A Calcium-Binding Protein, Calbindin-D9k, Is Regulated through an Estrogen-Receptor-Mediated Mechanism following Xenoestrogen Exposure in the GH3 Cell Line

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A variety of environmental chemicals may possess the potential to interact with various endocrine factors and consequently cause adverse effects on the reproductive, central nervous, and immune systems via the endocrine system(s). In this study, we used the GH3 cell line as an in vitro model to determine the effects of potential endocrine disruptors (EDs) on the induction of calbindin-D9k (CaBP-9k), a useful biomarker for detecting the estrogenic activities of EDs. A rat pituitary cell line, GH3, was treated with octyl-phenol (OP), nonyl-phenol (NP), and bisphenol A (BPA) in a dose-dependent manner (10⁻⁵, 10⁻⁶, and 10⁻⁷M) for 24 h. To determine the time dependency, the cells were exposed to a high concentration (10⁻⁵M) of OP, NP, and BPA and harvested at different time points (1, 3, 6, 12, and 24 h). An antiestrogen, ICI 182,780, was used to examine the potential involvement of the estrogen receptor (ER) in the induction of CaBP-9k by EDs via an ER-mediated pathway. Treatment with OP, NP, and BPA induced a significant increase in CaBP-9k expression at both the transcriptional and translational levels in a dose-dependent manner. Interestingly, ED exposure caused a significant increase in CaBP-9k messenger RNA (mRNA) expression at 6 h, whereas induction of CaBP-9k protein was observed as early as 1 h after treatment. However, both CaBP-9k mRNA and protein expression peaked at 24 h following treatment. The differential response of CaBP-9k mRNA and protein to EDs may be explained by translational efficiency. Cotreatment with ICI 182,780 significantly reversed ED-induced CaBP-9k expression in GH3 cells, suggesting that EDs may trigger the induction of CaBP-9k via an ER-mediated pathway in these cells. Taken together, these results demonstrate for the first time that a single in vitro exposure to OP, NP, or BPA results in an increase in CaBP-9k expression in GH3 cells, after 24 h. These results may contribute to our understanding of the relationship between the molecular events evoked by ED exposure and its biological effects on the pituitary gland in humans and animals. This in vitro model, in combination with a single injection in vivo method developed by us, may be important for elucidating additional details of the mode of action of xenoestrogens and may provide reliable tests for screening estrogenic agonists and antagonists.

Key Words: GH3 cell line; endocrine disruptors; estrogenicity.

Environmental chemicals can alter functions of the endocrine system via various mechanisms. It has been suggested that some chemicals may bind to steroid hormone receptors, mimicking or blocking the action of these hormones. Other chemicals may stimulate or inhibit various enzymes that play essential roles in the synthesis of a hormone (Dang et al., 2007a). These actions may lead to altered functioning of the endocrine system and cause abnormal hormone regulation and gene expression (Dang et al., 2007a; Waring and Harris, 2005). These environmental chemicals, both naturally occurring and man-made, are referred to as endocrine disruptors (EDs). Phthalate acid ester, alkylphenols (APs), polychlorinated biphenyls (PCBs), phytoestrogens, and methoxychlor are rapidly metabolized EDs (Elshby et al., 2001a,b). It has been documented that phthalates, some PCBs, dichlorodiphenyl trichloroethane, and its derivatives, certain insecticides and herbicides such as kepone and methoxychlor, plastic components such as bisphenol A (BPA), and components of detergents and their biodegradation products, such as APs, possess estrogen-like activities and can bind estrogen receptors (ERs) to induce or modulate an ER-mediated response (Choi and Lee, 2004; Gray et al., 1997; Laws et al., 2000; Roy et al., 1997). The potencies of EDs, which can be detrimental to humans and animals, are distinct and dependent upon their estrogenicity (Dang et al., 2007a). Whereas some substances, such as diethylstilbestrol, are well known for high-potency endocrine disruption (Cupp and Skinner, 2001; Newbold, 2004), others, including APs and BPA, have been reported to be weak estrogenic agonists (Bolger et al., 1998; Hong et al., 2004a;
CaBP-9k mRNA and protein in immature rats and that we demonstrated that a single injection of OP, NP, or BPA results in increased CaBP-9k messenger RNA (mRNA) and protein levels induced by EDs are considered to be very useful tools for screening environmental estrogenic compounds in the immature rat model (Choi and Jeung, 2003). In a previous study, we demonstrated that the GH3 cell line is a good candidate for investigating the estrogenicity of EDs in vitro. OP, NP, and BPA were selected for this study because we have tested them previously (An et al., 2002; Dang et al., 2007b; Hong et al., 2004a). GH3 cells were treated with OP, NP, and BPA (10^{-5}, 10^{-6}, and 10^{-7}M) and harvested 24 h after treatment. The cells also were exposed to high concentrations (10^{-5}M) of OP, NP, and BPA and harvested at different time points (1, 3, 6, 12, and 24 h). The effects of these EDs on the induction of CaBP-9k mRNA and protein were examined by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) and Western blot assays, respectively. Additionally, an antiestrogen, ICI 182,780, was used to examine the potential involvement of the ER in the induction of CaBP-9k in these cells.
**Western blot analysis.** Protein was extracted with Proprep solution (Intron Co., Seoul, South Korea) according to the supplier’s suggestions. Fifty micrograms of cytosolic protein was loaded on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinyl difluoride membranes (Amersham Pharmacia Biotech, Rockville, MD) using a tank transfer system (Bio-Rad) according to the manufacturer’s instructions. Membranes were blocked for nonspecific reactions overnight in PBS-Tween containing 5% skim milk, prior to incubation with a rabbit polyclonal antibody (1:500) specific for rat CaBP-9k. Horseradish peroxidase–conjugated secondary antibody (1:3000) from Santa Cruz Biotechnology (Santa Cruz, CA) was visualized using a Western light chemiluminescent system (Amersham Pharmacia Biotech). CaBP-9k levels were quantified by the Chemi Doc XRS system using Quantity One Software (Bio-Rad) and standardized against the beta-actin levels of each sample.

