Cytosolic phospholipase A_2 in rat decidual cells: evidence for its role in decidualization

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Abstract We investigated the existence and possible role of cytosolic phospholipase A_2 ($cPLA_2$) in rat decidualized uteri. PLA_2 activity in the cytosol of a decidualized uterine horn, induced by intraluminal oil infusion, was significantly higher than that in contralateral intact horn. The activity was almost completely depressed by $cPLA_2$ inhibitors including arachidonyl trifluoromethyl ketone (ATK). The immunoreactive signals for $cPLA_2$ were intense in decidua and glandular epithelial cells. In vivo administration of ATK (0.1–100 μg) caused a dose-dependent inhibition of decidualization. These results show the presence of $cPLA_2$ and its probable implication in decidualization in rat uterus.

Key words: Cytosolic phospholipase A_2; Immunohistochemistry; Decidualization; Rat

1. Introduction

Phospholipase A_2 (PLA_2) is a growing superfamily of enzymes that cleave glycerophospholipid into fatty acid and lysophospholipids [1]. When the fatty acid is arachidonic acid (AA), it not only acts as a multiple intracellular effector but also serves as the precursor of eicosanoids. On the other hand, lysophospholipids may be metabolized into a platelet-activating factor (PAF) and lysophosphatidic acid. Thus, PLA_2 has a central role in the biosynthesis of phospholipid-derived mediators.

Mammalian PLA_2s are largely classified into four groups [1]. The first group enzymes ($sPLA_2$) with 14 kDa are secreted and dependent on millimolar level Ca^{2+}. The second group ($cPLA_2$) with 85 kDa is cytosolic and activated by submicromolar Ca^{2+}. The third group ($iPLA_2$) with 80–85 kDa is also cytosolic but Ca^{2+}-independent. The last group enzymes are Ca^{2+}-independent and also have an acetylhydrolase activity for PAF. From the four groups, $cPLA_2$ has attracted much attention, since it preferentially hydrolyses AA-containing substrates. Moreover, its activity is stimulated by the elevated intracellular Ca^{2+} and mitogen-activated protein kinases cascades [2]. These enzymatic characteristics clearly implicate the fundamental roles of $cPLA_2$ in various cellular processes [2].

The uteri exhibit multiple reproductive functions through the generation and action of several prostaglandins (PGs). These include decidualization [3], the initiation of luteolysis [4], and smooth muscle contraction and cervical ripening at parturition [5]. Many studies indicated the intrauterine distribution and functional role of $sPLA_2$ [6–8] and cyclooxygenase (COX) which metabolizes AA to PGH_2 [9–11]. Recent evidence using Western or Northern blot analysis has shown $cPLA_2$ expression in uteri of several species [7,12–15]. However, the defined cellular distribution and the physiological intervention of $cPLA_2$ still remain unclear. To address this issue, we investigated the localization and characterization of uterine $cPLA_2$ activity and its implication in decidualization using rats.

2. Materials and methods

2.1. Antibody and chemicals

The specific antibody for $cPLA_2$ was donated by the Genetics Institute (Cambridge, MA, USA). Radiolabeled 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 $cPLA_2$ [16], methyl arachidonylfluorophosphonate (MAFP), which inhibits both $cPLA_2$ and $iPLA_2$ [17], and bromoenol lactone (BEL), which inhibits $iPLA_2$ [17], were purchased from Cayman (Ann Arbor, MI, USA). All other reagents were of analytical grade.

2.2. Animals and decidualization

Adult female rats of Wistar-Imamichi strain were used for this study. Pseudopregnancy was induced by mating with vasectomized male rats in the proestrous evening. To induce the artificial decidualization, sesame oil (50 μl) was introduced into one (right side) uterine horn using a syringe and a 23 G needle on day 4 of pseudopregnancy (PSP4). The contralateral intact horn served as control. Four days later (on PSP8), animals were decapitated, and the wet weight of each infused or non-infused uterine horn was measured. The fold increase in horn weight was used as an index of decidualization. Tissues were instantly stored frozen at −80°C until PLA_2 activity assay. Uteri were also sampled for histological examination and $cPLA_2$ immunohistochemistry.

