Localization of various secretory phospholipase A\textsubscript{2} enzymes in male reproductive organs

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Abstract

Current evidence suggests the presence of transcripts for several secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) enzymes in male genital organs. In this study, we examined by immunohistochemistry the localization of group IIA, IIC, IID, IIE, IIF, V and X sPLA\textsubscript{2}s in male genital organs. In sPLA\textsubscript{2}-IIA-deficient C57BL/6 mouse testis, sPLA\textsubscript{2}-IIC, -IID, -IIE, -IIF, -V and -X were diversely expressed in spermatogenic cells within the seminiferous tubules. Immunoblotting revealed the presence of these sPLA\textsubscript{2}s in mouse spermatozoa. In addition, sPLA\textsubscript{2}-IIF, -V and -X were localized in the interstitial Leydig cells. The same set of sPLA\textsubscript{2}s was detected in a mouse cultured Leydig cell line, and adenovirus-mediated transfer of these sPLA\textsubscript{2}s into Leydig cells resulted in increased prostaglandin production. sPLA\textsubscript{2}-IIC, -IID, -IIE, -IIF, -V and -X were also detected diversely in the epithelium of the epididymis, vas deferens, seminal vesicles, and prostate. In a sPLA\textsubscript{2}-IIA-positive FVB strain, weak expression of sPLA\textsubscript{2}-IIA was detected in Leydig cells. Notable differences in the sPLA\textsubscript{2} expression profiles were found in the seminal vesicles and prostate between mice and humans. Taken together, individual sPLA\textsubscript{2}s exhibit distinct or partially overlapping localizations in male reproductive organs, suggesting both specific and redundant functions.

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1. Introduction

Secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) is a group of enzymes with conserved catalytic site and Ca\textsuperscript{2+}-binding loop, and catalyzes the release of fatty acids and lysophospholipids from the sn-2 position of glycerophospholipids in the presence of Ca\textsuperscript{2+}. To date, 10 sPLA\textsubscript{2} enzymes (sPLA\textsubscript{2}-IIA, -IIB, -IIC, -IID, -IIE, -IIF, -III, -V, -X, and -XIIA) have been identified in mammals [1]. sPLA\textsubscript{2}s belonging to the I/II/V/X branch may have arisen from recent and successive gene duplication events, whereas group III and XII sPLA\textsubscript{2}s are distantly related. Since the genes for sPLA\textsubscript{2}-IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, and -XIIA have been identified in mammals [1], sPLA\textsubscript{2}s are found in diverse biological events, such as arachidonic acid metabolism, cell signaling, and inflammation.
(AA) metabolism [2–12], digestion of dietary phospholipids [13], atherosclerosis [14–16], anti-bacterial defense [17–20], and oncogenesis [21]. However, the precise physiological functions of individual sPLA₂S are still obscure.

During the last two decades, the presence of sPLA₂S in male genital organs and seminal plasma of several animal species has been demonstrated in several studies [22–33]. Initial biochemical analyses showed that a PLA₂ activity exists in spermatozoa and that PLA₂ may be an important factor for the acrosome reaction and fertilization [22–24]. An sPLA₂ purified from human seminal plasma was ascribed to sPLA₂-IIA on the basis of its N-terminal sequence [25]. Immunohistochemical analyses using antibodies raised against sPLA₂-IB or -IIA demonstrated positive staining in prostate [26, 27], cauda epididymal spermatozoa [28] and seminal vesicles [27], according to animal species. However, recent evidence for the presence of transcripts for several sPLA₂S (IIA, IIC, IID, IIE, IIF, V and X) in human and mouse male genital organs [29–31] has raised a question as to the cross-reactivity of the previously used anti-sPLA₂ antibodies among sPLA₂ isozymes. Although Langlais et al. [32] purified a 17-kDa PLA₂ from human spermatozoa, its N-terminal amino acid sequence does not fit that of any of the mammalian sPLA₂S so far known. Expression of sPLA₂-IIIC, a rodent-specific enzyme, in mouse spermatogenic cells within the testicular seminiferous tubules has been demonstrated by in situ hybridization [33].

In this study, we evaluated the localization of various sPLA₂S (IIA, IIC, IID, IIE, IIF, V and X) in mouse male reproductive organs. We show that individual sPLA₂S display unique cellular distributions in the testis, epididymis, vas deferens, seminal vesicles and prostate. There are some species-associated differences in the expression profiles of sPLA₂S, particularly in the prostate. On the basis of the results, possible functions of each sPLA₂ in the male genital organs are discussed.

