IN VITRO AND *IN VIVO* ESTROGENIC ACTIVITY OF ALKYLPHENOLIC COMPOUNDS

Seung Jun Kwack, Oran Kwon, Hyung Sik Kim, Soon Sun Kim, So Hee Kim, Kyung Hee Sohn, Rhee Da Lee, Eui Bae Jeung[1], beum-Soo An[1] and Kui Lea Park

Department of Toxicology, National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul, Korea [1]Department of Veterinary Medicine, College of Veterinary Medicine and Research Institute of veterinary Medicine, Chungbuk National University, Chung-Ju, Korea

Introduction

Alkylphenol polyethoxylates (APEs), which 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[1]. APEs released into sewage are metabolized by microbes in sewage sludge to alkylphenolic compounds (APs), which were relatively stable and have been found in both sediments surface water, and in fish fat[2,3]. The aims of this study were to (a) examine the estrogenicity of alkylphenolic compounds by *in vitro* and *in vivo* methods, as established by EDSTAC and OECD; (b) evaluate the sensitivity of each test methods; (c) identify a new biomarker, Calbindin-D_{9K} (CaBP-9K), for estrogenicity and its usefulness.

Methods and Materials

CaBP-9K mRNA Expression Test (Northern Blot & Dot Blot Assay): The uteri were obtained from uterotrophic assay and total RNA was extracted with Trizol (Life Technologies, inc.). 10μg of total RNA was electrophoresed on 1% agarose gels for 90 min at 110V. A dot blot assay was carried out at a loading concentration of 5μg and membranes were exposed to X-ray films.

Uterotrophic Assay: SD female rats were ovariectomized 1 week prior to administration. Each experiment was accompanied by a vehicle control group (corn oil) and a positive control group E2 (1.0 μg/kg/day) or APs (10, 50, 200, and 400 mg/kg/day) by S.C. injection. The animals received daily doses of the test compounds for 3 successive days and were sacrificed by cervical dislocation 24 hr after the final dose. The uterus and vagina were excised and subsequently weighted[4,5].

E-screen Assay: MCF-7 cells (passage No. 7) were adjusted to 5×10^4 cells/ml in growth media and 100μl seeded into 96-well culture plates. 24 hr after cell plating, cells were switched to 90μl wells of DMEM containing 5% CDA-FBS and 10μl of media, with or without the test compounds. The treated-plates were incubated for 6 days in the cell culture incubator at 37°C in 5% CO_2 and MCF-7 cell proliferation was determined by SRB assay[6].

Estrogen Receptor Binding Assay: MCF-7 cells were fed with 5% CDA-FBS for 3 days prior to the assay. Cells were diluted in DMEM with 1% CDA-FBS to 1×10^6 cells/ml. Diluted cells (100μl) were added to glass tubes containing test compounds (50μl) and [^{3}H]-estradiol (50μl). The contents were mixed by gently shaking and incubated at 37°C in 5% CO_2 for 45 min. After vigorously shaking, the supernatant was removed, the sediment was washed 2 times with PBS, and the radioactivity measured using a LSC[7].

Results and Discussion

CaBP-9K mRNA Induction by APs in an Ovariectomized Rat Model: The data shown in Figure 1 demonstrate that the message of CaBP-9K mRNA was observed in ovariectomized rats at doses of 4-pentylphenol of 400 mg/kg, 4-nonylphenol of 200 and 400 mg/kg, 4-phenylphenol or 4-t-octylphenol at 50, 100, 200, and 400 mg/kg. The CaBP-9K mRNA levels in the uterus at a dose of 200 mg/kg changed significantly in the group that received 4-t-octylphenol treatment. At doses of 400 mg/kg, the CaBP-9K mRNA expression was significantly increased in the groups that received 4-pentyl, 4-nonyl ($P<0.05$), 4-phenyl, and 4-t-octylphenol ($P<0.01$) (Figures 2 & 3).

Effect of APs on the Uterine and Vaginal Weight in Ovariectomized Rats: Among the AP compounds, 4-t-octylphenol, 4-nonylphenol, and 4-phenylphenol increased in uterus wet weight up to 1.9-, 1.7-, and 1.5-fold at the highest dose (400 mg/kg) (Figure 4).

Effect of APs on MCF-7 Cell Line Proliferation: Among the APs, 4-t-octylphenol and 4-nonylphenol were considerably more potent than any of the other compounds (Figure 5).

Binding of APs to Estrogen Receptor in Intact MCF-7 Cells: 4-t-Octylphenol and 4-nonylphenol dose-dependently inhibited the binding of [^{3}H]-E2 to the ER of MCF-7 cells (Figure 6).

