DIFFERENTIAL REGULATION OF MOUSE CALBINDIN-D_{9k} GENE IN PLACENTA AND EXTRA-EMBRYONIC MEMBRANE DURING PREGNANCY

Beum-Soo An and Eui-Bae Jeung

Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Chongju, Chungbuk, Korea.

Introduction

The mammalian duodenum, placenta and uterus express a 9kilodalton cytosolic calcium binding protein termed Calbindin-D_{9k} (CaBP-9K)[4]. Originally, this protein was discovered based on its vitamin D dependency in the duodenum and its relation to intestinal calcium absorption. In the uterus, its expression is controlled by 17β-estradiol (E2) in rat and by progesterone (P4) in mouse[1]. Placental CaBP-9K is also believed to participate in calcium transport events between the maternal and fetal organism[5]. Factors regulating expression of CaBP-9K in placenta and extra-embryonic membrane are unknown. In our recent study, mouse CaBP-9K expression in uterus was measured during pregnancy and lactation. It was found that during pregnancy, the levels of CaBP-9K mRNA were expressed highly at post implantation and late pregnancy, controlled by P4 and progesterone receptor levels in uterus. The present experiment was designed to monitor CaBP-9K gene expression and the correlation with steroid hormones (E2 and P4) in pregnant mouse placenta and extra-embryonic membrane. In this study, we analyzed the CaBP-9K expression in placenta and extra-embryonic membrane through pregnancy (day 7~birth in placenta and day 10~birth in extra-embryonic membrane) by Northern blot assay. The steroid hormones (E2 and P4) and their receptors were analyzed by ELISA and RT-PCR/Southern blot assay, respectively The steroid hormone antagonists such as Tamoxifen (TAM), ICI 182,780, and RU 486 were used to investigate the regulation of mouse CaBP-9K gene expression in early and late pregnant period when CaBP-9K was expressed highly in uterus. After treatment, the CaBP-9K and ER/PR expression levels were analyzed by Northern and RT-PCR/Southern blot, respectively.

Methods and Materials

In the first experiment, six week olds female ICR mice were mated with adult males overnight

and the following morning the presence of vaginal plug after mating was designated day 0 of pregnancy. Group of mice were killed on each day of pregnancy. In the second experiment, females were mated as described above and three groups of 5 animals were injected (s.c) with 2μg /mouse of RU 486, Tamoxifen and ICI 182,780 dissolved in sesame oil on pregnancy day 7 and 15. The mice injected with vehicle (sesame oil) were served as a control. After single injection of each chemical, the mice were sacrificed at 24, 48 and 72 hours. Blood was collected for hormone assay and their placenta and extra-embryonic membrane were removed for RNA isolation. Ten microgram of total RNA were loaded on 1% agarose gel for Northern blot analysis and 18S rRNA served as an indicator of quantity of total RNA. Five microgram of total RNA were reverse transcribed using M-MLV reverse transcriptase and random primer (9mer). For the PCR reaction, 10% of the RT products were used. The samples contained ER/PR primer for estrogen and progesterone receptor gene and also had primers for 1A gene as an internal standard. For southern blot assay, 50% of the PCR products were used.

Results and Discussion

In the first experiment, the expression of CaBP-9K was increased gradually and peaked at P17 (up to 80-fold) in placenta. In extra-embryonic membrane, the signals of CaBP-9K mRNA had not uniformity, but the expression levels of CaBP-9K declined generally (by 2.8 fold at P17) and increased again at P18. The expression of ER and PR mRNA could be considered to the regulatory factor correlated with that of CaBP-9K mRNA in placenta (Fig. 1A) and extra-embryonic membrane (Fig. 1B).

