehicle alone. Each animal was treated for 29 days. Uterine tissue was examined for PR expression using IHC. In the control primate, there was no detectable expression of PR in the endometrial stroma and glands (Figure 1). As expected, estradiol treatment increased the expression of PR in both the stroma and glands. BPA treatment increased PR expression minimally compared to the control. When BPA and estradiol were co-administered, PR expression was decreased in the endometrial stroma and glands compared to those exposed to estradiol alone.

An H-score was used to obtain a semi-quantitative measure of expression. Estradiol exposure led to a 49 fold increase in glandular PR expression ($p<0.01$) and an 11 fold increase in stromal PR expression ($p<0.01$) compared to vehicle treated control. BPA exposure led to a 5 fold increase in glandular PR expression and a 5 fold decrease in stromal

PR expression compared to control, however, the difference was not statistically significant. Exposure to a combination of estradiol and BPA decreased glandular PR expression to 62% ($p<0.01$) and decreased stromal PR expression to 50%, ($p<0.01$) of that seen in the endometrium of primates treatment with estradiol alone.

In order to determine if BPA affected endometrial PR gene expression directly, Ishikawa cells were exposed to estradiol, BPA, the combination of the two or vehicle control for 24 hours. Quantitative real time RT-PCR was performed on mRNA isolated from Ishikawa cells after treatment. Exposure to estradiol alone led to a 5.2 fold increase in PR expression compared to vehicle control ($p<0.01$). BPA acted as a weak estrogen; exposure to BPA increased the expression of PR 1.7 fold ($p<0.05$). However, when estradiol and BPA were used in combination, PR expression was increased compared to control ($p<0.01$), however PR expression was decreased to 61% of that seen in cells treated with estradiol alone ($p<0.01$). Evaluation of the PR-B isoform showed similar results. The expression of PR-B in the estradiol treated group was increased by 4.1 fold ($p<0.01$). The expressions of PR-B in the BPA treated group were increased by 1.9 fold ($p<0.05$). Yet, when estradiol and BPA were in used combination, the expression of PR-B was increased by only 2.8 fold ($p<0.05$) compared to control, and decreased to 70% of that seen in cells treated with estradiol alone ($p<0.05$).

Discussion

BPA is a xenoestrogen endocrine disruptor that is thought to act as a weak estrogen by binding to the estrogen receptor with low affinity. It is a common ingredient in the manufacture of polycarbonate plastics and production has grown rapidly, from 16 million pounds in 1991 to 2.3 billion pounds in 2004 in the United States alone (34). Several harmful health effects have been associated with BPA exposure including prostate cancer, breast cancer, obesity, early sexual maturation in females, and altered reproductive function (35). The effects of BPA are dependent on time of exposure and dose. Low dose neonatal exposure to BPA in mice causes abnormalities in the female reproductive tract including early vaginal opening and ovarian and reproductive tract abnormalities in females (36,37). Low levels of BPA exposure in-utero have also led to uterine disruption in rat offspring and altered vaginal morphology in postpubertal offspring (7,9) BPA leads to epigenetic alterations and an increase in HOXA10 mRNA expression; an essential gene associated with uterine development and fertility (38,39).

In humans there is an association between recurrent miscarriage and BPA exposure (12). This correlation is interesting given that mice exposed to low doses of BPA have high rates of meiotic failure, specifically an increase in aneuploid eggs and embryos (40,41). While the increased aneuploidy rates are expected contribute to adverse pregnancy outcomes and pregnancy loss, uterine effects of BPA are likely to have an impact on pregnancy as well.

Uterine proliferation and differentiation during the preimplantation period and in pregnancy have been well characterized and these processes are dependent on estrogen and progesterone. These two hormones are both necessary and sufficient to achieve endometrial development and to support pregnancy. Estrogen induces expression of progesterone receptor (PR). A balance between estrogen and progesterone is necessary for the normal and cyclic function of the endometrium, and disruption of this balance can lead to pathologies such as infertility, abnormal bleeding, endometriosis, pregnancy loss, and cancer (42). Rhesus monkeys treated with CDR01-85/287, a non-steroidal estrogen antagonist known for its anti-implantation effects, show markedly decreased progesterone receptor expression (43). Similarly, in humans an inadequate response to progesterone or an insufficient serum progesterone concentration may lead to pregnancy loss and infertility (40). The alteration of

PR expression by BPA could provide an explanation for how BPA exerts its estrogenic endocrine disrupting effects in early pregnancy.

BPA exposure has also been associated with disorders characterized by unrestrained endometrial growth such as endometriosis and endometrial hyperplasia (14,15,16,17). Estrogens typically cause endometrial growth and proliferation, while progestins cause endometrial differentiation. Estrogen exposure unopposed by progesterone leads to excessive endometrial growth hyperplasia and an increased risk of endometrial epithelial cancer. Similarly endometriosis is characterized by a loss of the usual inhibitory effects of progesterone and is considered progesterone resistant (13,19,20,21, 22,23,24,25,). Diminished PR expression and therefore diminished progesterone action would be expected to result in increased endometrial proliferation, seen in both of these conditions. The association of BPA with each of the previously described clinical conditions may be due to diminished PR expression, progesterone resistance, diminished opposition of estrogen and therefore, indirectly, a functional increase in estrogen action.

In summary BPA exposure results in antagonism of estradiol's effect on PR. By decreasing PR expression BPA decreases the ability of progesterone to inhibit estradiol action. BPA exposure in women may affect the balance between estrogen and progesterone action and in turn lead to a heightened estrogen response. Therefore, BPA indirectly leads to increased estrogen action. The effect of BPA on PR may explain the association of BPA exposure with diseases associated with increased endometrial proliferation and diminished differentiation, including spontaneous abortion, endometriosis, endometrial hyperplasia and cancer.

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FIGURE 1.

A) Endometrial PR expression using immunohistochemical analysis in the non-human primate. PR immunostaining was nearly absent either the glands or stroma of control animals. BPA treatment had minimal effect on endometrial PR expression. As expected, estradiol treatment led to a large increase in both glandular and stromal PR expression. Treatment with the combination of estradiol and BPA led to decreased glandular and stromal expression compared to treatment with estradiol alone. Representative photomicrographs taken at 400X magnification are shown.

B) The effect of estradiol and BPA on endometrial PR protein expression in the non-human primate. H score was used as a semi-quantitative measure of PR expression. Estradiol exposure led to a 49 fold increase in glandular PR expression and an 11 fold increase in stromal PR expression compared to vehicle treated control (*p<0.01). Exposure to a combination of estradiol and BPA decreased glandular PR expression to 62% and decreased stromal PR expression to 50% of the H-score in obtained in endometrium of animals treated with estradiol alone (**p<0.01).

FIGURE 2.

Total PR and PR-B mRNA expression in Ishikawa cell line after treatment with estradiol and/or BPA. Real-time PCR results show that total PR and PR-B expression increased 5.2 fold and 4.1 fold, respectively, in cells treated with estradiol compared to the control (* $p < 0.01$). PR and PR-B expression increased 1.7 fold and 1.9 fold, respectively, in cells treated with BPA when compared to the control (* $p < 0.05$). PR and PR-B expression decreased to 0.6 fold and 0.7 fold respectively, in cells treated with a combination estradiol and BPA when compared to those treated with Estradiol alone (** $p < 0.01$ total PR and $p < 0.05$ PR B).