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This study was undertaken to compare the sensitivity of screening test methods and to investigate the structure–activity relationships of the estrogenic activity of alkylphenolic compounds (APs) using in vitro and in vivo assays. Two in vitro systems, MCF-7 cell proliferation (E-screen assay) and competitive binding assay to estrogen receptor (ER), were selected to evaluate the estrogenic effects. Uterotrophic assay and Calbindin-D_{9K} (CaBP-9K) mRNA expression were also examined in ovariectomized Sprague-Dawley female rats. A series of APs with various alkyl groups were examined, namely, 4-propylphenol, 4-butylphenol, 4-t-butylphenol, 4-pentylphenol, 4-nonylphenol, 4-octylphenol, 4-t-octylphenol, and 4-phenylphenol, and 17β-estradiol (E2) was used as a positive control. In the E-screen assay, E2 was found to induce maximum proliferation of MCF-7 cells at 1 nM. Among the APs, 4-t-octylphenol and 4-nonylphenol were found to be considerably more potent than any other compound and estrogenic effects were detectable at 1 and 10 μM, respectively. 4-t-Octylphenol and 4-nonylphenol inhibited the binding of E2 to the ER of MCF-7 cells in a competitive ER binding assay. The uterotrophic effects to APs (10, 50, 200, and 400 mg/kg/d) were compared to E2 (1 μg/kg) in ovariectomized rats after treatment for 3 d. 4-Nonylphenol, 4-t-octylphenol, and 4-phenylphenol produced dose-dependent increases in the uterine weights of ovariectomized rats. In the CaBP-9K mRNA expression test, CaBP-9K mRNA levels were detected in the uteri of ovariectomized rats treated with 4-pentylphenol (400 mg/kg), 4-nonylphenol, 4-phenylphenol (200 and 400 mg/kg), and 4-t-octylphenol (50 mg/kg and above), respectively. In the dot blot assay, CaBP-9K mRNA levels were significantly increased in rats exposed to 4-t-octylphenol (200 and 400 mg/kg), 4-pentylphenol, 4-nonylphenol, and 4-phenylphenol

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(400 mg/kg), respectively. Among the APs, compounds with bulky alkyl groups or higher carbon numbers possessed higher estrogenic capacity. In addition, the pattern of CaBP-9K expression correlated with that of the 3-d uterotrophic assay. Therefore, our results suggest that the CaBP-9K gene might be used as a potential biomarker for the screening of endocrine disruptors.

Hormones play an important role in the differentiation of tissues, the development of reproductive functions, and the regulation of homeostasis. Endocrine disruptors (EDs) are synthetic or natural hazardous substances that interfere with the normal function of hormones when introduced into the body (Kelce et al., 1995; Bruder et al., 1997; Maness et al., 1998). The abnormal effects of EDs are suggested to be due to their ability to (a) mimic the effect of endogenous hormones, (b) antagonize the effect of endogenous hormones, (c) disrupt the synthesis and metabolism of endogenous hormones, or (d) disrupt the synthesis and metabolism of hormone receptors (Colborn et al., 1993). Moreover, the hormonelike activity of EDs may be sustained long after they are released into the environment. It has been pointed out recently by various studies that many chemicals in the environment contribute actions of EDs (Colborn & Smolen, 1996; Klotz et al., 1996; DeRosa et al., 1998). As a result, the U.S. Environmental Protection Agency (EPA) has established an EDs screening and testing program and formed the Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC) for consultation and research upon diverse EDs (Gray, 1998). In addition, the committee of the Organization for Economic Cooperation and Development (OECD) with responsibility for testing guidelines and regulations relating to EDs extended the scope of regulation pertaining to humans, to wildlife, fish, and invertebrates (Holmes et al., 1998). Recently, a number of environmental pollutants have been found to affect the endocrine system, and thus produce adverse effects in various living organisms (DeRosa et al., 1998). For example, the alkylphenol polyethoxylates (APEs) are used as nonionic surfactants in industrial and household cleaning agents, in the manufacture of paints and plastics, in pesticide formulations, and in the manufacture of rubber goods (Giger et al., 1984; Nimrod & Benson, 1996). APEs released into sewage are metabolized by microbes in sewage sludge to alkylphenolic compounds (APs), which are relatively stable and have been found in both sediments and surface water and in fish fat (Jobling & Sumpter, 1993; White et al., 1994; Ahel et al., 1994; Blackburn & Waldock, 1995). Several studies have demonstrated that APs have some estrogenic activity in various experimental test systems (Munkittrick et al., 1998). Although estrogenic potential was reported in these specialized studies, the mechanisms are unclear. Of the alkylphenols tested for their estrogenicity, octylphenol (OP) has been shown to possess approximately 1000 times less potency than 17β-estradiol (E2) in vitro (Mueller & Kim, 1978; White et al., 1994). Administration of OP to adult male rats was found to significantly suppress testicular function; in particular, there were reduced testes size, lower testosterone concentrations, and decreased spermatogenesis (Blake & Boockfor,

