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PII S0024-3205(99)00190-3

Life Sciences, Vol. 64, No. 25, pp. 2367-2373, 1999

0024-3205/99/$–see front matter

PROTEIN KINASE C ISOFORMS DURING THE DEVELOPMENT OF DECIDUOMATA IN PREGNANT RATS

Jyh-Cherng Shyu[1], Yih-Shou Hsieh[2], Chen-Lurng Chang[1], Chin-Chiu Tsai[1], Hui-Chen Liu[2], Ai-Chi Chang[2], Lien-Chuan Yang[3], Long-Yau Lin[4] and Jer-Yuh Liu[2]

Department of Physiology[1] and Institute of Biochemistry[2], Chung Shan Medical and Dental College, and Department of Pathology[3] and Obstetrics[4], Chung Shan Medical and Dental College Hospital, Taichung 40203, Taiwan, R.O.C.

(Received in final form March 3, 1999)

Summary

In this study, we determined the expression of protein kinase C (PKC) isoforms during pregnancy. At pregnant duration, PKCα was down-modulated in the deciduomata but not in the myometrium. Down-modulation was compatible with the increase in cell mitosis, which reached a maximum at 8-9 days. On the other hand, PKCζ was not down-modulated. It was increased both in the cytosolic and particulate fractions of the deciduomata, and paralleled the frequency of decidual cell mitosis. The other PKC isoform of δ was also increased, but it was associated with the cell regression. Therefore, these findings confirmed that the variable expression of PKC isoforms in decidualizing tissue may be involved in the modulation of decidual cell growth.

Key Words: protein kinase C, deciduomata, pregnant rats

Protein kinase C (PKC) plays a crucial role in the regulation of calcium-dependent cellular functions (1). In response to extracellular signals e.g. growth factors, hormones, and neurotransmitters (2,3), PKC may play an essential role in trans-membrane signal transduction. It is also found to induce many cellular responses including cell proliferation, differentiation, gene expression and tumor promotion (4). Over-expression of PKC activity can cause multiple growth abnormalities in rat fibroblasts (5).

To date, twelve isoenzymes of PKC (α,βI, βII, γ,δ,ε,ζ,η, θ,ι,λ and μ) have been identified (6-9). These isoenzymes exhibit differences in function and localization (10-13). Recent reports indicated that individual PKCs were involved in restoring cell proliferation. The PKCα level was increased in carbon tetrachloride-induced liver regeneration (14). Selective decreases in nuclear PKCα and increases in nuclear PKCδ were observed after partial hepatectomy (15). Redistribution of PKC from soluble to particulate fractions occurred during liver regeneration (16,17). Folic acid-induced kidney regeneration caused PKCα, but not PKCδ and PKCε, to decrease (18). Liver and kidney regeneration also induced primary response

Address for Correspondence: Ph.D. Jer-Yuh Liu, Institute of Biochemistry, Chung Shan Medical and Dental College, No.110, Sec. 1, Chien Kuo N. Road, Taichung 40203, Taiwan. **Tel:** (886) 04-3896190-50815 **Fax:** (886) 04-3890964. **E-mail:** jyl@mercury.csmc.edu.tw.

genes, fos, myc, TIS 1, TIS 8, and TIS 11 (19-22). These data suggest that PKC activation occur during chemical or mechanical-induced cell proliferation. However, the role of PKC in decidualizing tissue is ill defined.

In previous study, we had found that the various expressions of PKC isoforms were observed in decidualinzing tissue of pseudopregnancy (23). The decreased cytosolic PKC α and the increased PKC ζ was associated with the growth of deciduomata, while PKC δ was related with the depression of mitosis. In this study, we determined the PKC isoforms in the decidualized uterine tissue during pregnancy.

Methods

Chemicals:

Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), ethylenediamine-tetraacetic acid (EDTA), sucrose, ethyleneglycol bis(α-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor type I-S, β-mercaptoethanol, Triton X-100 and $MgCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal treatment:

Virgin Sprague-Dawley rats (National Science Council Animal Center, Taipei, Taiwan, R.O.C) weighing 250-300g were maintained in a temperature-controlled room (at 24 °C) and illuminated for 12 hours daily (lights on from 5 AM to 5 PM). Vaginal smears were taken daily at 08:00 - 09:00 h after at least 7 days of adaptation to the new environment. Only those animals showing two consecutive 4-day cycles were used and were placed with males of proven fertility on 17:00 - 18:00 proestrus day of the third consecutive cycle. Conclusive of successful pregnancy was that those were found with vaginal sperm on the following morning. Two independent experiments were proceeding. The animal was decapitated on days 1, 5, 11, 13 and 15 of pregnancy in the first experiment and on days 5, 8, 9 and 11 of pregnancy in the scondary experiment. On the indicated days of pregnancy the two uterine horns with conceptus were removed, weighed, and quickly frozen on dry ice for tissue extraction.

