Luteal phospholipase A_2 activity increases during functional and structural luteolysis in pregnant rats

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Abstract We determined cytosolic phospholipase A_2 activity of the corpus luteum during luteolysis in pregnant and post-partum rats. Phospholipase A_2 activity and its metabolite prostaglandin $F_{2\alpha}$ in the corpus luteum remarkably increased just before parturition and further rose transiently during post-partum structural luteolysis. The absence of a pups' suckling stimulus delayed corpus luteum involution, being associated with an altered fluctuation in phospholipase A_2 activity and depressed prostaglandin $F_{2\alpha}$ levels. Exogenous prolactin had a reversal effect. Pharmacological and immunochemical characterization suggests multiple isoforms of phospholipase A_2 in a pregnant corpus luteum. These results show the increased phospholipase A_2 activity and its possible implication in luteolysis in pregnant rats.

Key words: Phospholipase A_2; Luteolysis; Parturition; Rat; Prostaglandin $F_{2\alpha}$

1. Introduction

Phospholipase A_2 (PLA_2) is a key enzyme that generates a variety of lipid mediators. These biologically active substances include free fatty acids, lysophospholipids and eicosanoids such as prostaglandin (PG) and leukotriene. PLA_2 comprises a growing superfamily and those of mammalian origin are largely divided into four groups according to a recent classification [1]. The first group of enzymes ($sPLA_2$), 14 kDa, is secreted and dependent on a mM level Ca^{2+}, being further divided into subtypes IB, IIA, IIC, V, etc. The second group of enzymes ($cPLA_2$), 85 kDa, is cytosolic and activated by sub-μM Ca^{2+} levels. The third PLA_2 group ($iPLA_2$), 80–85 kDa, is also cytosolic but Ca^{2+}-independent. The last group of enzymes is Ca^{2+}-independent and they have an acetylhydrolase activity for platelet activating factor.

Eicosanoids exert a wide range of physiological and pathological effects in the body. $PGF_{2\alpha}$ acts on the cell surface specific receptor (FP) of the corpus luteum (CL) to induce luteolysis in mammals [2]. Luteolysis in rats is composed of functional luteolysis, characterized by a decline in the circulating progesterone level, and the subsequent structural luteolysis, marked by CL involution [3]. The potent luteolysin $PGF_{2\alpha}$ can be generated by CL itself [2] and rat CL expresses type IB and IIA PLA_2 and $cPLA_2$ [4–6]. Luteal cytosolic PLA_2 activity, mainly exhibited by $cPLA_2$, is shown to increase during functional luteolysis in pseudopregnant rats [5,7]. Mice lacking $cPLA_2$ [8,9] or the FP receptor [10] failed to deliver because of obstructed luteolysis at term. This suggests the indispensable role of $cPLA_2$ in $PGF_{2\alpha}$ generation which causes functional luteolysis and induction of delivery. However, the dynamics in luteal PLA_2 activity during functional luteolysis in pregnant animals, including rats, is not yet fully understood. In addition, it is unclear whether PLA_2 and one of its metabolites $PGF_{2\alpha}$ are also responsible for structural regression. A previous immunohistochemical study showed that the signal for $cPLA_2$ in CL became intensified with age in the cycling rat ovary [6]. This finding suggests an intimate association between PLA_2 and the structural involution of CL, however, there have been few reports relating them.

We here investigate the above two problems using pregnant and post-partum rats. Unlike the CL of cycling or pseudopregnant rat ovary, the pregnant CL becomes hypertrophied during gestation and is bound to undergo post-partum involution, thus being a good model to analyze the mechanism of structural luteolysis.

2. Materials and methods

2.1. Antibodies and chemicals

The specific antibodies for human $cPLA_2$ and rat PLA_2-IB were donated by Genetics Institute (Cambridge, MA, USA) [11] and Dr. K. Nomura (Shionogi Research Laboratory, Osaka, Japan) [4], respectively. Radiolabelled phosphatidylcholine (PC) (1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl PC) was obtained from DuPont-NEN (Boston, MA, USA). Arachidonyl trifluoromethyl ketone (ATK) which inhibits $cPLA_2$ [12], methyl arachidonylfluorophosphonate (MAFP) which inhibits both $cPLA_2$ and $iPLA_2$ [13] and bromoenol lactone (BEL) which inhibits $iPLA_2$ [13] were purchased from Cayman (Ann Arbor, MI, USA). An ELISA kit for $PGF_{2\alpha}$ was also purchased from Cayman. Ovine prolactin (PRL) was obtained from Sigma (St. Louis, MO, USA). All other reagents, including dithiothreitol (DTT) an $sPLA_2$ inactivator [14], were of analytical grade.

