Transgenic Expression of Group V, but Not Group X, Secreted Phospholipase A2 in Mice Leads to Neonatal Lethality because of Lung Dysfunction

Received for publication, August 21, 2006, and in revised form, September 25, 2006 Published, JBC Papers in Press, September 28, 2006, DOI 10.1074/jbc.M607975200

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In an effort to elucidate the functions of secreted phospholipase A2 (sPLA2) enzymes in vivo, we generated transgenic (Tg) mice for group V sPLA2 (sPLA2-V) and group X sPLA2 (sPLA2-X), which act potently on phosphatidylycholine in vitro. We found that sPLA2-V Tg mice died in the neonatal period because of respiratory failure. The lungs of sPLA2-V Tg mice exhibited atelectasis with thickened alveolar walls and narrow air spaces, accompanied by infiltration of macrophages and only modest changes in eicosanoid levels. This severe pulmonary defect in sPLA2-V Tg mice was attributable to marked reduction of the lung surfactant phospholipids, phosphatidylycholine and phosphatidylglycerol. Given that the expression of sPLA2-V is greatly elevated in human lungs with severe inflammation, our present results raise the intriguing possibility that this isozyme may contribute to ongoing surfactant hydrolysis often observed in the lungs of patients with respiratory distress syndrome. In contrast, sPLA2-X Tg neonates displayed minimal abnormality of the respiratory tract with normal alveolar architecture and surfactant composition. This unexpected result was likely because sPLA2-X protein existed as an inactive zymogen in most tissues. The active form of sPLA2-X was detected in tissues with inflammatory granulation in sPLA2-X Tg mice. These results suggest that sPLA2-X mostly remains inactive under physiological conditions and that its proteolytic activation occurs during inflammation or other as yet unidentified circumstances in vivo.

Phospholipase A2 (PLA2) hydrolyzes cellular glycerophospholipids to release lysophospholipids and free fatty acids. Because these lipid products and/or their metabolites take part in a number of biological responses, PLA2s have attracted much attention over the last 2 decades. A number of mammalian PLA2s have been identified to date, including 10 secreted PLA2s (sPLA2s; group IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA) (1, 2), 6 cytosolic PLA2s (cPLA2s; α, β, γ, δ, ε, and ζ) (3–6), 6 Ca2+-independent PLA2s (iPLA2s; β, δ, γ, ε, ζ, and η) (7–9), 4 platelet-activating factor acylhydrolases (PAF-AHs; plasma PAF-AH and intracellular PAF-AH-I (α) and -II (α)) (10), and 2 lysosomal PLA2s (acidic Ca2+-dependent lysosomal PLA2 (aiPLA2)/peroxiredoxin 6) and lysosomal PLA2 (LPLA2)/1-O-acylceramide synthase (11, 12).

The sPLA2 family represents a group of structurally related, disulfide-rich, Ca2+-dependent, low molecular weight enzymes with a catalytic histidine (1, 2). Individual sPLA2s exhibit unique tissue and cellular localizations and enzymatic properties, suggesting their distinct roles in various pathophysiological events. Several sPLA2 enzymes have the ability to hydrolyze cellular phospholipids to release arachidonic acid (AA), a precursor of eicosanoids, and lysophospholipids (13–18). Among them, group V sPLA2 (sPLA2-V) and group X sPLA2 (sPLA2-X) represent the most potent sPLA2 isoforms that have the ability to act on the phosphatidylcholine (PC)-rich outer plasma membrane of various cells (14–17). Because of the secreted moeity of sPLA2s, it is likely that their target substrates also exist in the extracellular milieu. In fact, several sPLA2s hydrolyze phospholipids in bacterial membranes (19–21), plasma lipoproteins (22–26), lung surfactant (27–35), and so on with distinct poten-

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Culture, Sports and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cies in vitro, suggesting that they play divergent roles in various biological events. However, the proposed roles of sPLA₂ enzymes have largely been inferred from their enzymatic and biochemical properties, the effects of their overexpression or exogenous addition in in vitro experimental settings, or the use of pharmacological inhibitors (whose specificity may not be strictly defined).

Given the complexity and diversity of the PLA₂ family, transgenic (Tg) overexpression (gain-of-function) and gene targeting (loss-of-function) strategies seem to be more appropriate for elucidating the functions of individual enzymes in vivo. Indeed, these approaches have proved fruitful for determining the crucial roles of cPLA₁α in eicosanoid and platelet-activating factor generation in a wide variety of pathological and physiological states (36–39); iPLA₂β in ischemic heart damage, sperm motility, nitric oxide-induced antiviral response, and diet-induced insulin secretion (40–43); iPLA₂ζ (adipose triglyceride lipase) in fat mobilization in adipose tissue (44); intracellular PAF-AH-I in spermatogenesis (45); and two lysosomal PLA₂s in steady-state lung surfactant turnover (11, 12). With regard to sPLA₂s, sPLA₂-IIA Tg mice (beyond that endogenous sPLA₂-IIA in mice is largely restricted to intestinal Paneth cells (e.g. BALB/c) or not expressed at all (e.g. C57BL/6 and 129/Sv)) showed marked resistance to bacterial infection (19, 20), thus substantiating the in vitro observations that this enzyme efficiently hydrolyzes bacterial membranes (21). In addition, sPLA₂-IIA Tg mice show increased lipoprotein hydrolysis and atherosclerosis (22–24), although sPLA₂-II and sPLA₂-X have also been implicated in these processes (25, 26). Mouse strains intrinsically deficient in sPLA₂-IIA because of a frameshift mutation in its gene are susceptible to intestinal tumorigenesis (45). Recent gene targeting studies of sPLA₂-IB and sPLA₂-V have provided unequivocal evidence for their roles in digestion of dietary phospholipids (47, 48) and eicosanoid production and phagocytosis by macrophages (49, 50), respectively. Furthermore, a gain-of-function study in Xenopus laevis has revealed the role of sPLA₂-XIIa in olfactory sensory neuron structure in a manner independent of its catalytic activity (51).

In this study, we generated Tg mice overexpressing sPLA₂-V or sPLA₂-X, the two most potent PC-hydrolyzing isozymes, in an effort to search for their suitable substrates in vivo. We found that all sPLA₂-V Tg neonates died within several hours after birth because of respiratory failure. This severe, lethal phenotype of sPLA₂-V Tg mice is attributable to aberrant hydrolysis of lung surfactant phospholipids, an event that has often been observed in the lungs of patients with respiratory distress syndrome (RDS). In contrast, sPLA₂-X Tg mice do not display such detrimental lung damage, because sPLA₂-X exists as an inactive zymogen in most tissues under physiological conditions. Proteolytic activation of sPLA₂-X in vivo occurs in particular situations such as inflammation.

