Differential Transcriptional and Translational Regulations of Calbindin-D$_{9k}$ by Steroid Hormones and Their Receptors in the Uterus of Immature Mice

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Abstract. Calbindin-D$_{9k}$ (CaBP-9k) is a cytosolic calcium binding protein mainly expressed in the duodenum, placenta and uterus, and intestinal CaBP-9k is regulated by 1, 25-dihydroxyvitamin D$_3$. However, despite the presence of vitamin D receptors, uterine CaBP-9k is not under the control of vitamin D, but seems to be regulated by sex steroids. This steroids-dependent regulation of CaBP-9k is not only limited to a tissue-specific manner but also extends to a species-specific manner. In this study, we examined the regulation of CaBP-9k gene at the transcriptional and translational levels, and also localized CaBP-9k protein in the uterus of immature mice. Treatment with progesterone (P4) resulted in the induction of CaBP-9k mRNA, and a co-treatment with estrogen (E2) plus P4 evoked a synergic effect on its mRNA level in this tissue. Interestingly, the translation of CaBP-9k protein was enhanced by E2, while no difference was observed at the transcriptional level. Not only P4 but also E2 itself induced an increase of CaBP-9k protein, and co-treatment with E2 and P4 showed a similar effect on its protein level in the uterus of immature mice. The CaBP-9k protein was localized in the glandular epithelium of stroma in the uterus of immature mice at diestrus, indicating that the expression of CaBP-9k protein is differentially regulated by sex steroids. A potential mechanism of synergic effect of P4 and E2 may be E2 action in the increase of progesterone receptor (PR), with up-regulated PR increasing P4-induced CaBP-9k expression. This complicated relationship between CaBP-9k and steroid receptors suggests that P4 regulates CaBP-9k gene in the uterus of immature mice, in addition, E2 also can affect the expression of CaBP-9k through the regulation of PR. The expression levels of ER$_{\alpha}$ and PR were further examined in this tissue. E2 stimulated the expression levels of ER$_{\alpha}$ and PR mRNAs and P4 inhibited the expression of these transcripts at an early time point (12 h) and increased them at 24 and 48 h, while co-treatments with both steroids increased transcripts of ER$_{\alpha}$ and PR at 24 h. In conclusion, P4 and PR may be dominant factors in the regulation of CaBP-9k. Also, E2 and ER$_{\alpha}$ can influence the expression of the CaBP-9k gene via an indirect pathway in the uterus of immature mice.

Key words: Calbindin-D$_{9k}$, Uterus, Mouse, Steroid hormone, Steroid receptor

Cabindin-D<sub>9k</sub> (CaBP-9k) is a cytosolic calcium binding protein mainly expressed in the duodenum, placenta and uterus [1, 2]. In adult rats, the highest concentration of CaBP-9k is observed in intestinal epithelial cells, with a concentration gradient along the gastrointestinal tract [3]. In our previous study, CaBP-9k was expressed in the intestine especially in the duodenum, not in the stomach or large intestine, with dependency on vitamin D [4]. Functionally, intestinal CaBP-9k is regulated by 1,25-dihydroxyvitamin D<sub>3</sub>, the hormonal or of vitamin D [5], and is involved in intestinal calcium absorption [3, 6]. However, despite the presence of vitamin D receptors, uterine CaBP-9k is not under the control of vitamin D, but seems to be regulated by the sex steroid hormones [7–10]. This differential regulation of CaBP-9k is not only limited just to a tissue-specific manner but also extends to a species-specific manner. In the rat uterus, the expression level of CaBP-9k gene is suppressed at diestrus, and increases at proestrus in response to the plasma estrogen levels [8, 11]. In ovariectomized rats, estrogen (E<sub>2</sub>) caused the rapid accumulation of CaBP-9k transcripts in the uterus [9], while progesterone (P<sub>4</sub>) inhibited the E<sub>2</sub>-induced CaBP-9k gene in E<sub>2</sub> primed ovariectomized rat [8]. In ovariectomized gilts, E<sub>2</sub> induced an increase in CaBP-9k mRNA level, whereas P<sub>4</sub> decreased this level [12]. In contrast, it has been demonstrated that P<sub>4</sub> up-regulated CaBP-9k gene in the bovine [12] and porcine uterus [13].