2. Materials and methods

2.1. Materials

Inbred C57BL/6 and FVB strains of mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Procedures for animal handling and preparation of mouse and human tissue sections were approved by the ethical committee of our Faculty. Mouse Leydig cell line I-10 were obtained from Human Science Research Resources Bank (Osaka, Japan) and were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10% (v/v) fetal calf serum (FCS; Böseserum). Specific antibodies for various mouse and human sPLA₂S were described previously [34,35]. Goat antibodies against human cylooxygenase (COX)-1 and human COX-2, and a rabbit antibody against human group IVA cytosolic PLA₂ (cPLA₂α) were purchased from Santa Cruz. Antibodies against cytosolic PGE synthase (cPGEs) and microsomal PGE synthases (mPGES-1 and -2) were described previously [36–38]. Preparation of cDNAs for sPLA₂S and cPLA₂α was described previously [2–5]. The enzyme immunoassay kit for PGE₂ was purchased from Cayman Chemicals. Human interleukin (IL)-1β was purchased from Genzyme. Primers for reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Greiner Japan. Other reagents of analytical grade were from Wako Chemicals.

2.2. RT-PCR

Synthesis of cDNA was performed using 0.5 µg of total RNA from cells and tissues and AMV reverse transcriptase, according to the manufacturer’s instructions supplied with the RNA PCR kit (Takara Biomedicals). Subsequent amplification of the cDNA fragments was performed using 0.5 µl of the reverse-transcribed mixture as a template with specific primers for each sPLA₂. For amplification of sPLA₂-IIA, -IIC, -IID, -IIE, -IIF, -V, and -X, a set of 23-bp oligonucleotide primers corresponding to the 5’- and 3’- nucleotide sequences of their open reading frames were used as primers [29–31]. The PCR conditions for sPLA₂-IIA, -IIC, -IID, -IIE, -IIF, -V, and -X were 94 °C for 30 s and then 30 cycles of amplification at 94 °C for 5 s and 68 °C for 4 min, using the Advantage cDNA polymerase mix (Clontech). The PCR conditions for sPLA₂-IIIF were 94 °C for 40 s and then 30 cycles of amplification at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with ExTaq polymerase (Takara Biomedicals). The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide. The gels were further subjected to Southern blot hybridization with sPLA₂ cDNAs as probes, as required for the experiments.

2.3. Northern blotting

Equal amounts (10 µg) of total RNA obtained from cells by TRIzol reagent (Invitrogen) were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with the respective cDNA probes that had been labeled with [32P]dCTP (Amersham Bioscience) by random priming (Takara Biomedicals). All hybridizations were carried out as described previously [2–5].

2.4. SDS-PAGE/immunoblotting

Lysates from 2×10⁵ cultured cells or 50-µg protein equivalents of tissue or sperm homogenates in phosphate-buffered saline (PBS) were subjected to SDS-PAGE using 7.5% (for cPLA₂α and COXs), 12.5% (for PGESs) and 15% (for sPLA₂s) gels under reducing (for cPLA₂α, COXs and PGESs) and non-reducing (for sPLA₂s) conditions. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotter.
Immunohistochemistry

Immunostaining of mouse genital organs was performed as described previously [39]. After deep anesthesia with ether, mice were perfused via the left ventricle of the heart with PBS containing 4% (w/v) sucrose and 4% (w/v) paraformaldehyde and then with Bouin’s solution. Genital organs were removed and soaked overnight at 4 °C in Bouin’s fixative. After being embedded in paraffin, the tissues were sectioned and mounted on slides. The sections were then deparaffinized in xylene and rehydrated in ethanol with increasing concentrations of water. Endogenous peroxidase activity was quenched with 0.3% (v/v) hydrogen peroxide in PBS for 30 min at room temperature. The tissues were then treated for 1 h at room temperature with 10% (v/v) sheep serum in PBS containing 0.1% (v/v) Triton X-100 and 0.1% (w/v) sodium azide for blocking. The tissue sections were then sequentially treated with antisera for individual mouse sPLA2s (1:200 dilution) for 1 day at 4 °C and with biotinylated anti-rabbit antibody (1:500 dilution) for 2 h at room temperature. Primary and secondary antibodies were diluted in PBS containing 1% (v/v) sheep serum (to avoid nonspecific binding) and 0.1% Triton X-100. A Vectastain Elite avidin-biotin-peroxidase kit (Vector Laboratories, Inc.) with diaminobenzidine substrate was used according to the manufacturer’s protocol. The tissues were counterstained with hematoxylin. Finally, sections were dehydrated, rinsed in xylene, and mounted with Mount-Quick (Daido Sangyo, Tokyo, Japan).

Immunohistochemical staining of human tissue sections was performed as described previously [37,38]. Briefly, the tissue sections were incubated with Target Retrieval Solution (DAKO) as required, incubated for 10 min with 3% (v/v) H2O2, washed three times with PBS for 5 min each, incubated with 5% (v/v) skim milk for 30 min, washed three times with PBS–TWEEN for 5 min each, and incubated for 2 h with anti-human sPLA2 antibodies (1:100 dilution in PBS). Then the sections were treated with the CSA system staining kit (DAKO) with diaminobenzidine substrate.