1997). OP was found to be toxic to aquatic animals and to exert adverse effects on murine splenocytes, events seen in the absence of estrogen (McLeese et al., 1981; Nair-Menon et al., 1996). Recent data also suggest that the estrogenic potential of nonylphenol (NP) occurs at concentrations well below lethality and is thus an environmental hazard (Lech et al., 1996). NP competes with estrogen for binding to the estrogen receptor (ER) (Flouriot et al., 1995; Danzo, 1997) and thus induces reproductive and developmental toxicity (Colborn et al., 1993; Christiansen et al., 1998). The aims of this study were to (a) examine the estrogenicity of alkylphenolic compounds using in vitro and in vivo methods, as established by EDSTAC and OECD, (b) evaluate the sensitivity of each test method, and (c) identify a new biomarker for estrogenicity and its potential usefulness. Estrogen response genes have been considered the most specific and critical methods to determine estrogenicity. Thus, the level of an estrogenic response gene, CaBP-9K, as a potential candidate biomarker to screen EDs, was measured.

MATERIALS AND METHODS

Materials

17β-Estradiol (E2) and dimethyl sulfoxide (DMSO) were purchased from Sigma Co. (St. Louis, MO). 4-Nonylphenol, 4-octylphenol, 4-*t*-octylphenol, 4-butylphenol, 4-*t*-butylphenol, 4-propylphenol, 4-pentylphenol, and 4-phenylphenol were purchased from the Aldrich Chemical Co. (Milwaukee, WI). [^{3}H]-Estradiol (TRK 322, 86 Ci/mmol) was obtained from Amersham Life Science Co. (Buckinghamshire, UK).

Animals

Sprague-Dawley rats (6 wk, about 180 g), acquired from Laboratory Animal Resources, National Institute of Toxicological Research (NITR) (Seoul, Korea), were maintained in accordance with the NITR of the Korea Food and Drug Administration guidelines for the care and use of laboratory animals. Animals were acclimated for 1 wk before use.

Cell and Culture Conditions

MCF-7 cell, a human breast cancer cell line, was obtained from Dr. Soto, A.M. (Tufts University, Boston). This cancer cell line was grown and passaged routinely as monolayer cultures in T-25 flasks in Dulbecco's modification of Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) containing 1 m*M* sodium pyruvate and 7.5% $NaHCO_3$ at 37°C in 5% CO_2.

E-Screen Assay

MCF-7 cells (passage number 7) were removed from their growth flask with trypsin/ethylenediamine tetraacetic acid (EDTA), counted, diluted to 5×10^4 cells/ml in growth media, and 100 µl was seeded into 96-well culture plates. Twenty-four hours after cell plating, the media was completely

removed and cells were switched to 90-µl wells with DMEM containing 5% charcoal–dextran activated FBS (CDA-FBS) and 10 µl of media, as already described, with or without the test compounds in a final DMSO concentration of 0.2%. Controls contained only 0.2% DMSO. The treated plates were incubated for 6 d in the cell culture incubator at 37°C in 5% CO_2, and MCF-7 cell proliferation was determined by sulforhodamine-B (SRB) assay (Soto et al., 1995).