2.3. Histology and immunohistochemistry of uterine $cPLA_2$

Uteri were fixed in Bouin's fixative containing $HgCl_2$, dehydrated, and embedded in paraffin. Tissues were serially sectioned (6 μm in thickness), deparaffinized, and stained. Some sections were stained by hematoxylin and eosin (H.E.). Immunohistochemistry of uterine $cPLA_2$ was performed as described previously [18,19]. Briefly, endogenous peroxidase activity was blocked by pretreatment with 0.3% H_2O_2 in methanol for 30 min. Sections were pretreated with 0.4% non-specific rabbit serum (NRS) in an antiserum binding buffer (0.15 M NaCl, 5 mM EDTA, 0.05 M Tris, 0.25% gelatin, and 0.05% Nonidet P40) for 1 h to reduce the non-specific background staining. Tissue sections were then treated with the antibody (1:250) at 4°C for 12 h, followed by incubation with horseradish peroxidase-labeled Protein A for another 2 h. Bound peroxidase was revealed by treatment with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 for 15 min. The control method without the antibody or using NRS (1:250) instead of the antibody gave no positive reaction (data not shown), as noted before [18,19].

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Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; PG, prostaglandin; AA, arachidonic acid; COX, cyclooxygenase; PAF, platelet-activating factor

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2.4. Assay of PLA_2 activity in uterine cytosol

PLA_2 activity in uterine cytosol was measured by the previous method with a slight modification [19,20]. Briefly, uteri were minced 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 $105\,000 \times g$ for 1 h, and the supernatants (cytosol) were processed for protein determination using a Bio-Rad protein assay kit. Liposome substrate was prepared with radiolabeled and non-radiolabeled 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, and 2 μM PC. In the experiment to pharmacologically characterize PLA_2 activity, the mixtures further contained 5 μM ATK, 20 μM MAFP, 50 μM BEL, or 5 mM dithiothreitol (DTT), the $sPLA_2$ inactivator [21]. The reaction was performed at 37°C for 1 h and terminated by adding 1.0 ml Dole's reagent. The released fatty acid was extracted and measured for its radioactivity by liquid scintillation counting.

2.5. Administration of ATK in vivo

A single injection of ATK (0.1–100 μg), dissolved in oil, was administered into the right side horn on PSP4. The contralateral untreated horn served as control. To test possible reversal effects of exogenous PGs on ATK-inhibited decidualization, PGE_2, $PGF_{2\alpha}$, or carbaprostacyclin, a stable analogue of prostacyclin (PGI_2), (each 10 or 100 μg) combined with ATK (100 μg) was injected into the left side horn. In this experiment, the other horn receiving only the inhibitor was used as control. Animals were killed on PSP8, and the wet weights of their uterine horns were measured.

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 significant.

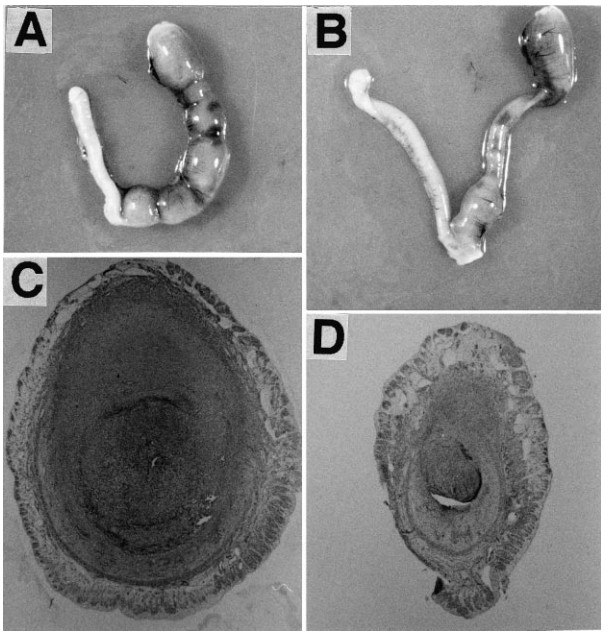

Fig. 1. Photograph and histology of decidualized uteri. Photos of uteri showing normal decidual reaction to oil (A) and a uterus injected with ATK (100 μg) (B). Cross sections stained by H.E. of a decidualized horn (C) and the middle portion of ATK-treated horn (D). Magnifications, A and B, ×1; C and D, ×10.