2.6. Expression of PLA2s by the adenovirus system

Adenoviruses bearing individual PLA2 cDNAs were prepared with the ViralPower Adenovirus Expression System (Invitrogen) according to the manufacturer’s instruction. Briefly, the full-length cDNAs for sPLA2s and cPLA2α were subcloned into the pENTER/D-TOPO vector by using the pENTER Directional TOPO Cloning kit (Invitrogen). After purification of the plasmids from the transformed Top10 competent cells (Invitrogen), the cDNA inserts were transferred to the pAd/CMV/V5-DEST vector (Invitrogen) by means of the Gateway system using LR clonase (Invitrogen). The plasmids were purified and digested with PacI (New England BioLabs). The linearized plasmids (1–2 µg) were then mixed with 4 µl of Lipofectamine 2000 (Invitrogen) in 200 µl of Opti-MEM medium (Invitrogen) and transfected into subconfluent 293A cells (Invitrogen) in 1 ml of Opti-MEM in six-well plates (Iwaki Glass). Then the 293A cells were cultured for 1–2 weeks in RPMI 1640 medium containing 10% FCS, with replacement of the medium every 2 days. When most cells became detached from the plates, the cells and culture medium were harvested together, freeze-thawed twice, and centrifuged to obtain the adenovirus-enriched supernatants. Then aliquots of the supernatants were added to fresh 293A cells and cultured for 2–3 days to amplify adenoviruses. After two to four cycles of amplification, the resulting adenovirus-containing media were used as virus stocks. Viral titers were determined by the plaque-forming assay with 293A cells. As a control, the pAd/CMV/V5-GW/lacZ vector (Invitrogen) was digested with PacI and transfected into 293A cells to produce LacZ-bearing adenovirus.

Aliquots of the adenovirus-containing medium were added to I-10 cells grown in 24-well plates (Iwaki Glass) and cultured for up to 36 h in the presence of 1 ng/ml IL-1β at 37 °C in a CO2 incubator. Then the supernatants were subjected to the sPLA2 enzyme assay and PGE2 enzyme immunoassay, and the cells were taken for Northern blotting to assess the expression of PLA2s.

2.7. Measurement of sPLA2 activity

sPLA2 activity was assayed by measuring the amounts of radiolabeled linoleic acid released from the substrate 1-palmitoyl-2-[14C]linoleoyl-phosphatidylethanolamine (Amersham Bioscience). The substrate in ethanol was dried under a stream of N2 and was dispersed in water by sonication. Each reaction mixture (total volume 250 µl) consisted of appropriate amounts of the required sample, 100 mM Tris–HCl (pH 7.4), 4 mM CaCl2 and 10 µM substrate. After incubation for 20 min at 37 °C, [14C]linoleic acid was extracted by Dole’s method, and the radioactivity was quantified by liquid scintillation counting, as described previously [2].

3. Results

3.1. Detection of sPLA2s in mouse genital organs by RT-PCR and immunoblotting

Expression of transcripts for various sPLA2s in genital organs of male C57BL/6 mice (testis, epididymis, vas
deferens, seminal vesicles and prostate) was examined by RT-PCR (Fig. 1A). "sPLA2-IID was expressed almost equally in the testis, epididymis, vas deferens and seminal vesicles, and weakly in the prostate. Although the expression of sPLA2-IIE was lower than that of other sPLA2s, it could be detected throughout the genital organs, with expression in the vas deferens being highest. Expression of sPLA2-IIF was highest in the testis, followed by the epididymis and vas deferens, whereas its expression in the seminal vesicles and prostate was weak, although significant. Expression of sPLA2-V was high in the testis and epididymis, and modest in the vas deferens, seminal vesicles and prostate. Thus, sPLA2-IIF and -V were preferentially distributed in the upper genital organs. Expression of sPLA2-X was intense in the testis and seminal vesicles, faint in the epididymis and vas deferens, and rather weak in the prostate. In the testis, vas deferens and seminal vesicles, the sPLA2-X transcript appeared as a doublet; sequencing of each DNA fragment revealed that the upper band corresponded to an alternative splicing variant that has a 99-bp insertion between exons 3 and 4 [40]."

We next investigated the expression of sPLA2 proteins in the testis, epididymis, and mature spermatozoa of C57BL/6 mice by immunoblotting with antibodies raised against individual sPLA2s. Specificity of these antibodies was confirmed by immunoblotting with authentic recombinant sPLA2s as well as with cell lysates transfected with individual sPLA2s [34,35]. As shown in Fig. 1B, sPLA2-IIC, -IID, -V and -X, but not sPLA2-IIE (probably because its expression level is too low to be detected by immunoblotting) or sPLA2-IID, were each detected in the testis and epididymis at the expected molecular sizes (14–18 kDa) (Fig. 1B). These four sPLA2s were also detected in spermatozoa (Fig. 1C). Of note, sPLA2-IIF was detected as a major 25-kDa band (larger than the expected 18-kDa size) and a minor 45-kDa band in the testis and epididymis (Fig. 1B). This result suggests that a portion of this enzyme may exist as a homodimer (as has been proposed [30]) or that this enzyme may undergo posttranslational modification (such as glycosylation) in these tissues. In contrast, only the smaller form of sPLA2-IIF was detected in spermatozoa (Fig. 1C).

