Maternal Exposure to Bisphenol A during Late Pregnancy Resulted in an Increase of Calbindin-D$_{9k}$ mRNA and Protein in Maternal and Postnatal Rat Uteri

Eui-Ju HONG$^{1}$, Kyung-Chul CHOI$^{2}$ and Eui-Bae JEUNG$^{1}$

$^{1}$Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk 361–763, Republic of Korea, $^{2}$Department of Obstetrics and Gynecology, British Columbia Children’s and Women’s Hospital, British Columbia Research Institute for Children’s and Women’s Health, University of British Columbia, Vancouver, BC V6H 3V5, Canada

Abstract. It has been reported that Calbindin-D$_{9k}$ (CaBP-9k) is rapidly and strongly induced by environmental estrogenic compounds, possibly through estrogen receptors (ER$_{α}$) in the uterus of mammals. CaBP-9k can be evaluated as an early gene marker for assaying estrogenic effects of putative environmental chemicals in the rat uterus. This study was undertaken to investigate CaBP-9k mRNA and protein expression in the postnatal rat uterus following maternal exposure to 17$β$-estradiol (E2) and bisphenol A (BPA) during the neonatal period. Treatment with a high dose of BPA (600 mg/kg body weight (BW) per day) resulted in a 3-fold increase in CaBP-9k mRNA expression for 3 days, while a single dose of E2 (40 µg/kg BW per day) induced 2-fold increase of this gene in the maternal uterus. In an agreement with maternal CaBP-9k mRNA, postnatal CaBP-9k mRNA in the uterus increased 4-fold when treated with BPA (600 mg/kg BW per day). In addition, treatment with increasing concentrations of BPA resulted in significant increases in CaBP-9k protein in the maternal rat uterus. It is of interest that increasing doses of BPA induced a significant ER$_{α}$ mRNA increase in the postnatal uterus. Furthermore, immunohistochemistry revealed that treatment with BPA induced CaBP-9k protein in the maternal uterus. We demonstrated that maternal exposure to BPA during late pregnancy induced CaBP-9k mRNA and protein in maternal and postnatal rat uterus. These results suggest that rapid absorption and distribution of environmental estrogenic compounds occurs in maternal and neonatal rat uterus and these chemicals can easily pass though the placenta during pregnancy to affect postnatal reproductive functions.

Key words: Bisphenol A, Calbindin-D$_{9k}$, Placental transfer, Postnatal

(J. Reprod. Dev. 51: 499–508, 2005)

Calbindin-D$_{9k}$ (CaBP-9k) and calbindin-D$_{28k}$ belong to a group of cytosolic calcium binding proteins [1]. CaBP-9k is a 9kDa cytoplasmic protein that binds to calcium with high affinity and is expressed predominantly in the mammalian uterus, intestine, and placenta [1, 2]. The human CaBP-9k gene, which spans approximately 5.5 kb on the X-chromosome, was cloned and sequenced. It was reported to consist of 3 exons and to carry 4 Alu repeats [3]. Human CaBP-9k is most homologous to the porcine and bovine homologs (88.6%), followed by rat (78.5%) and murine CaBP-9k (75.9%) [4]. In the rat uterus, it has been demonstrated that the CaBP-9k gene is highly regulated by estrogen (E2). CaBP-9k mRNA

Accepted for publication: April 26, 2005
Published online: June 10, 2005
Correspondence: E.-B. Jeung (e-mail: ebjeung@chungbuk.ac.kr)
increased during proestrus, when the E2 concentration was high, and was repressed during diestrus in response to the decrease in plasma E2 [5].

