Lung endothelial cell platelet-activating factor production and inflammatory cell adherence are increased in response to cigarette smoke component exposure

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Running title: CSE increases endothelial PAF production

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ABSTRACT

An early event in the pathogenesis of emphysema is the development of inflammation associated with accumulation of polymorphonuclear leukocytes (PMN) in small airways, and inflammatory cell recruitment from the circulation involves migration across endothelial and epithelial cell barriers. Platelet-activating factor (PAF) promotes transendothelial migration in several vascular beds and we postulated that increased PAF production in the airways of smokers might enhance inflammatory cell recruitment and exacerbate inflammation. To examine this possibility, we incubated human lung microvascular endothelial cells (HMVEC-L) with cigarette smoke extract (CSE) and found that CSE inhibits PAF-acetylhydrolase (PAF-AH) activity. This enhances HMVEC-L PAF production and PMN adherence and adherence is blocked by PAF receptor antagonists (CV3988 or ginkgolide B). CSE also inhibited PAF-AH activity of lung endothelial cells isolated from wild type (WT) and iPLA$_2\beta$ knockout mice, and with WT cells CSE enhanced PAF production and RAW 264.7 cell adherence. In contrast, CSE did not affect PAF production or RAW 264.7 cell adherence to iPLA$_2\beta$-null cells, suggesting that iPLA$_2\beta$ plays an important role in PAF production by lung endothelial cells. These findings suggest that inhibition of PAF-AH by components of cigarette smoke may initiate or exacerbate inflammatory lung disease by enhancing PAF production and promoting accumulation of inflammatory cells in small airways. In addition, iPLA$_2\beta$ is identified as a potential target for therapeutic interventions to reduce airway inflammation and the progression of chronic lung disease.

INTRODUCTION

Smokers are at higher risk of heart attack, stroke and chronic obstructive pulmonary disease (COPD). COPD is the fifth leading cause of death worldwide and it is estimated that there are 15-17 million people with COPD in the US alone (34). Human COPD includes emphysema and chronic bronchitis and is marked by several pathological features, such as small airway remodeling, vascular remodeling with pulmonary hypertension and mucus overproduction (7,15,16). The progression of chronic lung diseases is marked by migration of circulating inflammatory cells into the airway, accumulation of neutrophils and macrophages, production of inflammatory mediators, and the release of proinflammatory cytokines and proteases (16). Activation of endothelial and epithelial cells in the airway and accumulation of leukocytes creates a milieu in which additional chemoattractant mediators are produced to perpetuate the influx of inflammatory cells that leads to tissue remodeling and destruction (16).

The molecular mechanisms by which inflammatory cells affect small airway remodeling are incompletely delineated at present (22,41), but cigarette smoke promotes release of oxidants from neutrophils and macrophages, which may trigger or potentiate TGF-β release and collagen production (11,32,33).

Recruitment of circulating inflammatory cells into the airways involves migration across the endothelial and epithelial cell barriers, and such migration is promoted by mediators that include platelet-activating factor (PAF). PAF is a membrane phospholipid-derived mediator produced by endothelial cells that causes transient adherence of neutrophils (37). Net PAF production is governed by its relative rates of biosynthesis and degradation. The remodeling pathway for PAF synthesis is activated during inflammation and involves tightly coupled phospholipase A_2

(PLA$_2$)-catalyzed hydrolysis of membrane phospholipids to produce lyso-PAF (37,44,46) and acetylation of lyso-PAF at the *sn*-2 position catalyzed by acetyl-CoA:lyso-PAF acetyltransferase (21,28,47). The principal pathway for PAF degradation is its hydrolysis by the enzyme PAF-acetylhydrolase (PAF-AH). Components of cigarette smoke inhibit PAF-AH activity (9,38), and this could result in PAF accumulation on the pulmonary endothelial cell surface *in vivo* and facilitate adherence and transmigration of circulating neutrophils.

We have previously reported that stimulation of human pulmonary microvascular endothelial cells (HMVEC-L) with protease-activated receptor agonists, e.g., tryptase, activates an intracellular Group VI phospholipase A$_2$ (iPLA$_2$) and that this results in increased release of arachidonic acid, PAF production and adherence of polymorphonuclear leukocytes (PMN) to HMVEC-L (42). We have also demonstrated that the iPLA$_2$ isoform responsible for endothelial cell PAF production is the Group VIA PLA$_2$ (iPLA$_2\beta$) and not the Group VIB PLA$_2$ (iPLA$_2\gamma$) (44). In the studies reported here, we have examined the hypothesis that cigarette smoke may exacerbate inflammatory airway diseases by inhibiting PAF-AH activity, which results in increased endothelial cell PAF production and enhanced inflammatory cell recruitment to the lung.