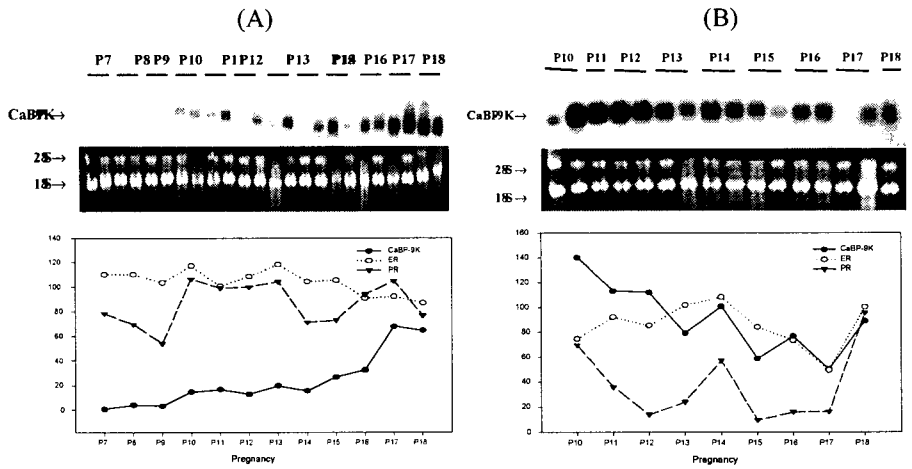

Fig. 1. Northern blot analysis for CaBP-9k mRNA expression during pregnancy (vaginal plug = day 0) in normal pregnant mouse placenta (A) and extra-embryonic membrane (B). Total RNA (10μg) was

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).

In the second experiment, Fig. 2 shows that effect of the steroid hormone antagonists on early pregnant mouse placenta. In early pregnant mouse placenta, the expression of CaBP-9K mRNA was blocked significantly at 24h (2.7 fold *vs* vehicle) by TAM which has partial agonist effect for ER and PR and RU486, antagonist of P4, at 72h (4 fold *vs* vehicle) after single injection.

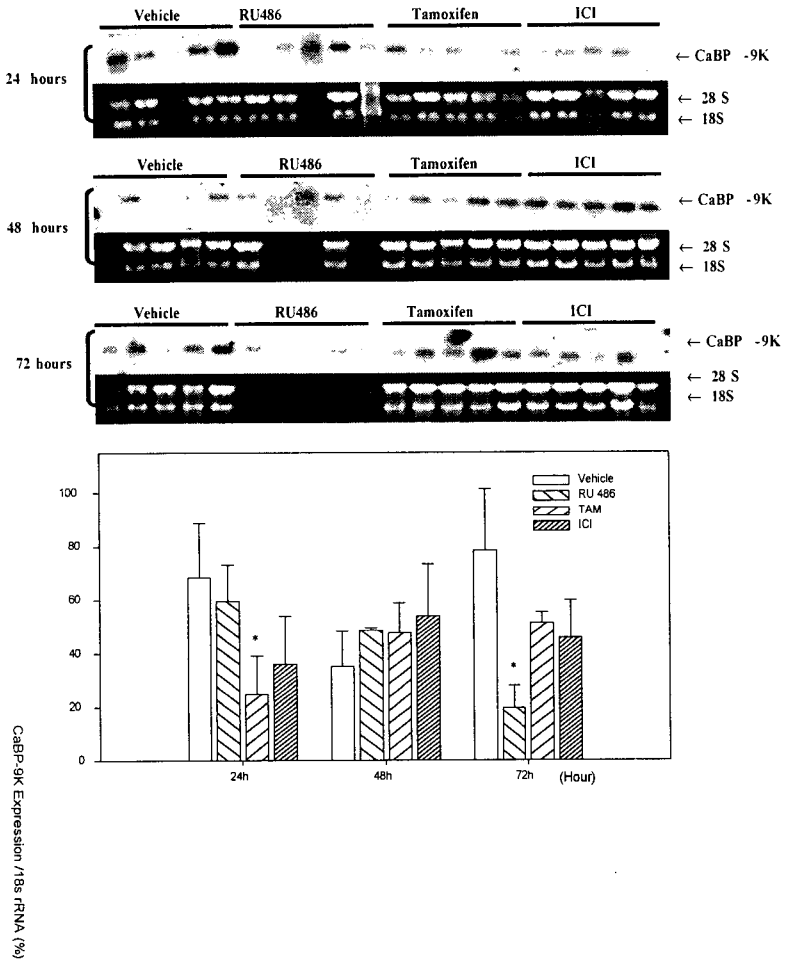

Fig. 2. Effect of TAM, RU486 and ICI on CaBP-9K mRNA in the placenta of early pregnant mouse. Pregnant mice were injected with TAM, RU486 and ICI at P7 and total RNA was extracted in placenta at 24, 48, or 72h after injection. Total RNA (10 μg) was subjected to Northern blot analysis. After hybridization 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). *;

$p < 0.05$, **; $p < 0.01$.