**EXPERIMENTAL PROCEDURES**

**Generation of sPLA₂ Tg Mice**—All mice were housed in climate-controlled (21 °C) specific pathogen-free facilities with a 12-h light-dark cycle, with free access to standard laboratory food (Picolab mouse diet 20; Laboratory Diet, Brentwood, MO) and water. All procedures involving animals were performed according to protocols approved by the faculties.

The strategy for the generation of sPLA₂ Tg mice is illustrated in Fig. 1A. The cDNA for mouse sPLA₂-V or human sPLA₂-X was inserted into the EcoRI site of the pcDNA3 vector (52) (Fig. 1). The plasmid, in which the transgene was flanked downstream of a neomycin cassette with LoxP sites at both ends, was excised at the HindIII and Sall sites to produce a 5.1-kb CAG-loxP-neo'-loxP-sPLA₂ (LNL-sPLA₂) fragment. Female 6–8-week-old C57BL/6 mice (Japan SLC) were treated with 5 units of horse gonadotropin (Teikokuzoki Pharmaceutical) and 48 h later with 5 units of human chorionic gonadotropin (Teikokuzoki Pharmaceutical) and then mated with male C57BL/6 mice. The purified LNL-sPLA₂ fragment was microinjected into 0.5-day fertilized eggs using a micromanipulator (Nikon), and the eggs were transferred to the fimbrae of uterine tubes in female ICR mice (Japan SLC) that had been mated with vasoligated male mice the previous day. The tails (5 mm in length) of the pups (4-week-old) were cut off and lysed in 500 μl of tissue lysis buffer comprising 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS, and 0.4 mg/ml proteinase K (Sigma) at 55 °C overnight. DNA was purified from the lysates using an automated DNA isolation system (NA-2000; Kurabo Industries), and aliquots were taken for PCR for genotyping (see below). Male founders were mated with female C57BL/mice to confirm germ line transmission by PCR genotyping, and those with successful germ line transmission were then crossed with female CAG-Cre Tg mice, which carry the Cre recombinase transgene under the control of the ubiquitously active cytomegalovirus immediate early enhancer/chicken β-actin hybrid (CAG) promoter (53, 54). This step resulted in removal of the neo’ cassette from the LNL-sPLA₂ transgene, thereby allowing activation of the sPLA₂-V or sPLA₂-X transgene in the whole body of the offspring (sPLA₂ Tg mice) (Fig. 1). As required, the male founders were mated with female pgk2-Cre Tg mice, in which the transcription of the Cre recombinase transgene was controlled by the spermatogenic cell-specific promoter pgk2, allowing generation of the testis-specific sPLA₂ Tg mice (55). All the sPLA₂ Tg mice were inbred with C57/B1/6 mice.

**PCR Genotyping**—Approximately 0.1 μg of genomic DNA obtained from the mouse tails was subjected to PCR amplification with ExTaq polymerase (Takara Biomedicals) and a set of primers of CAG-F1 (5’-CTGCTAACCAGTGTTGCTC-3’) and CAGV-R1 (5’-CATTCTTCCTGGTACCTC-3’) (for sPLA₂-V Tg mice) or CAG-F1 and CAGX-R1 (5’-GGGCTTACGAGTTCACATC-3’) (for sPLA₂-X Tg mice). The PCR primers were obtained from Fasmac. The PCR conditions were 95 °C for 5 min and then 35 cycles of 95 °C for 30 s and 68 °C for 3 min on a thermal cycler (Applied Biosystems). The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide.

**Northern Blotting**—Equal amounts (~10 μg) of total RNA obtained from tissues by use of 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 appropriate cDNA probes that had been labeled with [32P]dCTP (PerkinElmer Life Sciences) by random priming.
**Group V and X Phospholipase A$_2$ Transgenic Mice**

(Takara Biomedicals). Hybridization and subsequent membrane washing were carried out as described previously (14).

**RT-PCR**—Synthesis of cDNA was performed using 0.5 μg of total RNA from tissues and avian myeloblastosis virus 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 (23-bp oligonucleotide primers corresponding to 5’- and 3’-nucleotide sequences of the open reading frame) for mouse sPLA$_2$-V. The PCR conditions were 94 °C for 30 s and then 35 cycles of amplification at 94 °C for 5 s and 68 °C for 4 min, using the Advantage cDNA polymerase mix (Clontech). RT-PCR for β-actin as a control was performed according to the manufacturer’s instructions (Sigma). The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide.

**Immunoblotting**—Tissues (100 mg) were soaked in 500 μl of SET buffer comprising 20 mm Tris-HCl (pH 7.4), 0.25 μm sucrose, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (Sigma) and then homogenized with a Polytron homogenizer and sonicator. The homogenates (20 μg of protein equivalents) were subjected to SDS-PAGE on 13% gels under reducing conditions with 2-mercaptoethanol. Protein concentrations in the samples were determined with a BCA protein assay kit (Pierce) using bovine serum albumin (Sigma) as a standard. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) with a semi-dry blotter (Trans-blot SD; Bio-Rad). After blocking with 5% (v/v) skim milk in TBS for 30 min, washed three times with 3% (v/v) H$_2$O$_2$, washed three times with TBS for 5 min each, and incubated with anti-sPLA$_2$-V antibody at 1:200 dilution in TBS overnight at 4 °C. Alternatively, anti-F4/80 (Serotec) and anti-surfactant protein C (SP-C; Santa Cruz Biotechnology) antibodies were used at 1:50 dilution, respectively. The sections were then treated with a catalyzed signal-amplified system staining kit (DAKO) with diaminobenzidine substrate, followed by counterstaining with hematoxylin and eosin. The cell type was identified from conventional hematoxylin and eosin staining of serial sections adjacent to the specimens used for immunohistochemistry.

For periodic acid-Schiff (PAS) staining (59), tissue sections were deparaffinized, rehydrated, and placed in 0.5% periodic acid solution for 5 min. After washing in water, the sections were placed in Coleman’s Schiff reagent for 15 min, washed in water for 10 min, and counterstained with hematoxylin solution for 15 min. After washing in water for 15 min, the sections were dehydrated and mounted with resins medium.

