SECRETORY PHOSPHOLIPASES A2 (sPLA2) are a family of lipolytic enzymes that catalyze the cleavage of fatty acids from the sn-2 position of phospholipids generating free fatty acids, including arachidonic acid (AA), and lysophospholipids leading to the generation of eicosanoids, including leukotrienes (LT). There are more than 19 different isoforms (groups) of PLA2 in humans, denoted by Roman numerals. PLA2s acting on membrane phospholipids have been implicated in intracellular membrane trafficking, differentiation, proliferation, and apoptosis.

sPLA2 activity is increased in the serum and bronchoalveolar lavage fluid (BALF) of patients with asthma (1) or the acute respiratory distress syndrome (ARDS) (14, 37) and in animals with acute lung injury (7-9).

SECRETORY PHOSPHOLIPASES A2 (sPLA2) are a family of lipolytic enzymes that catalyze the cleavage of fatty acids from the sn-2 position of phospholipids generating free fatty acids, including arachidonic acid (AA), and lysophospholipids leading to the generation of eicosanoids, including leukotrienes (LT). There are more than 19 different isoforms (groups) of PLA2 in humans, denoted by Roman numerals. PLA2s acting on membrane phospholipids have been implicated in intracellular membrane trafficking, differentiation, proliferation, and apoptosis.

sPLA2 activity is increased in the serum and bronchoalveolar lavage fluid (BALF) of patients with asthma (1) or the acute respiratory distress syndrome (ARDS) (14, 37) and in animals with acute lung injury (7-9).
containing 0.5 or 5 U/ml sPLA2 with or without an inhibitor or KHS alone (period 2).

**Tracheal secretion in calcium-free medium.** MG secretion in Ca2+-free medium was tested because sPLA2 requires millimolar concentrations of Ca2+ for enzymatic activity, whereas receptor-mediated sPLA2 activity has been reported to be calcium independent (13, 19). Ferret tracheal segments were first rested in KHS (1.3 mM Ca2+, pH 7.4). After resting for 1 h, the segments were incubated for 30 min with KHS (period 1) and then incubated for another 30 min in KHS containing porcine pancreatic sPLA2 at 5 U/ml or KHS alone in the presence (+) or absence (−) of 2 mM EGTA (period 2). Secreted MG was analyzed as described below.

**Intratracheal sPLA2 exposure.** Ferrets were anesthetized with 40 mg/kg ketamine and 5 mg/kg xylazine and intubated for 30 min once per day for 3 days using an endotracheal tube (ETT) coated with 20 units of porcine pancreatic sPLA2 mixed in 300 μl of water-soluble K-Y jelly (Johnson & Johnson, New Brunswick, NJ). The position of the ETT was checked with a neonatal bronchoscope. Trachea from larynx to carina were removed 1, 5, or 10 days after the last sPLA2 exposure (sPLA2 day 1, sPLA2 day 5, sPLA2 day 10). Control animals were intubated with an ETT coated with 300 μl of K-Y jelly alone for 30 min per day for 3 days, and trachea were removed 1 day after the last intubation. Each trachea was divided into eight roughly equal segments, and the segments were rested in KHS. A middle segment was fixed in 10% formalin and processed for histological evaluation.

After resting for 1 h, ferret tracheal segments were incubated for 30 min with KHS to measure constitutive MG secretion (period 1) and then incubated for 30 min in KHS with a secretagogue or KHS alone to measure stimulated MG secretion (period 2). To evaluate MG secretion in response to cholinergic or serine protease stimulation, methacholine (MCh) at 10−7 M or human neutrophil elastase (HNE; Elastin Products, Owensville, MO) at 10−8 M was used based on our previous studies (16).

**MG secretion.** Ferret tracheal mucins have high blood group titers, reflecting an abundance of galactose-N-acetyl-a1–3 (fucose-a1–2) galactose-R (21). These blood group antigens can be detected by Dolichos biflorus agglutinin (DBA) lectin. DBA immunohistochemistry has shown specific binding to goblet cells and submucosal glands in ferret trachea (16).