In previous studies using Northern and Western blot assays, we demonstrated that the estrogenic compounds, octylphenol (OP), nonylphenol (NP), and bisphenol A (BPA), induced CaBP-9k mRNA and protein in the rat uterus [6, 7]. In the rat uterus, CaBP-9k mRNA is predominantly expressed in the endometrial and myometrial stromal cells during the non-pregnant estrous cycle, but in pregnant rats, this gene is expressed in the luminal epithelium, juxtaposed yolk sac epithelium, and intraplacental yolk sac [8, 9]. Environmental chemicals that disrupt endocrine function have been suspected for their adverse effects on wild animal and human reproductive systems [10, 11]. Among these chemicals, BPA is recognized as a potential environmental estrogenic chemical that may interfere with endogenous estrogen. It is widespread in the environment and commonly ingested by humans since it is used in the manufacture of polycarbonate and other plastics, including food-storage containers. BPA has an estrogenic activity in vitro and in vivo [12], acting only as an agonist of estrogen via ERß, but as both an agonist and antagonist via ERα in some tissues. Thus, BPA activity may depend on the ER subtype and the tissues involved [13]. In general, environmental estrogenic activity is low compared to E2 or diethylstilbestrol (DES), but exposure to these compounds through diet or the environment has led to the detection of significant quantities of these substances in human urine and tissue samples [14]. Although the impact of environmental estrogens on reproductive health is not well defined, these chemicals are found to disrupt the mammalian reproductive system, which would confirm their estrogen-like activity in vitro [15]. In an in vitro binding study, the binding affinity of BPA to both ERα and ERβ has been demonstrated to be approximately 10,000-fold weaker than the E2 binding affinity and 20,000-fold weaker than the DES binding affinity [16]. In vivo estrogenic activities (400–1,000 mg/kg/day) have been recognized in immature or ovariectomized rats and mice [17, 18]. Thus, the doses of BPA used in this study should be expected to show an effect equivalent to the effects of steroid hormones.

Transplacental absorption may require both lipophilic and hydrophilic properties. DES was rapidly absorbed and distributed to maternal mouse organs. It accumulated in the liver and was rapidly distributed to the fetus where it induced ERα expression in the newborn mouse uterine epithelium [19, 20]. BPA appeared to be transferred rapidly across the placenta, much like thiabendazole, and salicylic acid, and its maximal fetal concentration was 30–100% of its maximal maternal plasma level [21, 22]. In our previous study, we demonstrated that maternal exposure to APs, OP, and NP during late pregnancy increased the expressions of CaBP-9k mRNA and protein in maternal and neonatal uteri, suggesting that these chemicals can easily pass through the placenta during pregnancy to affect the functions of neonatal reproductive tissues [23]. In addition to OP and NP, we further investigated CaBP-9k mRNA and protein expression in maternal and postnatal rat uteri following maternal BPA exposure during late pregnancy. Furthermore, BPA-induced ERα mRNA expression was analyzed in maternal and postnatal rat uteri following maternal exposure to BPA.

Materials and Methods

Treatment

Pregnant Sprague-Dawley (SD) rats were obtained from Dae Han Biolink Co., Ltd. (Chungbuk, Korea). All experimental procedures and animal care were approved by the Ethics Committee of the Chungbuk National University. All animals were housed in polycarbonate cages and acclimatized to an environmentally controlled room (temperature: 23 ± 2 C, relative humidity: 50 ± 10%, frequent ventilation, and 12 h light cycle). They were fed soy-free pellet food (Dyets, Inc., Bethlehem, PA, U.S.A.). In the experiment, three groups of five animals (n=15) were given a subcutaneous injection with BPA at doses of 200, 400, or 600 mg/kg BW per day on days 17, 18, and 19 of pregnancy (P17–19). Another two groups of five animals (n=10) were injected with a single dose of E2 (40 µg/kg BW) as positive controls or with a corn oil vehicle (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) as a negative control on P17–19, as indicated above. On the fifth day after delivery, maternal and postnatal rats were euthanized and the uteri were removed and fixed (Fig. 1).
Northern blot analysis