**Electrospray Ionization/Mass Spectrometry (ESI/MS)**—Lungs of newborn mice were lavaged with 1 ml of SET buffer, and the resulting bronchoalveolar lavage fluid (BALF) was used after centrifugation to remove the cells and tissue debris. Lipids were extracted from the BALF by the method of Bligh and Dyer (60). Before the lipid extraction, PC with C14:1–14:1 (diacyl) and phosphatidylglycerol (PG) with C14:0–14:0 (diacyl) were added to the BALF (1 nmol of internal standard per 1 mg of protein equivalents) as internal standards. MS spectra were obtained on a Quattro Micro tandem quadrupole mass spectrometer (Micromass) equipped with an ESI, as described previously (61). Lipid extracts obtained from BALF were reconstituted in 2:1 chloroform/methanol (100–300 μmol phosphorus/liter), and 2 μl of the sample was injected per run. The samples were introduced by means of a flow injector into the ESI chamber at a flow rate of 4 μl/min in a solvent system of acetonitrile/methanol/water (2:3:1; v/v) containing 0.1% (v/v) ammonium formate (pH 6.4). The mass spectrometer was operated in the positive and negative scan modes. The flow rate of the nitrogen drying gas was 12 liters/min at 80 °C. The capillary and cone voltages were set at 3.7 kV and 30 V, respectively; argon at 3–4 × 10$^4$ torr was used as the collision gas, and a collision energy of 30–40 V was used for obtaining fragment ions for precursor ions.

**Measurement of Eicosanoids**—The contents of prostaglandin E$_2$ (PGE$_2$) and leukotriene C$_4$ (LTC$_4$) in the BALF were quantified with PGE$_2$ and LTC$_4$ enzyme immunoassay kits (Cayman Chemicals).
AA Release—Culture of the human bronchial epithelial cell line BEAS-2B and its stable transfectants for various PLA$_2$s (produced by the lentivirus method (Invitrogen)) was done as described previously (58). Cells grown to near confluency in 48-well plates (Iwaki Glass) were incubated with $[^3]$HAA (Amersham Biosciences) (0.1 μCi/ml) overnight. After three washes with fresh medium, 100 μl of culture medium with or without tert-butylhydroperoxide was added to each well, and the radioactivities released into the supernatants after incubation for appropriate periods were measured. The percentage release was calculated using the formula (S/(S + P)) × 100, where S and P are the radioactivities measured in the supernatant and cell pellet, respectively, as described (14).

Cotton Thread-induced Granulation Tissue Formation—Cotton threads (model no. 8; Araiwa) were washed overnight with ethyl acetate, dried at room temperature before being cut into 1-cm lengths (3 mg weight), and sterilized by dry heat at 180 °C for 2 h. The cotton threads were implanted subcutaneously into the dorsum of anesthetized mice by using a 13-gauge implant needle (Natsume). After appropriate periods, the mice were anesthetized and killed, and the granulation tissues were dissected together with the cotton threads and weighed. The isolated granulation tissues were washed, cut into small pieces with scissors, and homogenized with a Polytron homogenizer in SET buffer. The homogenates were taken for immunoblotting and PLA$_2$ enzyme assay, as mentioned above.

RESULTS

Generation of sPLA$_2$ Tg Mice—Tg constructs for sPLA$_2$-V or sPLA$_2$-X (Fig. 1A) were microinjected into the pronuclei of fertilized eggs of C57BL/6 females and transferred into the oviducts of ICR pseudopregnant females. The offspring were examined for expression of the transgenes (LNL-V or LNL-X) by PCR genotyping. Tail biopsies were taken on day 28, and the genomic DNAs isolated were subjected to PCR genotyping. The PCR genotyping were performed on their F1 progeny. Fragments of 0.3 and 0.6 kb were not detected in WT littermates (where S is the transgene-positive, but $\frac{P}{S}$ are the transgene-negative, pups revived after cesarean section). More sensivas, and died within the first 8 h on average after birth (Fig. 2A). PCR genotyping showed that all the dying pups carried the active sPLA$_2$-V transgene, whereas the survivors did not. Pups from two independent lines of LNL-V Tg (see Fig. 1B) mated with CAG-Cre Tg mice displayed a similar phenotype, whereas the LNL-V Tg × C57BL/6 offspring grew healthy. There was no evidence for abnormality in the LNL-V Tg × CAG-Cre Tg pups before birth (E18.5), yet the sPLA$_2$-V transgene-positive, but not transgene-negative, pups revived after cesarean section again all died within ~8 h. These results clearly indicate that Tg overexpression of sPLA$_2$-V in the whole body leads to neonatal death in mice.

In agreement with the PCR genotyping, RNA blotting demonstrated marked expression of sPLA$_2$-V mRNA in all the tissues of transgene-positive neonates (sPLA$_2$-V Tg mice), whereas it was below the detection limit in transgene-negative WT neonates in this experimental setting (Fig. 2B). More sen-
sitive RT-PCR demonstrated the expression of endogenous sPLA₂-V mRNA in the hearts of newborn WT mice (an organ in which sPLA₂-V shows the highest expression in various species (1, 2)), and sPLA₂-V expression in the heart was increased 15-fold in Tg mice compared with that in WT mice (supplemental Fig. S1). Increases of PLA₂ enzymatic activity in the homogenates of tissues from sPLA₂-V Tg mice toward an exogenous substrate (1-palmitoyl-2-linoleoyl-PE) (Fig. 2 C) roughly correlated with the results of RNA blotting (Fig. 2 B). Thus, as compared with the PLA₂ activities in the brain, heart, and lung of WT mice, which reflect the combined activities of various PLA₂ enzymes intrinsically present in each tissue, those of sPLA₂-V Tg mice were increased 2-, 2.5-, and 7-fold, respectively. Judging from the enzyme activity (in comparison with pure recombinant sPLA₂-V expressed by Escherichia coli and under the assumption the activity was not perturbed by endogenous substances), and given that the tissue homogenates had been diluted 5-fold with the buffer during preparation, the concentration of sPLA₂-V in the Tg mice was estimated to be within the range 10–100 ng/ml, depending on the tissue.

The short gasping breaths, tachypnea, and cyanotic skin color (indicative of impaired oxygenation) observed in newborn sPLA₂-V Tg mice suggested that they had a profound defect of the respiratory system. To confirm this, we monitored the respiration rates of sPLA₂-V Tg and control mice using plethysmography. As compared with newborn WT mice that exhibited a regular and constant rate of respiration that declined gradually to reach a steady state after 5–6 h, the breathing rate of sPLA₂-V Tg mice was shallow at birth and became more rapid after 3–4 h, thereafter becoming labored, irregular, and gasping after 5–6 h (Fig. 3 and Table 1). However, ventilation (in terms of minute volume) in Tg and WT mice was similar over the first 6 h (Table 1).

**Histological Abnormalities in the Lungs of sPLA₂-V Tg Mice**—Histological examination of the lungs of sPLA₂-V Tg mice and their littermate controls (8 h after birth) revealed pronounced defects in the former (Fig. 4). Tissues other than the lung appeared normal in sPLA₂-V Tg mice, except for signs of skin inflammation such as dermal edema and macrophage infiltration (supplemental Fig. S2). Because of the marked respiratory defect in Tg mice, we subsequently focused on lung histology.

