PHYSIOLOGY AND USE OF CALBINDIN-D_{9k} AS A BIOMARKER TO DETECT ALKYLPHENOLIC COMPOUNDS

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Introduction

Calbindin-D_{9k} (CaBP-9k) is a member of a large family of intracellular calcium binding proteins that have high affinities for calcium. The expression of the CaBP-9k gene in rat uterus is under strict estrogen regulation. It has been demonstrated that estrogen injection to 21-day old rats increased the expression of CaBP-9k mRNA up to 300-fold when compared with non-treated rats[1, 2]. Furthermore, CaBP-9k mRNA in uterus is known to fluctuate during the estrus cycle of the rat where serum estrogen level is also under fluctuation[5]. At diestrus, the CaBP-9k mRNA was not detectable. At proestrus, the mRNA increased and reached to the highest level at estrus and then decreased as the cycle entered metestrus. To date, there was no evidence that the CaBP-9k gene was regulated by estrogen in other tissues or cells other than the female reproductive tract.

Considering its strict regulation by estrogen, in the present study, we hypothesize that the CaBP-9K gene has the possibility as a biomarker for estrogenic response of the environmental estrogens. We have analyzed the time- and dose-dependent CaBP-9K gene expression in the uterus for three-days injection of E_2, octyl-phenol (OP), nonylphenol (NP) and bisphenol (BPA) in the immature rats and compared with uterotropic response of the compounds. Furthermore, we studied the effect of the compound on the ERa mRNA level in order to investigate the possible mechanisms by which the compounds regulate CaBP-9k mRNA expression.

Methods and Materials

Three groups of five immature rats animals were given subcutaneous (SC) injection with OP, NP and BPA at the dose of 10, 100 and 1000 mg/kg BW/day dissolved in corn oil for 3 days and sacrificed after at day 4. Control rats were given E_2 (40 μg/kg BW) or corn oil for the same period of time. In the second experiment, three groups of five animals were given E_2 (40 μg /kg BW) or BP, OP and NP (1,000 mg/kg BW) for 3 days, and each group was sacrificed at 3, 6, 12, 24, 48 and 72 h after final injection.

The uterus was rapidly excised, and washed in cold sterile 0.9% NaCl solution. Total RNA was extracted with Trizol (Life Tech., Rockville, MD) and the concentration of RNA was determined by the

absorbance at 260 nm. Dot blot assay (5 μg total RNA) was carried out. For Northern blot analysis, ten microgram of total RNA were electrophoresed on 1% formaldehyde denaturing agarose gels for 90 min at 110 V, and 18S rRNA served as an indicator of quantity of total RNA. RNA was then transferred from the agarose gel to the nylon membrane with Vacuum blotter (Bio-Rad) according to manufacturer's suggested procedure. The CaBP-9k signal was detected by hybridizing the membrane with the radio-labeled probe and exposing to x-ray film.

Quantification of ERα by RT-PCR

Since ERα is known to mainly mediate E2 responses in the uterus, we measured only ERa mRNA level in the present study. Five microgram of total RNA from the uteri of rats treated with the estrogen compound and E2 in a dose- and time-dependent manner were reverse transcribed using M-MLV reverse transcriptase and random primer (9 mer). The samples contained ERα primers (ER1; 5'-ATGATCAACTGGGCAAAGA-3' and ER2; 5'-AGATGCTCCATGCCTTTGTT-3') and primers for IA gene (IA-1; 5'-GATATAGCATTCCCACGAATA-3' and IA-2; 5'-GGGCTTTTGCTCATGTGTCAT-3'). PCR for 1A gene was amplified to rule out the possibility of RNA degradation and was used to control the variation in mRNA concentrations in the RT reaction. The products were visualized by agarose gel electrophoresis stained with ethidium bromide, and the photograph was scanned and analyzed using molecular analysis program version 1.5.

Data analysis

Data are presented as the mean ± SD. The data were analyzed by ANOVA followed by Tukey's multiple comparison test. $P< 0.05$ was considered statistically significant.

Results and Discussion

Calbindin-D_{9k} (CaBP-9k) (Mr 9000) is a cytosolic calcium binding protein mainly expressed in duodenum, placenta and uterus. In the uterus, it has been demonstrated that the CaBP-9k gene expression is highly regulated by estrogen[5]. The present study demonstrates for the first time that CaBP-9k mRNA is rapidly and strongly induced by environmental estrogenic compounds, possibly through regulation of ERα mRNA expression in the rat uterus. These results provide novel evidence that CaBP-9k can be used as a marker gene for assaying the estrogenicity of putative estrogenic compounds.