In Southern analysis, the expression of ER mRNA was blocked by RU486 at 72h, while that of PR mRNA was also down regulated by RU486 at 48 and 72h (data was not shown). In these results, 18S rRNA was degenerated due to abortion in groups treated with RU486, thus we measured the CaBP-9K mRNA by RT-PCR and normalized with 1A. The PCR analysis of CaBP-9K mRNA was identical to results of its Northern blot analysis (data was not shown). The effects of the antagonists were also investigated in placenta (A) and extra-embryonic membrane (B) at late pregnant mice.

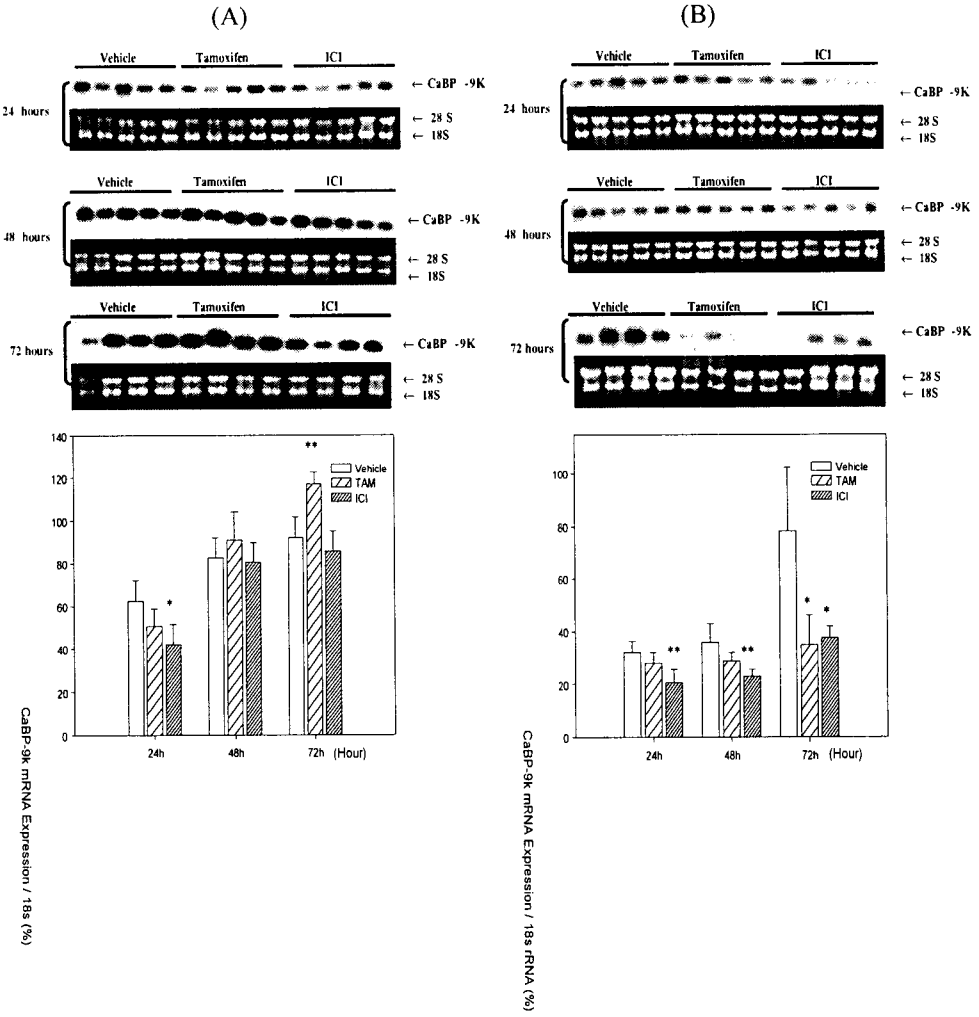

Fig. 3. . Effect of TAM, RU486 and ICI on CaBP-9K mRNA in the placenta (A) and extra-embryonic membrane (B) of late pregnant mouse. Pregnant mice were injected with TAM, RU486 and ICI at P15 and total RNA were extracted in placenta and extra-embryonic membrane at 24, 48, or 72h after injection. Total 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). *; $p < 0.05$, **; $p < 0.01$

In placenta (A), the expression of CaBP-9K mRNA was down regulated up to 1.5 fold by ICI at 24h and up regulated up to 1.3 fold by TAM at 72h after single injection, while expression of ER mRNA was down regulated by ICI in every sacrificing time but significant only at 48h (1.3 fold *vs* vehicle). ICI, antagonist of ER reduced the CaBP-9K gene expression in all sacrificing time significantly up to 2.1 fold (at 72h) and TAM also reduced that only at 72h up to 2.2 fold in extra-embryonic membrane (B), but any significant fluctuant expression of ER/PR gene has not been detected by southern analysis. In our previous study, the expression of CaBP-9K mRNA in pregnant mouse uterus was mainly regulated by P4 and PR, and the expression was blocked by RU486. However, in placenta and extra-embryonic membrane, the regulation system of CaBP-9K expression may have not similar repertory at uterus. During late gestation in rat and mice, placental level of CaBP-9K was increased, and it is response to the calcium demands of the developing skeleton. By biochemical and immunochemical means, high concentrations of