Group X secretory PLA2 in neutrophils plays a pathogenic role in abdominal aortic aneurysms in mice

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Watanabe K, Fujioka D, Saito Y, Nakamura T, Obata J, Kawabata K, Watanabe Y, Mishina H, Tamaru S, Hanasaki K, Kugiyama K. Group X secretory phospholipase A2 in neutrophils plays a pathogenic role in abdominal aortic aneurysms in mice. Am J Physiol Heart Circ Physiol 302: H95–H104, 2012. First published October 7, 2011; doi:10.1152/ajpheart.00695.2011.—Group X secretory PLA2 (sPLA2-X) is expressed in neutrophils and plays a role in the pathogenesis of neutrophil-mediated tissue inflammation and injury. This study tested the hypothesis that sPLA2-X in neutrophils may contribute to the pathogenesis of abdominal aortic aneurysms (AAA) using sPLA2-X−/− mice. AAA was created by application of CaCl2 to external surface of aorta. As a result, the aorta of sPLA2-X−/− mice had smaller diameters (percent increase from baseline: 24.8 ± 3.5% vs. 49.9 ± 9.1%, respectively; P < 0.01), a reduced grade of elastin degradation, and lower activities of elastase and gelatinase (26% and 19% lower, respectively) after CaCl2 treatment compared with sPLA2-X+/+ mice. In sPLA2-X−/− mice, immunofluorescence microscopic images showed that the immunoreactivity of sPLA2-X was detected only in neutrophils within aortic walls 3 days, 1, 2, and 6 wk after CaCl2 treatment, whereas the immunoreactivity was not detected in macrophages or mast cells in aortic walls. In sPLA2-X−/− mice, immunoreactivity also was colocalized in cells expressing matrix metalloproteinase (MMP)-9. Neutrophils isolated from sPLA2-X−/− mice had lower activities of elastase, gelatinase, and MMP-9 in response to stimuli compared with sPLA2-X+/+ mice. The attenuated release of elastase and gelatinase from sPLA2-X−/− neutrophils was reversed by exogenous addition of mouse sPLA2-X protein. The adoptive transfer of sPLA2-X−/+ neutrophils days 0 and 3 after CaCl2 treatment reversed aortic diameters and elastin degradation grades in the lethally irradiated sPLA2-X−/− mice reconstituted with sPLA2-X−/+ bone marrow to an extent similar to that seen in sPLA2-X+/+ mice. In conclusion, sPLA2-X in neutrophils plays a pathogenic role in AAA in a mice model.

leukocytes; proteases; eicosanoids

PROINFLAMMATORY LEUKOCYTES and their products, such as proteolytic enzymes, play critical roles in the pathogenesis of abdominal aortic aneurysms (AAA) (2, 5, 13, 18, 22, 26). Leukocytes-generating eicosanoids modulate the pathogenic role of leukocytes in autoinflammatory and paracrine manners (9, 14, 16). A recent report (34) did not determined which type of the cells in the arterial walls was a source of sPLA2-X in their mice.

We have shown previously that sPLA2-X in neutrophils modulates their cytotoxic activities and that sPLA2-X in neutrophils plays an important role in the pathogenesis of myocardial ischemia-reperfusion injury (11). It is well known that neutrophils critically contribute to AAA formation (5, 13, 18, 22, 26). Thus this study examines the possible roles of sPLA2-X in neutrophils in the pathogenesis of AAA using sPLA2-X deficient (sPLA2-X−/−) mice.

METHODS

Materials. The purified recombinant mouse sPLA2-X protein and the rabbit anti-human sPLA2-X polyclonal antibodies were prepared as in a previous report from our laboratory (11, 12). The following antibodies were used: rat monoclonal antibody to mouse neutrophils (ab2557; Abcam, Cambridge, UK), rat monoclonal antibody to mouse CD68 (ab53444–100; Abcam), goat polyclonal antibody to human matrix metalloproteinase (MMP)-9 (sc-6840; Santa Cruz, CA), goat polyclonal antibody to mouse mast cell tryptase (sc-32474; Santa Cruz, CA), rabbit polyclonal antibody to human α-smooth muscle actin (α-SMA; ab5694; Abcam), and rat monoclonal antibody to mouse CD31 (550274; BD Pharmingen). Cell culture reagents were from Sigma (Tokyo, Japan) and Invitrogen (Carlsbad, CA). Other chemicals were purchased from Sigma unless otherwise indicated.