To determine CaBP-9k mRNA expression in the maternal uterus, Northern blot analysis was performed as described previously [24]. Briefly, RNA was isolated from the maternal uterus using Trizol Reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, U.S.A.). Total RNA (10–20 µg) was electrophoresed on agarose gel, and 28S rRNA was used as an indicator of the quantity of total RNA. The RNA was transferred onto a nylon membrane (Amersham Pharmacia Biotech, Morgan, Ontario, Canada), UV cross-linked, and prehybridized for 2 h at 42 C in buffer containing 50% formamide, 5X Denhardt’s solution (0.1% polyvinylpyrrolidene, 0.1% BSA, 0.1% Ficoll), 5X SSPE, 0.1% SDS, and denatured salmon sperm DNA (100 µg/ml). CaBP-9k cDNAs were labeled with [32P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) using a Random Primed DNA labeling kit (Takara Biotech, Kyoto, Japan) as per the manufacturer’s suggested protocol. Hybridization with labeled probes was performed in fresh buffer for 18 h at 42 C with 32P-labeled cDNA. After stringent washes, the membrane was exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, U.S.A.) at –80 C.

Reverse transcription-polymerase chain reaction

Total RNA (5 µg) from the postnatal uterus was used for reverse transcription/polymerase chain reaction (RT/PCR) analysis. Total RNA was subjected to first strand cDNA synthesis using M-MLV reverse transcriptase (Kosco Inc., Seoul, Korea), dNTPs (2.5 mM), RNase inhibitor (20 units), and random primers (9 mer) according to manufacturer’s suggested protocol. A 1 µl aliquot was used for PCR under standard conditions. A 20-µl PCR reaction contained 1 unit of Taq polymerase (Intron Co., Seoul, Korea), 1.5 mM MgCl2, 2 mM dNTPs, and 100 pmol of each specific primer. The primers for CaBP-9k were 5’-AAG AGC ATT TTT CAA AAA TA-3’ (sense) and 5’-GTC TCA GAA TTT GCA TTA TT-3’ (antisense). The primers for the ERα gene were 5’-ATG ATC AAC TGG GCA AAG A-3’ (sense) and 5’-TGT ACA CTC CGG AAT TAA GC-3’ (antisense). The primers for the 1A gene were 5’-GAT ATA GCA TTC CCA CGA ATA-3’ (sense) and 5’-GGG CTT TTG CTC ATG TTG CAT-3’ (antisense). Each PCR cycle consisted of denaturation at 95 C for 60 sec, annealing at 55 C of 60 sec, and extension at 72 C for 90 sec. To determine the conditions for logarithmic phase PCR amplification for CaBP-9k (314-bp), ERα (325-bp), and cytochrome c oxidase subunit 1 (1A, 968-bp), aliquots were amplified using different numbers of cycles. The IA gene was amplified to rule out the possibility of RNA degradation and was used to control the variation in mRNA concentrations in the reverse transcription reaction.

A linear relationship between PCR products and amplification cycles was observed in CaBP-9k, ERα and IA mRNAs. For quantification, we used 25 cycles for CaBP-9k and ERα and 18 cycles for IA. Ten µl of each PCR product was fractionated on a 2% agarose gel stained with ethidium bromide. The photograph was scanned and analyzed using Molecular Analysis Program version 1.5 (Gel Doc 1000, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR products were transferred to a nylon membrane using a Vacuum Blotter (Bio-Rad Laboratories, Inc.) and UV cross-linked to the membrane using a Gene Cross-Linker (Bio-Rad Laboratories, Inc.). Membranes were prehybridized in a solution containing 50% formamide, 5X SSPE, 5X Denhardt’s solution, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA for 3 h at 42 C. [32P]-dCTP-labeled probes were added to the hybridization solution and incubated for 16 h at 42 C. Membranes were washed three successive times at 42 C in 2X SSC, 0.1% SDS, at 54 C in 1X SSC, 0.1% SDS, and at 68 C in 0.1X SSC, 0.1% SDS, respectively. Membranes were exposed to X-ray film (Eastman Kodak), developed, scanned, and analyzed by Molecular Analysis Program version 1.5.