Remarkably, thinning of the alveolar septa at birth, a prerequisite for blood-gas exchange, was impaired in sPLA₂-V Tg mice (Fig. 4A). This, as compared with the well organized lung architecture of WT mice (Fig. 4A, panels a and b), sPLA₂-V Tg mice exhibited extensive atelectasis with formation of hyaline membranes lining the airway surfaces, and the alveolar walls were abnormally thickened (Fig. 4A, panels c and d). Although microvessels were embedded within the thickened alveolar walls, there was no sign of vascular leakage or endothelial cell abnormalities. Immunostaining of WT lungs with anti-sPLA₂-V antibody revealed the localization of endogenous sPLA₂-V in some, if not all, bronchial epithelial cells (Fig. 4B, panel a), a staining pattern similar to that in normal human lungs (58). In Tg mice, the whole lung area was markedly stained with anti-sPLA₂-V (Fig. 4B, panel b) but not with con-
trol (Fig. 4B, panel c) antibody, with the alveolar surfaces being most intensely stained. Although the mechanism whereby overexpressed sPLA2-V tended to accumulate on the alveolar surfaces is unclear, one possible explanation is that sPLA2-V secreted from alveolar cells might be preferentially associated with heparan sulfate proteoglycan on the alveolar surface, because sPLA2-V has high affinity for heparan sulfate proteoglycan (14, 17) and because the alveolar surface is enriched in the proteoglycan that plays a crucial role in the regulation of alveolar differentiation (62).

Although there were few F4/80-positive alveolar macrophages in the lungs of WT neonates (Fig. 4C, panel a), scattered staining of these cells was found in the lungs of sPLA2-V Tg mice (Fig. 4C, panel b), suggesting the presence of inflammation in the latter. However, no migration of granulocytes and lymphocytes was observed in the lungs of either Tg or WT mice. Immunohistochemistry for SP-C, a surfactant apolipoprotein produced by type II alveolar epithelial cells, showed equivalent staining in the lungs of WT (Fig. 4D, panels a and b) and Tg (Fig. 4D, panels c and d) mice, unlike several gene-manipulated mice that exhibit neonatal death because of respiratory failure resulting from impaired differentiation of alveolar epithelial cells (62–67), suggesting that terminal differentiation of type II pneumocytes did occur normally in the lungs of sPLA2-V Tg mice. To further address this issue, lung sections were subjected to PAS staining, because immature epithelial cells contain abundant PAS-positive gly-
This characteristic of sPLA2-V Tg mice differs from that of 
and showed no difference between Tg and WT mice (Fig. 5).

Mice phenotype of sPLA2-V Tg mice. Prostanoid levels in the lungs could fully account for the lethal 
cells are markedly elevated and participate in the pathogenesis 
mice or humans with asthma or lung fibrosis, in which levels of 
(14, 17). We therefore analyzed the levels of PGE2, a prod-
to promote the generation of eicosanoids in various cells 
using cultured lung epithelial cells that sPLA2-V, in comparison 
relative to that of WT mice, even though the decrease in PE was 

Modest Changes in Eicosanoid Levels in BALF of sPLA2−V Tg 
Mice—Eicosanoids, particularly LTs, are known to profoundly influence lung pathology (68–71), and sPLA2-V has the ability to promote the generation of eicosanoids in various cells in vitro (14, 17). We therefore analyzed the levels of PGE2, a product of the cyclooxygenase pathway, and LTC4, a product of the lipoxygenase pathway, in BALF obtained from sPLA2−V Tg and WT neonates at 8 h after birth. As shown in Fig. 5, the BALF of sPLA2−V Tg mice contained about twice as much PGE2 as that of WT mice. This result is in line with our in vitro observations using cultured lung epithelial cells that sPLA2−V, in comparison with other PLAs, has the particular ability to augment AA release from cells undergoing membrane-oxidative stress relative to that produced by undamaged cells (supplemental Fig. S3). In contrast, the levels of LTC4 in BALF were somewhat low and showed no difference between Tg and WT mice (Fig. 5). This characteristic of sPLA2−V Tg mice differs from that of mice or humans with asthma or lung fibrosis, in which levels of cysteinyl LTs produced from infiltrating leukocytes and mast 
cells are markedly elevated and participate in the pathogenesis (69–71). Overall, it is unlikely that only modest changes in prostanoid levels in the lungs could fully account for the lethal phenotype of sPLA2−V Tg mice.

Aberrant Reduction of Lung Surfactant in sPLA2−V Tg Mice—Recent reports have shown that the neonatal death observed in several gene-manipulated mice because of pulmonary collapse is attributable to disturbed homeostasis of pulmonary surfactant (62–67). The principal function of lung surfactant, which is a mixture of phospholipids (90%) and surfactant proteins (10%), is to reduce the work of breathing by lowering alveolar surface tension during respiration. PC with saturated and monosaturated fatty acids (palmitic acid in particular) is predominant in surfactant phospholipids (~80% of total lipid) (27), and PG (~10% of total lipid) plays a role in phospholipid-protein interactions that maintain the alveolar surfactant layer, especially through interaction with SP-B (72, 73). Because changes in the ratio of the surfactant components can dramatically alter the surface tension in small airways and alveoli, compromising airway patency, gas exchange, and host defense, any surfactant abnormality can have severe path-
ological consequences in terms of lung function. Interestingly, previous studies have indicated a role of sPLA2s in inflammation-mediated surfactant malfunction through hydrolysis of surfactant phospholipids (27–35).

We therefore extracted and analyzed surfactant phospholipids in BALF (1 ml) from sPLA2−V Tg and WT neonates. Protein concentrations in BALF from WT and Tg mice were similar (0.22 ± 0.05 mg/ml and 0.28 ± 0.09 mg/ml (n = 9–12), respectively) over 8 h after birth (i.e. just prior to death). Both genotypes contained 15–25 nmol of phospholipids in their BALF. In a primary study, phospholipids extracted from BALF of both genotypes at term were separated on two-dimensional thin layer chromatography, followed by quantification of phospho-
ropeus Fig. 5, lower panel). Comparable internal standard peaks in individual MS spectra between both genotypes indicate that the lipid extraction from BALF was successfully done. Thus, the overall reduc-
tion of PC in BALF of sPLA2−V Tg mice as compared with 
that of control mice. PE was also decreased substantially 
(19.1 ± 0.6% reduction, n = 2) in BALF of sPLA2−V-Tg mice relative to that of WT mice, even though the decrease in PE was less obvious than that in PC.