Estrogen Receptor Binding Assay

MCF-7 cells were incubated with 5% CDA-FBS for 3 d prior to the assay. Cells were removed with trypsin/EDTA and, after dispersion to single cells, diluted in DMEM with 1% CDA-FBS to 1×10^6 cells/ml. Diluted cells (100 µl) were added to test compounds (50 µl) and [^{3}H]estradiol (50 µl). The contents were mixed by gently shaking and incubated at 37°C in 5% CO_2 for 45 min, with mixing every 15 min. Following incubation, the cells were centrifuged at 600 × g for 5 min at 4°C. The supernatant was removed and the tubes were plunged into an ice bath. Within 5 min, 1 ml of ice-cold TPSG (0.2% Triton X-100 in PBS, pH 7.0, containing 0.1 *M* sucrose and 10% glycerol) was added. After vigorously shaking, the tubes were incubated for 5 min and centrifuged at 600 × g for 5 min at 4°C. The supernatant was removed, the sediment was washed two times with PBS, and the radioactivity was measured using a liquid scintillation counter (LSC) (Zava et al., 1997).

Uterotrophic Assay

Sprague-Dawley female rats (6 wk, approximately 180 g) were ovariectomized 1 wk prior to administration and randomly assigned to control and test groups. Each experiment was accompanied by a vehicle control group (corn oil) and a positive control group E2 (1 µg/kg/d) or APs (10, 50, 200, and 400 mg/kg/d) by subcutaneous injection. A dosing volume of 4 ml/kg was used for all administrations. The animals received daily doses of the test compounds for 3 successive days and were sacrificed by cervical dislocation 24 h after the final dose. The uterus and vagina were excised, trimmed free of fat, and subsequently weighed. The body weights of the animals were recorded prior to the administration of the test compounds and immediately before sacrifice (Odum et al., 1997; OECD, 1999).

CaBP-9K mRNA Expression Test (Northern Blot and Dot Blot Assay)

The uteri, obtained from uterotrophic assay, were rapidly excised and washed in cold sterile 0.9% NaCl. Total RNA was extracted with Trizol (TEL-TEST, Inc., Friendswood, TX) according to the supplier's instructions. RNA was denatured by heating at 65°C for 15 min, and 10 µg of total RNA was electrophoresed on 1% agarose gels for 90 min at 110 V. 18S rRNA served as an indicator of quantity of total RNA. RNA was then transferred from the agarose gel to a nylon membrane with a vacuum blotter (Bio-Rad, Hercules,

CA). A dot blot assay was carried out with a Bio-Rad instrument at a loading concentration of 5 μg. RNA was ultraviolet (UV) cross-linked to the membrane using a gene cross-linker. Membranes were prehybridized in 50% formamide, 5 × standard saline phosphate EDTA (SSPE), 5 × Denhardt's, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml of salmon sperm DNA for 3 h at 42°C. The [^{32}P]-dCTP-labeled probes (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) were added to the prehybridization solution and incubated overnight. Membranes were then washed at 42°C in 2 × standard saline citrate (SSC), 0.1% SDS, then at 54°C in 1 × SSC, 0.1% SDS, and at 68°C in 0.1 × SSC, 0.1% SDS. Membranes were exposed to x-ray films.

Statistics

Data were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA) and Duncan's one-way test (SigmaStat, Jandel Scientific Co., Germany). Differences were considered to be significant at $p < .05$.

