Identification of a Soluble-Form Phospholipase A$_2$ Receptor as a Circulating Endogenous Inhibitor for Secretory Phospholipase A$_2$

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Summary

Venomous snakes have various types of phospholipase A₂ inhibitory proteins (PLIs) in their circulatory system in order to protect them from attack by their own phospholipase A₂s (PLA₂s). Here we show the first evidence for the existence of circulating PLI against secretory PLA₂s (sPLA₂s) in mammals. In mouse serum, we detected specific binding activities of group IB and X sPLA₂s (sPLA₂-IB and sPLA₂-X), which was in contrast with the absence of binding activities in serum prepared from mice deficient in PLA₂ receptor (PLA₂R), a type I transmembrane glycoprotein related to the C-type animal lectin family. Western blot analysis after partial purification with sPLA₂-IB-affinity column confirmed the identity of serum sPLA₂ binding protein as a soluble form of PLA₂R (sPLA₂R) that retained all of the extracellular domains of the membrane-bound receptor. Both purified sPLA₂R and the recombinant soluble receptor having all of the extracellular portions blocked the biological functions of sPLA₂-X, including its potent enzymatic activity and its binding to the membrane-bound receptor. Protease inhibitor tests with PLA₂R-overexpressing Chinese Hamster Ovary (CHO) cells suggested that sPLA₂R is produced by cleavage of the membrane-bound receptor by metalloproteinases. Thus, sPLA₂R is the first example of circulating PLI that acts as an endogenous inhibitor for enzymatic activities and receptor-mediated functions of sPLA₂s in mice.
Introduction

Phospholipase A$_2$ (PLA$_2$) is an enzyme that catalyzes the hydrolysis of the $sn$-2 ester bond of glycerophospholipids (1, 2). Since its discovery, PLA$_2$ has been a molecular target of extensive research because of its critical involvement in physiological and pathological events such as phospholipid turnover and production of proinflammatory lipid mediators (3). To date, a number of mammalian intracellular and extracellular PLA$_2$s have been identified and classified into different families according to their biochemical features (4). Among them, sPLA$_2$s have several common characteristics including a relatively low molecular mass (13-18 kDa), the presence of 6 to 8 disulfide bridges, and an absolute catalytic requirement for millimolar concentrations of Ca$^{2+}$ (5, 6). At present, mammalian sPLA$_2$s are classified into ten different groups (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XII) depending on the primary structure characterized by the number and positions of cysteine residues (5, 7-12). Given their potent enzymatic activities and enhanced expression in various inflammatory conditions, sPLA$_2$s are thought to play crucial roles in the development of various disease states. For example, high levels of group IIA sPLA$_2$ (sPLA$_2$-IIA) were detected in the plasma of patients with sepsis, and sPLA$_2$-IIA plays a critical role in the hydrolysis of lung surfactant phospholipids during the progression of acute lung injury (13, 14). We and other groups have shown that group X sPLA$_2$ (sPLA$_2$-X) possesses stronger hydrolyzing activity toward phosphatidylcholine than sPLA$_2$-IIA, and elicits marked release of arachidonic acid linked to the production of various lipid mediators in macrophages and colon cancer cells (15, 16).

In addition to its digestive function, sPLA$_2$ can exert various biological responses via its binding to the PLA$_2$ receptor (PLA$_2$R) (17). PLA$_2$R is a type I
transmembrane protein with a molecular mass of 180 kDa. Its overall molecular organization is related to a unique member of the C-type animal lectin family (subgroup VI), which includes the macrophage mannose receptor (18). PLA₂R is composed of a large extracellular portion consisting of an N-terminal cystein-rich region, a fibronectin-like type II domain, and a tandem repeat of 8 carbohydrate-recognition domains (CRDs) and a short intracellular C-terminal region (19). In mice, group IB sPLA₂ (sPLA₂-IB) was identified as the first endogenous ligand of PLA₂R (20). Recently, sPLA₂-X was also identified as a high affinity ligand of PLA₂R (21). Although sPLA₂-IIA can bind PLA₂R with about 10-fold lower affinity compared to sPLA₂-IB and X, some inbred mouse strains possess a natural mutation in the sPLA₂-IIA gene (5, 6). We have shown that sPLA₂-IB exerts various biological responses via binding to PLA₂R, including cell proliferation, lipid mediator productions, and chemokinetic migration in various cell types (22-24). Furthermore, our recent analysis of PLA₂R-deficient mice has revealed that PLA₂R may play a role in the production of pro-inflammatory cytokines during the progression of endotoxic shock (25).