Preparation of cytosolic and particulate fractions:

All procedures were performed at 4°C. On days 9, 11, 13 and 15 of pregnancy, the conceptuses were collected and were slit open. The trophoblast and fetus were removed. The deciduomata were separated from the myometrium by gentle scraping with a glass slide. On day 15 the deciduomata can not be obtained, because it had been regressed. Others, both deciduomata and myometrium samples were then homogenized by thirty strokes using a Konetes homogenizer at a ratio of 1 g tissue/10 ml homogenization buffer A. The homogenization buffer A consisted of 20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.3 M sucrose, 2 mM PMSF, 10 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor type I-S and 50 mM β-mercaptoethanol at pH 7.5. On days 1, 5 and 8 of pregnancy, the deciduomal tissue can not be removed individually. As a result, whole uterine tissues were homogenized as above. These homogenates were centrifuged at 100,000g for 1 hour at 4°C. The supernatant was stored at -70°C for the cytosolic fraction and the pellet was suspended in buffer B (20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, 10 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor type I-S, 50 mM β-mercaptoethanol and 0.1% Triton X-100 at pH 7.5.) and homogenized with thirty strokes. A 30 min exposure to 0.1% Triton X-100 at 0°C has been demonstrated to extract PKC from tissue suspensions of rat brain, liver, and kidney (24). This procedure was modified and the homogenates were incubated at 4°C in a rotating mixer for 1 hour. Centrifugation (100,000 g, 1 hour) was performed to remove any nonsolubilized material and the supernatant was then referred to as the particulate fraction and stored at -70°C. The enzyme was stable for at least 1 month.

Electrophoresis and Western Blot:

Tissue extract samples were prepared as described above. The protein content was determined using the Bio-Tad assay with bovine serum albumin as a standard. Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (25) using 10% polyacrylamide gels and the equal amounts of protein were loaded. After samples were electrophoresed at 140 V for 3.5 hour, the gels were equilibrated for 15 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol, as described by Brockenbrough et al. (26). Electrophoresed proteins were transferred to nitrocellulose paper (Amersham, Hybond-C Extra Supported, 0.45 Micro) using a Hoefer Scientific Instruments Transphor Units at 100 mA for 14 hour. Nitrocellulose papers were incubated at room temperature for 2 hour in blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 and 3% (v/v) fetal bovine serum. Monoclonal antibodies to PKC isoforms (Transduction Laboratories) were diluted 1:200 in antibody binding buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1%(v/v) Tween-20 and 1% (v/v) fetal bovine serum. Incubations were performed at room temperature for 3.5 hour. The immunoblots were washed three times in 50 ml blotting buffer for 10 min and then immersed in the second antibody solution containing alkaline phosphatase goat anti-rabbit IgG (Promega) for 1 hour and diluted 1000-fold in binding buffer. The filters were then washed three times in blotting buffer for 10 min. Color development was presented in a 20 ml mixture consisting of 7 mg nitro blue tetrazolium, 5 mg 5-bromo-4-chloro-3-indolyl-phosphate, 100 mM NaCl and 5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 9.5. The band density was quantified by densitometer (Alphalmager 2000, Alpha Innotech Corporation)

Histology examination:

After removal of the uterus, one or two conceptuses were immediately fixed in 10% formalin and stained with hematoxylin and eosin. For the mitotic index, at least 10000-15000 nuclei were scored for each time point.

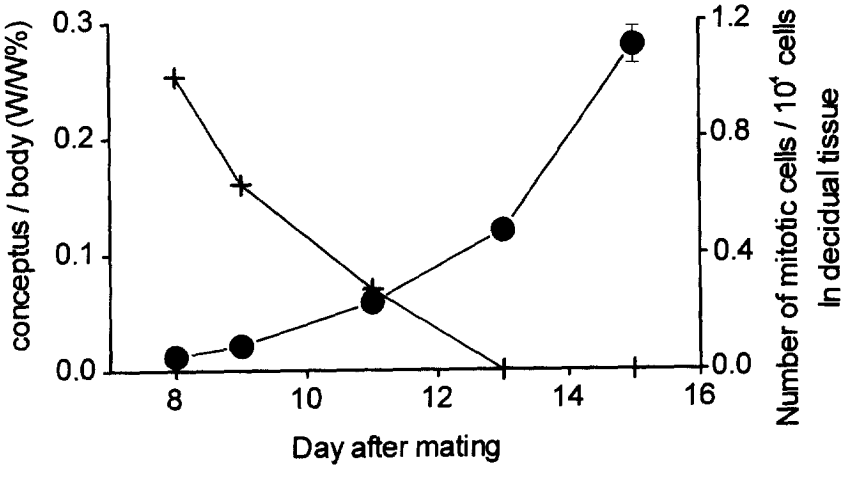

Fig. 1.