Mouse. The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (No. 19-35), and

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procedures were carried out in accordance with Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). We have generated sPLA$_2$-X knockout mice as described in a previous report from our laboratory (11). Male sPLA$_2$-X$^{-/-}$ mice with a C57BL/6 background from F13 to F15 (10–12 wk old, 23–28 g) and the littermate sPLA$_2$-X$^{+/-}$ male mice as controls were analyzed.

Experimental AAA formation. The sPLA$_2$-X$^{+/-}$ mice, the sPLA$_2$-X$^{-/-}$ mice, or the chimeric mice were subjected to experimental AAA formation (2, 4). In brief, mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). For analgesia, buprenorphine (0.05 mg/kg; Otsuka Pharmaceutical, Tokyo, Japan) is subcutaneously administered 30 min before the surgery and every 8 h after surgery for 2 days. The adequacy of anesthesia and analgesia was determined by limb muscular relaxation and no reaction to the toe pinch test. Mice underwent laparotomy. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal structures. The diameter of the aorta was measured in triplicate midway between the renal artery origin and iliac artery bifurcation under a dissection microscope (Olympus, Tokyo, Japan) using a calibrated ocular grid. After baseline measurements, a strip of cotton gauze soaked with CaCl$_2$ (0.5 M) was applied to the external surface of the aorta. NaCl (0.9%) was substituted for CaCl$_2$ in sham control mice. After 15 min, the gauze was removed and the aorta was rinsed with 0.9% sterile saline and the incision was closed; mice were returned to their cages after recovery. Six weeks later the mice underwent laparotomy and dissection. Anesthesia and analgesia were performed in the same manner as the first surgery. Measurements of the aortic diameter were repeated at the same location in the midinfrarenal aorta. The measurements were made by an observer (K. Kawabata) unaware of the experimental groups. An AAA was defined as an increase in aortic diameter ≥50% since a commonly used clinical standard to diagnose AAA is an increase in aortic diameter of 50%. The entire infrarenal aorta (or aneurysm, when present) was removed for histological examination of elastin degradation. In some of the experiments, the aortas were harvested for examination of real-time PCR, proteases activities, immunofluorescence analysis 3 days, 1, 2, and 6 wk after the treatment with NaCl or CaCl$_2$.

Preparations of neutrophils, macrophages, and smooth muscle cells. Mice were anesthetized with 5% isoflurane in the anesthetic chamber to the point they were nonresponsive to toe pinch and then euthanized by cervical dislocation. Neutrophils were purified from bone marrow cells by using a MACS selection system using biotin-labeled anti-Gr-1 (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. Neutrophil purity was 97% as assessed by cytopsin. Peritoneal macrophages were elicited by intraperitoneal injection of 2 ml of 3% thioglycollate for 3 days. Thioglycollate-elicited peritoneal macrophages and resident peritoneal macrophages were harvested by peritoneal lavage with 5 ml PBS. To isolate aortic SMC, the aortas excised from sPLA$_2$-X$^{+/-}$ mice were cut into pieces on a gelatin dish and cultured with DMEM and 10% FBS. The confluent cells were verified by an immunohistochemical analysis using antibodies to α-SMA and to mouse CD31, and these cells consisted mostly of vascular SMC (≥99%).

Bone marrow transplant and adoptive neutrophil transfer. Bone marrow transplantation was performed as described previously (11). Briefly, sPLA$_2$-X$^{+/-}$ mice and sPLA$_2$-X$^{-/-}$ mice (10–12 wk of age) were anesthetized and euthanized as described above. The marrow from the tibia and femur was harvested under sterile conditions. Cells were washed and followed by hypotonic lysis of erythrocytes, yielding ∼5 × 10$^7$ nucleated bone marrow cells per mouse. The sPLA$_2$-X$^{+/-}$ mice (6 wk of age) were lethally irradiated with 8 Gy. Immediately after irradiation, 1 × 10$^7$ bone marrow cells from sPLA$_2$-X$^{+/-}$ mice or sPLA$_2$-X$^{-/-}$ mice were injected through the tail vein. Six weeks after transplantation, the resulting chimeric mice were subjected to experimental AAA induction, as described above. In parallel, genomic DNA was extracted from peripheral blood, bone marrow, and tail, and the efficiency of reconstitution was confirmed by genomic PCR.

