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Research Article

Targeting the PAF:PAF Receptor Interaction to Manage PMN Recruitment to the Lung of Smokers

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Abstract

Cigarette smoking is associated with a high degree of mortality and morbidity and is associated with damage to almost every organ in the body, particularly the lungs. Smokers are at increased risk for pulmonary diseases such as chronic obstructive pulmonary disease (COPD) which involves a robust migration of leukocytes. We have demonstrated previously that adherence of polymorphonuclear leukocytes (PMN) to lung endothelial cells exposed to cigarette smoke extract (CSE) is mediated via inhibition of platelet-activating factor acetylhydrolase (PAF-AH) activity, leading to PAF accumulation. We now show that CSE incubation of human small airways epithelial cells (HSAEC) results in inhibition of PAF-AH activity, increased PAF accumulation and PMN adherence and transmigration across the HSAEC monolayer. PMN adherence and transmigration is inhibited by bromoenol lactone pretreatment of HSAEC to inhibit calcium-independent phospholipase A_2 ($iPLA_2$), or by inhibiting the PAF receptor on PMN using ginkgolide B. The number of cells in the bronchoalveolar fluid from the lungs of $iPLA_2\beta$ knockout mice was significantly lower than in wild type mice exposed to cigarette smoke. We propose that targeting PAF accumulation or blocking the PAF receptor may represent therapeutic targets to manage inflammatory cell infiltration in the lungs of smokers.

Keywords: Inflammation; Platelet-Activating Factor; Cigarette Smoking; Lung Epithelium

Abbreviations

BEL	:Bromoenol Lactone
BAL	:Bronchoalveolar Lavage
COPD	:Chronic Obstructive Pulmonary Disease
HSAEC	:Human Small Airway Epithelial Cells
iPLA2β	:Calcium-Independent Phospholipase A2β
iPLA2β-KO	:Calcium-Independent Phospholipase A2β knockout
MPO	:Myeloperoxidase
PAF	:Platelet-Activating Factor
PAF-AH	:Platelet-Activating Factor Acetylhydrolase
PMN	:Polymorphonuclear Leukocytes
WT	:Wild Type

Introduction

Smoking damages almost every organ of the body and is associated with a high degree of morbidity and mortality. It is estimated that as many as 42 million people in the U.S. are smokers [1, 2]. 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 up to 24 million individuals with COPD in the US alone. Human COPD consists of several anatomic changes-emphysema, small airway remodeling, vascular remodeling with pulmonary hypertension, mucus overproduction and chronic bronchitis. The disease process in the lungs of COPD patients involves a robust migration of leukocytes, the production of inflammatory mediators and the release of potentially destructive proinflammatory cytokines and proteases. The combination of activated leukocytes, endothelial and epithelial cells results in the generation of additional chemo attractant mediators that can lead to the influx of additional inflammatory cells. This leads to tissue remodeling and destruction. Small airway remodeling (increases in bronchiolar wall fibrous tissue, muscle, inflammatory cells and luminal mucus) is an important cause of airway obstruction in cigarette smokers but little is known about the pathogenesis of this disease. The role of inflammatory cells in small airway remodeling is uncertain, but since neutrophils and macrophages release oxidants in response to smoke, these cells may potentiate oxidant-mediated activation of TGF-β and increase collagen production.

In recent studies, we have determined that cigarette smoke is associated with increased platelet-activating factor (PAF) accumulation in human lung-derived endothelial cells as a result of inhibition of its metabolic enzyme PAF acetylhydrolase (PAF-AH) [3]. PAF is a membrane phospholipid-derived metabolite that is involved in inflammatory reactions. The enhanced cell surface expression of PAF on activated endothelial cells results in increased adherence of inflammatory cells. We hypothesize that cigarette smoking can increase recruitment of inflammatory cells to the small airways via PAF accumulation in epithelial cells and that management of PAF accumulation or disruption of the PAF-PAF receptor interaction may provide effective therapeutic options to manage inflammatory diseases such as COPD.

Materials and Methods

Cell Culture

Human small airway epithelial cells (HSAEC) were grown in SAGM growth medium (Lonza, Walkersville, MD) and maintained at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Cells were treated with CSE (20μg/mL) for indicated times.

Measurement of PAF Production

HSAEC grown to confluence were incubated with Hanks' balanced salt solution (135mM NaCl, 0.8mM $MgSO_4$, 10mM HEPES, pH 7.4, 1.2 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4 and 6.6 mM glucose) containing 10 μCi of [^{3}H] acetic acid for 20 min at room temperature. Total lipid extracts were resuspended in 9:1 $CHCl_3$:MeOH and applied to TLC plates. Plates were developed in 100:50:16:8 chloroform, methanol, acetic acid and water. The region corresponding to PAF was scraped and measured by liquid scintillation counting.

Measurement of PAF-AH Activity

HSAEC were grown to confluence, harvested in 1.2mM Ca^{2+} HEPES buffer and sonicated on ice. Cellular protein (25 μg) was incubated with 0.1mM [acetyl-^{3}H] PAF (10mCi/mmol) for 30 min at 37°C. The reaction was stopped by adding 50 μL 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.