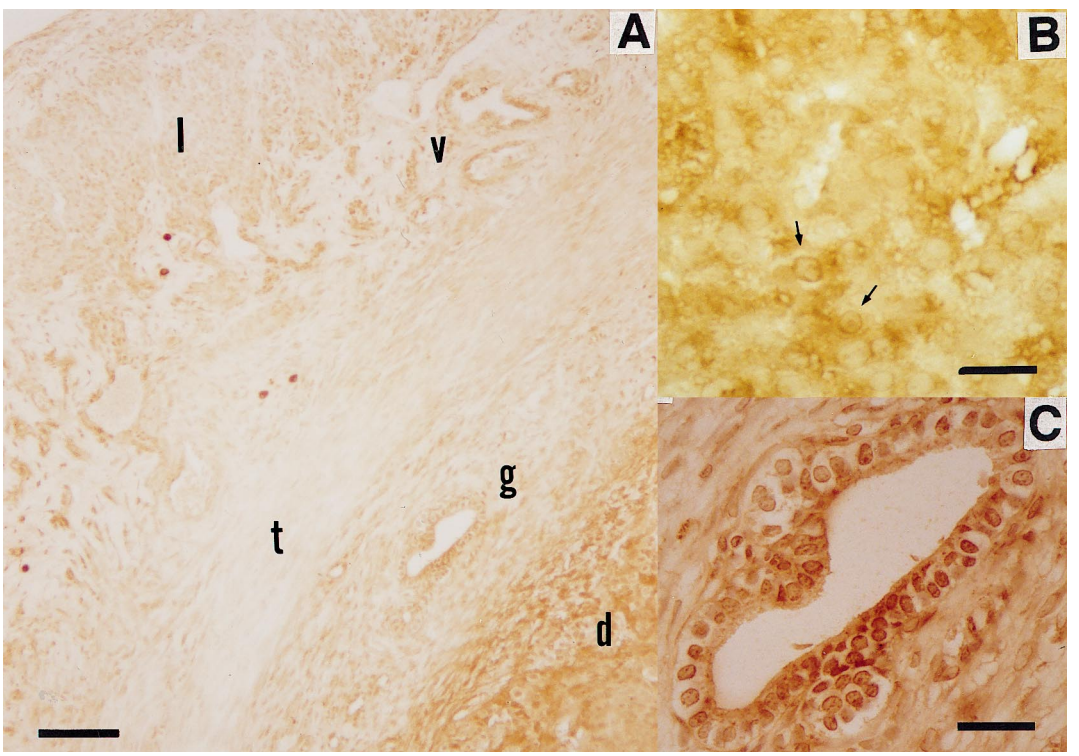

Fig. 2. Immunohistochemical distribution of $cPLA_2$ in uterus. A: Immunoreactive signals were intense in decidua (d) and uterine gland (g), limited in longitudial muscles (l) and blood vessels (v), and negative in transverse muscles (t). B: Positive signals were scattered in the cytoplasm in most cells and localized to nuclear membranes in some cells (arrows) of decidua. C: Epithelial cells of uterine glands were characteristic to nuclear localization of $cPLA_2$. Bars: A, 10 μm; B, C, 2.5 μm.

3. Results

3.1. Immunohistochemical demonstration of cPLA$_2$ in decidualized uteri

The current method successfully induced decidualization with the contralateral horn intact (Fig. 1A). The weight ratios of the injected horn to the another ranged 3.8 to 7.9 (5.4 ± 0.7, $n = 7$). Histology of the transverse section in the treated horn revealed massive proliferation of the stromal cell, which is the hallmark of decidualization (Fig. 1C). The immunohistochemistry demonstrated positive signals for cPLA$_2$ in decidua, uterine glands, and in blood vessels in rat uterus (Fig. 2A). Positive signals were scattered in the cytoplasm of most decidual cells as well as in the nucleus of some cells (Fig. 2B). Glandular epithelial cells showed a remarkably preferential immunoreaction in nuclear envelopes (Fig. 2C). Interestingly, longitudinal smooth muscles were faintly positive, while transverse smooth muscles were negative (Fig. 2A).