Change in conceptus weight and the rate of the decidual cell mitosis during pregnant duration. Values expressed are the mean ± S.D. for 3-5 measurements. -●- : conceptus weight versus body weight, -+- : the rate of mitosis in decidual tissue.

Results

Evaluation of the mitotic rate of the Deciduomata in Rat Uterus:

After mating, the conceptus undergoes a increase in weight, especially from day 8 (Fig. 1). The proliferation of the decidual cell was evaluated by the histology examination. The mitotic cell was designated as the previously described (23). The rate of the decidual cell mitosis was increased at 7 day (data not shown), and reached a maximum (0.0101%) at 8-9 days, then quickly dropped down. On day 13 the decidual cell was regressed to a fine layer of cells between the embryonic cavity and the uterine wall and did not express almost the mitotic cells.

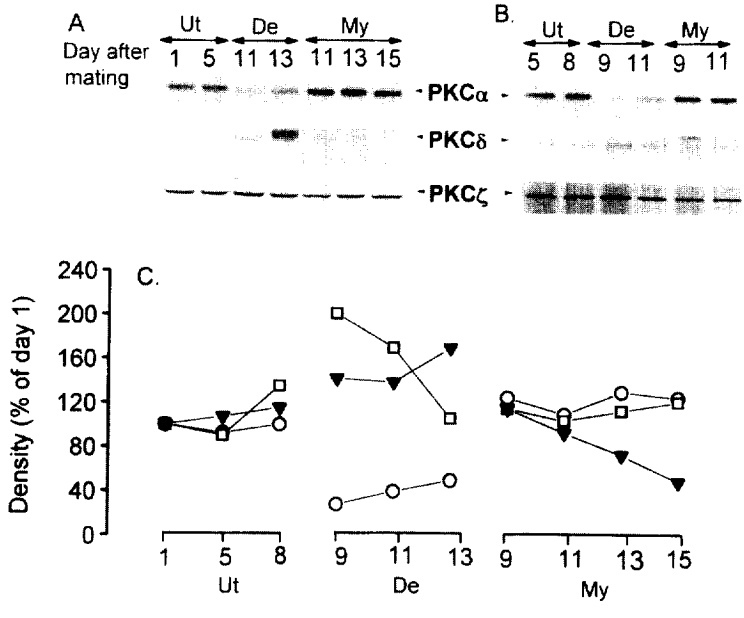

Fig. 2.

Immunoblot analysis of PKC α, δ and ζ isoenzymes in the cytosolic fraction. Uterine cytosolic fraction was prepared as described in "Materials and Methods". Aliquots of the uterus homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with PKC isoenzyme-specific antibodies. Samples in each lane were from individual rats in the first experiment (A). Samples in each lane were from individual rats in the secondary experiment (B). The quantitative data of the cytosolic fraction from all experimental animals are presented (C). On days 9, 11, 13 and 15, the samples were the homogenates of the deciduomata (De) or myometrium (My). On days 1, 5 and 8, samples were of the whole uterine tissue (Ut). The levels of PKC isoforms on day 1 were defined as 100%. Values expressed were the mean for 3-5 measurements. –○– : PKC α, -▼– : PKC δ, –□– : PKC ζ.

Characterization of PKC Isofroms in deciduomata:

Because the three isoforms (α, δ and ζ) of PKC were significantly correlated with the decidualization of pseudopregnant rat (23), we then examined the expression of these isoforms during pregnancy. These data revealed that PKCα content decreased 50%-72% in the cytosolic fraction of the deciduomata on days 9-13 as compared to day 1 (Fig. 2A, 2B and 2C), as well as decreased 23%-50% in the particulate fraction on days 8-9 as compared to day 1(Fig. 3A, 3B and 3C). Since the sample extract on day 8 is composed of the decidual tissue and myometrium, the level of the cytosolic-PKCα may decrease absolutely in the decidual tissue. This phenomenon was designated as down-modulation (18) and preceded the increase in cell mitosis which was maximal at 8-9 days. Both the cytosolic- and particulate-PKCα contents were slight increased in the myometrium as compared to day 1(Fig. 2 and 3).