**Data analysis.** The data were represented as the means ± SD and analyzed by one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test. p < 0.05 was considered statistically significant.

**RESULTS**

**Effects of EDs on Regulation of CaBP-9k mRNA Expression**

The dose- and time-dependent effects of OP, NP, and BPA on the induction of CaBP-9k mRNA were examined by semi-quantitative RT-PCR as described in the “Materials and Methods.” As shown in Figure 1, a significant increase in CaBP-9k mRNA expression was observed with the highest concentration (10⁻⁵M) of OP, NP, or BPA at 24 h after a single exposure. At a concentration of 10⁻⁶M, OP and NP also evoked a marked rise in CaBP-9k mRNA expression, whereas a modest increase was observed with BPA exposure. As a positive control, E2 induced a significant rise in CaBP-9k mRNA expression at all concentrations, with the highest response at 10⁻⁷M. In the time-dependent experiments, OP, NP, and BPA (10⁻⁵M) induced an increase as early as 3 h after treatment and CaBP-9k mRNA peaked at 12 h, as shown in Figure 2. This upregulation continued until 24 h after treatment. As expected, E2 exposure (10⁻⁷M) also evoked an elevation in CaBP-9k mRNA expression as early as 3 h and peaked at 24 h (Fig. 2). Coadministration of the pure antiestrogen, ICI 182,780, completely reversed the ED-induced increase in CaBP-9k mRNA in GH3 cells, as seen in Figure 3, suggesting that the biological effects of EDs on the induction of CaBP-9k mRNA may involve an ER-mediated physiological response in vitro.

**Effects of EDs on Regulation of CaBP-9k Protein Expression**

The effects of EDs on the expression of CaBP-9k protein were determined by Western blot analysis. As shown in Figure 4, increasing concentrations of OP, NP, or BPA resulted in a dose-dependent elevation in CaBP-9k protein in these cells. A significant induction of CaBP-9k protein was observed at 24 h after 10⁻⁵M ED exposure. Whereas the effects of OP and NP were observed at the lower concentration (10⁻⁶M), there was no difference between the response to 10⁻⁷M BPA and the negative control. As expected, E2 induced high-level expression of CaBP-9k protein after 24 h in a dose-dependent manner. It is of interest that treatment with a high concentration (10⁻⁵M) of OP or NP significantly increased the expression of CaBP-9k protein as early as 1 h, whereas BPA induced only a weak response at the same concentration (Fig. 5). The responses fell after 6 h, but then peaked after 24 h. As a positive control, E2 also resulted in a significant increase in CaBP-9k at 1 h, with induction falling after 12 h and then peaking at 24 h after exposure. As shown in Figure 6, the level of CaBP-9k protein increased significantly after 24 h in response to OP, NP, and BPA, whereas cotreatment with ICI 182,780 significantly attenuated ED-induced CaBP-9k protein in GH3 cells.

**DISCUSSION**

In this study, we examined the estrogenic effects of OP, NP, and BPA on the regulation of CaBP-9k mRNA and protein in...
Interestingly, a marked rise in CaBP-9k expression was observed at high (10⁻⁵M) and medium (10⁻⁶M) concentrations of OP and NP, whereas a significant dose-dependent induction was observed only at a high concentration of BPA. As expected, all concentrations of E₂ enhanced CaBP-9k expression. It has been suggested that the estrogenic potency of OP is about 10⁻³–10⁻⁷M as compared to 10⁻⁹M of E₂ (Arnold et al., 1996; Dang et al., 2007a; Nagel et al., 1997; White et al., 1994).

The identification of estrogen-responsive genes is a critical step toward understanding the mechanism underlying estrogenic effects (Watanabe et al., 2003). It is well-documented that the CaBP-9k gene is controlled by sex hormones. In the rat uterus, the expression of the CaBP-9k gene is upregulated by estrogen and downregulated by progesterone during the estrous cycle and early pregnancy (Krisinger et al., 1992, 1994; L’Horset et al., 1993, 1994). In addition, a marked upregulation of CaBP-9k following E₂ treatment in vitro has been reported by Fujimoto et al. (2004), indicating that this gene can be a useful biomarker for screening the estrogenicity of environmental estrogen-like compounds.