3.2. cytosolic PLA$_2$ activity in decidualized uteri

The characterization of cytosolic PLA$_2$ activity in decidualized uteri is shown in Fig. 3. PLA$_2$ activities in decidualized and intact horns were 3.32 ± 0.63 pmol/mg/min and 1.220.51, respectively, generating a significant difference ($P < 0.05$). ATK almost repressed PLA$_2$ activity in decidualized uteri ($P < 0.01$). In the presence of MAFP, the activity was below the detectable limit. BEL and DTT had slightly and no inhibitory effect, respectively.

3.3. Inhibitory actions of ATK on decidualization

Effects of ATK challenge in vivo on decidualization are shown in Figs. 1 and 4. Administration of 100 μg ATK-inhibited decidualization, whose typical specimen is shown in Fig. 1B. The drug-treated horn was generally thin in the middle and, occasionally, in the lower part adjacent to the cervix as well. Histological examination revealed a subtle proliferation of stromal cells, confirming reduced decidual reaction (Fig. 1D). As the uterus showed normal decidualization in both edges of the horn, ATK effect might be spatially limited. The inhibitory effect of ATK was clearly dose-dependent, and the significant decrease in the decidualization index was achieved with as little as 1.0 μg drug (Fig. 4A). Plasma progesterone levels, determined by radioimmunoassay [20], were not altered among experimental groups (data not shown). No experimental groups of exogenous PG induced a significant increase in the horn weight compared to that of the inhibitor alone (Fig. 4B). However, PGE$_2$ or prostacyclin (each 100 μg) showed the modest up-regulatory effect, without significant differences compared to that of the vehicle group.

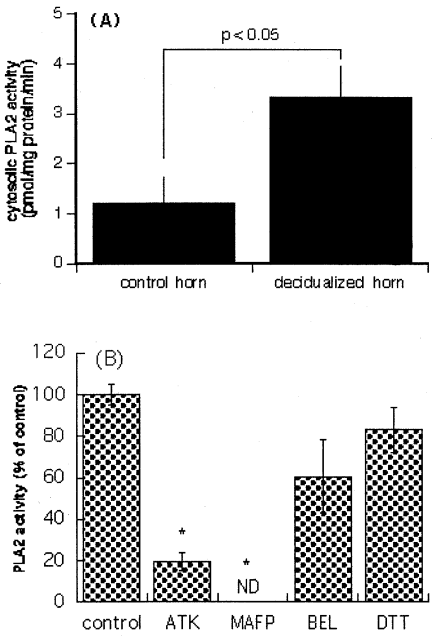

Fig. 3. Characterization of cytosolic PLA$_2$ activity in decidualized uteri. A: Comparison of cytosolic PLA$_2$ activities between decidualized and intact horns. Data are mean ± S.E.M. ($n = 4$). B: Effects of ATK (10 μM), MAFP (20 μM), BEL (50 μM), and DTT (5 mM) on cytosolic PLA$_2$ activity in decidualized uteri. Data are mean ± S.E.M. ($n = 4$). *, $P < 0.01$ vs. control group. ND, not detectable.

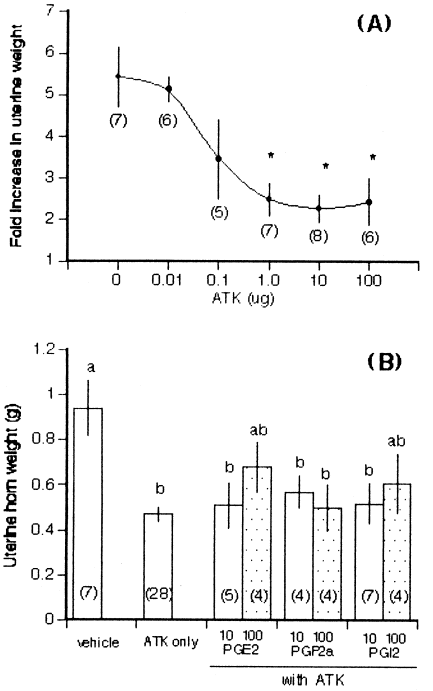

Fig. 4. Effects of ATK with or without PGs on decidual reaction. A: Dose-dependent inhibition of decidualization by ATK (0.1–100 μg). Data are mean ± S.E.M. of indicated numbers of samples. *, $P < 0.05$ vs. vehicle-treated group. B: Effects of a vehicle, ATK (100 μg) with or without PGE$_2$, PGF$_{2\alpha}$, or PGI$_2$ (10, 100 μg) on uterine horn weights. Data are mean ± S.E.M. of indicated numbers of samples. Different alphabetical letters show the significant differences among the groups ($P < 0.05$).