MATERIALS AND METHODS

Materials. Human lung microvascular endothelial cells (HMVEC-L) were obtained from Lonza (Walkersville, MD). Cigarette smoke extract (CSE) was obtained from Murty Pharmaceuticals, Inc. (Lexington, KY). Methyl arachidonyl fluorophosphonate (MAFP) was obtained from Cayman Chemical Co. (Ann Arbor, MI). [^{3}H] acetic acid, sodium salt and hexadecyl-2-acetyl-sn-glyceryl-2-phosphoryl-choline, 1-O-[acetyl-^{3}H(N)] were from Perkin Elmer (Boston, MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Endothelial cell culture. Human lung microvascular endothelial cells (HMVEC-L, Lonza, Walkersville, MD) were grown to confluence in endothelial cell basal medium supplemented with growth factors (Lonza, Walkersville, MD) and incubated at 37˚C, in an atmosphere of 95% O_2, 5% CO_2. Cells from passages 3-4 were used for experiments.

The generation of mice deficient in iPLA$_2$β has been described previously (4). Mice were housed in a pathogen-free facility and studies were conducted under protocols approved by Saint Louis University Animal Care and Use Committee. Endothelial cells were isolated from mouse lung by collagenase digestion. The diced lung tissue was incubated in 1 mg/ml collagenase for 1 h @ 37°C and the digested tissue was passed through a cell strainer. Endothelial cells were isolated by incubating with anti-mouse platelet/endothelial cell adhesion molecule 1 (PECAM-1) coupled to magnetic beads. Cells were washed, resuspended in EGM-2MV cell culture medium (Lonza) and plated in 25 cm^2 culture flasks. Non-adherent cells were removed the next day and cells were grown to confluence and passaged at a 1 to 3 dilution. Cells from passage 3-4 were used for experiments. Isolation purity was verified by staining with anti-factor VIII antibody and preparations with greater than 85% endothelial purity were used.

Phospholipase A₂ activity. The surrounding medium was removed from confluent endothelial cells and immediately replaced with ice-cold buffer containing 250 mmol/L sucrose, 10 mmol/L KCl, 10 mmol/L imidazole, 5 mmol/L EDTA, 2 mmol/L DTT, and 10% glycerol (pH 7.8) (PLA_2 assay buffer). Endothelial cells suspended in ice-cold PLA_2 assay buffer were sonicated on ice six times for 10 s, and the sonicate was centrifuged at 14000g for 10 min. PLA2 activity in the supernatant was assessed by incubating the enzyme with a synthetic 100 μM (16:0,[³H]18:1 plasmenylcholine substrate (specific activity of 150 dpm/pmol, ~68 μCi/μmol) in assay buffer containing 100 mM Tris, 4 mM EGTA, and 10% glycerol (pH 7.0) at 37 °C for 5 min in a total volume of 200 μL. Reactions were initiated by adding the radiolabeled phospholipid substrate as a concentrated stock solution in ethanol. Reactions were terminated by the addition of butanol, and the released radiolabeled fatty acid was isolated by application of an aliquot of the butanol phase to channeled Silica Gel G plates, development in a petroleum ether/diethyl ether/acetic acid mixture (70/30/1, v/v), and subsequent quantification by liquid scintillation spectrometry with appropriate quench correction.

Acetyl-CoA:lyso-PAF acetyltransferase activity. HMVEC grown to confluence were removed from the tissue culture plate in ice-cold $NaCl/PO_4$ buffer (139 mM NaCl, 5 mM Na_2HPO_4, 5 mM $NaH_2PO_4.H_2O$, pH=7.4). Cellular protein (40 μg) was incubated with 40 μM 16:0 lyso-PAF, 200 μM [³H] acetyl-CoA (0.3 μCi/100 nmol) at 37°C for 15 min in buffer containing 4.2 mM HEPES (pH 7.4), 137 mM NaCl, 2.6 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.25% bovine serum albumin (w/v). Unreacted [³H] acetyl-CoA was removed using Dowex X1-8 resin columns and [³H] acetyl PAF was quantified by liquid scintillation spectrometry. Loss of [³H] acetyl PAF was corrected by adding a known amount of [¹⁴C]palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine as an internal standard.