Some of the sPLA$_2$-X$^{+/-}$ mice reconstituted with the sPLA$_2$-X$^{-/-}$ marrow were tail- vein injected with bone marrow neutrophils from sPLA$_2$-X$^{+/-}$ mice (1 × 10$^7$ neutrophils in 200 μl of PBS) on days 0 and 3 after the CaCl$_2$ treatment. In a separate adoptive neutrophil transfer experiment, we evaluated whether adoptively transferred neutrophils localized to the aortic wall. The isolated bone marrow neutrophils from sPLA$_2$-X$^{+/-}$ mice (1 × 10$^7$) were labeled with cell tracker orange (CMTMR at 2.5 μM; Invitrogen, Carlsbad, CA) for 15 min at room temperature and tail-vein injected into the sPLA$_2$-X$^{+/-}$ mice 3 days after the CaCl$_2$ treatment. Four hours later, the abdominal aorta was harvested and embedded in optimal cutting temperature (OCT) compound. Frozen sections were examined for the presence of cells with CMTMR under a confocal microscope (Olympus FV-1000; Tokyo, Japan) equipped with a × 60/1.0 numerical aperture oil-immersion objective. A serial section was stained with anti-mouse neutrophil polyclonal antibody (Abcam), and the proportion of the CMTMR-labeled neutrophils in aortic tissue after CaCl$_2$ treatment was calculated.

Measurement of mRNA expression levels. Total RNA was extracted from tissues and cells with a Qiagen RNeasy kit and DNase I (Qiagen, Tokyo, Japan). The mRNA expression levels in 0.1 μg of total RNA were quantified by a real-time two-step RT-PCR assay using SYBR Green I chemistry and a 7500 Real-Time PCR System at 40 cycles (Applied Biosystems) (11, 31). The PCR primers are listed in Table 1. The GAPDH housekeeping gene was used for normalization of gene expression. In some analyses, the quantification of the mRNA target was obtained by calculating the relative expression of the reference gene compared with GAPDH (31). Data were expressed as Δcycle threshold (Ct), which was calculated as the number of PCR cycles for liftoff for the target mRNA of interest minus the number of PCR cycles for liftoff for GAPDH mRNA: ΔCt = Ct (gene of interest) − Ct (GAPDH). The Ct values for GAPDH were typically around 20.

Histological study. For immunofluorescence analysis, aortas after CaCl$_2$ or NaCl treatment were perfusion-fixed with 4% paraformaldehyde in PBS (pH 7.4) at 60 mmHg, harvested, and then sliced into sections of 10 μm thickness (11). These sections were fixed with acetone at −20°C and then incubated with the primary antibodies followed by secondary antibodies. The secondary antibodies for the immunofluorescence study included Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Carlsbad, CA). For other histological analysis, the aortas were perfusion-fixed with 4% paraformaldehyde at 60 mmHg, harvested, and immersion-fixed in 4% paraformaldehyde overnight. They were then embedded in paraffin and sliced into sections of 5 μm thickness. These sections were fixed with acetone for 5 min at −20°C and then incubated with the primary antibodies followed by secondary antibodies. The secondary antibodies for the immunofluorescence study included Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Carlsbad, CA).