Venomous snakes have PLA₂ inhibitory proteins (PLIs) in their blood sera in order to protect them from leakage of their own venom PLA₂s into their circulatory system (26). Mammalian sPLA₂s exhibit a wide variety of pathological functions via enzymatic activities or receptor-mediated responses, however, the existence of PLI molecules in mammalian circulation has not yet been demonstrated. In our recent analysis by sandwich enzyme-linked immunosorbent assay (ELISA), we found the presence of a soluble form of PLA₂R (sPLA₂R) in mouse plasma, although its biochemical features and biological significance has not been clarified yet (27). In the present study, we definitely identified the sPLA₂ binding protein in mouse sera as
sPLA$_2$R which retains all of the extracellular domains of the cell-associated receptor. We found that circulating sPLA$_2$R can block both the enzymatic activity and receptor binding activity of sPLA$_2$-X. Thus, we report here that sPLA$_2$R is the first example of circulating PLI in mammals.
Experimental Procedures

Materials.

Sodium [125I] iodine (carrier-free, 3.7 GBq/ml) was purchased from Amersham Pharmacia Biotech. Porcine pancreatic sPLA2-IB was obtained from Boehringer Mannheim. Recombinant mouse sPLA2-X, recombinant soluble PLA2R (rsPLA2R) and biotin-labeled rabbit anti-sPLA2R antibody (Ab) were prepared as described in the previous paper (27, 28). LPS (Salmonella Typhosa 0901) was purchased from Difco Laboratories. Bovine serum albumin (BSA) and bovine γ-globin were purchased from Sigma. DPPC (1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphocholine) was obtained from Avanti Polar Lipids, Inc., Alabaster, AL. Polyethylenglycol (PEG) 6000 was purchased from Nacalai Tesque. Protease inhibitors were obtained from Peptide Institute, Inc. and Wako Pure Chemical Industries. BB2516 was synthesized at Shionogi Research Laboratories. The generation of PLA2R-deficient mice was described in our previous paper (25), and they were backcrossed more than eleven times. In each experiment, C57BL/6J mice matched for gender and age were used as wild-type littermates.

Binding of sPLA2-IB and sPLA2-X to Mouse Serum.

Iodination of porcine sPLA2-IB or mouse sPLA2-X was performed by the chloramine-T method (29), and the specific radioactivities of 125I-sPLA2-IB and 125I-sPLA2-X were about 7000 and 4600 cpm/fmol, respectively. The serum prepared from wild-type or PLA2R-deficient mice (100 µl) was incubated with 3 nM 125I-sPLA2-IB or 125I-sPLA2-X in the binding buffer (20 mM Tris-HCl (7.4) containing 2 mM EDTA and 0.1% BSA). After incubation for 1 h at 4°C, 600 µg of bovine γ-globin and 20% PEG 6000 were added. After further incubation for 30 min at 4°C, the reaction mixture was
filtered through Whatman GF/B glassfilter and the radioactivity of the filter was measured. Specific binding was determined as the differences between the presence and absence of unlabeled 500 nM porcine sPLA2-IB.

**Western blot analysis of serum sPLA2-R.**

Serum sPLA2-R was partially purified from wild-type or PLA2R-deficient mice with a sPLA2-IB-affinity column as described previously (30), and then concentrated with ultrafree (Millipore). Chinese Hamster Ovary (CHO) cells that stably express the membrane-bound mouse PLA2R (PLA2R-CHO cells) and their crude membrane lysates were prepared as described previously (28, 29). The serum sPLA2-R materials, the membrane fractions of PLA2R-CHO or parent CHO cells, and purified rsPLA2-R protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-20% gradient gel (Daiichi Chemical Co., Ltd.). Western blot analysis was performed according to our previous paper (27) with biotin-labeled rabbit anti-sPLA2-R Ab (1 µg/ml) and streptavidin-conjugated horseradish peroxidase (Boehringer Mannheim). The blot was incubated with a chemiluminescent detection reagent (ECL Western blotting detection reagents, Amersham Pharmacia Biotech) according to the manufacturer’s instructions and analyzed using a Fluor-S MAX MultiImager (Bio-Rad).