PAF Acetylhydrolase Activity. HMVEC-L or mouse lung endothelial cells grown to confluence in 35 mm dishes were removed from the tissue culture plate in 1.2mM Ca^{2+} HEPES buffer and sonicated on ice. Cellular protein (25µg) was incubated with 0.1mM [acetyl-^{3}H] PAF (10mCi/mmol) for 30 min at 37°C. The reaction was stopped by adding 50µL 10M acetic acid and 1.5mL 0.1M sodium acetate. Released [^{3}H]acetic acid was isolated by passing the reaction mixture through a C_{18} gel cartridge (Baker Chemical Co., Phillipsburg, NJ) and radioactivity was measured using a liquid scintillation counter.

PAF production. HMVEC-L or mouse lung endothelial cells grown in 35mm dishes were washed twice with Hanks' balanced salts solution (HBSS). Cells were incubated with 10 µCi [^{3}H] acetic acid/well for 20 min. After experimental conditions, cell lipids were extracted by the method of Bligh and Dyer (10). The chloroform layer was concentrated under N_2, resuspended in 9:1 $CHCl_3$/MeOH, applied to a silica gel 60 TLC plate, and developed in chloroform /methanol/ acetic acid/water (50/25/8/4 v/v). The region corresponding to [^{3}H] PAF was scraped and radioactivity quantified using liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by adding a known amount of [^{14}C] PAF as an internal standard.

In selected experiments, human PAF was measured directly using an ELISA kit (Biotang Inc., Waltham, MA). HMVEC-L monolayers were washed with ice-cold D-PBS and frozen at -20°C. After two freeze-thaw cycles, aliquots of the suspension were added to microtiter plates with a biotin-conjugated polyclonal antibody specific for PAF. PAF content in samples was determined spectrophotometrically at 450 nm using a Synergy 2 microplate reader (Biotek, Winooski, VT).

Neutrophil adherence.   80ml of blood was obtained from healthy adults and layered over an equal volume of Polymorphprep (Axis-Shield, Oslo, Norway) in 50 ml conical tubes. Tubes were spun at 500 x g for 30 min at 20°C. The buffy coat at the sample/medium interface consisting of PMN was removed, washed and re-suspended in 5 ml of ice cold HBSS and cells counted. HMVEC-L grown on a 12 mm plate were washed twice with HBSS. After appropriate pre-treatment of PMNs with CV3988 or ginkgolide B, 0.5 ml of PMN suspension (4×10^6 cells/ml) in HBSS were added to each of the wells and incubated for 10 min at room temperature. Media and unbound cells were removed and discarded. Plates were washed twice with pre-warmed Dulbecco's PBS. Adherent PMN and endothelial cells were lysed in 1 ml of 0.2% Triton X-100. For maximal binding 0.5 ml aliquot of PMN suspension + 0.5 ml of 0.2% Triton X-100 was used. To measure MPO activity, 400µl of cell lysate was added to 1ml of PBS, 1200 µl Hanks Buffer + BSA, 200 µl 3,3'-dimethyoxybenzidine, and 200 µl of 0.05% H_2O_2 added. 200 µl of 1% NaN_3 was added after 15 min. to stop the reaction and absorbance measured using a 4050 UV/Visible Spectrophotometer (Biochrom, Cambridge, England) at 460 nm.

RAW 264.7 cell adherence. RAW 264.7 cells were grown to confluence in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. Cell suspensions (10×10^6/ml) were labeled with 4 µg/ml calcein-AM for 45 mins @ 37^oC. Cells were washed three times with HEPES buffer, resuspended at a concentration of 4×10^6/ml and 0.5 ml was added to confluent mouse lung endothelial cell monolayers. At the end of the incubation time, non-adherent cells were removed and adherent cells and endothelial cells were lysed in 1 ml of 0.2% Triton X-100. Calcein fluorescence in each sample was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The percent RAW 264.7 cell adherence in each sample was calculated based upon the fluorescence measured in 0.5 ml of RAW 264.7 cell suspension.