RESULTS

Effect of APs on MCF-7 Cell Line Proliferation

The effects of E2 (1×10^{-14}–1×10^{-8} *M*) and APs (1×10^{-8}–1×10^{-3} *M*) on MCF-7 cell proliferation are shown in Figure 1. E2, a positive control, stimulated maximum proliferation of MCF-7 cells at 1×10^{-9} *M*. Among the

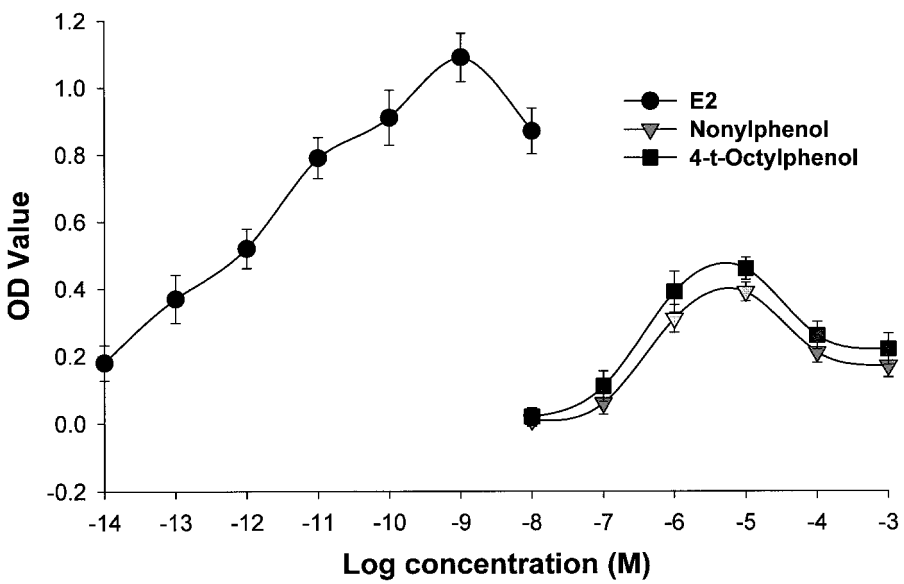

FIGURE 1. Effects of alkylphenolic compounds on the proliferation of MCF-7 human breast cancer cells. Cells were exposed for 6 d to AP compounds or E2 in DMEM medium supplemented with 5% CDA-FBS. Cell proliferation was determined by SRB assay. Data are mean ± SD of six experiments.

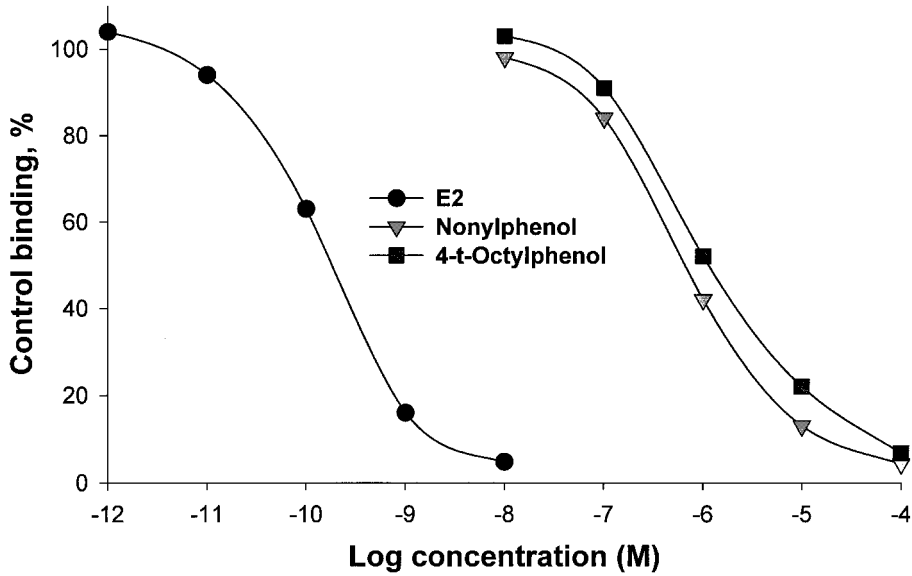

FIGURE 2. Competitive inhibition of [^{3}H]-E2 binding to the ER of MCF-7 cells. Cells were incubated with [^{3}H]-E2 and increasing concentrations of the test compounds as described in Materials and Methods. Results were expressed as a percentage of the inhibition of [^{3}H]-E2 binding to ER in the presence of the test compound.