**Inhibition of sPLA2-X enzymatic activity by sPLA2-R.**

Mouse sPLA2-X (0.14 nM) was preincubated with various concentrations of rsPLA2-R in the assay buffer (0.1 M Tris-HCl, pH 8.0, containing 0.15 M NaCl, 0.01 M CaCl2 and 1 mg/ml BSA) for 1 h at 37°C. In separate experiments, mouse sPLA2-X was preincubated in the absence or presence of serum sPLA2-R prepared from wild-type or PLA2R-deficient mice. The enzymatic activity was evaluated using 0.1 mM DPPC as a
substrate. After incubation for 1 h at 37°C, the reaction was stopped by the addition of Dole’s reagent (heptane, 2-propanal, 2 N sulfuric acid = 10:40:1, v/v/v), and the released fatty acids were quantified by reverse-phase HPLC on a LiChroCART 75-4 Superspher 60 RP-8 column (Merck), as described by Tojo et al. (31).

**Effect of rsPLA2R on sPLA2-X-induced fatty acid release in mouse splenic cells.**

Splenic cells were prepared from male C57BL/6J mice (10 weeks) according to the method of Funk et al. (32). The prepared cells were washed with Hanks’ buffered saline (pH 7.6) containing 0.1% BSA and suspended in the same buffer at a density of 9.5 x 10^6 cells/ml. Aliquots of cell suspension (0.4 ml) were preincubated for 10 min at 37°C and then stimulated with 2 nM mouse sPLA2-X with or without various concentrations of rsPLA2R in a final volume of 0.5 ml. The reaction was stopped by addition of 2 ml of Dole’s reagent. The released fatty acids were extracted and quantified as described previously (31).

**Inhibition of sPLA2 binding to PLA2R by sPLA2R.**

PLA2R-CHO cells were cultured in 24-well plates in 10% FCS/DMEM. At confluence, the cells were washed three times with phosphate-buffered saline, and incubated with 1 nM ^125^I-sPLA2-X in the absence or presence of various concentrations of rsPLA2R in 0.4 ml of the binding buffer (Hanks’ balanced salt solution, pH 7.4, containing 0.1% BSA) for 2 h at 4°C. After the incubation, cells were washed with the binding buffer three times, and the cell-bound radioactivity was measured. In separate experiments, the membrane fractions of PLA2R-CHO cells were incubated with 0.2 nM ^125^I-sPLA2-X in the absence or presence of serum sPLA2R prepared from wild-type or PLA2R-deficient mice, or 12.5 nM rsPLA2R. After incubation for 2 h at room temperature, the reaction mixture was filtered through Whatman GF/C glassfilter. The
specific binding was determined as the differences between the presence and absence of unlabeled 500 nM mouse sPLA\textsubscript{2}-X.

*Effects of protease inhibitors on the release of sPLA\textsubscript{2}R from PLA\textsubscript{2}R-CHO cells.*

After cultivation in 96-well plates, PLA\textsubscript{2}R-CHO cells were washed three times with PBS and then incubated with or without protease inhibitors at 37°\textdegree C for various times. After the incubation, the supernatant was collected by centrifugation at 8,000 rpm for 10 min at 4°\textdegree C and the released sPLA\textsubscript{2}R was measured with established sandwich ELISA (27). In this assay, purified rsPLA\textsubscript{2}R was used as a conventional standard because of its molecular weight being similar to that of serum sPLA\textsubscript{2}R (the detection range from 0.1 to 100 ng). In separate experiments, the membranes of PLA\textsubscript{2}R-CHO cells were incubated in the absence or presence of protease inhibitors for 1 h at 37°\textdegree C. After centrifugation at 40,000 rpm for 1 h at 4°\textdegree C, the released sPLA\textsubscript{2}R in the supernatant was measured with sandwich ELISA.