Cell surface expression of adhesion molecules. HMVEC-L were grown to confluence in 16 mm culture dishes. Cells were incubated with CSE for selected time periods at 37^oC in 95% O_2/5% CO_2. At the end of the incubation period, the medium was quickly removed, the cells were washed with PBS, immediately fixed in 1% paraformaldehyde and incubated at 4^oC overnight. Cells were washed three times with PBS and then blocked with TBST supplemented with 0.8% BSA (wt/vol) and 0.5% fish gelatin (wt/vol) for 1 h at room temperature. Cell cultures were incubated with appropriate primary antibodies (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA). Cultures were incubated in the dark for 30 mins with 3,3',5,5'-tetramethylbenzidine liquid substrate. Reactions were stopped by the addition of sulfuric acid, and color development was measured with a microtiter plate spectrophotometer at 450 nm.

Caspase activity. Caspase activity was measured using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI). After CSE treatment, endothelial cell monolayers were washed, trypsinized and centrifuged. Cells (2.5×10^4) were resuspended in 75 μl medium and aliquoted into 3 wells of a 96 well plate. An equal volume of Caspase-Glo was added to each well and samples were incubated at room temperature for 30 min. Samples were analyzed for luminescence using a Victor 1420 Multilabel Counter (Perkin Elmer, Waltham, MA). Media only samples were used as controls. Control luminescence was subtracted and luminescence in each sample calculated relative to that in untreated cell controls.

Cell viability assay. Cell viability and CSE cytotoxicity was evaluated using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). After CSE incubation, HMVEC-L were washed with Dulbecco's phosphate-buffered saline (D-PBS) and incubated with 4 μM

ethidium homodimer-1 and 2 µM calcein-AM for 45 mins at 37°C. Fresh D-PBS was added to the cells and fluoresecence was examined using an Olympus IX81 motorized inverted microscope (Olympus, Center Valley, PA).

Statistical analysis. All studies were repeated with at least four separate cell cultures. Data were analyzed using Students t-test or one-way analysis of variance followed by post hoc analysis using Dunnett's test. Differences were regarded as significant at $p < 0.05$ and highly significant at $p < 0.01$. Error bars in the figures represent the standard error of the mean.

RESULTS

Endothelial cell PAF-AH activity is inhibited by CSE.

We incubated (8 h) human lung microvascular endothelial cells (HMVEC-L) with increasing concentrations of cigarette smoke extract (CSE) and measured platelet-activating factor acetylhydrolase (PAF-AH) activity. Significant inhibition of PAF-AH activity was observed with CSE concentrations > 4 μg/ml (Figure 1, upper panel) and the IC_{50} was approximately 11 μg/ml. Tithof et al have shown that polycyclic aromatic hydrocarbons found in cigarette smoke cause apoptosis in human coronary artery endothelial cells by a PLA_2-dependent mechanism (54). We therefore incubated HMVEC-L with increasing concentrations of CSE for 8 h and measured caspase 3/7 activity (Figure 1, lower panel). No significant increase in caspase 3/7 activity at a CSE concentration of 25 μg/ml was observed, indicating that under those exposure conditions, apoptosis was not induced. We therefore used a CSE concentration of 20 μg/ml in all subsequent experiments to avoid inducing endothelial cell apoptosis.

In subsequent experiments, we measured the activity of endothelial PAF synthesis (PLA_2 and acetyl-CoA:lyso-PAF acetyltransferase) and degradation (PAF-AH) enzymes in response to CSE exposure. Incubation with CSE (20 μg/ml) has no significant effect on $iPLA_2$ activity (Figure 2, upper panel) or acetyl-CoA:lyso-PAF acetyltransferase activity (Figure 2, middle panel), but resulted in a time-dependent reduction in PAF-AH activity that was significant after 4 h and progressive over 24 h, when about 93% inhibition was achieved (Figure 2, lower panel). As a positive control, HMVEC-L were incubated with a known PAF-AH inhibitor (methyl arachidonyl fluorophosphonate, 5 μM, 10 min) (27), which achieved 98% inhibition of PAF-AH activity (0.05 ± 0.07 nmol/mg protein/min). Thus, incubation with CSE results in a time- and

concentration-dependent inhibition of endothelial cell PAF-AH activity but has little effect on PAF synthesis enzymes.

We have previously demonstrated that inhibition of PAF-AH with MAFP results in increased endothelial cell PAF production by preventing its degradation (27). Here, we measured PAF accumulation in HMVEC-L incubated with CSE (20 µg/ml) for increasing time intervals. A time-dependent increase in PAF measured by incorporated [^{3}H] acetate or by ELISA was observed and found to be significant after 4 h and increased progressively over 24 h (Figure 3). Inhibiting PAF-AH activity with CSE thus augments net PAF production by HMVEC-L. We examined HMVEC-L cell death over time and demonstrated that there was no significant cell death associated with enzyme inhibition and PAF accumulation at 20 µg/ml CSE over 24 hours (Figure 4).