In the uterus, CaBP-9k is expressed mainly in endometrial stroma and the myometrium of rat [7, 14, 15]. However, in pregnant rats, it is also expressed in the uterine epithelium [16]. In the non-pregnant cow, it has been demonstrated that CaBP-9k is expressed in the luminal and glandular epithelium of the endometrium, not in the myometrium, nor in the stromal cells of the endometrium [17]. The mRNA level of CaBP-9k is predominantly localized in the luminal epithelium in pregnant mice, and the expression of CaBP-9k is increased in the uterus during early pregnancy [18]. In the present study, we examined the regulation of the CaBP-9k gene in the uterus of immature mice in which steroid hormones are not detectable before maturation. Immature mice were injected with E<sub>2</sub>, P<sub>4</sub>, or E<sub>2</sub> and P<sub>4</sub> together to investigate the role of steroid hormones on the regulation of the CaBP-9k gene, and also the relationships of steroid hormone receptors with the expression of CaBP-9k.

### Materials and Methods

**Chemicals**

Progesterone (P<sub>4</sub>) and 17β-estradiol (E<sub>2</sub>) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

**Experimental animals and treatments**

Immature female mice (18 days old) were obtained from Hanlim Co., Ltd. (Daejun, Korea). All animals were housed in polycarbonate cages, and used after acclimation to an environmentally controlled room (temperature: 23 ± 2°C, relative humidity: 50 ± 10%, frequent ventilation and 12 h light cycle). They were fed with soy-free pellet food (Samyang Ltd., Seoul, Korea) and tap water *ad libitum* throughout the experimental period. Four groups of twenty animals (total n=80) were given subcutaneous (SC) injections with E<sub>2</sub> (40 µg/kg/day), P<sub>4</sub> (500 µg/kg/day), combination of E<sub>2</sub> with P<sub>4</sub>, or vehicle for 3 days, and from each group, five animals were euthanatized 12, 24, 48 and 72 h after the final injection. All experimental procedures and animal use were approved by the Approval of Ethics Committee of the Chungbuk National University.

**Northern blot analysis**

The mice were euthanatized, and their uteri were rapidly excised. After washing in cold sterile 0.9% (w/v) NaCl solution, total RNA was extracted with Trizol (Life Technologies, Inc., Rockville, MD, USA) according to manufacturer’s suggested procedure, and the concentration of RNA was determined by the absorbance at 260 nm. The RNA was denatured by heating at 65°C for 15 min. Ten micrograms of total RNA were electrophoresed on 1% (w/v) formaldehyde denaturing agarose gels for 90 min at 110 V, and 18S rRNA served as an indicator of quantity of total RNA. The RNA was then transferred from the agarose gel to a nylon membrane with Vacuum blotter (Bio-Rad, Hercules, CA, USA) according to manufacturer’s suggested procedure. The RNA was UV cross-linked to the membrane using a gene cross-linker (Bio-Rad). The membranes were prehybridized in 50% (w/v) formamide, 5 × SSPE, 5 × Denhardt’s, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.1 mg/ml salmon sperm DNA for 3 h at 42°C. Partial fragments (167 bp) of specific mouse CaBP-9k cDNA (reference gene; accession number: **AN et al.**
AY034822) were used for template of the probe. A radioactive-labeled probe was prepared from the Random Primer Labeling Kit (Life Technologies), according to manufacturer’s suggested procedure. The contents of the primer labeling mixture were as follows: 25 ng cDNA, 2 µg random primer dCTP, 2.5 µl dNTP mixture (0.2 mM each dGTP, dATP and dTTP), 50 µCi [32P]-dCTP, and 1 µl Klenow enzyme (2 units/µl). After mixing with other reagents, the mixture was incubated at 37 C for 1 h. Before using, the probe mixture was denatured at 95 C for 3 min and immediately chilled on ice. [32P]-labeled probe to detect CaBP-9k transcript was added to 25 ml hybridization solution and incubated for 16 h at 42 C. The membranes were washed three times at 42 C in 2 × SSC 0.1 % (w/v) SDS, at 54 C in 1 × SSC 0.1% (w/v) SDS, and at 68 C in 0.1 × SSC 0.1% (w/v) SDS. The membranes were then exposed to X-ray films (Eastman Kodak Co., Rochester, NY, USA). The films were scanned and analyzed by Molecular Analysis Program version 1.5 (Gel Doc 1000, Bio-Rad). This assay was performed in triplicate.