It has been shown that intestinal CaBP-9k is regulated at the transcriptional level and posttranscriptional level by 1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$), the hormonal form of vitamin D[6]. In contrast, the CaBP-9k gene is not under the control of vitamin D in the uterus despite the presence of vitamin D receptors in this tissue; instead it is predominantly controlled by estrogen. Earlier study demonstrated that CaBP-9k synthesis fell drastically in the uterus of ovariectomized rats, but was greatly enhanced by low physiological doses of E2 in a dose-dependent manner as assessed by CaBP-radioimmunoassay (RIA). Other report showed that E2 stimulates the transcription of the CaBP-9k gene in

the uterus of mature ovariectomzied and

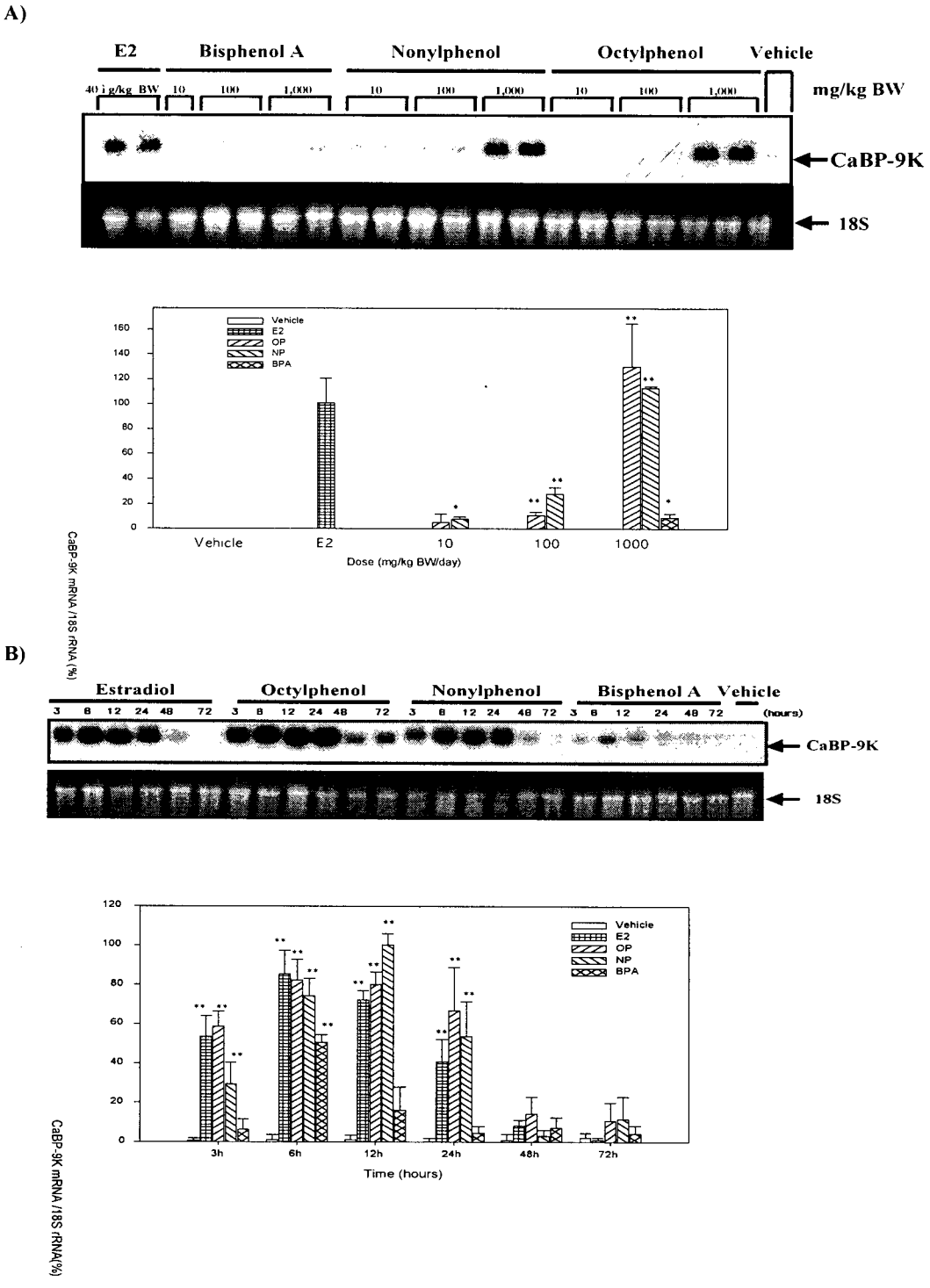

Figure 1. Northern blot analysis for CaBP-9k mRNA expression. Animals were treated with the compounds in a dose (A)- and time (B)-dependent manner. Total RNA (10 μg) was subjected to Northern