To gain more insight into the change in surfactant of V-Tg mice, we carried out ESI/MS analysis. Before lipid extraction 
from BALF, internal standards (see “Experimental Proce-
dures”) were added to BALF (1 nmol of standards per 1 mg of protein equivalents) for calibration. ESI/MS revealed that surf-
factant obtained from WT neonates just at birth (0 h) contained PC molecular species with C16:0–C16:0, C16:0–16:1, and to a 
lesser extent C16:0–18:1 fatty acyl chains as well as other minor molecular species (Fig. 6A, upper panel), consistent with a previous report (65). These PC species were markedly reduced (by ~75, 60, and 35% in PC species with C16:0–16:1, C16:0–16:0, and C16:0–18:1, respectively, relative to those in littermate WT mice) in the surfactant of sPLA2−V Tg mice (Fig. 6A, lower panel). Comparable internal standard peaks in individual MS spectra between both genotypes indicate that the lipid extraction from BALF was successfully done. Thus, the overall reduc-
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ESI/MS seemed consistent with that shown by the thin layer chromatographic study (see above). Reduction of surfactant phospholipids in sPLA2−V Tg mice at 8 h after birth was more prominent (Fig. 6, B and C). Thus, in contrast to WT littermates in which surfactant PC levels were nearly constant (Fig. 6B, upper panel), sPLA2−V Tg neonates at 8 h had lost most of their surfactant PC (Fig. 6B, lower panel), indicating that the hydroly-
ysis of surfactant PC paralleled the ongoing lung damage in Tg mice. It is noteworthy that the marked reduction of surfactant PC over time was not accompanied by a concomitant increase in lyso-PC in Tg mice (data not shown), even though the ratio of lyso-PC to PC increased from <5% in WT mice to >90% in Tg mice because of a marked decrease of PC in the latter. This is probably because lyso-PC is taken up rapidly and re-acylated by

cogen stores that are subsequently converted to surfactant phospholipids following cell maturation (64). As expected, 
alveoli of control neonates had no PAS staining (Fig. 4E, 
panel a), because glycogen is almost entirely converted to surf-
actant phospholipids at this developmental stage (64). Although most areas of the lungs in Tg mice were largely PAS-
negative (data not shown), weak staining of a few epithelial cells 
was focally evident (Fig. 4E, panel b). Thus, although most type 
II epithelial cells were terminally differentiated in the lungs of 
sPLA2−V Tg mice, there was a small population of PAS-positive 
cells that remained immature.

Aberrant Reduction of Lung Surfactant in sPLA2−V Tg Mice—
Recent reports have shown that the neonatal death observed in several gene-manipulated mice because of pulmonary collapse is attributable to disturbed homeostasis of pulmonary surfactant (62–67). The principal function of lung surfactant, which is a mixture of phospholipids (90%) and surfactant proteins (10%), is to reduce the work of breathing by lowering alveolar surface tension during respiration. PC with saturated and monosaturated fatty acids (palmitic acid in particular) is predominant in surfactant phospholipids (~80% of total lipid) (27), and PG (~10% of total lipid) plays a role in phospholipid-protein interactions that maintain the alveolar surfactant layer, especially through interaction with SP-B (72, 73). Because changes in the ratio of the surfactant components can dramatically alter the surface tension in small airways and alveoli, compromising airway patency, gas exchange, and host defense, any surfactant abnormality can have severe path-
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ropeus Fig. 5, lower panel). Comparable internal standard peaks in individual MS spectra between both genotypes indicate that the lipid extraction from BALF was successfully done. Thus, the overall reduc-
tion of PC species in V-Tg mice relative to WT mice revealed by 
ESI/MS seemed consistent with that shown by the thin layer chromatographic study (see above). Reduction of surfactant phospholipids in sPLA2−V Tg mice at 8 h after birth was more prominent (Fig. 6, B and C). Thus, in contrast to WT littermates in which surfactant PC levels were nearly constant (Fig. 6B, upper panel), sPLA2−V Tg neonates at 8 h had lost most of their surfactant PC (Fig. 6B, lower panel), indicating that the hydroly-
ysis of surfactant PC paralleled the ongoing lung damage in Tg mice. It is noteworthy that the marked reduction of surfactant PC over time was not accompanied by a concomitant increase in lyso-PC in Tg mice (data not shown), even though the ratio of lyso-PC to PC increased from <5% in WT mice to >90% in Tg mice because of a marked decrease of PC in the latter. This is probably because lyso-PC is taken up rapidly and re-acylated by
alveolar cells, as has been reported for patients with inflammatory lung diseases in which increased sPLA₂ activity and surfactant dysfunction have typically not been associated with concomitant elevation of lyso-PC (74).

Fig. 6C shows the results of ESI/MS analysis of PG, a minor but important component of surfactant, in BALF from mice 8 h after birth. As in the case of PC above, PG molecular species in the surfactant were markedly decreased in sPLA₂-V Tg mice (Fig. 6C, lower panel) as compared with those in WT littermates, which showed PG species with C16:0–16:0, C16:0–18:1, and C18:0–18:2 (Fig. 6C, upper panel).

Taken together, we conclude that the lethal lung dysfunction observed in sPLA₂-V Tg mice is caused by aberrant hydrolysis of lung surfactant by sPLA₂-V. The reduction in both PC and PG (as well as PE as assessed by thin-layer chromatography) in Tg mice (Fig. 6) agrees with a recent study that sPLA₂-V hydrolyzes these phospholipids derived from lung surfactant in vitro (33, 35). Testis-specific sPLA₂-X Tg Mice—Because a previous study has shown that sPLA₂-IIA Tg mice are infertile because of impaired spermatogenesis (75), we generated testis-specific sPLA₂-V Tg mice to ask if Tg overexpression of sPLA₂-V in the testis would also result in the same phenotype. For this purpose, we crossed LNL-V Tg mice with pgk2-Cre Tg mice, in which Cre recombinase is expressed only in spermatogenic cells, allowing Cre/LoxP recombination and thereby activation of the sPLA₂-V transgene under control of the CAG promoter only in these cells. The LNL-V Tg × pgk2-Cre Tg litter size was normal, and all the pups were viable and grew normally over 1 year. The transgene-positive pups (pgk2-V-Tg mice) overexpressed sPLA₂-V mRNA only in the testis, as assessed by RNA blotting in this setting (supplemental Fig. S4A). Furthermore, RT-PCR demonstrated increased expression of sPLA₂-V mRNA in the testis but not in other tissues of the Tg mice in comparison with respective WT tissues with (e.g. spleen) or without (e.g. liver) endogenous sPLA₂-V expression (supplemental Fig. S4B), thus confirming that the sPLA₂-V transgene was activated by the CAG promoter only in germ cells of pgk2-V Tg mice. These results indicate that overexpression of sPLA₂-V in spermatogenic cells does not lead to infertility and does not affect the life of male mice.