Real-time polymerase chain reaction (PCR)

Total RNA (4 µg) was reverse transcribed into first strand cDNA using M-MLV reverse transcriptase (Ambion Inc., Austin, Texas, USA) and random primer (9 mer). The standard curve was generated by serial dilution (1, 1/10, 1/100, 1/1,000 and 0) of a standard preparation of total RNA isolated for cDNA from the intestine of a female mouse. The real-time PCR reaction was carried out in a 25 µl final volume containing the followings: dH2O up to 25 µl, 5 µl of 5 X Taq DNA polymerase (Takara Bio Inc., Kyoto, Japan), 2.5 µl of diluted (1:30,000) SYBR Green (Takara Bio), 0.5 µl of forward and reverse primers, and 2 µl of cDNA. To detect the mouse ERα mRNA, oligo sequences of primers were employed: 5'-ATG ATC AAC TGG GCA AAG A-3' (sense, ER1) and 5'-TGT ACA CTC CGG AAT TAA GC-3' (antisense, ER2). The primers for PR were: 5'-CTG TGC CTT ACC ATG TGG CA-3' (sense, 5'PR) and 5'-TTC ACC ATG CCC GCC AGG AT-3' (antisense, 3'PR). The primers for Cytochrome C oxidase subunit I (1A) gene were: 5'-GAT ATA GCA TTC CCA CGA ATA-3' (sense, A-1) and 5'-GGG CTT TTG CTC ATG TGT CAT-3' (antisense, A-2). Three steps of PCR amplification were carried out for 45 cycles by Smart Cycle System (Takara Bio) under the following conditions: initial denaturation at 95 C for 30 sec, denaturation at 95 C for 5 sec, annealing at 55 C for 7 sec, and extension at 72 C for 12 sec. Calculation of relative expression levels of each sample was conducted based on the Cycle threshold (Ct) and growth curve at the monitoring. PCR amplification curves were evaluated through the fluorescence of double stranded DNA-specific dyes (SYBR Green I) vs. the amount of standardized PCR product. Expression levels of ERα and PR were normalized by expression level of 1A mRNA.

Western blot analysis

The mice were euthanatized, and the uteri were rapidly excised, and washed in cold sterile 0.9% (w/v) NaCl. Protein was extracted with Proprep (Intron Co., Seoul, Korea) according to the supplier’s instruction. Protein content of the supernatants was determined using a Bradford assay (Bio-Rad). Fifty micrograms of cytosolic protein was run on 15% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Rockville, MD, USA) using a Semi-dry transfer cell (Bio-Rad) according to the manufacturer’s protocol. The membrane was immunoblotted using a rabbit polyclonal antibody (Swant, Bellinzona, Switzerland, 1:2,000) specific to the mouse CaBP-9k. Alternatively, the membrane was stripped, and probed with a rabbit polyclonal antibody for beta-actin (Santa Cruz Biotech. Co., Santa Cruz, CA, USA; 1:2,000) in order to control the amount of protein loaded. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody, and visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech), followed by autoradiography. The level of CaBP-9k protein was quantitated by densitometry (NIH image β), and standardized against the level of β-actin per sample. Each assay was repeated three times.

Immunohistochemical staining

The localization of CaBP-9k protein was examined by immunohistochemistry. Small pieces of mouse uteri were embedded in paraffin, and paraffin sections (5 µm in thickness) were deparaffinized in xylene and hydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol for 20 min.
Incubation of the sections in 3% (w/v) bovine serum albumin (BSA) blocked nonspecific reaction for 1 h at room temperature (22–25 °C) and then incubated with a rabbit polyclonal antibody (Swant; 1:1,000) specific to mouse CaBP-9k dissolved in 1% (w/v) BSA at 4 °C over night. After washing, the sections were incubated with the secondary biotinylated antibody (rabbit IgG, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min and then incubated with ABC-Elite for 30 min at room temperature. Diaminobenzidine (DAB; Sigma) was used as chromogen, and the sections were counterstained with haematoxylin followed by mounting with Canada balsam.

Data analysis
Data are presented as the mean ± SD. The data were analyzed by the non-parametric procedure of the Kruskal-Wallis test, followed by Dunnett’s test for two-pair comparisons. Each value of Dunnett’s test was converted to rank for statistical analysis. All statistical analyses were performed with SAS. A value of p<0.05 was considered statistically significant.