APs, 4-*t*-octylphenol and 4-nonylphenol were considerably more potent than any other compound, and their estrogenic effects were detected at 1×10^{-6} *M* and 1×10^{-5} *M*, respectively. However, at the higher concentration ($>1 \times 10^{-4}$ *M*), 4-*t*-octylphenol and 4-nonylphenol induced slight cytotoxicity in the MCF-7 cells.

Binding of APs to Estrogen Receptor in Intact MCF-7 Cells

The ER binding capacity of E2 (1×10^{-14}–1×10^{-8} *M*) and APs (1×10^{-8}–1×10^{-3} *M*) are shown in Figure 2. Results are represented as the percentage of [^{3}H]-E2 binding inhibition to ER. Among the AP compounds tested, only 4-*t*-octylphenol and 4-nonylphenol concentration-dependently inhibited the binding of [^{3}H]-E2 to the ER of MCF-7 cells.

Effect of APs on the Uterine and Vaginal Weight in Ovariectomized Rats

The results of the uterotrophic assay are shown in Figure 3. E2 produced a significant increase in wet uterus and vaginal weights up to 7.3- and 1.4-fold, respectively. Among the AP compounds, 4-*t*-octylphenol, 4-nonylphenol, and 4-phenylphenol increased uterus wet weight up to 1.9-, 1.7-, and 1.5-fold at the highest dose (400 mg/kg). No significant effect of APs on vaginal weight was observed in this experiment.

CaBP-9K mRNA Induction by APs in an Ovariectomized Rat Model

The data shown in Figure 4 demonstrate that CaBP-9K mRNA was induced in ovariectomized rats at doses of 400 mg/kg 4-pentylphenol, 200

and 400 mg/kg 4-nonylphenol, and 50, 100, 200, and 400 mg/kg 4-phenylphenol or 4-*t*-octylphenol. The levels of mRNA from uterus were determined with total RNA in the amount of 5 μg/dot. Total RNAs were extracted from the uterus in ovariectomized rats after treatment with various alkylphenolic compounds. As determined by the molecular analysis program of the autoradiograms, the CaBP-9K mRNA levels in the uterus at a dose of 200 mg/kg were significantly increased in the group that received 4-*t*-octylphenol treatment. At dose of 400 mg/kg, the CaBP-9K mRNA expression was significantly increased in the groups that received 4-pentyl-, 4-nonyl-, 4-phenyl-, and 4-*t*-octylphenol (Figures 5 and 6).

DISCUSSION

Many testing methods have been introduced for the screening and testing of EDs (Garrison et al., 1996; Chen et al., 1997; Andersson et al., 1999; Balmelli et al., 1999). The reliable detection of EDs using in vivo and in vitro experiments is complicated, and to date no completely reliable testing method has been identified (Andersen et al., 1999). Therefore, both the sensitivity of screening and testing methods for EDs and the structure–activity relationships of AP estrogenic activity using in vitro and in vivo assays were determined. Generally, alkylphenolic compounds are estrogenic, and the activities of these chemicals have been shown to closely depend on the nature of the alkyl substitutions. One of the most widely used in vitro