To determine whether inhibition of PAF-AH results in potentiation of agonist-stimulated increases in endothelial cell PAF production, we stimulated HMVEC-L that had been incubated with CSE (20 µg/ml, 18 h) with thrombin (1 IU/ml, 10 mins) or tryptase (20 ng/ml, 10 mins) and found that CSE increases PAF accumulation alone and potentiates the increases induced by thrombin or tryptase (Figure 5).

CSE exposure results in increased PMN adherence to HMVEC-L.

Increased endothelial cell PAF production is associated with increased PMN adherence to the endothelial cell wall (37,44,56). In addition to the PAF-PAF receptor interaction, adherence of PMN to activated endothelial cells and their transmigration across the endothelium also requires interactions between adhesion molecules on the endothelial cell surface and corresponding ligands on PMN (37). We examined the effects of CSE on HMVEC-L cell surface expression of

adhesion molecules and observed sequential, transient expression of P-selectin, E-selectin, ICAM-1, and then VCAM-1 in that order on the cell surface over time (Figure 6).

We isolated PMN from peripheral blood and incubated them with HMVEC-L that had been exposed to CSE. PMN adherence to HMVEC-L was found to increase progressively as a function of the duration of exposure to CSE (Figure 7). As a positive control, HMVEC-L were incubated with PMA (100 nM, 20 mins), which is known to stimulate HMVEC-L PAF production and PMN adherence. As expected, PMA did promote PMN adherence to HMVEC-L, and this was blocked by the PAF receptor antagonists ginkgolide B or CV3988 (10 μM ,10 mins) (Figure 7).

Taken together, these findings demonstrate that CSE increases cell surface expression of adhesion molecules in addition to promoting PAF production, and these effects cooperate to increase inflammatory cell adhesion to the endothelium. The fact that pretreating PMN with PAF receptor antagonists completely inhibited their adherence to the endothelium reflects the requirement that PAF on the endothelial cell surface interact with the PAF receptor on the PMN surface for adhesion to occur, even though other adhesion molecules are also expressed on the endothelial cell surface.

CSE does not increase PAF production in the absence of iPLA$_2$β in mouse lung endothelial cells

We have previously demonstrated that protease-activated receptor (PAR) agonists such as thrombin or tryptase stimulate human lung endothelial cell PAF production in a process that requires activation of Group VIA PLA$_2$ (iPLA$_2$β) and that PAR agonists fail to stimulate PAF production by lung endothelial cells isolated from iPLA$_2$β knockout (iPLA$_2$β-KO) mice (46). Since the defect in iPLA$_2$β-KO cells reflects a deficiency in PAF biosynthesis, it might be

expected that inhibiting degradation with CSE would have little effect on net PAF production by iPLA$_2$β-KO cells. To examine that possibility, we first incubated lung endothelial cells from wild type (WT) and iPLA$_2$β-KO mice with CSE and measured PAF-AH activity as a function of exposure time and observed a progressive, time-dependent reduction in activity in cells of both genotypes with little difference between them (Figure 8, upper panel).

We then measured PAF production under these conditions and found that incubation of WT lung endothelial cells with CSE resulted in progressive time-dependent accumulation of PAF, but with iPLA$_2$β-KO cells no significant PAF accumulation occurred with increasing incubation intervals (Figure 8, lower panel). These data reflect the requirement for iPLA$_2$β in lung endothelial PAF production and indicate that, although iPLA$_2$β-null cells express PAF-AH activity that is inhibited by CSE in the same manner as for WT cells, net PAF production by iPLA$_2$β-null cells is not affected by CSE because they fail to synthesize PAF. Interference with PAF degradation thus fails to cause PAF accumulation with the iPLA$_2$β-null cells. These results suggest that CSE might also fail to promote inflammatory cell adherence to iPLA$_2$β-KO cells.