**Results**

*Binding of sPLA\textsubscript{2}s to mouse serum.*

To explore the existence of circulating PLI in mammals, we first examined the binding potency of endogenous sPLA\textsubscript{2}s to mouse serum. We used two types of \textsuperscript{125}I-labeled sPLA\textsubscript{2}s (sPLA\textsubscript{2}-IB and sPLA\textsubscript{2}-X) and evaluated their bindings to mouse serum proteins using PEG precipitation assay. As shown in Fig. 1, specific binding activities were detected with both ligands (10.5 fmol/ml and 7.3 fmol/ml of serum, respectively). The specific binding linearly increased depending on the serum volume added up to 100 µl (data not shown). We have previously shown that sPLA\textsubscript{2}-IB and sPLA\textsubscript{2}-X can specifically bind to the PLA\textsubscript{2}R expressed in alveolar type II epithelial cells and splenic
lymphocytes in mice (21). In addition, our recent analysis with sandwich ELISA suggested the presence of sPLA₂R in mouse plasma (27). In order to examine the relationships between serum sPLA₂ binding proteins and putative sPLA₂R, we next examined the binding potencies of ¹²⁵I-labeled sPLA₂S to serum prepared from PLA₂R-deficient mice, in which there was no positive signal for sPLA₂R in sandwich ELISA (27). As shown in Fig. 1, there were few, if any, specific binding activities of both sPLA₂ ligands in the serum prepared from knockout mice. These findings suggest that serum sPLA₂R protein(s) are representative of the specific binding activities of sPLA₂ ligands.

Identification of serum sPLA₂R protein.

We next performed the partial purification of serum binding proteins with sPLA₂-IB-affinity column. The specific binding activities of ¹²⁵I-labeled sPLA₂S were detected in the acid-eluted materials in contrast to their absence from the pass-through fractions. Conversely, there was little protein in the eluted fractions of serum prepared from PLA₂R-deficient mice (data not shown), demonstrating the binding proteins to be serum sPLA₂R(s). In order to clarify their identities, we performed western blot analysis with anti-PLA₂R Ab, which can specifically detect the membrane-bound PLA₂R protein (ca. 200 kDa) in the membrane fractions of PLA₂R-CHO cells compared to those of the parent CHO cells (Fig. 2, lane 1 and 2). The recombinant soluble form of PLA₂R (rsPLA₂R) composed of all of the extracellular domains of the membrane-bound receptor (1-1365 amino acids) showed a slightly smaller molecular mass (ca. 180 kDa in lane 3) than that of the membrane-bound form. Serum sPLA₂R was detected as a single band at the same position with rsPLA₂R (lane 4), which contrasted with no
visible bands in the eluted fractions prepared from PLA2R-deficient mouse serum (lane 5). These results demonstrate that serum sPLA2R contains all of the extracellular domains of PLA2R with high binding activity to sPLA2-IB and sPLA2-X.

Functions of serum sPLA2R.

There are two biological responses elicited by sPLA2s; one is dependent on the phospholipid-hydrolyzing activity and the other is mediated via the PLA2R binding (17). Since sPLA2-X was proven to elicit both responses in mice (28), we next examined the effects of serum sPLA2R on sPLA2-X-induced responses. First, we examined the effects of rsPLA2R and serum sPLA2R on sPLA2-X enzymatic activity toward DPPC as a substrate. In this in vitro assay system, the enzyme activity of about 0.1 nM mouse sPLA2-X can be detected, and the purified rsPLA2R markedly blocked the enzymatic activity with an IC50 value of 0.04±0.01 nM (Fig. 3A). The acid-eluted materials prepared with sPLA2-IB-affinity column (Fig. 2) were found to contain the sPLA2R at the concentration of 0.37 nM by sandwich ELISA using rsPLA2R as a conventional standard. As shown in Fig. 3B, the serum sPLA2R fractions effectively blocked the enzymatic activity of sPLA2-X up to 70%, which contrasted with no significant suppression with sPLA2R fractions prepared from PLA2R-deficient mouse serum. We then examined the effect on enzymatic activity of sPLA2-X toward intact cell membranes. We have previously reported that mouse sPLA2-X induced prompt and marked release of arachidonic acid from mouse spleen cells (28). However, a relatively higher concentration of sPLA2-X (over 0.5 nM) was required for detection of sufficient responses in intact cell systems. As shown in Fig. 3C, rsPLA2R dose-dependently blocked the arachidonic acid release evoked by mouse sPLA2-X (2 nM). However,
significant suppression could not be observed using the serum sPLA₂R fractions, possibly due to its lower quantities of sPLA₂R protein (data not shown).