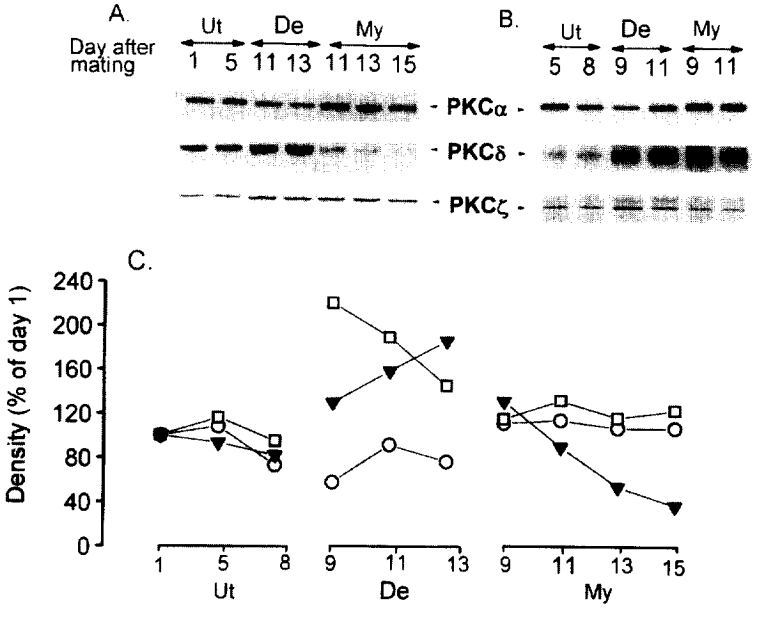

Fig. 3.

Immunoblot analysis of PKCα, δ and ζ isoenzymes in the particulate fraction. Uterine particulate fraction was prepared as described in "Materials and Methods". Aliquots of the uterus homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with PKC isoenzyme-specific antibodies. Samples in each lane were from individual rats in the first experiment (A). Samples in each lane were from individual rats in the secondary experiment (B). The quantitative data of the cytosolic fraction from all experimental animals are presented (C). On days 9, 11, 13 and 15, the samples were the homogenates of the deciduomata (De) or myometrium (My). On days 1, 5 and 8, samples were of the whole uterine tissue (Ut). The levels of PKC isoforms on day 1 were defined as 100%. Values expressed were the mean for 3-5 measurements. –○– : PKCα, -▼– : PKCδ, –□– : PKCζ.

PKC ζ, like pseudopregnancy, was not down-modulated. It was increased both in the cytosolic and particulate fractions of the decidual tissue, and slight increased in myometrium (Fig. 2 and 3). The level of PKC ζ in the cytosolic and particulate fractions of deciduomata increased 201%-221% on day 9 relative to day 1 and paralleled with the proliferative potent of the decidual cell. The other isoform of PKC δ was also increased both in the cytosolic and particulate fractions of the decidual tissue, but not in that of the myometrium (Fig. 2 and 3). The level of this isoform in the cytosolic and particulate fractions of the deciduomata increased 170%-180% on day 13 relative to day 1, which was associated with the cell regression.

Discussion

Our previous data showed that the level of the cytosolic PKC α was down-modulated during the development of deciduomata in the pseudopregnant rats (23). According to the present data, the down-modulation was also observed in the decidual tissue of pregnancy, and paralleled with the increase of the mitotic index. By the immunohistochemistry, the PKCα (but not the PKCζ) was mainly located in the surrounding nuclear of the decidual cell (31), which was designed as the activation and down-modulation (32), suggesting that PKC α may be the only down-modulated isoform expressed in the deciduomata.

Our data also indicated that PKCζ, a considered mitogen (27, 28), was highly expressed predominantly in the deciduomata of the proliferative stage, and PKCδ, a considered negative-regulator (29, 30), was also highly expressed predominantly in the deciduomata but in the regressive stage. The overexpression of PKC δ and ζ coexisted on days 3 and 5 in pseudopregnancy (23) and on days 9 and 11 in pregnancy (Fig. 2 and 3), suggesting an important physiological equilibrium of growth control by these isoforms.

In conclusion, we suggested that the various expression of PKC isoforms may play an important role in the regulation of the development of the deciduomata during pseudopregnancy and pregnancy, although the level of PKC isoforms mRNA have not been detected.

Acknowledgements

This study was supported by a grant from Chung Shan Medical and Dental College (CSMC 86-OM-A-032 and CSMC 86-OM-B-026), Taichung, and the National Science Council (NSC 87-2314-B-040-029), Taipei, Republic of China.

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