Results
Effect of sex steroids on CaBP-9k mRNA level
The effect of sex steroids on CaBP-9k transcript was examined in the uterus of immature mice following treatments with E2, P4, or E2 plus P4. The expression level of CaBP-9k mRNA was analyzed at different time points (12, 24, 48 or 72 h) after 3 days of treatment by Northern blot assay. To normalize the variation of CaBP-9k expression levels, transcripts of 28S and 18S rRNAs were quantitated as internal controls. The treatment with P4 resulted in the induction of CaBP-9k mRNA at 12 and 24 h, whereas E2 did not induce any significant change in the expression of CaBP-9k as seen in Fig. 1. It is of interest that co-treatment with E2 plus P4 evoked a synergic effect on the induction of CaBP-9k mRNA in the uterus of immature mice at 12 and 24 h compared with P4 treatment alone (Fig. 1). However, no significant difference was observed in the expression level of CaBP-9k mRNA induced by E2, P4 or both at the 48- and 72-h time points.

Effect of sex steroids on CaBP-9k protein level and its localization
The expression level of CaBP-9k protein was further evaluated following treatments with sex steroids. The immature mice were treated with E2, P4 or both E2 and P4 and the protein level of CaBP-9k was examined by immunoblot analysis. In contrast to its mRNA level, the expression level of CaBP-9k protein was enhanced by E2 treatment at 24 h as shown in Fig. 2. In addition, treatments with P4 and E2 plus P4 stimulated the expression of CaBP-9k protein at 24 h in parallel with its mRNA level. Even though E2 increased CaBP-9k protein, P4 effect on the induction of CaBP-9k protein was much greater than the E2 effect (Fig. 2). We further analyzed the localization of CaBP-9k protein during the estrous cycle and the effect of endogenous sex steroids by immunohistochemistry. As expected, the expression level of CaBP-9k protein was highly expressed in the uterus at diestrus, in which the P4 concentration is relatively high, while it was undetectable at proestrus and estrus (Fig. 3). CaBP-9k protein was mainly localized in the endometrial stromal cells of stroma in the uterus of immature mice as shown in Fig. 3.

Effect of sex steroids on steroid receptors
Twenty-four hours after final injections with E2, P4 or E2 plus P4, total RNAs were extracted from the uterus, and transcriptional levels of ERα and PR were analyzed by real time PCR. As seen in Fig 4, E2 induced a significant increase of ERα at 12–24 h and PR at 12–48 h of treatment. Interestingly, P4 decreased the ERα and PR transcripts at 12 h, while it increased them at 24 and 48 h after final treatment. In addition, co-treatment with E2 and P4 resulted in an increase in ERα at 24 h and PR at 12, 24 and 48 h after final injection (Fig. 4). It appears that P4 may suppress E2-induced stimulation of ERα transcript at 24 and 48 h after final treatment.

Discussion
The regulation of the CaBP-9k gene in an E2-dependent manner has been demonstrated during the estrous cycle in the uterus of ovariectomized rats [8, 11]. Because the CaBP-9k gene has been shown to be sensitive to estrogen, we recently
hypothesized that the expression of CaBP-9k mRNA and protein could be used as biomarkers to detect estrogenic chemicals in the uteri of rats [19–21]. Even though the regulation of the CaBP-9k gene is well characterized in the rat uterus, its regulation is unknown in the reproductive tissues of mice. During the estrous cycle in mice, the highest expression of CaBP-9k mRNA was observed at diestrus and metestrus, in which the P4 level is enhanced, whereas only basal levels of its expression were observed at proestrus and estrus, indicating that mouse CaBP-9k gene is primarily regulated by P4, not E2 in the uterus [18]. In our previous study, the expression levels of porcine
Fig. 2. Induction of CaBP-9k protein by steroids. Immature female mice were injected with steroids, E2, P4, or E2 plus P4, for 3 days, and the expression level of CaBP-9k protein was analyzed at 24 h after the final injection by immunoblot analysis. Data are presented as the mean ± SD. a; statistically significant vs. vehicle at P<0.05.

Fig. 3. Localization of CaBP-9k protein in the estrous cycle. The localization of CaBP-9k was examined by immunohistochemical staining as described in Materials and Methods. S, stroma cells; LE, luminal epithelial cell. Magnification × 100.
CaBP-9k mRNA and protein were highly expressed in the uterus during the luteal phase compared to the follicular phase. In addition, porcine CaBP-9k was dominantly expressed in the epithelium and glandular structure of the pig uterus during the luteal phase [13]. To date, the mechanism of distinct regulation of the CaBP-9k gene in the rat and mouse has not been made clear. In rats, the regulation of this protein is mediated through an estrogen-response element (ERE) identified in intron A of the mouse CaBP-9k 5′-flanking region.