Elastase and gelatinase activities in CaCl$_2$-treated aortas and isolated neutrophils. The isolated neutrophils were suspended at a density of 1 × 10$^7$/ml in HBSS and stimulated at 37°C for 30 min with PMA (1 μM) or for 5 min with cytochalasin B (CB; 5 μg/ml) followed by an additional incubation for 30 min with N-formyl-methionyl-leucyl-phenylalanine (FMLP; 1 μM) (11). In some experiments, neutrophils were stimulated in the presence of mouse sPLA$_2$-X protein (1 ng/ml) or vehicle. The cell suspensions were centrifuged (300 g for 5 min at 4°C), and the supernatants were harvested. The pelletted cells were lysed by an addition of an equivalent volume of HBSS containing 0.5% CHAPS (3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate). The elastase and ge-
latinase activities in the harvested supernatants and cell lysates were determined using EnzCheck Elastase Assay kit and EnzChek Gelatinase Assay kit (Invitrogen), according to protocols provided by the manufacturer. The gelatinase activity measured by EnzChek Gelatinase Assay kit was represented as total gelatinase activity since this assay kit measures net activity of MMP-9 and other MMPs including MMP-2. The lower limitation of detection was 5 mU/ml and 0.3 mU/ml, respectively. Release of elastase and total gelatinase activities of the isolated neutrophils into the supernatant was determined using EnzCheck Elastase Assay kit and EnzChek Gelatinase Assay kit was represented as total gelatinase activity since this assay kit measures net activity of MMP-9 and other MMPs including MMP-2.

Leukotriene B4 release from neutrophils. The isolated neutrophils were stimulated as described above. Leukotriene B4 (LTB4) levels in the supernatants were measured by Enzyme Immunoassay as described by the manufacturer of the kit (Biotrak, RPN 223, Amersham, UK). Absorbances were measured at 450 nm using the Spectra Max 340 Microplate Reader (Molecular Devices). The lower limitation of detection was 6 pg/ml. Results are expressed as picograms per 107 cells.

Gelatin zymography of isolated neutrophils. Supernatants from the neutrophils were resolved in 7.5% SDS-PAGE containing 1 mg/ml gelatin (Sigma, Tokyo, Japan) and separated by electrophoresis. Subsequently, SDS was removed from the gels by two washes (15 min) with 2.5% Triton X-100. Gels were incubated for 24 h (37°C) in zymography buffer containing (in mM) 50 Tris-HCl, 50 Tris-Base, and 5 CaCl2 and stained with Coomassie Brilliant Blue. Recombinant MMP-9 (R&D Systems, Minneapolis, MN) was used as a positive control. Densitometric analysis was performed with ImageJ software from the NIH.

Respiratory burst function in isolated neutrophils. Production of active oxygen metabolites during neutrophil activation was measured by the method of luminol-dependent chemiluminescence using the SpectraMax L Luminescence Microplate Reader (Molecular Devices) as described in a previous report from our laboratory (11). The light emission was recorded after addition of luminol (0.1 mM), followed by the addition of HBSS as a vehicle or one of the activators including fMLP (10 μM) plus CB (5 μg/ml), or serum-opsonized zymosan (OZ, 100 μg/ml). Integrated counts were determined as the sum of chemiluminescence counts integrated for 5 min (fMLP plus CB) or 20 min (OZ) after activation.

Statistical analysis. Results are expressed as means ± SE. Mean values were compared between two groups with an unpaired t-test. Comparisons among ≥3 groups were performed by one-way ANOVA with the Scheffé F procedure for post hoc analysis. P < 0.05 was considered statistically significant.

### RESULTS

Characterization of sPLA2-X−/− mice. There was no significant difference in baseline characteristics of sPLA2-X−/− mice compared with sPLA2-X+/+ mice, as shown in previous reports from our laboratory (11). Expression levels of mRNA for sPLA2-IIIC, -IID, -IIE, sPLA2α (IVA), sPLA2-V, iPLA2β (VI), and sPLA2-XIIA in the abdominal aorta at baseline were similar between the sPLA2-X+/+ and sPLA2-X−/− mice (data not shown). Expression of mRNA for sPLA2-IB, -IIF, -III, and sPLA2-III were 100 ± 27, 109 ± 33, and 100 ± 27, respectively, in sPLA2-X+/+ and sPLA2-X−/− mice. The expression of mRNA for sPLA2-IIIC, -IID, -IIE, and sPLA2α (IVA) was 90 ± 27, 86 ± 27, 90 ± 27, and 90 ± 27, respectively, in sPLA2-X+/+ and sPLA2-X−/− mice. The expression of mRNA for sPLA2-V, iPLA2β (VI), and sPLA2-XIIA was 90 ± 27, 90 ± 27, and 90 ± 27, respectively, in sPLA2-X+/+ and sPLA2-X−/− mice. The expression of mRNA for sPLA2-IIIC, -IID, -IIE, and sPLA2α (IVA) was 90 ± 27, 86 ± 27, 90 ± 27, and 90 ± 27, respectively, in sPLA2-X+/+ and sPLA2-X−/− mice.