Next, we examined the effects of rsPLA₂R and serum sPLA₂R on sPLA₂-X binding activity. As shown in Fig. 4A, rsPLA₂R protein blocked the sPLA₂-X binding to PLA₂R-CHO cells with an IC₅₀ value of 1.2 ± 0.2 nM, which coincided with the binding affinity of sPLA₂-X to the cell-surface PLA₂R in mouse osteoblastic MC3T3-E₁ cells (Kd = 4.6 nM) (21). We next examined the effect of concentrated sPLA₂R materials prepared from a sPLA₂-IB-affinity column, which was shown to have the sPLA₂R concentration of 1.36 nM by ELISA. As can be seen from Fig. 4B, the sPLA₂-X binding was blocked up to 48% by these materials, which contrasted with no significant inhibition by the eluted fractions prepared from PLA₂R-deficient mouse serum. Taken together, these findings demonstrate that serum sPLA₂R is functional in terms of the suppression of sPLA₂-X-induced biological responses.

Study of the mechanisms underlying sPLA₂R production.

In order to investigate the mechanisms underlying the production of sPLA₂R, we used PLA₂R-CHO cells that spontaneously release sPLA₂R protein into the culture medium. We confirmed that sPLA₂R protein isolated from the conditioned medium of PLA₂R-CHO cells has the same molecular weight as the serum sPLA₂R by western blot analysis (data not shown). As shown in Fig. 5A, the sPLA₂R levels in the supernatant were time-dependently increased during the cultivation of PLA₂R-CHO cells. This release response was suppressed over 80% by the addition of BB2516, a specific broad-spectrum metalloproteinase inhibitor (33), in contrast to no significant inhibition by p-APMSF, a serine protease inhibitor (34). Next, the effects of various types of protease
inhibitors were evaluated for the spontaneous release of sPLA₂R from the membrane fractions of PLA₂R-CHO cells in order to eliminate their toxic effects on intact cells. The concentration used was optimized for their efficient and specific inhibition against target proteases in vitro. After incubation in the presence of inhibitors for 1 h, the released sPLA₂R in the supernatant was evaluated with sandwich ELISA. As shown in Fig. 5B, EDTA, a metal chelator, and BB2516 blocked the sPLA₂R release up to 78 and 69%, respectively. Especially, an IC₅₀ value of BB2516 was evaluated to be 4.7 nM (data not shown). In contrast, there was no significant suppression by other protease inhibitors, including p-APMSF, leupeptin, and pepstatin that are known as inhibitors for serine, cysteine or asparate proteases (35). These findings suggest that metalloproteinases are involved, at least in part, in the proteolytic cleavage of the membrane-bound receptor in PLA₂R-CHO cells.
Discussion

Venomous snakes have PLIs in their plasma to protect themselves from their own venomous PLA2s that elicit a wide variety of toxicities, such as neurotoxicity and myotoxicity (36). In mammals, there are diverse types of sPLA2s that can be released outside of the cells and exist in the tissue fluids and blood plasma (3). The present study is the first to identify mouse sPLA2-R as a circulating PLI for sPLA2s in mammals. Western blot analysis revealed that serum sPLA2-R retained all of the extracellular domains of the membrane-bound form of the receptor being similar to rsPLA2-R (Fig. 2). We have previously shown that rsPLA2-R retained the same binding dissociation constant (Kd = 0.97 nM for sPLA2-IB) and ligand specificity as the membrane-bound receptor (17, 37). Scatchard plot analysis has revealed that rsPLA2-R can bind one sPLA2 ligand per molecule, like the native cell-surface receptor (37). In fact, rsPLA2-R protein strongly blocked the sPLA2-X binding to the membrane-bound receptor with an IC50 value of 1.2 nM (Fig. 4A), which coincided with the binding affinity of sPLA2-X to the cell-surface PLA2-R (21). In the present study, serum sPLA2-R was found to possess binding activity for sPLA2-IB and sPLA2-X (Fig. 1). In addition, serum sPLA2-R blocked the enzymatic activity and receptor binding activity of sPLA2-X (Fig. 3B and 4B). These findings demonstrate that serum sPLA2-R has similar characteristics with rsPLA2-R in terms of biochemical properties as well as functional activities, and thus confirmed our previous estimation of the plasma sPLA2-R concentration in normal mice with sandwich ELISA (0.13 nM), in which rsPLA2-R was used as a conventional standard (27).