3.2. Immunohistochemistry of sPLA2s in the testis

To determine the cellular origins of sPLA2s in these tissues, we performed immunohistochemistry with antibodies specific for individual enzymes. sPLA2-IIA (used as a negative control) provided no detectable staining signals in C57BL/6 mouse testis (Fig. 2A). sPLA2-IIC was stained in the whole area of spermatogenic cells within the seminiferous tubules, with spermatogonia providing the Fig. 1. RT-PCR and immunoblot analyses of sPLA2 expression in male genital organs and spermatozoa of mice. (A) Total RNAs extracted from individual tissues were subjected to RT-PCR using primers specific for each sPLA2. Amplified bands were visualized in agarose gels with ethidium bromide. (B and C) Immunoblotting of mouse testis and epididymis (B) as well as spermatozoa (C) with anti-sPLA2 antibodies. Samples (50 μg protein equivalents) were applied to 15% SDS-PAGE gels and then immunoblotted with the respective antibodies.
Fig. 2. Immunohistochemical localization of sPLA₂s in mouse testis. (A) sPLA₂-IIA, (B) sPLA₂-IIC, (C) sPLA₂-IID, (D) sPLA₂-IIE, (E) sPLA₂-IIF, (F) sPLA₂-V, and (G) sPLA₂-X. sPLA₂-IIC was stained in spermatogenic cells, including spermatogonia (dark arrowheads). sPLA₂-IID was detected in the central part of the seminiferous tubules, which are enriched in late spermatids and spermatozoa. Scattered signals for sPLA₂-IIE, -V and -X were detected in the acrosomes of spermatocytes (blue arrows). Leydig cells (red arrows) were positively stained for sPLA₂-IIF and -V as well as weakly for sPLA₂-X. sPLA₂-IIF was also detected in Sertoli cells (green arrows). sPLA₂-IIA was undetectable. Panels a, ×40; panels b, ×100; and panels c, ×400.
most intense staining (Fig. 2B), consistent with a previous in situ hybridization study [33]. sPLA2-IIID gave string-like and elongated staining signals in the central part of the seminiferous tubules (Fig. 2C), suggesting its presence in elongating late spermatids and spermatozoa. Although staining of sPLA2-IIE was weak, scattered signals with crescent and elongated shapes were detectable in spermatogenic cells (Fig. 2D). These signals represent a typical feature of location in the acrosomes [41]. sPLA2-IIF (Fig. 2E) and -V (Fig. 2F) were clearly detected in Leydig cells of the interstitium. Late spermatids or spermatozoa near the central part of seminiferous tubules, as well as Sertoli cells, were also positive for sPLA2-IIF (Fig. 2E). Crescent and elongated signals for sPLA2-V, which were similar to but more intense than those for sPLA2-IIE, were seen in the seminiferous tubules, suggesting its localization in the acrosomes of spermatocytes and/or round and elongating spermatids (Fig. 2F). sPLA2-X was uniformly stained in spermatogenic cells (Fig. 2G). Again, crescent and elongated signals for sPLA2-X were obvious in the area enriched in spermatocytes and/or round and elongating spermatids (Fig. 2G), as seen with sPLA2-V (Fig. 2F). In addition, Leydig cells were weakly stained with sPLA2-X (Fig. 2G).

3.3. Studies using cultured Leydig cells

Leydig cells constitute the principal endocrine component of the testis, occupying most of the extravascular space of the interstitium. The primary role of Leydig cells is to synthesize and secrete androgenic steroids in response to gonadotropic hormones. Previous studies have shown that Leydig cells produce PGE₂, which can modify their own functions and alter seminiferous tubule contractility in autocrine and paracrine fashions [42–46]. In order to assess the potential contribution of sPLA2s to PGE₂ production in Leydig cells, we took advantage of the mouse Leydig cell line I-10. As assessed by RT-PCR, sPLA2-IIF was most intensely detected in I-10 cells (Fig. 3A). Weak expression of sPLA2-V and -X was also evident in these cells, whereas other sPLA2s (IIA, IID, and IIE) were below the detection limit (Fig. 3A). Thus, detection of the transcripts for sPLA2-IIF, -V and -X in I-10 cells was consistent with the immunohistochemical analysis of mouse testis (Fig. 2). To the best of our knowledge, this is the first demonstration of a cell line that expresses sPLA2-IIF endogenously. As assessed by immunoblotting, I-10 cells expressed cPLA2, COX-1 and -2, mPGES-1 and -2, and cPGES constitutively (data not shown), confirming that all components for PGE₂ biosynthesis are present in these cells.