In addition, cloning of intron A of the mouse CaBP-9k gene has revealed a single-base difference in ERE compared to that of rats [10, 12]. This may partially explain the difference observed in the hormonal regulation of the CaBP-9k gene in the uterus of rats and mice. As another possible mechanism of species- and tissue-specific regulation of CaBP-9k by sex steroids, we can consider different expression levels of co-activators, and different interaction of those co-regulators with ERα and PR in a tissue- and species-specific manner. Co-activators have been known to regulate transcriptional activities of steroid receptors via protein-protein interaction and chromatin modification due to their intrinsic enzymatic activities, such as histone acetyltransferase activity [22]. It has been shown that the ratios and expression levels of co-activator/co-suppressor modulate ERα and PR mediated transcription activities [23, 24]. In this study, for the first time, we characterized the effects of sex steroids, E2, P4 and E2 plus P4, on the regulation of the CaBP-9k gene in the uterus of immature mice. In addition, the effects of these steroids on their receptors and the relationship between steroid hormone receptors and CaBP-9k expression were further investigated. The mRNA level of CaBP-9k was significantly stimulated by P4, and co-treatment with E2 plus P4 evoked a synergic effect on the induction of CaBP-9k mRNA in the uterus of immature mice. Not only P4, but also E2 alone, induced an increase of CaBP-9k protein, but combination of E2 and P4 did not show a synergic effect on the induction of CaBP-9k in this tissue. It is of interest that the expression of CaBP-9k protein was enhanced by E2 alone, while E2 did not induce the expression of CaBP-9k mRNA, suggesting that the protein of CaBP-9k may be induced by a post-transcriptional modification in the uterus of immature mice.

Treatment with E2 induced a synergic effect on the expression of the CaBP-9k gene with P4. As a possible mechanism of the synergic effect of P4 plus E2, we suggest that E2 may induce an increase of PR expression, and up-regulated PR gene may increase CaBP-9k in a P4-dependent manner. This complicated relationship between CaBP-9k and sex steroid receptors suggests that P4 regulates the CaBP-9k gene, and that E2 also can affect the expression of CaBP-9k through the regulation of PR in the uterus of immature mice. PR is one of the
potent E2 response genes, whereas P4 seems to antagonize E2-induced transcriptional activity or gene expression in the uterus and breast. In real-time PCR results, E2 significantly induced the expression levels of ERα and PR at early time points, and co-treatment with P4 plus E2 may inhibit the effect of E2. Although E2 stimulated ERα and PR mRNA levels, P4 inhibited the mRNA levels of ERα and PR at 12 h and increased their expression levels after 24 h, while co-treatment of these steroids increased PR and ERα at 24 h. These differential regulations of ERα and PR were confirmed by reverse transcription (RT)-PCR/Southern blot analysis (Data not shown). A possible explanation for these differential regulations of steroid hormones at different time points may be direct and indirect regulation of steroid hormones. In new-born mice, diethylstilbestrol (DES) also induced differential regulation of ER, depending on time point, with a similar pattern [25]. There is some evidence of direct and indirect hormonal regulation in various tissues. In primates and cats, the expression of ERα and PR is increased by E2 in the uterus, whereas P4 reduces their expression [26]. In addition, it has been shown that E2 has dual effects on the expression of PR; i.e., it decreased PR in the luminal epithelium but increased the level of PR in the stroma and myometrium of the mouse uterus [27]. We further analyzed the localization of CaBP-9k protein during the estrous cycle and the effect of endogenous sex steroids by immunohistochemistry. The CaBP-9k protein was highly expressed in the uterus at diestrus, in which the P4 concentration is relatively high, while it was undetectable at proestrus and estrus, indicating that the P4 level is critical in the regulation of CaBP-9k. It was mainly localized in the glandular epithelium of stroma in the uterus of immature mice, and it was expressed mainly at the glandular epithelium in the stroma where the PR gene would be up-regulated by E2 [27]. This result supports our hypothesis of complicated regulation of CaBP-9k by steroids and their receptors.

Taken together, these results indicate that P4 and PR may be dominant factors in the regulation of CaBP-9k. In addition, E2 and ERα can influence the expression of the CaBP-9k gene via an indirect pathway in the uterus of immature mice.

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References

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