A sandwich enzyme-linked lectin assay was used to measure MG secretion as previously described (16–18). A 96-well microtiter plate was coated with 60 μl of DBA (6 μg/ml in PBS) and incubated at 4°C overnight. After rinsing five times with PBS with 0.5% Tween 20 (PBS-Tween), the plate was exposed to sample buffer and incubated at room temperature for 2 h. It was then incubated for 1.5 h with 50 μl of DBA conjugated with horseradish peroxidase (0.25 μg/ml) in PBS containing 1% bovine serum albumin. Before and after this step, the plate was washed five times with PBS-Tween. One hundred and fifty microliters of tetramethylbenzidine (0.42 mM) in citrate-acetate buffer (pH 6.0) was added to each well and incubated for 10 min. The reaction was stopped by adding 50 μl of 4.7 N H2SO4. Color development was read as the difference in absorbance at 450 and 650 nm. The concentration of MG was calculated by comparison with asialo ovine submaxillary mucin at 20–200 ng/ml. The amount of MG was expressed as mucin weight in the incubated buffer per trachea tissue weight (ng/gm tissue).

The relative increase of MG in each tracheal segment was calculated as the ratio of the actual amount of secreted MG in period 2 incubation divided into that of period 1. A secretory index (SI) for stimulated MG secretion was calculated using the ratio of stimulated MG secretion divided by that of unstimulated secretion (both period 1 and 2: KHS) in each animal. A percent (%) change of SI was calculated using the SI of stimulated MG secretion divided by that of unstimulated (KHS) secretion in each animal.

**Histological evaluation.** A middle segment of trachea was fixed in 10% formalin, embedded in paraffin, and processed for histological analysis using light microscopy. The tissue was cut in 4-μm sections, and the slides were stained with hematoxylin and eosin to measure the accumulation of inflammatory cells and overall severity of inflammation. The accumulation of inflammatory cells was variable, possibly related to the degree of tissue exposure to the sPLA2-coated tubes. Thus we could not accurately quantify the amount of inflammation by cell counts so we determined the type of cellular infiltrate and how long the inflammatory cells and loss of epithelial integrity persisted after sPLA2 exposure ceased.

**Statistical analysis.** Statistical analysis of data was performed using the StatView 5.0 statistics package (SAS, Cary, NC). After confirming that data were normally distributed, data were analyzed using ANOVA Scheffe’s F test or two-tailed unpaired t-test. P values of ≤ 0.05 were considered statistically significant. Data are presented as means ± SE.

**RESULTS**

**sPLA2 stimulates MG secretion in the ferret trachea in vitro.** sPLA2 rapidly stimulated MG secretion in the excised ferret trachea in a dose- and a time-dependent manner (Fig. 1). sPLA2 at a concentration of 0.5 (P < 0.05) and 5 (P < 0.0001) U/ml significantly increased MG secretion compared with KHS control.

**sPLA2 and LO inhibitors decrease sPLA2-induced MG secretion.** Atropine at 10−4 M had no effect on sPLA2-induced MG secretion. sPLA2-induced MG secretion was significantly decreased by p-BPB (an sPLA2 inhibitor) at 10−4 M (P < 0.01), quercetin (a LO inhibitor) at 10−4 M (P < 0.01), or MK-886 (a specific 5-LO inhibitor) at 5 × 10−3 M (P < 0.01) (Fig. 2). MK-886 at 10−4 M also inhibited sPLA2-induced MG secretion (P < 0.005) as shown in Fig. 3, top. Indomethacin (a cyclooxygenase inhibitor) at 10−4 M did not affect sPLA2-induced MG secretion (Fig. 2).

**Mucin secretion in calcium-free KHS.** sPLA2 at 5 U/ml significantly increased MG secretion in the ferret trachea (KHS in 1.3 mM Ca2+), and secretion was significantly decreased in Ca2+-free medium (KHS in 1.3 mM Ca2+ + 2.0 mM EGTA) as shown in Fig. 3. Constitutive MG secretion was also decreased in Ca2+-free medium compared with KHS control (P < 0.01).