To achieve efficient and prompt transfection of sPLA2s into cultured cells, we constructed adenovirus harboring each sPLA2 cDNA. Then I-10 cells were infected with adenoviruses for individual sPLA2s for 24 h, and PGE₂ released into the supernatants was quantified. Appropriate expression of each enzyme was verified by Northern
blotting (Fig. 3B). The levels of individual sPLA2s released into the supernatants of adenovirus-infected cells ranged from 10 to 20 ng/ml, as estimated by their enzymatic activities in comparison with the activities of their respective authentic recombinant sPLA2s (data not shown). As shown in Fig. 3C, there were marked increases in PGE2 production by I-10 cells transfected with sPLA2-IIF, -V and -X, a group of enzymes intrinsically expressed in mouse epididymis.

Fig. 4. Immunohistochemical localization of sPLA2s in mouse epididymis. (A) sPLA2-IIA, (B) sPLA2-IIIC, (C) sPLA2-IIID, (D) sPLA2-IIIE, (E) sPLA2-IIF, (F) sPLA2-V, and (G) sPLA2-X. Staining of the caput portion of the epididymis is shown. Panels a, ×40; panels b, ×100; and panels c, ×400. sPLA2-IIIE, -IIF, -V and -X, but not sPLA2-IIA, were located in the tubular epithelial cells of the epididymis. sPLA2-IIIE and -IIF were particularly evident in the apical epithelial cells (arrowheads). Spermatozoa (asterisks) were positive for sPLA2-IIIC, -IID, -IIF, -V and -X.
Leydig cells (see above), as well as those transfected with cPLA$_2$α, which was used as a positive control for increased AA metabolism [47]. sPLA$_2$-IIA and -IID, which were immunohistochemically undetectable in Leydig cells (Fig. 2A and C), failed to increase PGE$_2$ generation appreciably (Fig. 3C). Time course experiments showed that the increased production of PGE$_2$ after transfection with sPLA$_2$-IIF and -X occurred kinetically in parallel with their inducible expression (Fig. 3D and E). PGE$_2$ production reached a plateau peak at 12 h (Fig. 3E), at which time the expression of sPLA$_2$-IIF and -X also peaked (Fig. 3D).

3.4. Immunohistochemistry of sPLA$_2$s in other genital organs

Spermatozoa leave the testis through the efferent ductules and enter the initial segment of the epididymis. Immunostaining of different parts of the epididymis revealed that sPLA$_2$-IIC (Fig. 4B), -IID (Fig. 4C), -IIF (Fig. 4E), -V (Fig. 4F) and -X (Fig. 4G) were located in the tubular epithelial cells of the epididymis. This staining pattern was similar between the caput (Fig. 4) and other parts (the initial segment, corpus and cauda) (data not shown) of the epididymis. sPLA$_2$-IIF often provided a very strong signal in the apical epithelial cells in the caput and cauda (Fig. 4E, panels b and c). Spermatozoa within the tubules were noticeably stained with sPLA$_2$-IIC, -IID, -IIF, -V and -X (Fig. 4, panels b).

When small round slices of the vas deferens between the epididymis and seminal vesicles were immunostained, sPLA$_2$-IIC (Fig. 5B), -IID (Fig. 5C), -IIF (Fig. 5E), -V (Fig. 5F) and -X (Fig. 5G) were detected in the luminal epithelium and the cilia. More intense signals for sPLA$_2$-IID

![Image of immunohistochemical localization of sPLA$_2$s in mouse vas deferens.](image-url)
and -V were recognized in the cilia than in the epithelial cell layer; the reverse was true for sPLA$_2$-IIIF. sPLA$_2$-X was intensely stained in both the apical and basolateral surfaces of epithelial cells, and the smooth muscle cell layer was also weakly sPLA$_2$-X-positive (Fig. 5G). sPLA$_2$-IIIE was detected faintly in the apical surface of the epithelium (Fig. 5D).

In the seminal vesicular gland, sPLA$_2$-IIC (Fig. 6B), -IID (Fig. 6C), -IIF (Fig. 6E), -V (Fig. 6F), and -X (Fig. 6G) were expressed in epithelial cells. Expression of sPLA$_2$-IIIE in epithelial cells was weak but significant (Fig. 6D). In the prostate, staining for sPLA$_2$-IIIF was most intense in epithelial cells (Fig. 6E), where sPLA$_2$-IIC (Fig. 6B), -IID (Fig. 6C) and -IIE (Fig. 6D) were also positive.

![Immunoistochemical localization of sPLA,6 in mouse seminal vesicles and prostate. (A) sPLA,-IIA, (B) sPLA,-IIC, (C) sPLA,-IID, (D) sPLA,-IIIE, (E) sPLA,-IIIF, (F) sPLA,-V, and (G) sPLA,-X. Panels a indicate seminal vesicles and panels b and c show prostate. In the seminal vesicles, sPLA,-IIC, -IID, -IIF, -V, and -X were expressed in epithelial cells. Expression of sPLA,-IIIE in epithelial cells was weak but significant, whereas sPLA,-IIA was not detected. In the prostate, sPLA,-IIC, -IID, -IIE and -IIF were detected in epithelial cells, whereas sPLA,-IIA, -V and -X were undetectable. Panels a and b, ×40; and panels c, ×100.](image-url)
Although transcripts for sPLA2-V and -X were faintly detected in the prostate by RT-PCR (Fig. 1), their immunoreactivity was barely detectable (Fig. 6, F & G). As expected, sPLA2-IIA was undetectable throughout the epididymis (Fig. 4A), vas deferens (Fig. 5A), seminal vesicles and prostate (Fig. 6A).