It was expected that if male pgk2-V-Tg mice were crossed with female C57BL/6 mice, the CAG promoter-regulated sPLA₂-V transgene would be transferred to the whole tissues/cells of their progeny, thereby causing early death of the transgene-positive neonates because of lung collapse. Indeed, the transgene-positive F1 neonates exhibited gasping behavior, tachypnea, and cyanosis, and all died soon after birth because of respiratory failure (data not shown), similar to the outcome in the LNL-V Tg × CAG-Cre Tg pups described above. These results further support our conclusion that overexpression of sPLA₂-V in the lungs facilitates surfactant hydrolysis, eventually leading to respiratory failure and thereby early death. Lack of Respiratory Abnormality in sPLA₂-X Tg Mice—Current biochemical and cell biological studies have established that sPLA₂-X is the most active sPLA₂ isozyme (sPLA₂-V is the next) on PC-rich membranes (14–16). We therefore anticipated that, as in the case of sPLA₂-V Tg mice, sPLA₂-X Tg mice would also display a lethal respiratory defect because of aberrant surfactant hydrolysis. Most unexpectedly, however, all the pups of LNL-X Tg (both of two independent founders; see Fig. 1B) × CAG-Cre Tg showed no neonatal death and grew to adulthood. RNA blotting clearly indicated the expression of sPLA₂-X mRNA in all tissues from the sPLA₂-X transgene-positive pups (sPLA₂-X Tg mice) (Fig. 7A). When PLA₂ enzymatic activities in the tissue homogenates from sPLA₂-X Tg and WT mice were measured using 1-palmitoyl-2-arachidonoyl-PC as a substrate, the activities in tissues of the Tg mice were similar to those of WT mice, with only a modest (1.3-fold) increase in the lung (Fig. 7B). Also, there was no increase in PLA₂ activity in

FIGURE 6. ESI/MS analysis of surfactants from WT and sPLA₂-V Tg mice. ESI/MS of PC (A and B) and PG (C) in surfactants from WT (upper panels) and sPLA₂-V Tg (lower panels) mice at birth (A) and 8 h after birth (B and C). Peaks for major molecular species and the internal standard (I.S.) are indicated. Representative results of 2–3 independent experiments are shown.
To assess whether sPLA2-X mRNA would be transcribed to its protein in sPLA2-X Tg mice, we carried out immunoblotting of these homogenates with anti-sPLA2-X antibody, using the culture supernatants and cell lysates of PC12 cells transfected with sPLA2-X as a positive control. We have reported previously that, in cultured cells (e.g. PC12 cells as shown in Fig. 7C), the active form of sPLA2-X, which was produced after proteolytic elimination of the N-terminal propeptide, and its highly glycosylated form, which gave higher and more heterogeneous molecular masses, were present mainly in the culture supernatant, whereas the inactive proenzyme before processing, as well as its glycosylated form, resided in the cells (61). As shown in Fig. 7C, two main bands ( approximate 23 kDa) appeared in most tissues of sPLA2-X Tg mice, verifying that the Tg overexpression of sPLA2-X in mice was indeed successful. Comparing these bands with those in the positive control, together with the result of the PLA2 enzyme assay (Fig. 7B), we realized that the two bands found in the Tg-derived tissues corresponded to the inactive zymogen and its glycosylated form (Fig. 7C). Thus, it is reasonable to consider that sPLA2-X exists mainly as a zymogen and is barely processed to the active form in most tissues under physiological conditions. An additional point to note was that only a minor but significant fraction of sPLA2-X was converted to the active form in the lung homogenate from Tg mice. In addition, the active form appeared to predominate in the Tg brain, even though the level of the overexpressed sPLA2-X in this tissue was lower than that in other tissues (Fig. 7C).

The histology of the lungs of sPLA2-X Tg and WT mice is shown in Fig. 7D. As in the case of WT mice (Fig. 7D, left panel), the alveolar architecture of Tg mice appeared well organized, and atelectasis was scarcely observed, even though moderately thickened alveolar walls were evident locally (Fig. 7D, right panel). Furthermore, analysis of lung surfactant by ESI/MS showed that there were minimal changes in the levels of surfactant PC (Fig. 8, upper panel) and PG (Fig. 8, lower panel) of sPLA2-X Tg mice compared with those of WT mice. Thus, the presence of almost intact lung surfactant could account for the absence of the lethal respiratory defect in sPLA2-X Tg mice, consistent with the notion that sPLA2-X remained largely inactive in vivo.

**Activation of sPLA2-X during Inflammation**—As the amino sequence of the N-terminal cleavage site for sPLA2-X is similar to that cleaved by several inflammatory proteases (76), we next examined if proteolytic activation of sPLA2-X would occur at inflamed sites. We employed a model in which subcutaneous implantation of a cotton thread in the dorsum of mice induces...
iformation of granulation tissue within 5 days (77). As assessed by immunoblotting, we found that, in addition to the bands for the proenzyme and its glycosylated form, a band corresponding to the active enzyme appeared in the granulation tissue of sPLA2-Tg mice on day 5, where nearly half of the overexpressed sPLA2-X was processed to the active form (Fig. 9A). Accordingly, the PLA2 enzymatic activity was significantly increased (3–4-fold) in the homogenate of the granulation tissue in sPLA2-Tg mice compared with that in WT mice (Fig. 9B), confirming the activation of sPLA2-X in this inflammation model.

**DISCUSSION**

In an effort to gain new insights into the in vivo functions of sPLA2 enzymes, we analyzed Tg mice for sPLA2-V and sPLA2-X, two very potently active enzymes on mammalian cell membranes because of their high interfacial affinity for PC (14–17). We have obtained unequivocal evidence that sPLA2-V targets lung surfactant, leading to catastrophic lung damage similar to RDS, a phenotype that has not been observed in sPLA2-IIA Tg mice (19, 20, 22–24, 75, 78). We have also shown that sPLA2-X, an enzyme whose activation requires cleavage of an N-terminal propeptide, remains inactive in a physiological milieu and that its proteolytic activation occurs in particular situations such as inflammation.