2.2. Animals

Mature female rats of the Wistar-Imamichi strain were used in this study. Animals showing a regular 4 day estrous cycle were mated with fertile male rats on the pro-estrous evening. The day of fertilization (vaginal estrus with sperm positive) was designated as day 1 of pregnancy (PRG1). The duration of pregnancy in our colony was 23.0 ± 0.1 days (mean ± S.E.M., $n = 13$). To induce delayed structural luteolysis of pregnant CLs, mothers in some groups were removed from pups immediately after delivery in order to avoid the occurrence of any suckling stimulus. To some of these mothers removed from pups, PRL (10 IU, intraperitoneal) was injected twice daily from day 0 post-partum (PP0, the delivering day) to PP2. Animals were killed at indicated times. Blood plasma was stored at −20°C until the progesterone assay. Ovaries were stored at −80°C until analyses of the PLA_2 activity and $PGF_{2\alpha}$ level in CLs were performed.

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Abbreviations: PLA_2, phospholipase A_2; PG, prostaglandin; CL, corpus luteum; PRL, prolactin

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2.3. Assay of PLA_2 activity in CL cytosol

PLA_2 activity in CL cytosol was measured by the previous method with a slight modification [7,15]. Briefly, pregnant CLs that were large, round-shaped and rather whitish were separated from the ovary. With post-partum ovaries, care was taken not to contaminate a new generation of CLs, which were small and reddish, since rats have post-partum ovulation. CLs were measured for wet weights and homogenized in 0.25 M sucrose, 0.25 mM EDTA and 0.05 M Tris-HCl (pH 9.0). The homogenates were centrifuged at $105000 \times g$ for 1 h and the supernatants (cytosol) were determined for their protein concentration using a Bio-Rad assay kit. Liposomes for substrate were prepared with radiolabelled and non-radiolabelled PC at a molar ratio of 1:4. The assay mixtures (200 μl in total volume) contained 0.1 M Tris-HCl (pH 9.0), 4 mM $CaCl_2$, 1 mg/ml fatty acid-free bovine serum albumin, 2 μM PC and cytosol. In experiments to pharmacologically characterize the PLA_2 activity, the mixtures further contained 5 μM ATK, 2 μM MAFP, 50 μM BEL or 5 mM DTT. The reaction was performed at 37°C for 1 h and stopped by adding 1.0 ml Dole's reagent. The released fatty acid was extracted and measured for its radioactivity by liquid scintillation counting.

2.4. Assay of plasma progesterone and luteal $PGF_{2\alpha}$

Plasma progesterone was assayed using a radioimmunoassay as described before [5]. Luteal $PGF_{2\alpha}$ was determined according to Higuchi et al. [16] using a commercial ELISA kit. Briefly, pregnant CLs were homogenized in 0.05 M phosphate buffer (pH 7.3) containing 0.1% gelatin and 0.01% merthiolate and then centrifuged at $10000 \times g$ for 20 min at 4°C. The supernatant was directly assayed for the $PGF_{2\alpha}$ content without any extraction or separation from other eicosanoids or lipids.

2.5. Immunohistochemistry of $cPLA_2$ and PLA_2-IB

The mother with suckling stimulus was killed on PP8 and ovaries were fixed in Bouin's fixative containing $HgCl_2$, dehydrated and embedded in paraffin. Tissues were serially sectioned (6 μm in thickness), deparaffinized and examined histologically and immunochemically. Some sections were stained by hematoxylin and eosin (HE). Immunohistochemistry of PLA_2 was performed by the previous methods with a slight modification [4,6,15]. Endogenous peroxidase was blocked by pretreatment with 0.3% H_2O_2 in methanol. Sections were incubated with anti-$cPLA_2$ (1:1000) or anti-PLA_2-IB (1:3000) for 1.5 h at room temperature. Visualization of the complex of the objective enzyme and the antibody was performed using the Vectastain Elite ABC staining method and 3,3′-diaminobenzidine tetrahydrochloride as peroxidase substrate. The control method using the non-specific rabbit serum gave no positive reaction (data not shown).

2.6. Statistical analysis

All data were presented as mean ± S.E.M. of indicated numbers of samples. The means among different groups were analyzed by one-way ANOVA and Student's *t*-test. A P value less than 0.05 was considered to be significant.