Fig. 7. Immunohistochemical localization of sPLA2-IIA in male genital organs of FVB mice. (A) sPLA2-IIA expression was detected in Leydig cells (arrows) in the testis. (B) sPLA2-IIA was undetectable in seminal vesicles. Panels a, ×40; and panels b, ×100.

Fig. 8. Immunohistochemical localization of sPLA2s in human seminal vesicles and prostate. (A) sPLA2-IIA, (B) sPLA2-IID, (C) sPLA2-IIE, (D) sPLA2-IIF, (E) sPLA2-V, and (F) sPLA2-X. Panels a and b indicate seminal vesicles and prostate, respectively (×40 magnification). In the seminal vesicles, sPLA2-IID and -V yielded positive staining in epithelial cells. sPLA2-IIA was also weakly positive, whereas sPLA2-IIE, -IIF and -X were poorly expressed, in epithelial cells. In the prostate, sPLA2-IIA and -IID, but not sPLA2-IIE, -IIF and -V, were expressed in epithelial cells. sPLA2-X was partly positive in these cells.
3.5. Expression of sPLA₂-IIA in male genital organs in a mouse strain with normal sPLA₂-IIA gene

Immunohistochemistry of sPLA₂-IIA in male genital organs of FVB mouse strain, in which the sPLA₂-IIA gene is intact, revealed that significant sPLA₂-IIA immunoreactivity was localized in Leydig cells in the testis (Fig. 7A). However, its expression was pretty low in the lower genital organs, as exemplified in the seminal vesicles (Fig. 7B). These results were confirmed by RT-PCR analysis of sPLA₂-IIA, where it was detected only in the testis (data not shown). The expression patterns of other sPLA₂s in these organs of FVB strain were essentially similar to those of C57BL/6 strain (data not shown).

3.6. Localization of sPLA₂s in human male reproductive organs

We next examined the locations of sPLA₂s in human male genital organs as well. Immunohistochemistry with antibodies specific for individual human sPLA₂s revealed that the expression profiles of each enzyme in the testis and epididymis of humans were similar to those of mice (data not shown), except sPLA₂-IIC, which is not expressed in humans [29]. However, notable species-associated differences in the sPLA₂ expression profile were found in the seminal vesicular glands and prostate. In human seminal vesicles, sPLA₂-IID (Fig. 8B) and -V (Fig. 8E) displayed intense staining in the epithelium. Staining for sPLA₂-IIA was also weakly positive in epithelial cells (Fig. 8A), whereas sPLA₂-IIE (Fig. 8C), -IIF (Fig. 8D) and -X (Fig. 8E) were poorly expressed. Thus, in contrast to mice (Fig. 6), sPLA₂-IIA is present and sPLA₂-IID and -X are not expressed in human seminal vesicles. In human prostate, intense immunoreactivity for sPLA₂-IIA was detected in epithelial cells and concrements of the gland (Fig. 8A). The prostate epithelium was also positive for sPLA₂-IID (Fig. 8B) and partially for sPLA₂-X (Fig. 8F), whereas sPLA₂-IIE (Fig. 8C), -IIF (Fig. 8D) and -V (Fig. 8E) were undetectable. Thus, the expression of sPLA₂s (IIE, IID and X) in human prostate is markedly distinct from that (IIC, IID, IIE, and IIF) in mouse prostate.

4. Discussion

In the present study, we performed immunohistochemistry to determine the cellular origins of several sPLA₂s (IIA, IIC, IID, IIE, IIF, V and X) in mouse and human male genital organs. We provide evidence that individual sPLA₂s exhibit unique cellular localizations in these tissues and that some enzymes are localized in the same cell types. Since the antibodies against mouse and human sPLA₂s used in this study are highly specific (even not cross-reacting with orthologs from other animal species) [34,35] and since the staining patterns of individual orthologs in testis and epididymis are similar between mouse and human, it is likely that the observed immunoreactivities truly reflect the locations of individual sPLA₂s. Previous immunohistochemical analyses using antibodies raised against sPLA₂-IIA or -IIB have shown that the immunoreactivities are located in various sites of the male genital organs, including hamster epididymal spermatozoa [28], bovine seminal vesicles and connective tissues [27], and human prostate and testicular round spermatids [26,27]. Beyond the species-associated differences in sPLA₂ expression profiles, our present results suggest that some of the previous immunohistochemical studies may have looked at multiple sPLA₂s in these organs. The localization of each sPLA₂ in mouse male reproductive organs, as clarified in the present study, is summarized in Fig. 9.