Table 1. Sequences of PCR primers

<table>
<thead>
<tr>
<th>Forward Primers</th>
<th>Reverse Primers</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>sPLA-2-II</td>
<td>5'-TGGCAAGGAGGATGATTTC-3'</td>
</tr>
<tr>
<td>sPLA-2-IIC</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>sPLA-2-III</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>sPLA-2-X</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>sPLA-2-XIIA</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>sPLA-2-XIIB</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>F4/80</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>5'-GAGGCCAGGGATGATTTC-3'</td>
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sPLA, secretory PLA.

Fig. 1. Comparison of the abdominal aortic diameters of Group X secretory phospholipase A2 (sPLA2-X+/+) mice [wild-type (WT)] and sPLA2-X−/− mice [knockout (KO)] 6 wk after treatment with NaCl or CaCl2. A: comparison of percent changes in aortic diameters from pretreatment to 6 wk after treatment with NaCl or CaCl2. Statistical analysis. Results are expressed as means ± SE. Mean values were compared between two groups with an unpaired t-test. Comparisons among ≥3 groups were performed by one-way ANOVA with the Scheffé F procedure for post hoc analysis. P < 0.05 was considered statistically significant.
Table 2. Aortic diameter in sPLA2-X+/+ mice and sPLA2-X−/− mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>sPLA2-X</th>
<th>WT</th>
<th>KO</th>
<th>sPLA2-X</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>CaCl2</td>
<td>50 ± 15.8</td>
<td>490 ± 10.0</td>
<td>513 ± 12.5</td>
<td>531 ± 9.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-treatment, μm</td>
<td>560 ± 10.0</td>
<td>560 ± 10.0</td>
<td>763 ± 35.0</td>
<td>663 ± 20.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent increase</td>
<td>12.3 ± 2.5</td>
<td>14.4 ± 2.7</td>
<td>49.9 ± 9.1</td>
<td>24.8 ± 3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA, %</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE or percentage. Comparison of the abdominal aortic diameters of sPLA2-X+/+ mice [wild-type (WT)] and sPLA2-X−/− mice [knockout (KO)] 6 wk after treatment with NaCl or CaCl2 is shown. An abdominal aortic aneurysm (AAA) was defined as an increase in aortic diameter ≥50% from pretreatment. *P < 0.05, compared with CaCl2-treated KO mice.

Expression of sPLA2-X in sPLA2-X+/+ mice. For sPLA2-X+/+ mice, the immunofluorescence microscopic images showed that the immunoreactivity of sPLA2-X was found in the intimal and adventitial layers. A majority of the immunoreactivity of sPLA2-X was in the adventitial layer. The double immunofluorescence study showed that sPLA2-X was confined to neutrophils within aortic walls 3 days and 1, 2, and 6 wk after CaCl2 treatment (Fig. 3A). The sPLA2-X immunoreactivity was not detected in macrophages or mast cells in the aortic walls (Fig. 4A). The sPLA2-X immunoreactivity was colocalized in cells expressing MMP-9 (Fig. 4A).

The expression of sPLA2-X mRNA was significant but low in homogenates of whole aortic tissue after CaCl2 treatment in sPLA2-X+/+ mice (mean ΔCt, 18.6 ± 1.2; Fig. 3B). Expression of mRNA was not detectable in tissue homogenates of NaCl-treated aortas of sPLA2-X−/− mice [no detectable Ct (no liftoff of the amplification plot at 40 PCR cycles), Fig. 3B]. The expression of sPLA2-X mRNA was detectable in the isolated neutrophils (mean ΔCt, 16.7 ± 1.0; Fig. 3B), but it was not detectable in isolated peritoneal macrophages (either thioglycollate elicited-macrophage or resident macrophage) or the cultured aortic SMC from sPLA2-X−/− mice [no detectable Ct (no liftoff of the amplification plot at 40 PCR cycles); Fig. 4B].