To test that possibility, RAW 264.7 cells, which represent a murine macrophage-like cultured cell line, were incubated with WT mouse lung endothelial cells that had been exposed to CSE or with control WT cells that had not been exposed. Exposure to CSE resulted in a significant increase in adherence of RAW 264.7 cells to WT lung endothelial cells over control levels (43.5 $\pm$ 2.6% vs. 11.7 $\pm$ 0.4%, n=6, p<0.01), and, as expected, adherence was suppressed to control levels by pretreatment with the PAF receptor antagonist ginkgolide B (13.4 $\pm$ 1.3%, n=6), which reflects the requirement for PAF in the adherence process. In addition, pretreating the WT lung endothelial cells with the iPLA$_2$β inhibitor (*S*)-bromoenol lactone ((*S*)-BEL, 5 μM, 10 min) also suppressed adherence of RAW 264.7 cells to control levels, which reflects the requirement for

iPLA$_2\beta$ in PAF production. No increase in RAW 264.7 cell adherence was observed to lung endothelial cells isolated from iPLA$_2\beta$-KO mice when incubated with CSE ($5.7 \pm 0.5\%$ vs. $6.6 \pm 1.1\%$ for CSE treated cells, n=6).

DISCUSSION

Smoking is associated with increased morbidity and mortality and inflicts damage on multiple organ systems, including cardiovascular and lung tissue. In the United States, the Centers for Disease Control has estimated annual smoking-attributable mortality, years of potential life lost (YPLL) for adults and infants, and productivity losses for adults. Between 1997 and 2001, cigarette smoking and passive exposure to tobacco smoke resulted in approximately 438,000 premature deaths, 5.5 million YPLL, and $92 billion in productivity losses annually (1,35). Cigarette smoke elicits the recruitment and adherence of circulating leukocytes to the vascular wall, which is an early event in inflammation and the pathogenesis of emphysema (16). Migration of circulating neutrophils to sites of tissue inflammation is a multistep process that involves the interaction of adhesion molecules and their receptors.

Neutrophil-endothelial cell adhesion requires the interaction of PAF on the surface of the endothelial cell with the PAF receptor on the surface of the neutrophils. Other events involved in adhesion include an initial tethering of PMN to selectin on the endothelial surface via PMN cell surface receptors (13,25,36). Firm adherence and transmigration are mediated by ICAM-1 and VCAM-1 expression on endothelial cells and their corresponding receptors on leukocytes (31,39). Activation of leukocytes occurs via the interaction of endothelial cell PAF with the PAF receptor on the tethered cells, which then activates a program of adhesion and β-integrin-mediated leukocyte migration (14,29,43). PAF also primes leukocytes to interact with agonists they will encounter after migration from the vasculature (14,29,43).   When endothelial cells fail to express PAF on their surface, PMN activation does not occur and they can be released to re-enter the circulation.

PAF is implicated in the pathophysiology of a number of human diseases, including asthma, endotoxic shock, ischemic injury, diabetes and hypertension (55). That PAF might be involved in cigarette smoke-induced tissue injury was first suggested in 1989 by a report that the plasma of smokers contained higher PAF concentrations than observed in nonsmokers (24). PAF is normally maintained at low concentrations by its rapid hydrolysis catalyzed by PAF-AH enzymes (26,42,50) which are calcium-independent phospholipases A_2 (iPLA$_2$) that preferentially hydrolyze phospholipids with short chain or oxidized fatty acids at the *sn*-2 position (26,50), such as the *sn*-2 acetate residue of PAF. PAF-AH isoforms comprise PLA$_2$ Groups VII and VIII of the Dennis classification scheme (12,45).

Group VIIA PAF-AH is a secreted enzyme found in the plasma. Groups VIIB, VIIIA and VIIIB are intracellular PAF-AH enzymes (12,45). Inhibition of endothelial cell PAF-AH activity results in increased endothelial cell surface expression of PAF and enhanced recruitment of circulating leukocytes and their adherence to the vascular wall (27). In the lung, these events are among the early inflammatory steps in the pathogenesis of emphysema (53). Previous studies have determined that cigarette smoke inhibits plasma PAF-AH activity *in vitro* (9,38), although Lehr and co-workers found no inhibition of circulating PAF-AH activity in hamsters exposed to cigarette smoke (30). In the studies described here, we have demonstrated that exposure of HMVEC-L to CSE extract results in a significant inhibition of PAF-AH activity, which indicates that intracellular PAF-AH isoforms are sensitive to inhibition by CSE.