We have previously reported that sPLA2-IB can elicit various biological responses possibly via PLA2-R binding, such as proliferation and chemokinetic
migration of vascular smooth muscle cells and potent vasoactive actions in cerebral arteries (17, 38). In addition, we have recently demonstrated that sPLA₂-X elicits marked release of arachidonic acid leading to lipid mediator productions in various cell types, including monocytes/macrophages (15, 16, 39). At the normal circulating level (0.13 nM), rsPLA₂R can effectively block the enzymatic activity of sPLA₂-X (0.14 nM) toward the substrate DPPC (Fig. 3A), and the prepared serum sPLA₂R fractions (0.37 nM) was found to suppress the activity up to 70% (Fig. 3B). However, in mouse spleen cells, the inhibitory potency of rsPLA₂R for sPLA₂-X-induced arachidonic acid release was weaker compared to that in the DPPC hydrolysis, possibly due to the requirement of higher concentration of sPLA₂-X for detection of the released fatty acids in intact cell systems. For the sPLA₂-X binding, rsPLA₂R was found to block up to 20% at the normal circulating concentration (Fig. 4A), and its inhibitory potency can be greatly enhanced at the concentration over 0.3 nM. In fact, the concentrated serum materials containing higher levels of sPLA₂R (1.36 nM) could significantly block the sPLA₂-X binding (Fig. 4B). It has been reported that the normal serum concentration of sPLA₂-IB is 5.1 ng/ml (0.36 nM) in humans (40). Although the normal plasma level of sPLA₂-IB and sPLA₂-X has not yet been examined in mice, these findings suggest that the circulating sPLA₂R can play one of the endogenous inhibitors that block the biological functions of these sPLA₂s in the normal conditions. In patients with acute pancreatitis and renal failure, increased systemic levels of sPLA₂-IB have been observed (40). We have recently shown that the plasma sPLA₂R concentration is significantly elevated to up to 1.5-fold of the normal level during murine endotoxic shock (27), although this enhanced level is not enough for the effective suppression of the enzymatic activities and the receptor-mediated responses evoked by higher concentration of sPLA₂s. Further
analysis of the circulating sPLA₂R and sPLA₂s levels during various pathological states are required to establish the role of circulating sPLA₂R as an endogenous inhibitor to protect the pathological functions of sPLA₂s.

In the plasma of various snakes, three distinct types of PLIs (PLI-α, β, γ) have been identified. PLIα is a 75-kDa glycoprotein composed of a trimer of 20-kDa subunits having sequence homology to the CRD of C-type lectins and preferentially blocks group II acidic PLA₂s (41). Intriguingly, sPLA₂R contains all of the extracellular domains of the membrane-bound receptor including the tandem repeat of eight CRD-like domains. Previous deletion experiments have demonstrated that three CRD-like domains (CRD3, 4, 5) are representative of the sPLA₂ binding activity (42, 43). In addition, the CRD of PLIα present in the blood plasma of the Habu snake Trimeresurus flavoviridis has similarities with the CRD5 of PLA₂R (28%) (6). Also, the soluble lung surfactant protein SP-A has been reported to have CRD that shares sequence homology with PLIα, and blocks the Habu snake venom sPLA₂ activity (44). Notably, SP-A can also bind to guinea pig sPLA₂-IIA to suppress its enzymatic activity in contrast to the absence of inhibition of porcine sPLA₂-IB (45). However, the binding affinity of SP-A for sPLA₂-IIA has not yet been determined and SP-A can also interact with other molecules, such as carbohydrate ligands and its high-affinity receptor present in alveolar type II cells (46). In contrast, mouse PLA₂R specifically recognizes sPLA₂-IB and X with a high affinity (Kd of 1.43 and 4.6 nM, respectively) and does not possess lectin activity (20). Thus, further studies are required to ascertain the biological roles of SP-A in the inhibition of sPLA₂-IIA activity in the lung. Nevertheless, these findings suggest that other C-type lectin family members having CRD-like domains might also behave as physiological sPLA₂ inhibitors in circulation and/or local tissue areas.
Numerous types of soluble-form membrane receptors and cell adhesion molecules are known to exist in circulation as innate host defense systems against exaggerated receptor-mediated responses (47). One possible mechanism underlying the production of soluble molecules is the alternative splicing event. In fact, the potential production of sPLA₂R in human kidney was suggested based on the finding of an alternatively processed transcript encoding the ectodomains of PLA₂R (48). However, the existence of a soluble form of PLA₂R has not been confirmed in humans, and we have not detected the alternatively spliced transcript in any of the tissues examined by northern blot and RT-PCR analysis in mice (data not shown). In another setting, the ectodomains of many membrane proteins are released by regulated proteolytic cleavage (49). In the present study, BB2516 and EDTA efficiently suppressed the proteolytic cleavage of membrane-bound PLA₂R in both intact PLA₂R-CHO cells and the membrane preparations (Fig. 5). In particular, the IC₅₀ value of BB2516 for inhibition of the cleavage of membrane-bound PLA₂R was 4.7 nM, which corresponded well with its inhibitory potency on metalloproteinases activity in vitro (50). Because BB2516 can suppress all types of metalloproteinases (33), the subtypes involved in the shedding process could not be identified in this study. Tumor necrosis factor-α (TNF-α)-converting enzyme (TACE), one of the members of a distintegrin and metalloproteinase (ADAM) family (51), is the first identified “sheddase” that is involved in the cleavage of TNF-α as well as various membrane-bound proteins including the TNF p75 receptor, L-selectin and transforming growth factor-α (52). CHO cells are known to constitutively express active TACE on the cell surface (53), suggesting a potential participation of TACE in the cleavage of membrane-bound PLA₂R, although further studies are needed to elucidate the metalloproteinases involved in vivo. A similar
shedd deep mechanism has been observed with the mannose receptor, another member of
the subgroup VI of the C-type lectin family (18), as its soluble form in mouse serum
can be produced by cleavage of the membrane-bound form via ADAM or matrix
metalloproteinases (MMPs) (54). Thus, the biological function as a soluble receptor
after processing by metalloproteinases might be another critical implication for this
family member. Among the metalloproteinases, MMP-14 (MT1-MMP) and MMP-9
(gelatinase B) transcripts are constitutively expressed in the spleen where PLA₂R and
its ligands, sPLA₂-IB and X, also exist (20, 27, 28). In addition, the expressions of
MMP-14, MMP-13 (collagenase 3) and MMP-11 (stromelysin 1) transcripts were
elevated in mouse spleen at 1 h after LPS injection (55). Upon endotoxin challenge, the
expression of PLA₂R mRNA was also elevated in the splenic lymphocytes and alveolar
type II epithelial cells after 1 h, and the circulating sPLA₂R level increased after 2-3 h
(27). These findings suggest a possible contribution of these MMPs to the production of
sPLA₂R in the tissues and circulation under physiological and pathological states.
Further studies with specific inhibitors should help identify the metalloproteinase types
involved.