Elastase and total gelatinases activities, respiratory burst function, and LTB4 formation. The activities of elastase and total gelatinases in the aortas after CaCl2 treatment were lower in sPLA2-X−/− mice than in sPLA2-X+/+ mice (Fig. 5A). Neutrophils isolated from sPLA2-X−/− mice had lower release of elastase, total gelatinases, and MMP-9 gelatinase activities in response to PMA or fMLP plus CB compared with sPLA2-X+/+ mice (Fig. 5B and Fig. 6). The attenuated release of proteases from sPLA2-X−/− neutrophils was reversed by the exogenous addition of mouse sPLA2-X protein (1 ng/ml) (Figs. 5B and 6), which alone at this dose did not induce a release of these proteases. Activities of elastase and total gelatinases in cell lysates of unstimulated neutrophils were similar between...
sPLA2-X+/+ and sPLA2-X−/− neutrophils (elastase: 130.4 ± 6.0 mU/ml vs. 133.4 ± 8.7 mU/ml, respectively, P = 0.80; total gelatinases: 58.4 ± 4.2 mU/ml vs. 62.6 ± 3.5 mU/ml, respectively, P = 0.51).

Neutrophils from sPLA2-X−/− mice had lower release of LTB4 and lower respiratory burst compared with sPLA2-X+/+ mice, and these attenuated functions were reversed by the addition of sPLA2-X protein (1 ng/ml) (Fig. 5, C and D).

**Bone marrow transplantation and adoptive transfer of neutrophils.** The aortic diameters and elastin degradation grades were significantly reduced in the lethally irradiated sPLA2-X+/+ mice reconstituted with sPLA2-X−/− bone marrow compared with the irradiated sPLA2-X+/+ mice reconstituted with sPLA2-X−/− bone marrow (Fig. 7 and Table 3). The adoptive transfer of sPLA2-X−/− neutrophils reversed aortic diameters and elastin degradation in chimeric sPLA2-X−/− mice reconstituted with sPLA2-X−/− bone marrow to an extent similar to that seen in sPLA2-X+/+ mice. Therefore, sPLA2-X in neutrophils may

DISCUSSION

The present study showed that aortic dilation and elastin degradation after CaCl2 treatment were attenuated in sPLA2-X−/− mice compared with sPLA2-X+/+ mice. The results indicate that sPLA2-X has a role in the pathogenesis of AAA, which is in agreement with a previous report (34). The present immunofluorescence studies showed that expression of sPLA2-X was confined to neutrophils in aortic walls after CaCl2 treatment. Moreover, the aortic dilation and the elastin degradation after CaCl2 treatment were also reduced in the irradiated sPLA2-X−/− mice reconstituted with bone marrow from sPLA2-X−/− mice. In addition, the adoptive transfer of sPLA2-X−/− neutrophils reversed the aortic dilation and elastin degradation in chimeric sPLA2-X−/− mice reconstituted with the sPLA2-X−/− bone marrow to an extent similar to that seen in sPLA2-X+/+ mice. Therefore, sPLA2-X in neutrophils may
Critically contribute to the pathogenesis of CaCl₂-induced AAA in a mouse model. This is supported by the present findings that elastase and MMP-9 gelatinase activities were lower in sPLA₂-X<sup>−/−</sup> neutrophils than in sPLA₂-X<sup>+/+</sup> neutrophils. That observation is consistent with the decrease in elastase and total gelatinases activities in sPLA₂-X<sup>−/−</sup> aortas compared with sPLA₂-X<sup>+/+</sup> aortas. The present study and a previous study from our laboratory (11) showed that sPLA₂-X in neutrophils contributes to generation of reactive oxygen species (ROS) in an autocrine manner. ROS have several pro-aneurysmal effects including matrix degradation and apoptosis of smooth muscle cells (20). ROS might activate proteolytic enzymes in the milieu of the aortic walls (20). Thus sPLA₂-X in neutrophils induces both proteolytic enzymes and ROS in neutrophils themselves, and the activated proteolytic enzymes degrade arterial matrix, resulting in aortic dilation and aneurysm formation.