3. Results and discussion

Having determined the luteal PLA_2 activity and $PGF_{2\alpha}$ level during late pregnancy and post-partum, we here show their increase just before parturition and their further and transient increase during post-partum structural lutcolysis.

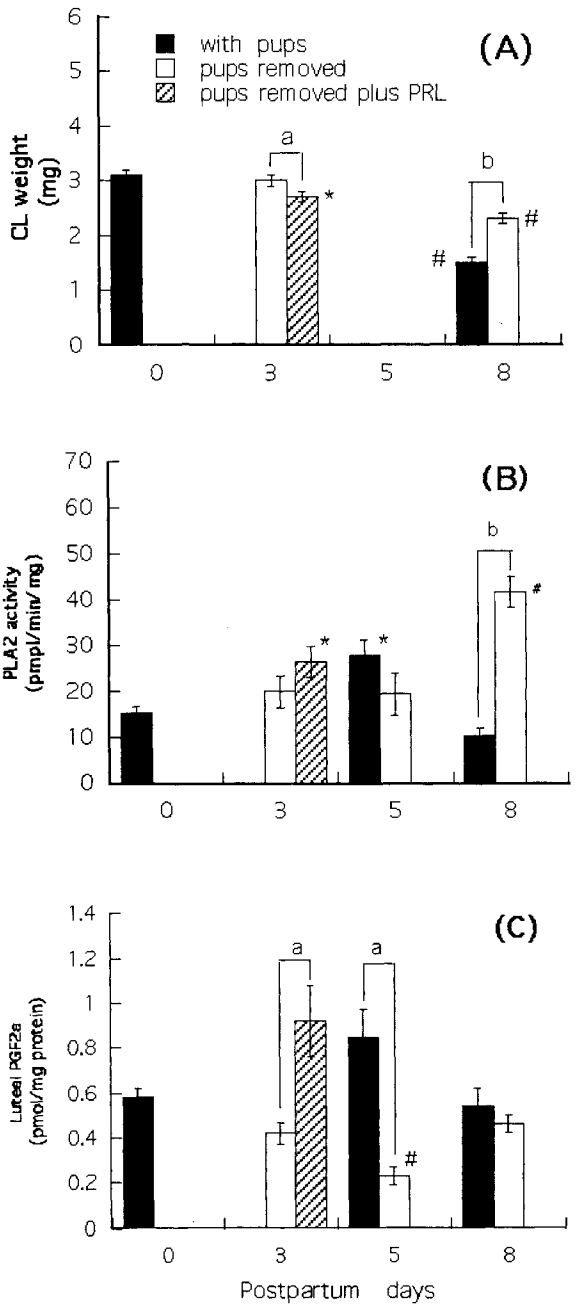

Fig. 1. Post-partum changes in wet weight (A), PLA_2 activity (B) and $PGF_{2\alpha}$ content (C) of CLs. Post-partum rats (with or without suckling stimuli or without suckling stimuli but PRL-administered) were killed at indicated times. CLs were separated, weighed and measured for cytosolic PLA_2 activity and $PGF_{2\alpha}$ levels as described in Section 2. Data are mean ± S.E.M. ($n > 15$ in A, $n = 3$–4 in B and C). *, $P < 0.05$ versus PP0; #, $P < 0.01$ versus PP0; a, $P < 0.05$; b, $P < 0.01$.

Table 1
Changes in plasma progesterone, luteal PLA_2 activity and $PGF_{2\alpha}$ content during late pregnancy

Period	Progesterone (ng/ml)	PLA_2 activity (pmol/min/mg)	$PGF_{2\alpha}$ (pmol/mg)
PRG19	138.2 ± 37.6	3.1 ± 0.6	Not tested
21	84.8 ± 20	3.5 ± 0.8	0.21 ± 0.04
22	33.5 ± 3.0	8.9 ± 3.7	1.00 ± 0.19*
23	26.3 ± 10.8	15.3 ± 1.5*	0.58 ± 0.04*

Pregnant rats were killed at indicated times. Plasma progesterone was measured by a radioimmunoassay. Luteal cytosolic PLA_2 activity and $PGF_{2\alpha}$ levels were determined as described in Section 2. Data are mean ± S.E.M. ($n = 3$, 4).
*$P < 0.05$ versus PRG21 within each column.