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

Acknowledgments

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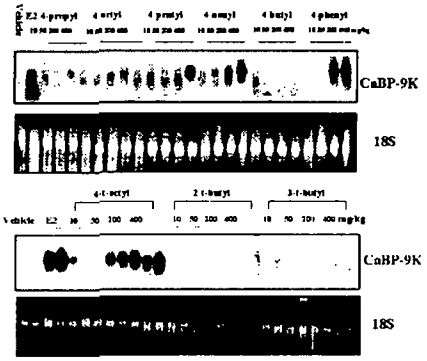

FIGURE 1. Northern blot analysis of CaBP-9K mRNA expression in ovariectomized rats treated with alkylphenolic compounds

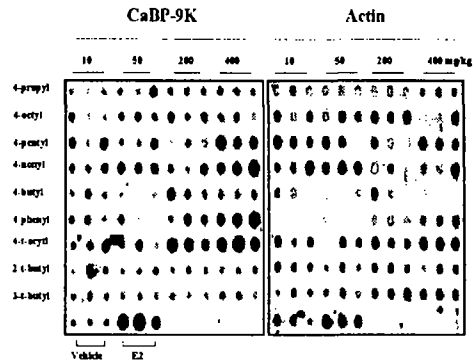

FIGURE 2. Dot blot analysis of CaBP-9K expression in ovariectomized rats treated with alkylphenolic compounds. Total RNA (5 μg) was dotted on the membrane and hybridzied to the random primed [^{32}P]-dCTP labeled probe.

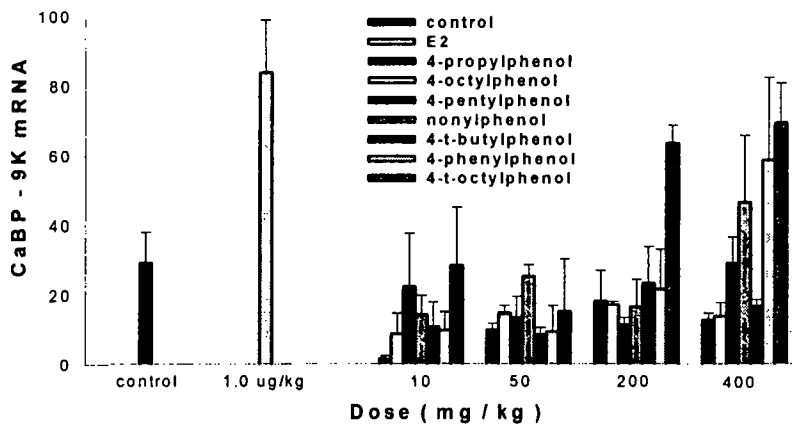

FIGURE 3. Schematic diagram from dot blot analysis of CaBP-9K expression in ovariectomized rats treated with alkylphenolic compounds.

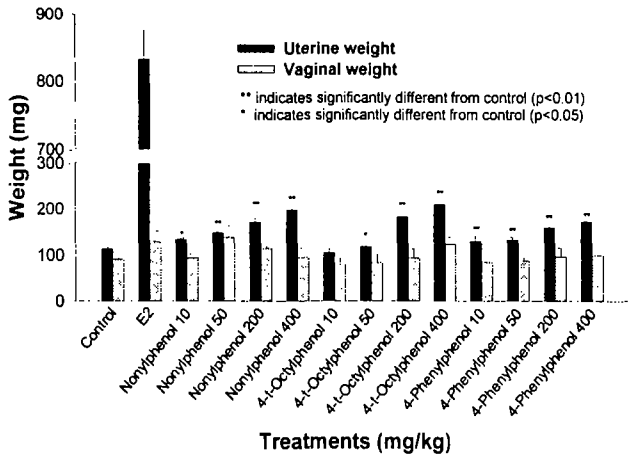

FIGURE 4. The effect of alkylphenolic compounds on the uterine and vaginal weight of female S.D. rats in the uterotrophic assay. Controls received only corn oil. Data represent group (mean ± SD).

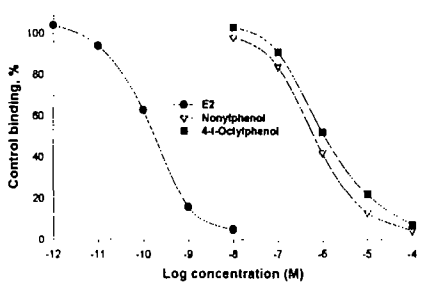

FIGURE 5. Effects of alkylphenolic compounds On the proliferation of MCF-7 cells. Cells were exposed for 6 days to AP compounds or E2 in DMEM medium supplemented with 5% CDA-FBS Cell proliferation was determined by SRB assay

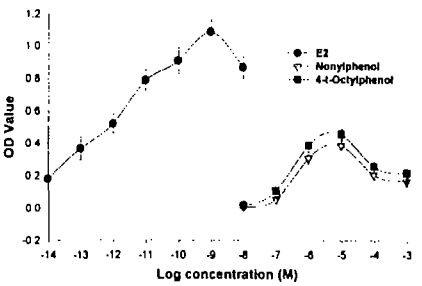

FIGURE 6. Competitive inhibition of [^{3}H]-E2 binding to the ER of MCF-7 cells. Resutls were expressed as a percentage of the inhibition of [^{3}H]-E2 binding to ER in the presence of the test Compounds