In conclusion, we have identified circulating sPLA₂R as an endogenous inhibitor
of sPLA₂s in mice. In this study, the use of PLA₂R-deficient mice has enabled us to
clearly show the existence of the receptor in serum. Since there is strict species
specificity in the relationships between sPLA₂ ligands and PLA₂R, further studies on
the identification of sPLA₂R and its ligand in humans are required for elucidation of
their physiological and pathological roles.
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Footnotes

1 The abbreviations used are: PLA₂, phospholipase A₂; sPLA₂, secretory PLA₂; sPLA₂-IB, IIA, X, group IB, IIA and X sPLA₂, respectively; PLA₂R, PLA₂ receptor; sPLA₂R, soluble form of PLA₂R; PLI, PLA₂ inhibitory protein; CRD, carbohydrate-recognition domain; CHO, Chinese Hamster Ovary; ELISA, enzyme-linked immunosorbent assay; BSA, Bovine serum albumin; PEG, Polyethylenglycol; DPPC, 1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphocholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; p-APMSF, (p-Amidinophenyl)methanesulfonyl Fluoride; TNF-α, Tumor necrosis factor-α; TACE, TNF-α-converting enzyme; ADAM, a distintegrin and metalloproteinase; MMP, matrix metalloproteinase.
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Figure Legends

FIG. 1. Binding of sPLA₂-IB and sPLA₂-X to mouse serum.

The binding of $^{125}$I-sPLA₂-IB or $^{125}$I-sPLA₂-X was evaluated with serum prepared from wild-type (WT) and PLA₂R-deficient mice (KO). The specific binding activity was determined as the differences between the presence and absence of 500 nM porcine sPLA₂-IB. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments.

FIG. 2. Characterization of serum sPLA₂R.

Serum sPLA₂R was partially purified with sPLA₂-IB-affinity column, and western blot was performed using rabbit anti-PLA₂R Ab, as described under “Experimental Procedures”. Lane 1, membrane fractions of CHO cells; Lane 2, membrane fractions of PLA₂R-CHO cells; Lane 3, rsPLA₂R protein; Lane 4, sPLA₂R materials purified from wild-type mouse serum; Lane 5, sPLA₂R fractions prepared from PLA₂R-deficient mouse serum. Molecular weight markers are indicated on the left of the figure.