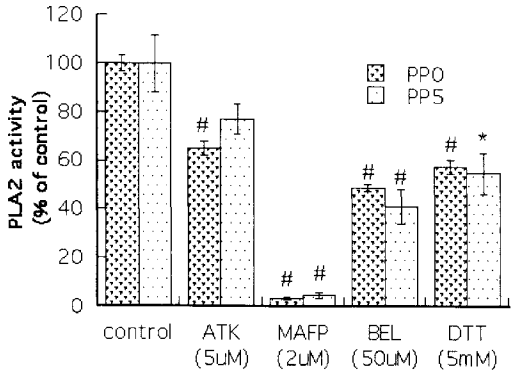

Fig. 2. Effects of several inhibitors on luteal PLA_2 activity. CL cytosol of mothers with suckling stimuli on PP0 or PP5 were incubated in 0.1 M Tris-HCl (pH 9.0), 4 mM $CaCl_2$, 1 mg/ml BSA, 2 μM PC substrate with or without each inhibitor for 1 h at 37°C. The free [^{3}H]AA released by the reaction was extracted and counted for its radioactivity. Data are mean ± S.E.M. ($n=4$). *, $P<0.05$ versus each control (no inhibitor); #, $P<0.01$ versus control.

First, we present evidence that the luteal PLA_2 activity and $PGF_{2\alpha}$ content increase just before parturition. Plasma progesterone was maintained at a high level until PRG21 and declined to the basal level on PRG22 (Table 1), indicating the accomplishment of functional luteolysis on PRG22. In contrast, PLA_2 activity was sustained at a basal level until PRG21, increased from PRG22 and further increased on PRG23 (to 494% of PRG19, $P<0.05$). Previous researchers did not detect a rise in PLA_2 activity until 1–2 days after parturition [17]. This discrepancy can possibly be explained by the fact that, in that study, the activity in a whole ovarian microsome was measured using a method somewhat different from more conventional ones, including ours [7–9,11,14]. We consider our present data to be more reliable and to reflect the luteal nature during functional regression with accuracy. This might be supported by the finding that luteal $PGF_{2\alpha}$ showed a marked (4.7-fold) increase from PRG21 to PRG22 followed by a slight decrease on PRG23 (Table 1). Olofsson et al. [18] demonstrated that in vivo levels of $PGF_{2\alpha}$, PGE_2 and prostacyclin in rat CLs were decreased from PRG7 and suppressed at a very low level until PRG19 and that all three prostanoids showed a quite similar pattern. Taken these findings, our data support the CL as the critical source of the luteolytic PG and indicate that luteal PG levels are suppressed in the late pregnancy and dramatically increase before parturition. Since induction of timely luteolysis is obligatory for a successful delivery, the tightly regulated mechanism of luteal PLA_2 activation and $PGF_{2\alpha}$ synthesis must operate in late pregnancy.

We also found the sustained increase in the PLA_2 activity and $PGF_{2\alpha}$ level during early post-partum. Functionally regressed rat CL undergo the following structural involution under the luteolytic influence of PRL [19] and its secretion by the pituitary in post-partum rats is induced by pups' suckling stimulus [20]. We confirmed that the weight of a post-partum CL of mothers with suckling stimulus decreased to 46.7% until PP8 (Fig. 1A). Histological examination verified the decreasing size and fibrotic replacement of a pregnant CL (Fig. 3A). Luteal PLA_2 activity showed a transient but significant increase on PP5 and decreased gradually on PP8 (Fig. 1B). Accordingly, the luteal $PGF_{2\alpha}$ level showed a quite similar temporal pattern (Fig. 1C). These findings may also implicate PLA_2 and its metabolite $PGF_{2\alpha}$ in structural luteolysis, especially in its early period.

Employing the delayed structural luteolysis model, we measured the luteal PLA_2 activity and $PGF_{2\alpha}$ level. The CL of the mother without suckling stimulus became smaller but significantly larger than that of the mother with suckling stimulus on PP8 (Fig. 1A). Histological studies established the existence of a large-sized pregnant CL and a slightly discernible appearance between the pregnant CL and the new CL derived from post-partum ovulation (data not shown). In these PRL-depleted mothers, the fluctuation in luteal PLA_2 activity was altered and appeared to be delayed by the augmentation of enzyme activity (Fig. 1B). It is more notable that luteal $PGF_{2\alpha}$ was potently suppressed as late as PP8 (Fig. 1C). Since these changes might be induced by the deficiency or