CaBP-9K have been localized to epithelial cells of the yolk sac and endodermal cells of the placenta[3]. A complementary DNA (cDNA) probe has also localized CaBP-9K mRNA in the trophoblast of the placenta. A role for CaBP-9K in calcium transfer is suggested by parallel gestational changes in placental calbindin-9K and fetal growth, which in turn reflects the fetal accumulation of calcium. The regulating factors of CaBP-9K in placenta and extra-embryonic membrane have not been known. In placenta, the regulation factor is different from other tissue such as intestine and kidney and the expression of this gene is not dependent on Vitamin D receptor[2]. The expression of CaBP-9K gene was increased gradually in normal pregnant mouse placenta, and these results were similarly correlated with peripheral plasma E2 concentration. The expression of CaBP-9K in placenta was down regulated by TAM at 24h and RU486 at 72h in early pregnant mouse and by ICI at 24h in late pregnant mouse. In late pregnant mouse, the expression of CaBP-9K also down regulated by ICI and TAM (at 72h only) in extra-embryonic membrane. In these results, we could conclude that the CaBP-9K mRNA is regulated by sex steroid hormones and their receptors through complex pathway both placenta and extra-embryonic membrane, and in extra-embryonic membrane, the regulation of CaBP-9K mRNA expression mediated by ER mainly.

Acknowledgments

This work was supported by NITR/Korea FDA Grant ED2000-4 for Endocrine Disruptors Research.

References

1. Tatsumi K., Higuchi T., Fujiwara H., Nakayama T., Itoh K., Mori T., Fujii S. and Fujita J. (1999) Mol Hum Reprod. 5(2);153.

2. Shamley D.R.,, Veale G., Pettifor JM. and Buffenstein R. (1996). J Endocrinol. 150(1);25.

3. Bruns ME., Kleeman E., Mills SE., Bruns DE and Herr JC. (1985) Anat Rec. 213(4);532.

4. Christakos S., Gabrielides C. and Rhoten WB. (1989) Endocr Rev. 10(1);3.

5. Mathieu CL., Mills SE., Burnett SH., Cloney DL., Bruns DE. and Bruns ME. (1989) Endocrinology; 125(5);2745.

6. Krisinger J., Setoyama T. and Leung PC. (1994) Mol Cell Endocrinol. 102(1-2);15.