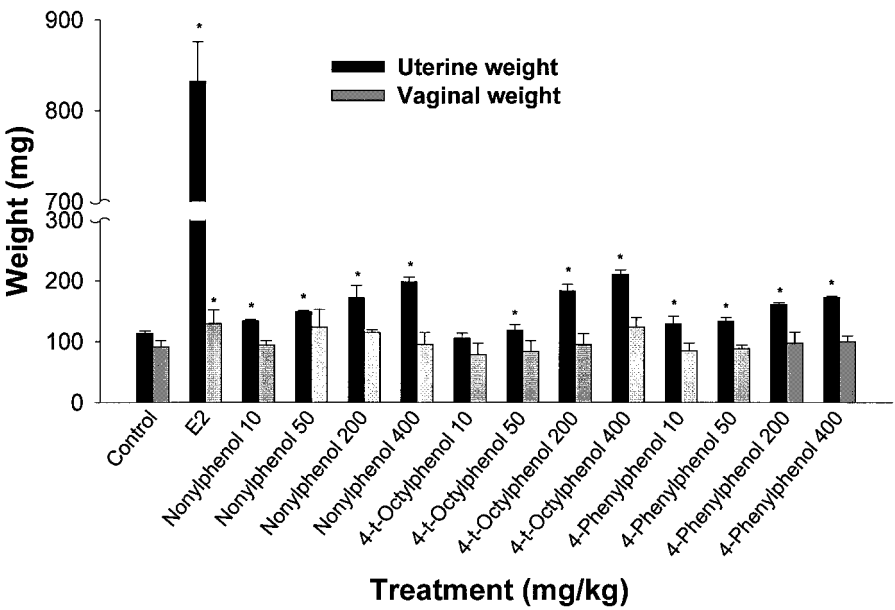

FIGURE 3. The effect of alkylphenolic compounds on the uterine and vaginal weight of female Sprague-Dawley rats in the uterotrophic assay. Controls received only corn oil. Data represent means ± SD of six animals per group. Asterisk indicates significant difference from control ($p < .05$).

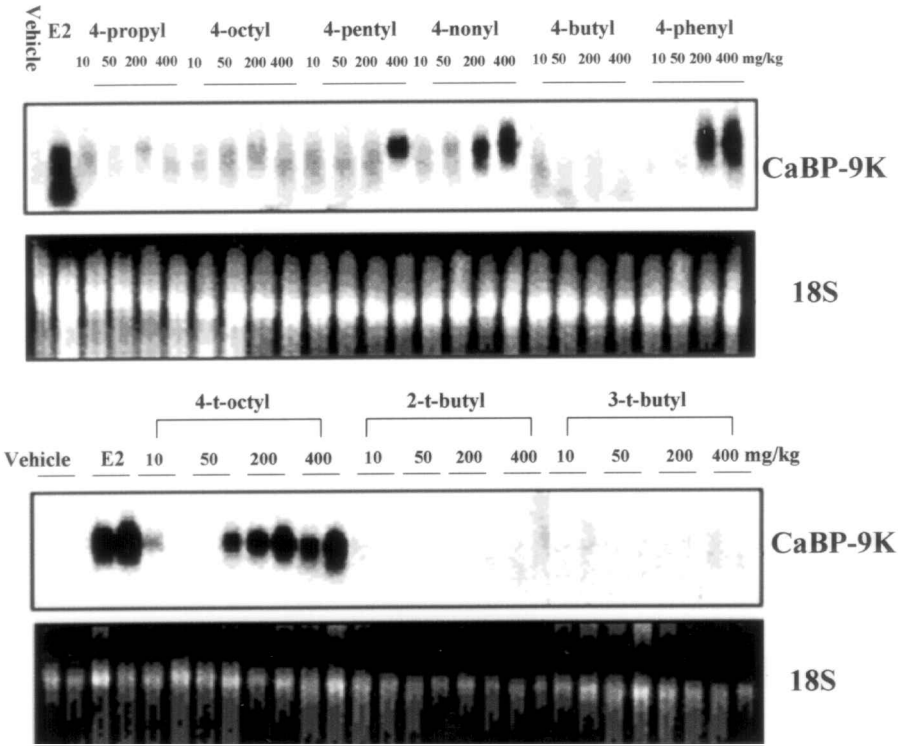

FIGURE 4. Northern blot analysis of CaBP-9K mRNA expression in ovariectomized rats treated with alkylphenolic compounds.