Western blot analysis

Rats were euthanized, and their uteri were rapidly excised and washed in cold sterile 0.9% NaCl. Protein was extracted with Proprep (Intron Co.) according to the manufacturer’s suggested instructions. Fifty µg of cytosolic protein per sample was resolved by electrophoresis on a 15%
SDS-polyacrylamide gel and transferred to PVDF transfer membrane (PerkinElmer Inc., Shelton, CT, U.S.A.) using a TransBlot Cell (Bio-Rad Laboratories, Inc.) according to the manufacturer’s suggested instructions. Membranes were blocked with phosphate-buffered saline containing 0.05% Tween-20 and 5% dry milk (w/v) overnight and were incubated sequentially with primary and secondary antibodies dissolved in 1% bovine serum albumin (BSA) for 1 h at room temperature. The primary antibody was a polyclonal antibody (Swant, Switzerland, 1:2,000) against rat CaBP-9k, and beta-actin antibody (42-kD, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as an indicator of total protein [7]. Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotech) was used as a secondary antibody.

Immunohistochemical staining (IHC)
CaBP-9k protein was localized by immunohistochemistry. Small pieces of rat uterus were paraffin-embedded and 5 µm sections were deparaffinized in xylene and hydrated in descending grades of ethanol. Nonspecific antibody interactions were blocked by incubating the sections in 3% (w/v) bovine serum albumin, dissolved in phosphate-buffered saline. The secondary biotinylated antibody (rabbit IgG; Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (w/v) in methanol for 20 min and the sections were incubated with ABC-Elite (Vector Laboratories, Inc.) for 30 min at room temperature. The chromogen was diaminobenzidine (Sigma-Aldrich Co.), and the sections were counterstained with haematoxylin and mounted with Canada balsam.

Data analysis
Data are presented as the mean ± SD. Data were analyzed by the non-parametric Kruskal-Wallis test, followed by Dunnett’s test for five-pair comparisons and multiple range test. Each value of Dunnett’s test was converted to rank for statistical analysis. All statistical analyses were performed with the Statistical Analysis System (SAS Institute, Cary, NC, U.S.A.). P < 0.05 was considered statistically significant.

Results
Calbindin-D9k mRNA expression in the maternal and postnatal uterus
Following maternal exposure to BPA (200, 400, or 600 mg/kg BW per day) during late pregnancy (P17-P19), we investigated maternal and postnatal CaBP-9k mRNA expression on day 5 after delivery by Northern blot analysis. Maternal exposure to BPA (600 mg/kg BW per day) resulted in an up to 6.4-fold increase of maternal uterine CaBP-9k mRNA vs. vehicle for 3 days (Fig. 2). In addition, a single dose of E2 (40 µg/kg BW) induced CaBP-9k mRNA (4.4-fold vs. vehicle) in the maternal uterus, as shown in Fig. 2. Postnatal uterine CaBP-9k mRNA expression was further examined by RT-PCR analysis following maternal injection with increasing doses of BPA. In agreement with maternal CaBP-9k mRNA, postnatal CaBP-9k mRNA was significantly increased when treated with BPA (4.4-fold vs. vehicle, 600 mg/kg BW per day), as shown in Fig. 3. These results indicated that CaBP-9k mRNA in the postnatal uterus was induced by a high dose of BPA, similar to CaBP-9k mRNA in the maternal uterus.
Effect of BPA in Postnatal Rat Uterus

Estrogen receptor mRNA expression in the maternal and postnatal uterus

Maternal and neonatal ERα mRNA expression was further analyzed by treating maternal rats with increasing doses of BPA to investigate the effect of BPA on ERα mRNA expression. There was no difference in ERα mRNA expression following maternal exposure to BPA or E2 (Fig. 4). In contrast, treatment with increasing doses of BPA (400 and 600 mg/kg BW) resulted in significant increases in ERα mRNA expression in the postnatal uterus (Fig. 5). The greatest increase in postnatal uterine ERα mRNA was observed with high doses of BPA (600 mg/kg BW), as shown in Fig. 5.