4.1. Testis

The PLA₂ reaction has been implicated in the acrosome reaction of spermatozoa and in plasma membrane fusion between sperm and oocytes [48,49]. The presence of particular sPLA₂(s) on the acrosomal surface and in the middlepiece mitochondria of hamster and human spermatozoa has been proposed [27,28]. The acrosome reaction of hamster spermatozoa and subsequent fertilization in vitro were blocked by an antibody against pancreatic sPLA₂-IB, and this effect was reversed by lysophosphatidylcholine (LPC), a major PLA₂ reaction product [50]. Accumulation of LPC occurred following in vitro incubation of spermatozoa, and LPC stimulated the fertilizing ability of spermatozoa and induced the changes in the zona pellucida and the oolemma that allowed sperm-egg fusion [51–54]. Moreover, the induction of sperm capacitation was delayed in mice lacking the receptor for platelet-activating factor, a lipid mediator that is synthesized from the sn-1 alkyl form of LPC [55].

We now show that several sPLA₂s are expressed in spermatogenic cells of mouse (and human) seminiferous tubules. In mouse testis, sPLA₂-IIC, -IID, -IIE, -IIF, -V and -X are expressed diversely in spermatogenic cells including spermatogonium, spermatocytes, spermatids and maturing spermatozoa (Fig. 2). It appears that sPLA₂-V, -X and -IIE are enriched in spermatocytes and/or round and elongating spermatids, whereas sPLA₂-IID and -IIF are enriched in the midpiece or tail portions of maturing spermatozoa. These sPLA₂s also provide positive staining in spermatozoa within the epididymis ducts (Fig. 4). Moreover, sPLA₂-IIC, -IID, -IIF, -V and -X are detected in mature spermatozoa by immunoblotting (Fig. 1A). Thus, it is tempting to speculate that these germ cell-associated enzymes may correspond to the sperm-associated sPLA₂ that had previously been reported [26,27]. It has been suggested that germ cell-associated sPLA₂s may play a role in the acrosome reaction.
and subsequent fertilization through producing LPC [48–54]. Indeed, staining signals for sPLA2-IIE, -V and -V in testicular spermatogenic cells (Fig. 2) could be attributed to the acrosome vesicles [41], although more detailed compartmentalization will need to be addressed by immunoelectron microscopy. The possible role of sPLA2s in the acrosome reaction, an exocytosis event by which the acrosomal content is released, is reminiscent of the ability of sPLA2s to promote exocytosis in mast cells and neuroendocrine cells [56,57].

We found that sPLA2-IIF, -V and -X are expressed in mouse Leydig cells (Figs. 2 and 3), suggesting a unique role for these enzymes in this steroid hormone-producing cell in the testis. Several reports have suggested the importance of AA and its metabolites for the function of Leydig cells. For instance, stimulation of Leydig cells with gonadotropin resulted in the release of AA, which was in turn required for optimal steroidogenesis [42–44]. In contrast, another study reported that PGE2 suppressed the gonadotropin-induced formation of testosterone in Leydig cells [45]. PLA2/COX-2 pathway products, such as PGE2 and PGF2α, modulated interleukin (IL)-1 and IL-6 production in Leydig progenitors [49]. PGE2 stimulated seminiferous tubule contractility through direct action on peritubular myoid cells [58]. Furthermore, immunoreactivities for the PGE2-biosynthetic enzymes downstream of PLA2s, such as COX-1 and mPGES-1, were detected in mouse Leydig cells [59]. The ability of sPLA2-IIF, -V, and -X to increase cellular AA release in several cell types [3–12] supports the idea that these enzymes may be involved in augmented AA release, and thereby hormonal production, in Leydig cells.

Indeed, irrespective of the limitation of the overexpression strategy, PGE2 production by a mouse Leydig cell line is markedly augmented by sPLA2-IIF, -V, and -X (Fig. 3). Previous studies have shown that sPLA2-V and -X [4,5], and probably -IIF [11], can elicit cellular AA release via acting on the outer leaflet of the plasma membrane (the external plasma membrane pathway). In contrast, sPLA2-IIA and -IID act on cells through association with heparan sulfate proteoglycans (HSPGs), where these HSPG-binding sPLA2s...
bind cell surface HSPG, internalized, and then function (HSPG-shuttling pathway), in a cell type-specific manner [3,5]. Thus, the ability of sPLA2-II, -V and -X, but not -IIA and -IID, to augment PGE2 production in Leydig cells may indicate that the external plasma membrane pathway, but not the HSPG-shuttling pathway, is operative in these cells. Alternatively, Leydig cells might have specific machinery to be susceptible to sPLA2-II, -V and -X (e.g., specific adaptors or receptors for sPLA2s might be expressed), a possibility that needs to be addressed. Even though sPLA2-IIA is weakly detected by immunohistochemistry in Leydig cells in FVB strain (Fig. 7), its contribution to PGE2 generation is unclear in this context. Importantly, the main sPLA2 expressed in Leydig cells is sPLA2-II, an enzyme that is poorly expressed in other tissues [60]. Thus, it would be important to clarify the dynamics, functions, and regulatory roles of this particular enzyme in this particular cell type.