The present quantitative RT-PCR analysis showed that low levels of sPLA₂-X mRNA were detected in whole AAA tissue from sPLA₂-X<sup>+/+</sup> mice. In line with this finding, the present

Fig. 4. Expression of sPLA₂-X and matrix metalloproteinase (MMP)-9 in sPLA₂-X<sup>−/−</sup> mice. A: representative immunofluorescence images of aortas 3 days after CaCl₂ treatment in sPLA₂-X<sup>−/−</sup> mice. Immunofluorescence images were stained with antibodies for sPLA₂-X (green, a, g, and j), MMP-9 (red, b and e), macrophages (Mφ) (red, h), and mast cell tryptase (red, k) and double-stained for sPLA₂-X and MMP-9 (c), neutrophils and MMP-9 (f), sPLA₂-X and Mφ (i), and sPLA₂-X and mast cell (l). Scale bars in images were 10 μm. Sections are representative of 5 mice. B: agarose gel electrophoresis of the amplified PCR products at 37 cycles from 0.1 μg of total RNA from peritoneal resident macrophages (Mφ), cultured aortic smooth muscle cells (SMC), and testis of sPLA₂-X<sup>−/−</sup> mice.
neutrophils, but it was expressed only in a subpopulation of neutrophils in bone marrow and in the aortic tissue. This may partly explain for low expression of sPLA2-X mRNA in total neutrophils. Under investigation in our laboratory are the characteristics and functional role of the subpopulation of sPLA2-X-positive neutrophils.

The secreted sPLA2-X from neutrophils is capable of activating neighboring cells (33). The secreted sPLA2-X might stimulate monocytes/macrophages in addition to neutrophils themselves in aortic walls to generate a variety of proinflammatory cytokines, leading to further recruitment of neutrophils and monocytes from circulating blood and their activation. sPLA2-X in neutrophils may trigger this inflammatory cascade on the basis of the present adoptive transfer experiment showing that the injected sPLA2-X+ neutrophils induced aortic diameters and elastin degradation despite their short life period in the aorta. Although the present study did not measure the aortic content of proinflammatory cytokines, a previous report (34) showed that deficiency of sPLA2-X was associated with a reduction in proinflammatory cytokines in the aortas of angiotensin II-induced AAA in ApoE−/− mice. Thus it is also possible that the weakened activation of neighboring monocytes/macrophages may contribute to a reduction of aneurysmal formation in sPLA2-X−/− mice. Recent studies have demonstrated several functional alterations in macrophages from sPLA2-X−/− mice compared with sPLA2-X+/+ mice (28, 29). The present study showed no detectable expression of sPLA2-X mRNA in macrophages from sPLA2-X−/− mice, but secreted sPLA2-X or lipid mediators from neutrophils may potentially alter the function of neighboring macrophages.

The present study did not elucidate the mechanisms by which the release of elastase and MMP-9 activities in response to external stimuli was suppressed in isolated sPLA2-X−/− neutrophils. We showed that the attenuated function of the isolated sPLA2-X−/− neutrophils was reversed by exogenous

study showed that the sPLA2-X immunoreactivity was limited to neutrophils within the arterial walls of AAA, whereas it was not detectable in monocytes/macrophages or mast cells in the AAA. Moreover, sPLA2-X mRNA was detected in isolated bone marrow neutrophils, whereas the present study failed to detect significant levels of sPLA2-mRNA in the isolated peritoneal macrophages or in cultured aortic SMC from sPLA2-X+/+ mice. Although previous reports showed that sPLA2-X was expressed in various tissues and cells in humans (15, 19, 23), sPLA2-X expression in the aortic walls seems to be limited to a small number of cells in mice. Our preliminarily experiment indicates that sPLA2-X was not expressed in all
addition of mouse sPLA2-X protein, which is consistent with a previous report from our laboratory (11). That previous report from our laboratory (11) also showed that extracellular release of arachidonic acid in response to fMLP was suppressed in sPLA2-X−/− mice, but its intracellular mechanisms remain unresolved.