The major role of surfactant is to reduce the surface tension at the air-liquid interface of the alveolus (27). Reduction of surfactant results in loss of alveolar stability and severe deterioration of gas exchange, leading to alveolar collapse. In this study, we found that sPLA2-V Tg mice died immediately after birth because of respiratory failure resulting from aberrant hydrolysis of lung surfactant phospholipids (Figs. 2–6). This neonatal death of sPLA2-V Tg mice is in many aspects similar, even if not entirely identical, to that observed in mutant mice manipulated for a panel of genes that have been implicated in surfactant homeostasis either directly or indirectly. These include, for example, mice deficient in Foxa2 (63), a transcription factor prerequisite for the expression of ABCA3 (for ATP-binding cassette A3) that plays a critical role in proper trafficking of surfactant in alveolar type II cells (79), mice ablated for hypoxia-inducible transcription factor-2α, in which maturation of type II cells and thereby surfactant synthesis are prevented (64), and mice null for endothelial nitric-oxide synthase, in which alveolar capillary dysplasia eventually causes marked thickening of saccular septa and reduction of surfactant material (65). Mice manipulated for surfactant apolipoprotein genes also display neonatal RDS, including mice with targeted disruption of SP-B, in which the routing, storage, and secretion of surfactant by type II cells are mitigated (67), and mice overexpressing SP-C, in which lung development is delayed or arrested because of cytotoxic aggregation of SP-C in the early surfactant secretory pathway (66). In all of these cases (as in the case of sPLA2-V Tg mice), mice are born normally because oxygen is supplied from the mother in utero, but they die shortly after birth because of RDS due to surfactant insufficiency. Likewise, newborn human infants with hereditary SP-B deficiency (80) or ABCA3 mutation (81) develop unremitting lethal RDS. A difference between these previously reported mouse models and the sPLA2-Tg mice analyzed here is that the former mostly arises from defects in surfactant synthesis and/or secretion, whereas the latter involves sPLA2-V-directed hydrolysis of surfactant after secretion. Although it remains obscure if sPLA2-V overexpression also affects surfactant homeostasis before secretion, virtually normal expression of SP-C in sPLA2-V Tg mice (Fig. 4D) suggests that terminal maturation of, and thereby surfactant synthesis by, alveolar type II cells may not be profoundly (even if not entirely because PAS-positive immature type II cells are focally present (Fig. 4E)) influenced by sPLA2-V.

Acute RDS is a clinically and pathologically complex syndrome because of acute life-threatening lung injury involving an alteration of pulmonary surfactant, and numerous predisposing factors can be involved in the etiology, including pneumonia and sepsis (27). Hydrolysis of surfactant phospholipids is an early pathological event of RDS, and hydrolysis of as little as 10–15% of surfactant can cause this serious condition (82, 83). Levels of PLA2 activity in BALF of patients with RDS are often positively correlated with disease severity (27–31), and a chemical sPLA2 inhibitor (S-5920/LY315920Na), which was initially developed to target sPLA2-IIA but subsequently shown to inhibit several sPLA2 isozymes with varied potency (84), protects animals against experimental RDS (32). These observations have led to the hypothesis that certain sPLA2 isozymes do contribute to surfactant hydrolysis associated with lung inflammation and injury. Several sPLA2 isozymes, including IB, IIA,
IID, III, V, and X are expressed in the lung (58, 85). Until recently, sPLA2-IIA has been thought to be primarily responsible for RDS pathogenesis, because it is markedly elevated in BALF of various animal species with RDS or related lung injury (27–32), and it hydrolyzes surfactant phospholipids, particularly PG, in vitro (35) and in vivo following intratracheal instillation (33). In contrast, however, SP-A, which is a homolog of sPLA2 inhibitory protein in snakes and does inhibit the activity of sPLA2-IIA (28), exerts an inhibitory effect on surfactant hydrolysis even in mouse strains that do not intrinsically express sPLA2-IIA (33), and it is now generally recognized that the hydrolytic action of sPLA2-IIA on PC is very weak (1, 2). Moreover, unlike sPLA2-V Tg mice (as used in this study), sPLA2-IIA Tg mice reportedly do not display any lung disorder (19, 20, 22–24, 75, 78). These observations suggest that sPLA2-IIA may not be the sole isozyme responsible for the hydrolysis of surfactant, particularly PC.

To our knowledge, this study is the first clear demonstration that sPLA2-V is capable of potently degrading surfactant phospholipids in vivo, and it lends support to the intriguing possibility that sPLA2-V may contribute profoundly, if not solely, to the hydrolysis of surfactant during RDS pathogenesis. In support of this notion, the expression of sPLA2-V is markedly elevated in bronchial and alveolar epithelial cells as well as in alveolar macrophages in the lungs of patients with severe pneumonia (58). sPLA2-V efficiently hydrolyzes PC, PG, and PE in surfactant reconstituted in vitro (35), consistent with our present in vivo study. sPLA2-V has a unique enzymatic property in showing a preference for PC with palmitic acid at the sn-2 position to that with AA in vitro (86) (even though it can release AA from cellular membranes in culture (14, 17)), which is in line with the fatty acid composition of surfactant PC. Moreover, beyond the limitations of the Tg overexpression strategy, only Tg mice for sPLA2-V, but not those for sPLA2-IIA (19, 20, 22–24, 75, 78), for sPLA2-X (Figs. 7 and 8), and for sPLA2-III,4 exhibit neonatal lethality because of RDS, implying the specific action of sPLA2-V on surfactant phospholipids in vivo. Nonetheless, definitive evidence that sPLA2-V does, in fact, play a role in inflammation-associated surfactant destruction in the lung awaits further studies employing proper RDS models in sPLA2-V-null mice.

On the other hand, the involvement of sPLA2-V in surfactant metabolism under “physiological” conditions is unlikely, because its expression in normal lung is low and limited to a population of bronchial epithelial cells in both mice (Fig. 4B, panel a) and humans (58), and more importantly, because our preliminary ESI/MS analysis has shown that surfactant levels are comparable between control mice and sPLA2-V-null mice.5 With regard to this issue, two lysosomal PLA2s, namely aiPLA2/peroxiredoxin 6 in pneumocytes (11) and LPLA2/1-O-acylceramide synthase in alveolar macrophages (12), have recently been implicated in physiological surfactant metabolism. Thus, gene targeting of aiPLA2/peroxiredoxin 6 has revealed that this enzyme is responsible for ~40% of steady-state surfactant PC degradation in the lysosomes of alveolar cells (11), and targeting of LPLA2/1-O-acylceramide synthase results in marked accumulation of lung surfactant, accompanied by macrophage phospholipidosis, by 1 year of age (12).

Application of the acute RDS model to cPLA2-α-null mice has demonstrated the crucial role of cPLA2-α-derived lipid eicosanoids in lung injury (38). Although sPLA2-V has the ability to promote eicosanoid generation by various cells in vitro (14, 17) and by macrophages in vivo as revealed in sPLA2-V-null mice (49), it is unlikely that eicosanoids participate in the lung injury of sPLA2-V Tg mice because the increases in eicosanoids in BALF of sPLA2-V Tg mice are only modest (for PGE2) or absent (for LTC4) (Fig. 5). This is probably because massive infiltration of eicosanoid-producing leukocytes does not occur in the lungs of sPLA2-V Tg neonates. A small increase in PGE2 might result from the direct action of sPLA2-V on macrophages, whose number is increased moderately in Tg mice (Fig. 4C), or on airway epithelial cells, particularly those undergoing membrane damage, as sPLA2-V is potently active on membrane-damaged cells following oxygen stress (supplemental Fig. S3). Alternatively, sPLA2-V might increase AA release from airway cells in concert with cPLA2-α, an event that has been observed in several cell culture studies (87–89). We do not rule out the possibility that sPLA2-V-derived eicosanoids do participate in the process of some pulmonary diseases such as those involving eosinophil infiltration, because sPLA2-V released from bronchial epithelial cells facilitates membrane hydrolysis of adjacent eosinophils in co-culture through the transcellular route (90).