**Intratracheal sPLA2 exposure induced MG hypersecretion.** Ferrets were intubated once per day for 3 days with an ETT coated with 20 units of sPLA2 mixed in water-soluble K-Y jelly. Constitutive MG secretion significantly increased 1 day after the last sPLA2 exposure compared with control (P < 0.0005) and returned to control levels at 5 days (sPLA2 day 5), as shown in Fig. 4, HNE at 10−8 M markedly and synergistically stimulated MG secretion in the sPLA2-exposed trachea (P < 0.01), and this hyperresponsiveness to HNE continued for 5 days (P < 0.01) and returned to control by day 10 (Fig. 5). MCh at 10−7 M increased MG secretion in the sPLA2-exposed trachea no more than from the nonexposed trachea.

**Histopathology of the sPLA2-exposed trachea.** One day after the last sPLA2 exposure (sPLA2 day 1), there was an influx of inflammatory cells in both the epithelium and the lamina propria. The inflammatory cells comprised a mix of polymorphonuclear and mononuclear cells. Five days after the last sPLA2 exposure, fewer inflammatory cells were in the epithelium, but there was increased airway mucus secretion. The lamina propria was relatively free of inflammation. Ten days after the last sPLA2 exposure (sPLA2 day 10), inflammatory cell numbers were similar to the airway not exposed to sPLA2. The lamina propria resembled control

AJP-Lung Cell Mol Physiol • VOL 292 • JANUARY 2007 • www.ajplung.org

Downloaded from http://ajplung.physiology.org/ by 10.220.33.6 on September 30, 2017
sPLA₂ activity is increased in the serum or BALF of patients with asthma (2) or ARDS (14, 37). We show here that sPLA₂ is a rapid and potent MG secretagogue, this effect is dose dependent, and it can be blocked by Ca²⁺ depletion or by LT inhibitors but not by cholinergic inhibitors. In vivo, sPLA₂ also induces a state of “secretory hyperresponsiveness” to HNE but not by cholinergic inhibitors. In vivo, sPLA₂ rapidly increases the thickness of interalveolar septa by edema and epithelial cell swelling (7, 8). We applied sPLA₂ directly to the tracheal epithelium by intubating ferrets with an ETT coated with sPLA₂ mixed in water-soluble jelly for 30 min once per day for 3 days. This technique was described previously by our group for exposure of the airway to bacterial endotoxin (18). Our results in vivo suggest that sPLA₂ induces local airway tissue injury and stimulates mucus hypersecretion.

sPLA₂ type IB is abundant in pancreatic fluid of many species and is sometimes referred to as pancreatic type sPLA₂. sPLA₂ type IIA is sometimes called inflammatory type, because it is highly expressed in the plasma or synovial fluids of patients with inflammatory diseases such as rheumatoid arthritis (30) and septic shock (38). sPLA₂ type IB is produced as an inactive proenzyme and activated by proteolytic enzymes (25, 26). sPLA₂ type IB was used in this study because it has similar enzymatic characteristics to sPLA₂ type IIA, it has a widespread tissue distribution including in the lung (31, 33, 36), and it has potent pathophysiological activity (1, 11, 15). Both sPLA₂ type IB and IIA are 14-kDa proteins (35) and require millimolar amounts of Ca²⁺ for enzymatic activity. Activated sPLA₂ type IB is detected in the serum and urine of subjects with acute lung injury but not in healthy controls (28).

sPLA₂ type IB has also been shown to regulate cellular function in vitro. It stimulates cell proliferation in Swiss 3T3 cells (1) and pancreatic cancer cells (11) and prostaglandin production and sPLA₂ type IIA expression in rat mesangial cells (15). These actions of sPLA₂ type IB are thought to be receptor mediated. A sPLA₂ type IB specific receptor has been identified (20). The receptor-mediated actions of this enzyme are not fully understood.

**DISCUSSION**

**tissues, and the basal cell layer of the epithelium was intact. There was no evidence of increased inflammatory cells in the control intubated airways.**