assays for detecting EDs is based on the induction of mitogenesis in estrogen responsive cells, particularly in the MCF-7 cell proliferation assay (E-screen) (Arcaro et al., 1999). Generally, MCF-7 cells highly express ER, AR, progesterone, glucocorticoid, vitamin D, and retinoic acid receptors (Gray et al., 1997). The E-screen assay, which was established by Soto et al. (1995), is considered to be one of the most sensitive assays for estrogenicity, as it is highly responsive to estrogens. Several studies have demonstrated that 4-*t*-octylphenol and 4-nonylphenol have weak estrogenic potency in vivo but also strongly induce MCF-7 cell proliferation by acting as agonists and binding to the ER (Verma et al., 1998). In addition, the ER competitive binding assay is also used as a screening method for EDs (Tong et al., 1997; Arcaro et al., 2001). In this study, MCF-7 cells were used for the screening of APs, and our results show that 4-*t*-octylphenol and 4-nonylphenol were more effective and potent than any other compound by in vitro assay. These results are consistent with those previously reported (Andersen et al., 1999). Although several in vitro methods for testing EDs have been developed, it is generally considered necessary to validate this data with in vivo animal study. The rodent uterotrophic assay was one of the original methods developed for studying estrogenicity some 70 yr ago, and even today, its

results are still regarded as the most useful indicators of EDs (Reel et al., 1997). It is common in this form of experiment to use ovariectomized rats, to achieve low basal levels of uterine weight as well as to minimize the effects of endogenous steroids. In our study, 4-nonylphenol, 4-*t*-octylphenol, and 4-phenylphenol produced dose-response increases in uterine weight of ovariectomized rats treated with APs, but vaginal weight was not shown to be significantly affected in any dosing group. CaBP-9K is an intracellular protein with high-affinity binding sites for calcium, like calmodulin, paralbumin, troponin C, and S 100 protein. CaBP-9K is a protein with a molecular weight of 9000 with 2 calcium-binding sites. The regulation of CaBP-9K in various tissues is not completely understood. Although its intestinal levels are clearly controlled by vitamin D, its placental and uterine concentrations do not respond to vitamin D depletion or vitamin D administration (Krisinger et al., 1992b). Experiments in rats showed 17β-estradiol responsiveness of uterine to CaBP-9K. A function of uterine CaBP-9K has been postulated based on its calcium-binding properties and cellular location, namely, that it may alter cytosolic calcium contraction and thereby affect myometrial activity. Rat CaBP-9K cDNA and the corresponding gene have been cloned. Ovariectomy caused a complete disappearance of CaBP-9K mRNA from the rat uterus, while the administration of 17β-estradiol led to a profound rebound of CaBP-9K mRNA expression (Krisinger et al., 1995). The CaBP-9K gene contains an estrogen response element (ERE), which mediates expression in the uterus. During the estrus cycle, rapid and