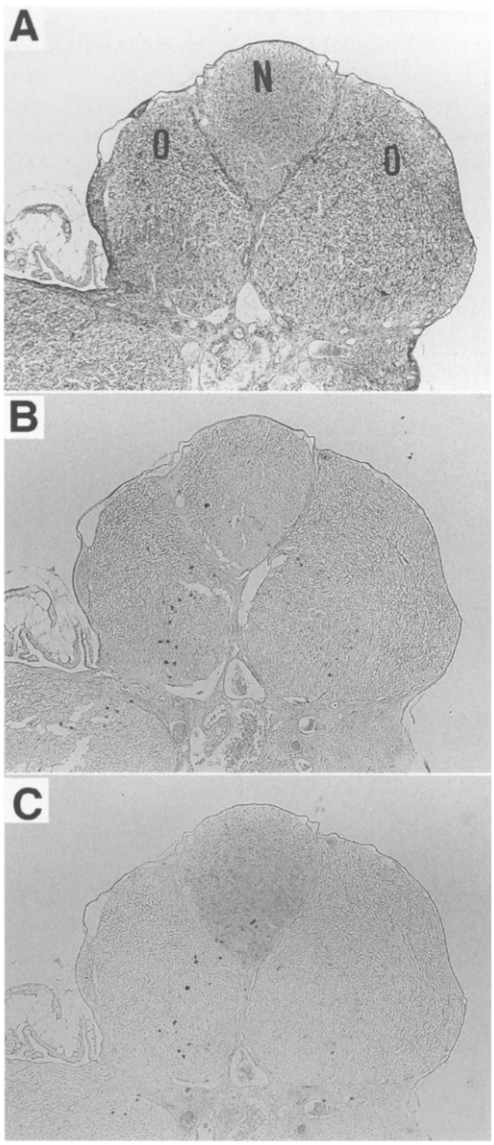

Fig. 3. Immunohistochemistry of $cPLA_2$ and PLA_2-IB in post-partum CLs. The mother with suckling stimuli was killed on PP8 and the ovaries were examined histologically. (A) HE staining of the ovary possessing pregnant CLs (O) and new CLs derived from post-partum ovulation (N). (B) anti-$cPLA_2$ staining (×1000), (C) anti-PLA_2-IB staining (×3000). Note that the pregnant and new CLs exhibit a similar immunoreactivity for $cPLA_2$ but distinct reaction for PLA_2-IB. Magnification, ×40.

absence of PRL, we subsequently tested the possible reversal effect of exogenous PRL. We found a small but significant decrease in weight, a subtle increased PLA_2 activity and a drastic rise in $PGF_{2\alpha}$ in PRL-treated CLs (Fig. 1). As PRL is shown to up-regulate PLA_2-IB and cyclooxygenase-2 (COX-2) expression, thus stimulating PGE_2 production in uterine stromal cells [21], the pituitary hormone may provoke luteal PG synthesis through activation of the COX system as well as the PLA_2 system. Collectively, our data provide evidence that PRL-induced structural luteolysis involves the enhanced PG synthesis, being partially mediated by PLA_2 activation.

We characterized PLA_2 enzymes in pregnant rat CLs using various inhibitors and immunohistochemistry. Although $cPLA_2$ was assumed to be the primary isoform in a pseudopregnant rat CL [5,7], ATK was not so effective in pregnant CLs on either PP0 or PP5 (25–35% inhibition) (Fig. 2). We identified a slight signal for $cPLA_2$ in pregnant CLs as well as in newly formed CLs (Fig. 3B). Our results suggest the presence but less important role of $cPLA_2$ in pregnant CLs. Rather, BEL and MAFP were more potent, suggestive of the substantial contribution of $iPLA_2$. Additionally, DTT was also more effective. Since immunoreactive PLA_2-IB was found exclusively in newly formed CLs (Fig. 3C), the DTT-sensitive activity may be expressed by PLA_2-IIA. From obtained results, we speculate that several isoforms of PLA_2 ($cPLA_2$, $iPLA_2$ and $sPLA_2$) may exist and function concertedly in pregnant rat CLs. Luteolysis is considered as the apoptotic cell death process. Stimulation of the apoptosis signalling receptors such as tumor necrosis factor receptor and Fas often leads to the activation of PLA_2, including $cPLA_2$ [22–24], $iPLA_2$ [25] and $sPLA_2$ [24,26]. Further studies are needed to clarify the complex mechanism of PLA_2 regulation in apoptosis of pregnant rat CLs.

In summary, this study is the first demonstration that the luteal PLA_2 activity in pregnant rats rises during functional luteolysis at term and more prominently during structural luteolysis after delivery. The increased activity may partially mediate PRL-induced structural regression through $PGF_{2\alpha}$ production.

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