**Fig. 1. Secretory PLA₂ (sPLA₂) stimulates mucous glycoconjugate (MG) secretion in the ferret trachea. Top: tracheal segments were immersed in Krebs-Henseleit solution (KHS). After resting for 1 h, segments were incubated in KHS for 30 min (period 1) and then for another 30 min in KHS with sPLA₂ at a concentration of 0.005 (n = 7), 0.05 (n = 7), 0.5 (n = 31), or 5 (n = 14) U/ml or KHS alone (n = 35) (period 2). Secreted MG was measured by enzyme-linked lectin assay (ELLA). The relative increase of MG in each tracheal segment was calculated as the ratio of the amount of secreted MG in period 2 incubation divided into that of period 1. A secretory index (SI) for stimulated MG secretion was calculated as the ratio of stimulated MG secretion divided by that of unstimulated secretion. All data are expressed as means ± SE. *P < 0.05 or **P < 0.0001 compared with KHS. Bottom: kinetics of sPLA₂-induced MG secretion. Tracheal segments were incubated in KHS with porcine pancreatic sPLA₂ at a concentration of 0.5 or 5 U/ml sPLA₂ or KHS alone (each: n = 6), and the buffers were collected at 5, 10, 15, and 30 min after incubation. All data are shown as concentration of secreted MG and expressed as means ± SE. *P < 0.0001 or **P < 0.0005 compared with each KHS group; +P < 0.01 compared with KHS group.**

**Fig. 2. Inhibition of sPLA₂-induced MG secretion. Tracheal segments were incubated for 30 min in KHS with an inhibitor or with KHS alone (period 1) and then for another 30 min in KHS alone or KHS containing sPLA₂ at 0.5 U/ml with or without an inhibitor (period 2). Shown are sPLA₂ at 0.5 U/ml alone (n = 23), atropine at 10⁻⁴ M (n = 3), p-bromophenacylbromide (p-BPB) at 10⁻³ M (n = 6) and 10⁻⁴ M (n = 6), quercetin at 10⁻⁴ M (n = 4), eicosatetraenoic acid (ETYA) at 10⁻⁴ M (n = 4), MK-886 at 5 × 10⁻⁴ M (n = 5), and indomethacin at 10⁻⁴ M (n = 6). Data are shown as percent change of SI compared with KHS group and expressed as means ± SE. MG secretion stimulated by sPLA₂ at 0.5 U/ml was significantly decreased by p-BPB at 10⁻⁴ M, quercetin at 10⁻⁴ M, and MK-886 at 5 × 10⁻⁴ M, compared with sPLA₂ at 0.5 U/ml alone (*P < 0.01).**
with EGTA at 2 mM, or KHS containing sPLA2 at 5 U/ml with or without MK-886 (1.3 mM Ca\textsuperscript{2+}) (period 1) and then for another 30 min in KHS alone or KHS containing sPLA2 at 5 U/ml with or without MK-886 at 10\textsuperscript{-4} M (period 2). Data are shown as SI and expressed as means ± SE. *P < 0.0001 and **P < 0.0005. Bottom: MG secretion in calcium-free medium. Tracheal segments were incubated for 30 min in KHS (1.3 mM Ca\textsuperscript{2+}) (period 1) and then for another 30 min in KHS alone, KHS with EGTA at 2 mM, or KHS containing sPLA2 at 5 U/ml with or without EGTA at 2 mM (each group: n = 4) (period 2). Data are shown as SI and expressed as means ± SE. *P < 0.0001, **P < 0.01, and ***P < 0.0005.

sPLA2-induced MG secretion, supporting the hypothesis that cyclooxygenase pathway products (e.g., prostaglandins) do not play an important role in sPLA2-induced MG secretion. Quercetin, an LO inhibitor, decreased sPLA2-induced MG secretion, suggesting that sPLA2 may stimulate MG secretion by activating the LO pathway. MK-886, a specific 5-LO inhibitor, decreased sPLA2-induced MG secretion in the ferret. MK-886 binds a membrane-bound 5-LO-activating protein, thereby inhibiting the translocation and activation of 5-LO (6, 29). MK-886 at 5 × 10\textsuperscript{-5} M also significantly decreased sPLA2-induced MG secretion. Thus sPLA2 probably stimulate tra-

Fig. 5. Human neutrophil elastase (HNE) and methacholine (MCh) stimulate MG secretion in the sPLA2-exposed trachea. Ferret tracheal segments were placed in KHS for 30 min to measure constitutive MG secretion (period 1) and then were incubated for another 30 min in KHS with HNE at 10\textsuperscript{-8} M, MCh at 10\textsuperscript{-7} M, or KHS alone (period 2). Shown are control unintubated (period 2); KHS and HNE at 10\textsuperscript{-8} M, MCh at 10\textsuperscript{-7} M; n = 15, 11, 10), control intubated (n = 14, 13, 13), sPLA2 day 1 (n = 14, 13, 13), sPLA2 day 5 (n = 10, 11, 11), and sPLA2 day 10 (n = 9, 12, 11). All data are shown as SI for MG secretion and expressed as means ± SE. *P < 0.01 compared with control unintubated.