Fig. 3. Inhibitory potency of sPLA₂R for sPLA₂-X enzymatic activity.

(A) Inhibitory potency of rsPLA₂R for sPLA₂-X enzymatic activity. Mouse sPLA₂-X (0.14 nM) was incubated with DPPC in the absence or presence of various concentrations of rsPLA₂R. (B) Inhibitory potency of circulating sPLA₂R for sPLA₂-X enzymatic activity. Mouse sPLA₂-X (0.14 nM) was incubated with DPPC in the absence (none) or presence of serum sPLA₂R fractions prepared from wild-type (WT) or PLA₂R-deficient (KO) mice, or 1 nM rsPLA₂R. (C) Inhibitory potency of rsPLA₂R on sPLA₂-X-induced arachidonic acid release from spleen cells. The spleen cells
prepared from male C57BL/6J mice were stimulated with mouse sPLA$_2$-X (2 nM) with or without various concentrations of rsPLA$_2$R, and the released arachidonic acid was quantified as described under “Experimental Procedures”. The results are expressed as the percentage of sPLA$_2$-X enzymatic activity in the absence of sPLA$_2$R. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments. The IC$_{50}$ value of rsPLA$_2$R in Fig. 3A was calculated to be 0.04 ± 0.01 nM from three separate experiments. Statistical significance in Fig. 3B was tested using Student’s $t$ test (*$P < 0.01$ versus control).

FIG. 4. Inhibitory potency of sPLA$_2$R for the sPLA$_2$-X binding activity.

(A) Inhibitory potency of rsPLA$_2$R for sPLA$_2$-X binding. PLA$_2$R-CHO cells in 24-well plate were incubated with $^{125}$I-sPLA$_2$-X (1.0 nM) in the absence or presence of various concentrations of rsPLA$_2$R. (B) Inhibitory potency of circulating sPLA$_2$R for sPLA$_2$-X binding activity. The membrane fractions of PLA$_2$R-CHO cells were incubated with $^{125}$I-sPLA$_2$-X (0.2 nM) in the absence (none) or presence of serum sPLA$_2$R fractions from wild-type (WT) or PLA$_2$R-deficient (KO) mice, or 12.5 nM rsPLA$_2$R. The specific binding activity was determined as the differences between the presence and absence of 500 nM mouse sPLA$_2$-X, and the results are expressed as the percentage of $^{125}$I-sPLA$_2$-X specific binding in the absence of sPLA$_2$R. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments. The IC$_{50}$ value of rsPLA$_2$R in Fig. 4A was calculated to be 1.2 ± 0.2 nM from three separate experiments. Statistical significance in Fig. 4B was tested using Student’s $t$ test (*$P < 0.05$ versus control).
FIG. 5. Effects of protease inhibitors on the release of sPLA$_2$R from PLA$_2$R-CHO cells. (A) PLA$_2$R-CHO cells were incubated with or without 1 µM BB2516 or 1 mM p-APMSF for various times at 37°C. After centrifugation, the supernatant was collected and the released sPLA$_2$R was quantified by sandwich ELISA. (B) The membrane fractions of PLA$_2$R-CHO cells were incubated with or without various protease inhibitors (20 mM EDTA, 1 µM BB2516, 1 mM p-APMSF, 10 µg/ml leupeptin and 10 µg/ml pepstatin A) for 1 h at 37°C. After centrifugation, the released sPLA$_2$R in the supernatant was quantified by sandwich ELISA. The results are expressed as the percentage of the released sPLA$_2$R in the absence of inhibitors. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments.
Fig. 1. Higashino, K. et al.

![Bar graph showing specific binding of 
$^{125}$I-sPLA2](chart.png)
Fig. 2. Higashino, K. et al.
Fig. 3A. Higashino, K. et al.
Fig. 3B. Higashino, K. et al.
Fig. 3C. Higashino, K. et al.

Recombinant sPLA2R (nM)

Released Arachidonic acid (% of control)

0 1 10 100

0 1 10 100

Recombinant sPLA2R (nM)
Fig. 4A. Higashino, K. et al.
Fig. 4B. Higashino, K. et al.
Fig. 5A. Higashino, K. et al.
Fig. 5B. Higashino, K. et al.
Identification of a soluble-form phospholipase A2 receptor as a circulating endogenous inhibitor for secretory phospholipase A2

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