Calbindin-D9k protein expression in the maternal uterus

CaBP-9k protein expression in the maternal uterus was examined by immunoblot analysis on day 5 after delivery following maternal exposure to BPA. Treatment of maternal rats with BPA (200, 400, and 600 mg/kg BW) resulted in significant increases in CaBP-9k protein (50, 40, and 50% vs. vehicle, respectively) in the maternal uterus, as shown in Fig. 6. However, there was no significant
change in CaBP-9k protein when rats were treated with E2 (Fig. 6). CaBP-9k protein in the neonatal uterus could not be determined since the total protein isolated from the neonatal uterus was not sufficient to perform Western blot analysis.

Localization of CaBP-9k protein in the maternal and postnatal uterus
On day 5 after delivery, CaBP-9k protein was localized by immunohistochemistry following treatment with increasing doses of BPA or E2. Similar to the Western blot analysis data shown in Fig. 6, the treatments with increasing doses of BPA increased CaBP-9k protein expression in the maternal uterus (Fig. 7). In addition, a single E2 treatment (40 µg/kg BW) increased CaBP-9k protein in this tissue, as indicated in Fig. 7. These results indicate that CaBP-9k protein is widely spread through stromal cells and induced by BPA in the endometrium of the maternal rat uterus.

Discussion
In our previous studies, the expression of CaBP-9k mRNA and protein was rapidly and strongly induced by environmental estrogenic compounds, possibly through ERα. CaBP-9k can be evaluated as an early gene marker for estrogenic effects caused by putative environmental chemicals in the rat uterus [6, 7]. E2 is a major factor controlling CaBP-9k gene expression in the rat uterus. The CaBP-9k gene is not expressed in the uterus of mature ovariectomized and immature rats that do not have circulating E2 from ovaries [25]. The CaBP-9k gene could be evaluated as a biomarker for the detection of environmental estrogenic compounds [6]. As previously studied by us, maternally injected estrogenic compounds may be transferred to neonates through breast milk, thus affecting uterine function, as shown by the induction of CaBP-9k gene expression in the neonatal uterus [26]. As a continuation of studies determining the effects of environmental endocrine disruptors, the present study was performed to investigate the involvement of CaBP-9k mRNA and protein in postnatal rats following maternal exposure to endocrine disruptors during the fetal period. Thus, the expression levels of CaBP-9k mRNA and protein were analyzed to determine the effect of BPA and E2 on the maternal and postnatal uteri on 5 day after delivery. We carried out prenatal exposure that resulted in an estrogenic inducing effect due to sufficient placental transfer of the chemicals during pregnancy and the feeding period after delivery.

During lactation in rats, maternal uterine CaBP-9k was determined, and a dramatic decrease in gene expression was detected on day 5 of lactation (L5). This suggested that a decrease in CaBP-9k mRNA resulted from the combination of high progesterone and low estrogen levels in the maternal uterus during lactation [5]. The E2 level was highest on day 21 of pregnancy (152 pg/ml) and decreased gradually during lactation starting at L2. This E2 decrease during the lactation period was significantly different when compared to the level on day 21 of pregnancy [27]. Thus, CaBP-9k mRNA expression was analyzed to determine the effect of BPA and E2 on the maternal uterus on day
5 post-delivery. Treatment with a high dose of BPA or E2 increased \(CaBP-9k\) mRNA in the maternal uterus. It appeared that injected BPA replaced endogenous estrogen in the uterus, so that the high dose of BPA enhanced \(CaBP-9k\) mRNA expression. In agreement with the maternal \(CaBP-9k\) mRNA, treatment with BPA caused a significant increase in postnatal \(CaBP-9k\) mRNA. It was assumed that injected BPA transferred to the fetuses and persisted. Orally administered BPA has been detected in maternal blood 10 min after treatment, reaching a maximal concentration at 20 min, and then gradually decreasing [28]. This extremely rapid absorption and clearance has been demonstrated at several BPA concentrations (100–800 mg/kg) [29, 30]. Transplacental exposure may enhance toxicity in newborn mice, and BPA was readily available to the fetus via transplacental uptake in mice [18]. We previously found that maternally-injected estrogenic compounds increased \(CaBP-9k\) mRNA and/or protein in the maternal tissues (uterus and placenta) and fetal uterus during late pregnancy, suggesting that, for the fetus, the placenta may not be a reliable barrier to these estrogenic compounds [31]. BPA transferring from maternal tissues or blood to the
fetus during late pregnancy increased postnatal CaBP-9k mRNA expression in this study.