We have shown that sPLA2 inhibitors suppressed myocardial ischemia-reperfusion injury in a mouse model (11). Previous reports showed that sPLA2 inhibitors also inhibited atherosclerosis and aneurysm formation of aortas in animal models (10). Recently, preliminary clinical studies (27) showed that varespladin, an oral inhibitor of sPLA2, reduced LDL-cholesterol and C-reactive protein levels. Therefore, sPLA2 inhibitors might have a therapeutic value for preventing cardiovascular events. The present data may provide a rationale for the study of the therapeutic effect of sPLA2 inhibitors on AAA.

This study has several limitations. First, there is a lack of a universally accepted animal model of AAA. The local application of CaCl2 to the adventitial arterial surface induced an inflammatory infiltraton and induction of metalloproteinases followed by aneurysmal dilation that is relevant to human AAA (6). It remains to be determined whether sPLA2-X in neutrophils has a role in AAA induction of angiotensin II infusion in apoe-deficient mice or in mice treated with intra-aortic elastase perfusion. In human AAA, neutrophils are mainly found in the intraluminal thrombus, whereas neutrophils are not abundant in the adventitia (14), which is in contrast with this mouse model. Our preliminary experiment shows high expression of sPLA2-X in neutrophils contained in human thrombus. Further study may reveal a potential role of neutrophils sPLA2-X in human AAA. Second, the expression level of sPLA2-X mRNA was relatively low in the isolated bone marrow neutrophils as well as whole AAA tissue, although low expression levels of mRNA can be functional. The expression levels of sPLA2-X protein should be quantified using Western blotting, but there are technical problems possibly due to post-translational modification such as glycation and binding to various intracellular molecules. Third, we did not measure the internal diameter of aortas using ultrasonography or angiography.

Table 3. Aortic diameter in sPLA2-X+/+ mice and sPLA2-X−/− mice with bone marrow transplantation and adoptive transfer

<table>
<thead>
<tr>
<th>sPLA2-X</th>
<th>WT→WT</th>
<th>KO→WT</th>
<th>KO→WT + WT Neutro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Pretreatment, μm</td>
<td>529 ± 10.0</td>
<td>536 ± 9.2</td>
<td>500 ± 10.9</td>
</tr>
<tr>
<td>Post-treatment, μm</td>
<td>814 ± 35.7*</td>
<td>693 ± 23.0</td>
<td>743 ± 17.0*</td>
</tr>
<tr>
<td>Percent increase</td>
<td>54.2 ± 6.7*</td>
<td>29.9 ± 6.1</td>
<td>49.1 ± 5.3*</td>
</tr>
<tr>
<td>AAA, %</td>
<td>57</td>
<td>29</td>
<td>57</td>
</tr>
</tbody>
</table>

Values are means ± SE or percentage. Comparison of aortic diameters 6 wk after CaCl2 treatment among sPLA2-X+/+ mice reconstituted with sPLA2-X−/− bone marrow (WT→WT), sPLA2-X−/− mice reconstituted with sPLA2-X−/− bone marrow (KO→WT), and sPLA2-X−/− mice reconstituted with sPLA2-X−/− bone marrow in combination with adoptive transfer of sPLA2-X−/− neutrophils (KO→WT + WT neutro) was shown. AAA was defined as an increase in aortic diameter ≥50% from pretreatment. *P < 0.05, compared with sPLA2-X−/− mice reconstituted with sPLA2-X−/− bone marrow (KO→WT).
In conclusion, sPLA2-X in neutrophils plays a critical role in the pathogenesis of AAA in a mouse model.

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DISCLOSURES
K. Hanasaki is an employee of Shionogi Research Laboratories, Shionogi (Osaka, Japan). He contributed to the development of sPLA2-X knockout mice, recombinant mouse sPLA2-X protein, and rabbit anti-human sPLA2-X polyclonal antibodies. Although he had no role in the study design, data collection, data analysis, data interpretation, or writing of the report, the other authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

REFERENCES