Here, we used MAFP as a positive control for PAF-AH inhibition. Although MAFP was developed initially as a PLA$_2$ inhibitor, we have demonstrated previously that it fails to inhibit thrombin-stimulated, membrane-associated endothelial cell iPLA$_2$ activity, although MAFP does inhibit PAF-AH activity and thereby potentiates thrombin-stimulated PAF production (27). As

we have previously reported, inhibition of PAF-AH alone by CSE is sufficient to result in a time-dependent increase in PAF production that correlates with PAF-AH inhibition in wild type cells with intact PAF biosynthetic capabilities. To determine whether increased PAF production also reflected an additional effect of CSE to activate $iPLA_2$ or acetyl-CoA: lyso-PAF acetyl;transferase activity, we measured activity of these enzymes in HMVEC-L incubated with CSE over the course of 24 h and failed to detect changes in activity after any duration of CSE exposure examined. This corroborates our hypothesis that inhibition of PAF-AH by CSE alone is sufficient to increase HMVEC-L PAF production even without a concomitant activating stimulus for $iPLA_2$, although activation of HMVEC-L $iPLA_2$ with the PAR agonists thrombin or tryptase amplifies the increase in PAF production by CSE.

We have previously demonstrated that the majority of HMVEC-L PLA_2 activity is membrane-associated, Ca^{2+}-independent and preferentially hydrolzes arachidonate-containing phospholipids (44). Stimulation of HMVEC-L $iPLA_2$ activity with PAR agonists results in increased PAF production, and this is associated with enhanced PMN adherence and release of arachidonic acid and prostaglandin I_2 (44).   We have also demonstrated that pretreating HMVEC-L with the $iPLA_2\beta$-selective inhibitor (S)-bromoenol lactone ((S)-BEL) completely inhibited thrombin- and tryptase-stimulated $iPLA_2$ activity, PGI_2 release and PAF production (46). Additionally, stimulation of lung endothelial cells from WT mice with the PAR agonists thrombin or tryptase results in increased PAF production, but lung endothelial cells from $iPLA_2\beta$-KO mice fail to produce PAF under these conditions (46). These findings demonstrate that $iPLA_2\beta$ is required for endothelial cell PAF production in response to the PAR agonists thrombin or tryptase. Here, we have shown that lung endothelial cells isolated from WT or $iPLA_2\beta$-KO mice exhibit similar levels of PAF-AH activity, and that the activity is inhibited by CSE and MAFP in cells of both

genotypes. Inhibiting PAF-AH activity in WT lung endothelial cells results in increased PAF production and adherence of mouse macrophages, but increased PAF production or macrophage adherence is not observed in iPLA$_2$β-KO lung endothelial cells under these conditions. This indicates that inhibition of PAF-AH alone fails to increase PAF production when there is a deficiency in PAF biosynthesis, as in cells that lack iPLA$_2$β. In future studies, we propose to determine whether the absence of iPLA$_2$β decreases CSE-induced inflammatory cell recruitment to the lung in a mouse model of emphysema. These studies would indicate whether iPLA$_2$β is a potential therapeutic target for smoking-induced inflammatory diseases.

Tobacco smoke is associated with many human diseases, including pulmonary and cardiovascular disease, and exerts a complex series of effects on release and inhibition of pro- and anti-inflammatory mediators. Cigarette smoke has been demonstrated to increase circulating levels of TNF-α, interleukin-1 (IL-1), IL-6 and IL-8 (8,19), but is associated with decreased mononuclear cell production of IL-1β, IL-2, TNF-α and interferon-γ (20). The net effect of cigarette smoke is to increase the PMN content of lung, and we propose that this results in part from increased endothelial cell PAF production. Smokers have higher levels of circulating PMN than non-smokers, primarily as a result of increased catecholamine secretion that stimulate release of leukocytes and platelets into the circulation from the bone marrow (48).   Smoking-induced impairment of immune function can lead to chronic pulmonary inflammation, with attendant remodeling and increased susceptibility to infection (3,50). Innate cellular immune responses such as recruitment of neutrophils and macrophages are designed to promote clearance of injurious agents and to effect removal of particulates and resolution of inflammation. Neutrophils are recruited first and then assist in recruiting monocytes, which differentiate into tissue macrophages (6,18). Although cigarette smoke enhances recruitment of these cells from

the circulation, they are functionally impaired and this can result in smoldering inflammation and increased susceptibility to chronic infection in smokers (49).