Importantly, comparison between sPLA2-V Tg mice and sPLA2-IIA Tg mice sheds light on the distinct roles of these two enzymes in vivo. As mentioned above, the lethal lung phenotype is peculiar to Tg mice for sPLA2-V but not to those for sPLA2-IIA (and several other sPLA2s), emphasizing a specific role of sPLA2-V in the lung. Besides the severe lung injury, sPLA2-V Tg mice show a symptom of dermal inflammation accompanied by edema and macrophage infiltration (supplemental Fig. S2). In contrast, no inflammatory phenotype has been reported in the skin (or any other tissue) of sPLA2-IIA Tg mice, which show permanent hair loss associated with hyperkeratosis (78). This difference might be related to the well-established view that sPLA2-V has the ability to elicit eicosanoid production more potently than does sPLA2-IIA in various cells (14, 17). Although male infertility because of impaired spermatogenesis has been reported for testis-specific sPLA2-IIA Tg mice (75), testis-specific sPLA2-V Tg mice are fertile. So far, no reproductive abnormality has been observed in sPLA2-V knock-out and Tg mice, even though sPLA2-V is expressed in the acrosomes of spermatogenic cells (57) and a potential role of the PLA2 reaction product, lyso-PC, in the acrosome reaction and fertilization has been proposed (91). By contrast, whether sPLA2-V does play a role in defense against bacterial infection and increased atherosclerosis, which are prominent phenotypes of sPLA2-IIA Tg mice that may be relevant to the physiological functions of this enzyme (19, 20, 22–24), is difficult to assess with sPLA2-V Tg mice because all die in the neonatal period. Because sPLA2-V exhibits bactericidal activity in vitro (less efficiently than sPLA2-IIA) (20, 21), promotes plasma

4 H. Sato, M. Ohtsuki, Y. Taketomi, S. Hatakeyama, I. Kudo, and M. Murakami, manuscript in preparation.
5 M. Murakami, S. Masuda, E. Kikawada, and J. P. Arm, unpublished observations.
lipoprotein hydrolysis (more potently than sPLA2-IIA) (26), and is detected in atherosclerotic lesions (as is sPLA2-IIA) (26), it is possible that sPLA2-IIA and sPLA2-V share redundant roles or act in a coordinated manner in anti-bacterial innate immunity and atherosclerosis development. Future studies using tissue-specific sPLA2-V Tg mice, as well as sPLA2-X knock-out mice, would be helpful for clarifying the roles of sPLA2-V in these situations.

Transfection of sPLA2-X in several types of mammalian cell results in spontaneous release of its active form into the culture medium (14, 58, 61), although the entity of the protease(s) responsible for the proteolytic processing of this enzyme is unknown. The amino acid sequence of the proteolytic cleavage site in sPLA2-X is similar to that cleaved by subtilisin-like intracellular processing proteases (76), and in fact, intracellularly activated sPLA2-X promotes AA release prior to secretion in HEK293 transfectants (90, 92). However, subsequent studies have shown that the active form of sPLA2-X is predominantly present in the culture supernatants of sPLA2-X-transfected bronchial epithelial cells, fibroblasts, and neuronal cells, leading to the suggestion that certain protease(s) present in serum, secreted from cells, or on cell surfaces may play a major role in the activation of sPLA2-X (61). This idea is supported by our present finding that sPLA2-X exists as a zymogen in most tissues of sPLA2-X Tg mice under physiological conditions (Fig. 7), thus largely explaining why sPLA2-X Tg mice do not exhibit the neonatal RDS phenotype (Figs. 7D and 8), regardless of the reported capacity of this enzyme to hydrolyze surfactant in vitro (33, 35). Moreover, the observation that sPLA2-X, but not sPLA2-V, is inhibited by SP-A (33) may also provide an additional explanation for the fact that only sPLA2-V Tg mice exhibit the RDS phenotype. Interestingly, activation (processing) of sPLA2-X was evident in the inflammatory granulation tissue of Tg mice (Fig. 9). Several inflammation-associated extracellular proteases, such as cathepsins and kallikreins, as well as type II transmembrane serine proteases, such as hepsin of which expression is elevated in cancer (93), have a potential to cleave the propeptide sequence of sPLA2-X. sPLA2-IB, another sPLA2 that possesses an N-terminal propeptide, can be converted to its active form by extracellular proteases such as trypsin and serum plasmin (94). We therefore propose that the activation of sPLA2-X occurs following the action of these disease-associated proteases, in particular pathological circumstances such as inflammation, tissue injury, or even cancer. This tightly regulated activation mechanism seems to be of great biological importance, as unregulated activation of sPLA2-X would be detrimental for the host because of its marked activity on cell membranes.

From this standpoint, we do not rule out the possibility that sPLA2-X could also participate in surfactant degradation and associated lung injury, in which the processing of sPLA2-X, and thereby surfactant hydrolysis, might occur. Indeed, some histological abnormality (alveolar wall thickness) was evident locally in sPLA2-X Tg neonates (Fig. 7D). This may be related to the presence of a small fraction of the active form of sPLA2-X in the Tg lung (Fig. 7C), even though the bulk surfactant composition in the Tg mice appears normal (Fig. 8). The fact that endogenous sPLA2-X is localized in type II epithelial cells (15) also supports a possible relationship of this enzyme with surfactant metabolism in certain situations. Another notable feature is that the active form of sPLA2-X was present in the brain of sPLA2-X Tg mice (Fig. 7C). Considering that sPLA2-X is located in neuronal fibers and that it is capable of facilitating neuritogenesis in cultured neuronal cells (61), processing of sPLA2-X might occur constitutively in neuronal cells. Likewise, endogenous sPLA2-X has been reported to be expressed in skin keratinocytes (95), leukocytes (76), and the gastrointestinal tract (96), and we have recently found that sPLA2-X Tg mice display some phenotypes unique to these tissues. Further studies using sPLA2-X Tg mice as well as sPLA2-X knock-out mice should help to reveal the tissue-specific roles of sPLA2-X, in association with its proteolytic processing and the identity of its activating protease(s).

Acknowledgments—We thank Drs. M. H. Gelb (University of Washington, Seattle) and G. Lambeau (CNRS-UPR 411, Sophia Antipolis, France) for providing cDNAs, recombinant proteins, and antibodies for sPLA2s. We also thank Drs. I. Saito (University of Tokyo, Tokyo, Japan) and J. Miyazaki (Osaka University, Suita, Japan) for kindly providing pCALNL5 plasmid and CAG-Cre mice, respectively.

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