4.2. Epididymis and vas deferens

It is well established that the epididymis and vas deferens produce a large amount of prostanoids. PGE2 is the most abundant prostanoid and exhibits potent smooth muscle relaxant activity. PGE2 has been thought to play an important role in the transport of sperm through the epididymis and its subsequent expulsion at coitus from the vas deferens [61,62]. In rodents, PGE2 is constitutively released into the semen from the vas deferens rather than from the prostate or seminal vesicles [63]. Expression of COX-1, COX-2 and mPGES-1 is observed in the epithelium of mouse epididymis and vas deferens [59]. Furthermore, the concentration of PGF2α, another seminal prostanoid, is also higher in the vas deferens than in other parts of the male genital organs [64].

We show that sPLA2-IIIC, -IID, -IIE, -IIF, -V and -X are expressed in the epithelium of mouse epididymis and vas deferens [59]. Furthermore, the concentration of PGF2α, another seminal prostanoid, is also higher in the vas deferens than in other parts of the male genital organs [64].

4.3. Seminal vesicles

PGE2 released from the seminal vesicles into the semen has been suggested to regulate sperm fertility [65], and COXs and mPGES-1 are expressed in epithelial cells of the seminal vesicles [59,66]. We show that sPLA2-IIIC, -IID, -IIE, -IIF, -V and -X are expressed in the seminal vesicular epithelium of mice (Fig. 6). sPLA2-IIID and -V are also expressed in human seminal vesicular gland, whereas sPLA2-IIIF and -X are detected only poorly (Fig. 8), thus revealing species difference. It is possible that these sPLA2s contribute to the amplification of lipid mediator production in the seminal vesicles. Although weak expression of sPLA2-IIA is seen in human seminal vesicular epithelium (Fig. 8), these cells do not appear to represent a major source of the sPLA2-IIA present in the seminal plasma [25], since this enzyme is far more intensely detected in the prostate gland, as discussed below.

4.4. Prostate

Several previous immunohistochemical studies reported that sPLA2-IIA is expressed in epithelial cells of human and bovine prostates, particularly in the posterior lobe and paraurethral glands [26,27]. Consistent with these reports, we now show that sPLA2-IIA is strongly expressed in epithelial cells of human prostate gland (Fig. 8), thus providing additional support for the notion that the major source of the sPLA2-IIA secreted in human seminal plasma is the prostate epithelium. It is notable that the sPLA2 enzymes expressed in human and mouse prostates are rather distinct. Thus, in addition to sPLA2-IIA, sPLA2-IID and partially sPLA2-X are expressed in human prostate epithelium (Fig. 8), whereas the sPLA2s detected in mouse prostate are sPLA2-IIIC, -IID, -IIE and -IIF (Fig. 6). However, as the expression of these sPLA2 mRNAs in mouse prostate is relatively low (Fig. 1A), the possibility that these sPLA2 proteins produced from the upper genital organs are secondary associated with the prostate epithelium should be also considered. Nonetheless, the epithelial location of these sPLA2s is rather distinct from the distribution of COX enzymes in human prostate [66], arguing against the potential contribution of these sPLA2s to prostanoid production in this organ. Given that several sPLA2s exhibit potent bactericidal activity [17–20], it is more likely that these sPLA2s, particularly the sPLA2-IIA abundantly secreted from the human prostate gland, may be a part of the anti-microbial arsenal to protect this organ and spermatozoa against microbial invasion. Similarly, various
sPLA2s secreted from the epithelium of the upper genital organs (epididymis, vas deferens and seminal vesicles) into the lumen may also contribute to anti-microbial defense throughout the genital ducts.

4.5. Conclusion

Our present study has demonstrated, for the first time, the detailed localization of individual sPLA2s in mouse male genital organs. Distinct sets of sPLA2s are distributed in spermatogenic and Leydig cells in the testis and epithelial cells throughout the ducts. In general, more than one sPLA2 is expressed in a single cell type, and these multiple enzymes may play redundant or distinct roles. As discussed above, possible functions of sPLA2s in male reproductive organs include (i) production of lipid mediators, (ii) regulation of spermatogenesis, (iii) regulation of sperm functions, including the acrosome reaction and fertilization, and (iv) defense against microorganisms invading these tissues. The ability of particular sets of sPLA2s (IIF, V, and X) are intrinsically expressed in Leydig cells to increase PGE2 production in a mouse Leydig cell line may be a reflection of their redundant function. Nevertheless, to understand the precise roles of individual sPLA2s in these tissues, a wide array of functional studies, including gene targeting, will be necessary. However, future studies with sPLA2 knockout mice should be interpreted with caution, because some functions of a particular sPLA2 may be compensated for by other sPLA2s and because there are several differences in the expression patterns of sPLA2s between mice and humans, as exemplified in seminal vesicles and prostate.

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References