Endothelial cell dysfunction is a principal consequence of smoking and is linked to oxidant stress (5,23), impaired nitric oxide production (51,57), apoptosis (2,17,40) and inflammation. Here we have demonstrated that inhibition of endothelial cell PAF-AH activity by CSE results in increased PAF production and then to inflammatory cell adhesion to the lung endothelium, and these effects occur at CSE concentrations below those required to trigger endothelial cell apoptosis. Inhibition of lung endothelial cell PAF-AH by cigarette smoke can result in increased pulmonary recruitment of inflammatory cells that contribute to chronic inflammation in the lungs of smokers. CSE-induced PAF production is blocked when endothelial cell $iPLA_2\beta$ is inhibited or absent, which reflects the requirement for $iPLA_2\beta$ as a potential target for therapeutic interventions to reduce airway inflammation and the progression of chronic lung disease.

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FIGURE LEGENDS

FIGURE 1. PAF-acetylhydrolase (PAF-AH) activity (upper panel) and caspase 3/7 activity (lower panel) measured in human lung microvascular endothelial cells (HMVEC-L) incubated with increasing concentrations of cigarette smoke extract (CSE) for 8 h. Data shown are mean $\pm$ SEM for 4 separate cell cultures. *p<0.05, **p<0.01 when compared to control.

FIGURE 2. Phospholipase A_2 (PLA_2) activity (upper panel), acetyl-CoA:lyso-PAF acetyltransferase activity (middle panel) and PAF-AH activity (lower panel) measured in HMVEC-L incubated with cigarette smoke extract (CSE, 20µg/ml) for up to 24 h. Data shown are means $\pm$ SEM for 6 separate cell cultures. *p<0.05, **p<0.01 when compared to control.

FIGURE 3. PAF production measured as [^{3}H] acetyl PAF (upper panel) or ELISA assay (lower panel) in HMVEC-L incubated with cigarette smoke extract (CSE, 20 µg/ml) for up to 24 h. Data shown are means + SEM for 4 separate cell cultures. *p<0.05, **p<0.01 when compared to control.

FIGURE 4. HMVEC viability measured using the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen). Cells were left untreated (A) or incubated with CSE (20 µg/ml) for 4h (C) or 24 h (D). Cells were treated with methanol (5 mins) as a positive control for cell death (B).

FIGURE 5. PAF production in HMVEC-L in response to thrombin (1 IU/ml 10 mins) or tryptase (20 ng/ml, 10 mins) is potentiated when HMVEC-L have been exposed to CSE (20 µg/ml, 18 h). Data shown are means + SEM for 6 separate cell cultures, **p<0.01 when compared to unstimulated controls. ++p<0.01 when comparing PAF production in the presence or absence of CSE.

FIGURE 6. Time course of changes in adhesion molecule expression in HMVEC-L incubated with CSE (20 µg/ml) for up to 24 h. Data shown are the mean fold changes over control for cell surface expression of P-selectin, E-selectin, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). Data shown represent means $\pm$ SEM for 8 separate experiments. **$p<0.01$ when compared to control.

FIGURE 7. Polymorphonuclear leukocyte (PMN) adherence to HMVEC-L monolayers incubated with CSE (20 µg/ml) for up to 24 h. HMVEC-L were incubated with PMA (100 nM, 20 mins) prior to the addition of PMN as a positive control. Where indicated, PMN were incubated with the PAF receptor antagonist CV3988 or ginkgolide B (10 µM, 10 mins) prior to addition to HMVEC-L. $2x10^6$ PMN were added to the wells for 10 min, adherence was measured by myeloperoxide activity and normalized to theoretical maximal binding. Data shown represent means + SEM for 4 separate cell culture experiments and PMN isolations. *$p<0.05$, **$p<0.01$ compared to control, ++$p<0.01$ when comparing data between treated and non-treated PMN.

FIGURE 8. PAF-acetylhydrolase (PAF-AH) activity (upper panel) and [^{3}H] acetyl PAF production (lower panel) measured in lung endothelial cells isolated from wild type or calcium-independent phospholipase A$_2$β (iPLA$_2$β)-knockout mice and incubated with cigarette smoke extract (CSE, 20µg/ml) for up to 24 h. Cells were incubated with methyl arachidonyl fluorophosphonate (MAFP, 5 µM, 10 mins) as a positive control for inhibition of PAF-AH activity. Data shown are means $\pm$ SEM for 6 separate cell cultures. *$p<0.05$, **$p<0.01$ when compared to corresponding control.