4. Discussion

This is the first characterization of the immunohistochemical distribution and enzymatic activity of cPLA$_2$ in mammalian decidualized uteri. We further show its probable implication in decidualization in vivo using a specific inhibitor.

Initially, we described the abundant distribution of immunoreactive cPLA$_2$ in decidua and epithelial cells of uterine glands in rat uterus. Moreover, our results provide, as far as we know, the first demonstration of the primary localization of cPLA$_2$ to nuclear envelopes in vivo. This was revealed in rat basophilic leukemia cells [22] and CHO cells overex-

pressing $cPLA_2$ [23] as well. The signal is also scattered in the cytoplasm of most decidual cells. The slight difference in its subcellular location between cells may be dependent on cellular activated states, since $cPLA_2$ translocate to nuclear envelopes upon Ca^{2+} mobilization. COX-2, an inducible-type isoform, also localizes mainly in nuclear envelopes of several cell types [24], suggesting its functional linkage with $cPLA_2$ [25]. Since COX-2 becomes expressed in stromal cells and epithelial cells surrounding the implanting site on day 4 of pregnancy (PRG4) in mice [10,11], decidua and luminal epithelium are reinforced as potential sources of PGs.

Next, we examined whether the present decidualization model involves the expression of PLA_2 activity. Cytosolic enzyme activity in the decidualized horn was significantly higher than that in the intact horn, the enhanced PG production being supported by decidua and in part by the uterine gland. The pharmacological study showed that the enhanced activity was mostly suppressed by ATK and, more intensely by MAFP. Since BEL showed a slight effect, the modest contribution of $iPLA_2$ is possible. These results, which are consistent with the immunohistochemical data, strongly suggest the increased activity and the primary role of $cPLA_2$ in decidualized uteri.

Finally, we explored the effect of the $cPLA_2$ specific inhibitor on decidualization in vivo. While MAFP effect was not examined, ATK caused a dose-dependent inhibition of decidualization. Its inhibitory effect was likely to be confined to the middle part of the horn, where the drug was primarily distributed. We histologically verified the impaired formation of decidua, but found the unaltered function to stimulate corpus luteum at least as late as PRG8. Although ATK has also been shown to inhibit the COX pathway [26,27], the challenged inhibitor must have affected $cPLA_2$. The intraluminal concentration of the agent, when injected with more than 1.0 μg, was probably above the level used in vitro (10 μM), and at that range we found the significant inhibition.

It is widely recognized that PGs play important roles in decidualization in rodents [3]. Kennedy found that the inhibition of decidualization by a COX inhibitor, indomethacin, was optimally reversed by a chronic infusion of PGE_2 or $PGF_{2\alpha}$ in rats [28]. In the present study, however, PGE_2 and prostacyclin had a slight effect in restoring ATK-inhibited decidualization. Recent study using COX-2-deficient mice also observed limited effects of prostacyclin and PGE_2 [11]. Our present dose was sufficient, as determined by the above study [28], although the use of a single injection and the instable nature of the ligands may be responsible. The inability of exogenous PG to optimally rescue $cPLA_2$ inhibition is possibly due to novel effects of intranuclear PGs. As supported by immunohistochemical data, $cPLA_2$ in combination with COX-2 may exert the nuclear mitogenic activity required for massive decidualization through nuclear PG generation. In addition to PGs, PAF also stimulates decidualization [3,29], further supporting a major role for $cPLA_2$ in this process.

In summary, this study shows increased $cPLA_2$ activity in rat decidualized uteri and immunoreactive $cPLA_2$ localization in decidual cells and glandular epithelial cells, preferentially in nuclear envelopes. Pharmacological inhibition of its activity resulted in impaired decidualization. These data collectively show its probable implication in decidualization through generation of bioactive lipid mediators such as PG.

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