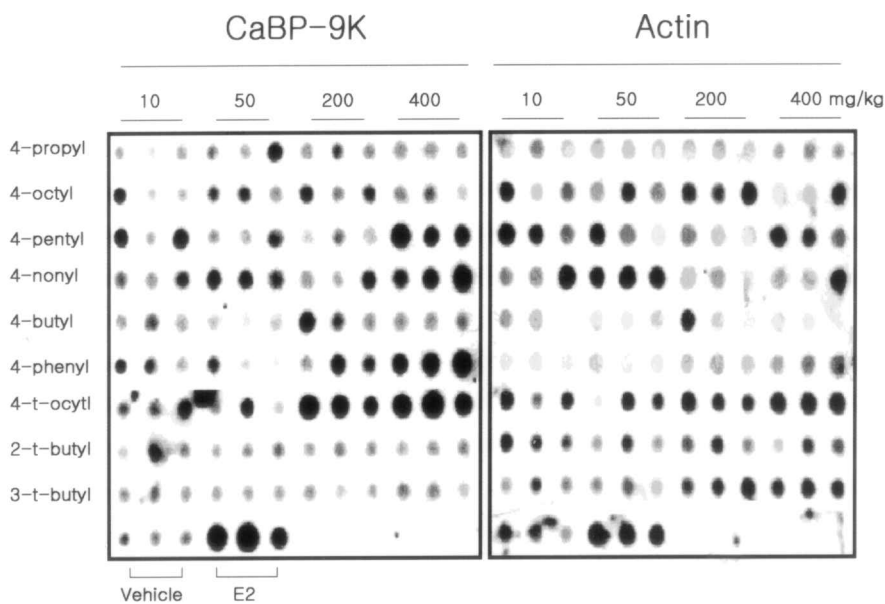

FIGURE 5. Dot blot analysis of CaBP-9K expression in ovariectomized rats treated with alkylphenolic compounds. Total RNA (5 μg) was dotted on the membrane and hybridized to the random primed [^{32}P]-dCTP labeled probe.

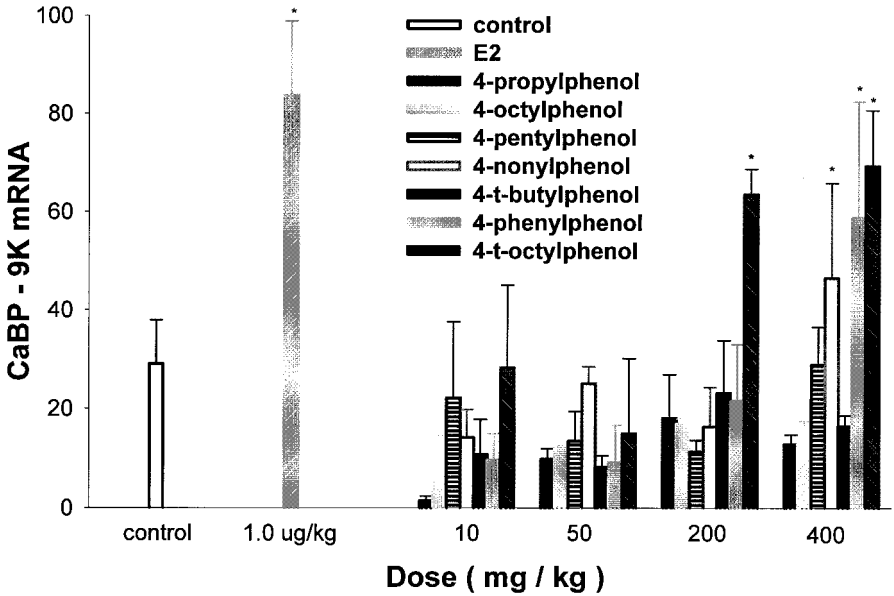

FIGURE 6. Schematic diagram from dot blot analysis of CaBP-9K expression in ovariectomized rats treated with alkylphenolic compounds. Data are mean ± SD of three experiments. Asterisk indicates significant difference from control ($p < .05$).

intense regulation at the mRNA level has been shown. During diestrus transcripts are at the detection limit but are dramatically increased at proestrus, showing about 10-fold difference between the periods. The potent and fast regulation of uterine CaBP-9K during the estrus cycle represents one of the few examples of an on/off regulation of gene expression (Krisinger et al., 1992a). In our results, 4-nonylphenol, 4-*t*-octylphenol, 4-phenylphenol, and 4-pentylphenol produced positive responses at the highest dose (400 mg/kg) as evidenced by an increase.

In summary, based on the results obtained so far, the in vivo uterotrophic assay and CaBP-9K expression assay seem to be sensitive screening methods for the estrogenic activity of APs. Among the APs, the estrogenicity of the compounds that have bulk alkyl substitution or a long carbon chain was higher than that of the others. In addition, the CaBP-9K gene might be used as a potential biomarker for the estrogenic response of environmental estrogens.

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