blot analysis. After hybridization with the radio-labeled probes and exposure to x-ray film, the signal was analyzed by molecular analysis program version 1.5 (Gel Doc 1000, BioRad). Data was analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests. The values represent means ± SD (n=5). *, $p < 0.05$, **; $p < 0.01$

immature rats as revealed by slot and Northern blot analysis[2]. Like previous reports, in the present study, we demonstrated a single dose of E2 showed a marked increase in CaBP-9k mRNA expression as demonstrated by dot and Northern blot assay. New finding of the present study is that environmental estrogens treatment also drastically increased the expression of CaBP-9k mRNA up to the similar level observed in E2 treatment. The change in CaBP-9k mRNA level did not observed at middle (100 mg/kg BW/day) and low dose (10 mg/kg/BW/day) of the compound when measured by dot blot analysis, whereas a small but significant increase in CaBP-9k mRNA expression was observed at middle and low dose of OP and NP when revealed by Northern blot assay. These results indicated that Northern blot assay has higher sensitivity than dot blot assay, suggesting that this assay can be used for weak estrogenic compounds. The effect of the estrogenic compounds in the present study was rapid such that change in CaBP-9k mRNA expression could be detected as early as 3 h after treatment, peaked at 6 h and continued until 72 h. Our results in a dose- and time-dependent experiment is consistent with previous reports in CaBP-9k mRNA expression by 17β-estradiol[1].

The endogenous ovarian estrogens are thought to initiate their physiological action in target tissues largely via interactions with a nuclear receptor system. Since CaBP-9k gene is regulated through the binding of ER/estrogen complex to estrogen response element (ERE), we studied the expression level of ERα mRNA in the uterus of rat uterus treated with the estrogenic compounds. The results showed that the alkylphenolic compounds as well as E2 stimulated the expression of ERα mRNA in a similar pattern to that of CaBP-9k mRNA in terms of dose- and time-dependent response. These results suggest that like the endogenous steroid, environmental estrogens may regulate CaBP-9k mRNA expression through modulating transcriptional activity of estrogen receptor.

Among the assays for the estrogenic activity of a chemical, an endogenous gene expression assay that measure estrogen-induced changes in the expression levels of endogenous genes either in cultured cells or in selected tissues from exposed animals, is proposed and widely being used. For example, Petit et al measured the induction of vitellogenin in trout hepatocyte cultures to identify estrogenic compounds. Jakowlew *et al.* [4] reported that rapid and strong induction of pS2 mRNA expression in the human breast cell. Like vitellogenin and pS2 gene, the rapid and strong regulation of CaBP-9k gene by estrogenic compounds in the present study suggests that CaBP-9k can be a marker gene for assaying estrogenicity. The widely used uterotropic assay was performed in comparison with CaBP-9k gene expression for estrogenicity of the chemical. The estrogenic compounds increased significantly the uterine wet weight at high dose excepting BPA and in middle dose of NP. When compared, a strong correlation between in vivo uterotropic assay and CaBP-9k mRNA expression assay. These results provide further evidence for the possibility of CaBP-9k

gene as a biomarker for the estrogenic assay.

In summary, we have demonstrated that CaBP-9k mRNA is strongly regulated by the endogenous estrogen and environmental estrogenic compound in the rat uterus. In addition, the estrogenicity as measured in term of CaBP-9k mRNA expression was correlated with in vivo uterotropic assay. Thus, in regard to risk assessment, our data demonstrate that CaBP-9k mRNA assay in the rat uterus represents a very sensitive and powerful tool identify substances with estrogenic activity when used with combination of the classical assay.

Acknowledgments

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References

1. Laws S.C., Carey S.A., Ferrell J.M., Bodman G.J. and Cooper R.L. (2000) Toxicol Sci. 54(1),154.
2. White R., Jobling S., Hoare S.A., Sumpter J.P. and Parker M.G. (1994) Endocrinology. 135(1):175.
3. Shelby M.D., Newbold R.R., Tully D.B., Chae K. and Davis V.L. (1996) Environ Health Perspect. 104,1296.
4. Jakowlew S.B., Breathnach R., Jeltsch J.M., Masiakowski P. and Chambon P (1984) Nucleic Acids Res. 12,2861.
5. Krisinger J., Dann J.L., Currie W.D., Jeung E.B. and Leung P.C. (1992) Mol Cell Endocrinol.86,119.
6. Darwish H.M. and DeLuca H.F. (1992) Proc Natl. Acad. Sci. 89,603.