A potential endocrine disruptor, BPA appears to have an estrogenic effect and is proposed to possess hormonal properties, such as mimicking natural hormones and inhibiting steroid hormone action. It can bind to ERs and induce an ER-mediated response [32]. No significant change in ERα mRNA expression in the maternal uterus was observed after treatment with BPA or E2 in this study. It can be postulated that physiological estradiol in all groups gradually decrease due to uterine atrophy during lactation. Thus, estrogenic compounds (BPA and E2) may not affect ERα mRNA during this period. However, ERα mRNA was significantly increased in the postnatal uterus by high doses of BPA. It has been shown that the CaBP-9k gene has an estrogen response element (ERE) mediating the E2-ER response in the rat uterus [33]. The CaBP-9k gene has an imperfect ERE, differing by one nucleotide, but despite this difference, this CaBP-9k ERE binds to ER to regulate this gene at the transcriptional level in target tissues [33, 34].

We further examined the effects of BPA on the CaBP-9k protein level in the maternal rat uterus. Treatment with increasing doses of BPA significantly increased CaBP-9k protein in the maternal uterus, suggesting that BPA may regulate translation of CaBP-9k protein during lactation. BPA has been demonstrated to compete with [3H]-estradiol for binding to ERs in the rat uterus, to induce progesterone receptors [35], and to promote cell proliferation in the cultured human mammary cancer cell line, MCF-7 [36]. BPA bound to both ERα and ERβ with low affinity and transactivated reporter genes in vitro [37, 38]. Treatment with DES significantly increased CaBP-9k protein in the postnatal uterus, indicating that maternally injected DES may transfer from the maternal side to the fetus during pregnancy (data not shown). Transplacental absorption may require both lipophilic and hydrophilic properties, so most organic compounds can transfer across the placenta [39]. For example, maternally administered DES was rapidly distributed to the fetus during pregnancy, resulting in a fetal level of DES that was 2 to 3-fold higher than that in maternal blood [40].

CaBP-9k protein was localized in the maternal rat uterus by immunohistochemistry following maternal exposure to BPA. CaBP-9k expression was previously detected mainly in the luminal epithelial cells of the uterus in pregnant rats, but the CaBP-9k gene in non-pregnant rats was mainly expressed in stromal cells of the myometrium and endometrium [41]. Immunohistochemical staining demonstrated that treatment with BPA increased CaBP-9k protein in the maternal uterus, confirming the mRNA level. The observation that stromal cells from the BPA-treated rats were immunopositive may reflect greater CaBP-9k protein expression in these cells, compared to epithelial cells. We could not deduce the effect of BPA treatment in the postnatal uterus following maternal exposure during late pregnancy because of high background staining and a low level of CaBP-9k protein.

In conclusion, we demonstrated that maternal exposure to BPA during late pregnancy induced CaBP-9k mRNA and/or protein expression in maternal and postnatal uteri. These results suggest that absorption and distribution of environmental estrogenic compounds by maternal and neonatal uteri are extremely rapid and that these chemicals can easily pass through the placenta to affect postnatal reproductive functions. Taken together, the present study implies that maternal exposure to environmental estrogenic chemicals during late pregnancy could result in chemical transfer from the mother to the fetus, thus influencing fetal reproductive health.

Acknowledgments

This work was supported by a Korea Research Foundation Grant (KRF 2004–041- E00335). We are grateful to Dr. Barb Conway at the British Columbia Research Institute for Children’s and Women’s Health, University of British, Columbia for a critical review of the manuscript.

References


26. Hong EJ, Choi KC, Jung YW, Leung PC, Jeung EB. Transfer of maternally injected endocrine disruptors...