It has been suggested that the timing of the exposure is very important for evaluating adverse health effects of EDs in humans and animals. In combination with dose responses, time-dependent relationships may provide credible information for the assessment of ED adverse effects. In the present study, time courses were performed to examine the potency of OP, NP, and BPA in the induction of CaBP-9k expression in GH3...
At a high concentration, OP, NP, and BPA induced a significant increase in CaBP-9k mRNA at 6 h after treatment and peaked at 12 h. It is of interest that a high concentration (10^{-5}/C255 5M) of OP or NP significantly increased the expression of CaBP-9k protein as early as 1 h after treatment, whereas BPA induced only a weak response at the same concentration. This increase was attenuated in a time-dependent manner and no statistical difference was noted between the 6-h levels of the treated and untreated groups. However, the upregulation recovered at 12 h and the peak response was observed at 24 h after ED exposure. The differential patterns of CaBP-9k mRNA and protein expression in response to EDs may be explained by translational efficiency. Evidence has suggested that since OP, NP, and BPA possess hormone-like activity, they can induce a physiological response via steroid hormone–mediated pathways. Previous studies have indicated that exposure of immature rat uteri to these synthetic compounds may lead to an increase in the expression levels of estrogen-regulated genes, including the CaBP-9k gene (Hong et al., 2006). In addition, ED contamination of offspring via the maternal placenta or breast milk has been reported (Hong et al. 2004a,b, 2005).

However, there are few in vitro or in vivo methods for determining whether a chemical is an ED (Choi and Jeung 2003). Several in vitro and in vivo methods for examining environmental estrogenic chemicals have been developed. In vitro methods include ER-binding assays, Michigan Cancer Foundation (MCF) cell-proliferation assays, and the yeast-estrogen-screen cell assay (Miller et al., 2000; Seifert et al., 1988). In vivo methods include a uterotrophic bioassay in ovariectomized (OVX) adult and immature female rodents, age at vaginal opening in prepubertal rats, vaginal cytology in adult OVX female rats, and estrous cyclicity in intact rats (Balaguer et al., 1999; Gray et al., 1997). Recently, induced biomarkers for detecting EDs have been widely used to characterize the estrogenicity of EDs. These biomarkers include pS2, MUC1,
These results indicate that the effects of OP, NP, and BPA on the induction of CaBP-9k expression may involve an ER-mediated physiological mechanism in vitro. Functionally, ERs are important for evoking pituitary-cell responsiveness to estrogens (Nguyen et al., 2005). In addition, they are considered to be necessary for modulating uterine CaBP-9k gene expression in rats (Krisinger et al., 1992, 1994). The classical mode of action of estrogen-like compounds is mediated via the ER and the ER-mediated pathway. However, recent studies have pointed out that nongenomic effects may also contribute to the potency of xenoestrogens in disrupting functional endocrine system(s) (Watson et al., 2007). Other evidence has shown that xenoestrogens can exert their effects by altering the synthesis or availability of endogenous hormones (Waring and Harris, 2005), suggesting that E2 and xenoestrogens may possess the potential to induce nongenomic responses. In a previous study, we demonstrated that the in vivo pathway induced by OP, NP, or BPA for the regulation of CaBP-9k expression involves the ER (Dang et al., 2007b). It has been well-documented that E2 treatment can induce a significant enhancement of pituitary CaBP-9k expression, whereas co-treatment with P4 completely attenuates this response, indicating that P4 may antagonize E2-induced CaBP-9k expression in the endocrine gland (Nguyen et al., 2005). The regulation of pituitary CaBP-9k expression evoked by steroid hormones is quite similar to the regulation of CaBP-9k expression in the uterus. It has been reported that induction of uterine CaBP-9k is influenced by EDs via the ERα-mediated pathway, but not by the ERβ pathway (Lee et al., 2005). ERα is highly expressed in the anterior lobe of the pituitary gland, whereas a very low ERβ expression level has been reported in this tissue (Kuiper et al., 1997). It is assumed that the ratio of ERα to β is about 380:1 in the GH3 cell line (Fujimoto et al., 2003), indicating that ERα is the predominantly expressed type in these cells. Although ERα and ERβ share some functional characteristics, distinct molecular mechanisms control their genes (Frasor et al., 2003).

In conclusion, we have demonstrated for the first time that a single in vitro exposure to xenoestrogens results in an increase in the induction of CaBP-9k expression at 24 h in the rat pituitary cell line GH3. These results may contribute to our understanding of the relationship between the molecular events evoked by ED exposure and their biological effects in human and animal pituitary glands. Furthermore, this model developed here may be important for elucidating in more detail the possible impacts of xenoestrogens and for the development of reliable tests for screening estrogenic agonists and antagonists.

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