Fig. 4. Constitutive MG secretion in the sPLA2-exposed trachea. Ferrets were intubated with an endotracheal tube (ETT) coated with 20 units of sPLA2 mixed in water-soluble K-Y jelly for 30 min once daily for 3 days. One (sPLA2 day 1), 5 (sPLA2 day 5), or 10 (sPLA2 day 10) days after the last sPLA2 exposure, tracheae were removed. Control animals were intubated with an ETT coated with jelly alone, and tracheae were removed 1 day after the third and last intubation (control intubated). After resting for 1 h in KHS, segments were placed in fresh KHS for 30 min. Secreted MG was measured by ELLA. Data are shown as secreted MG. Shown are control unintubated: no treatment (n = 36), control intubated (n = 40), sPLA2 day 1 (n = 40), sPLA2 day 5 (n = 32), and sPLA2 day 10 (n = 32). Data are shown as absolute amount of secreted MG and expressed as means ± SE. *P < 0.0005 compared with control unintubated.
cheal MG secretion through the activation of LO and the 5-LO pathway.

In persons with asthma, bronchial hyperresponsiveness is defined by the decrease in airflow, generally FEV1, to a variety of stimuli including MCh, histamine, hyperosmolar challenge, exercise, or cold air. In this study, we show that local tracheal exposure to sPLA2 exposure induced tracheal inflammation, producing a long-lasting enhanced secretory response to HNE but not to MCh stimulation (Fig. 5). We have termed this enhanced and synergistic secretory response secretory hyper-responsiveness. This suggests that sPLA2 causes hypertrophy or hyperplasia of secretory cells or glands (analogous to asthma increasing the amount of smooth muscle in the airway) and/or that the secretory apparatus is sensitized after sPLA2 exposure to produce a greater secretory response to HNE, analogous to the concept of “twitty airways.” Because secretory hyper-responsiveness to HNE returned to control levels 10 days after the last sPLA2 exposure, we hypothesize that in this model, the airway has been primed to respond to HNE in an exaggerated manner.

Bowton and colleagues (3) studied the effect of LY-333013, a potent inhibitor of group II sPLA2, on allergen-induced bronchoconstriction following inhaled allergen challenge in atopic asthmatics. Compared with placebo, LY-333013 had no impact on bronchial hyperresponsiveness defined by pulmonary function changes following inhaled allergen challenge (3). However, when Masuda and colleagues (24) examined the expression and function of sPLA2 in human lung-derived cells and in human lungs with pneumonia, they showed that whereas groups V and X sPLA2 were widely expressed in the airway epithelium, interstitium, and alveolar macrophages, group IIA sPLA2 was restricted to the pulmonary arterial smooth muscle layers and bronchial chondrocytes, and groups III and IIF sPLA2 were minimally detected. These results suggest that groups V and X sPLA2 are involved in disease pathogenesis in the human lung to a greater extent than the group II sPLA2 enzymes.

Inflammatory cells consisting of polymorphonuclear and mononuclear cells rapidly accumulated in the sPLA2-exposed trachea by day 1, but by day 10 after exposure, the sPLA2-exposed trachea appeared the same as control trachea. Neutrophils release chemical mediators that are potent airway secretagogues (5, 34). The number of neutrophils and the amount of neutrophil elastase is increased in sputum of patients with cystic fibrosis or severe asthma (10, 32). HNE enhanced MG hypersecretion in the sPLA2-exposed trachea compared with cystic fibrosis or severe asthma (10, 32). HNE enhanced amount of neutrophil elastase is increased in sputum of patients.

Acknowledgments

We thank Lauren Vannoy for technical assistance and Dr. David Bass for first suggesting these experiments.

Present address of K. Okamoto: Mie University School of Medicine, Department of Otolaryngology, Tsu, Japan; present address of J.-S. Kim: Department of Otolaryngology, College of Medicine, Kyungpook National University, Daegu, Korea.

REFERENCES