Measurement of PMN adherence

Blood (80 ml) was obtained from healthy donors and PMN isolated using Polymorphprep (Axis-Shield, Oslo, Norway). HSAEC grown to confluence in 34-mm dishes were incubated with CSE for indicated times and then 2 x 10^6 PMNs added for 20 mins. Non adherent PMNs were removed by washing the wells twice with Hanks' buffer. HSAEC and adherent PMNs were lysed with 0.2% Triton X-100. Myeloperoxidase (MPO) content was determined by adding 400μL of cell lysate to a tube containing 1mL of PBS, 1.2mL Hanks buffer with bovine serum albumin, 200μL of 0.125% 3,3'-dimethoxybenzidine, and 200μL of 0.05% H_2O_2. After samples were incubated at 37°C for 15 min, the reaction was stopped by the addition of 200 μL of NaN_3, and the absorbance of each tube was measured at 460 nm. MPO content in 2 x 10^6 PMNs was determined and used as the value for 100% adherence.

Measurement of PMN transmigration

HSAEC were grown to confluence on Transwell inserts. Inserts were inverted and cells were incubated with CSE for 18 hours. 2 x 10^6 PMNS were added to the Transwell insert and left for 4 hours. At the end of incubation, PMN were collected from the bottom of the culture dish and MPO content determined as described above.

Measurement of cells in bronchoalveolar lavage fluid from mouse lung

Wild type and iPLA$_2$β-knockout mice were exposed to cigarette smoke (48 mins/day, 5 days/week) for 2 or 4 weeks using the

SCIREQ inExpose system (SCIREQ, Montreal, Quebec). Mouse lungs were lavaged three times with 1 ml PBS with 1% FBS. Lavage fluid was centrifuged at 400 x g for 5 mins at 4°C and cell pellet resuspended in 150 µl PBS with 1% FBS. Cell numbers were counted using a hemocytometer.

Results and Discussion

Platelet-activating factor (PAF) is implicated in the pathophysiology of a number of human diseases, including asthma, endotoxic shock, ischemic disease, diabetes and hypertension. The relationship of PAF to cigarette smoking was first suggested in 1989 when it was reported that there was a higher concentration of PAF in the plasma of smokers compared to nonsmokers. PAF is produced by a variety of cells that participate in the development of inflammatory reactions, such as macrophages, polymorphonuclear leukocytes (PMN), eosinophils, basophils and platelets. Most of the cells that produce PAF also possess PAF receptors and are targets for PAF action. PAF promotes the aggregation, chemotaxis, granule secretion and oxygen radical generation from leukocytes and the adherence of leukocytes to the endothelium. PAF also increases the permeability of the endothelial cell monolayer and stimulates smooth muscle contraction. The enhanced expression of endothelial cell-associated PAF has been shown to cause transient adherence of neutrophils to the endothelium [3, 5-13]. Additionally, PAF receptor antagonists have been demonstrated to prevent neutrophil adherence and migration across endothelial cells [3, 14-16]. Together these studies have demonstrated the importance of PAF in the process of transmigration.

There is much less information available regarding the role of PAF in lung epithelial cells. We incubated human lung small airway epithelial cells (HSAEC) with cigarette smoke extract (CSE) for up to 72 hours and measured PAF-AH activity (Figure 1). HSAEC PAF-AH activity was significantly inhibited after 4 hrs of CSE exposure and remained inhibited for up to 72 hours. The inhibition of PAF-AH activity was accompanied by a significant increase in PAF accumulation that started at 1 hr following CSE incubation and progressively increased over the 72 hour time period studied (Figure 2). Increased cell surface expression of PAF is associated with increased inflammatory cell adherence. We incubated HSAEC with CSE for up to 72 hours, and added 2 x 10^6 PMN isolated from human peripheral blood. Non-adherent PMN were removed by washing after 20 min and adherence measured at each time point. Adherence of PMN to HSAEC increased progressively as a function of the duration of CSE exposure (Figure 3). The time course of PMN adherence was similar to that observed for PAF accumulation (Figure 2).

In previous studies using endothelial cells [3, 10, 13, 17-20], we have shown that PAF production is initiated by activation of calcium independent phospholipase A_2 ($iPLA_2$) and that pretreatment with the $iPLA_2$-selective inhibitor bromoenol lactone (BEL) inhibits PAF production. When HSAEC were pretreated with BEL prior to incubation with CSE, PMN adherence was significantly inhibited (Figure 4). The seeds and leaves of *Ginkgo biloba* have been used in traditional medicine to treat respiratory diseases, cardiovascular disorders, memory loss and loss of hearing [21, 22]. In vitro, *Ginkgo biloba* extract exhibits anti-infective, chemopreventive and anticancer effects [23-26]. *Ginkgo biloba* is also a PAF receptor antagonist [27-30]. In this study we used an extract of *Ginkgo biloba*, ginkgolide B, to decrease inflammatory cell adherence to lung epithelial cells exposed to cigarette smoke. When PMN were incubated with ginkgolide B, PMN adherence was minimal (Figure 4).

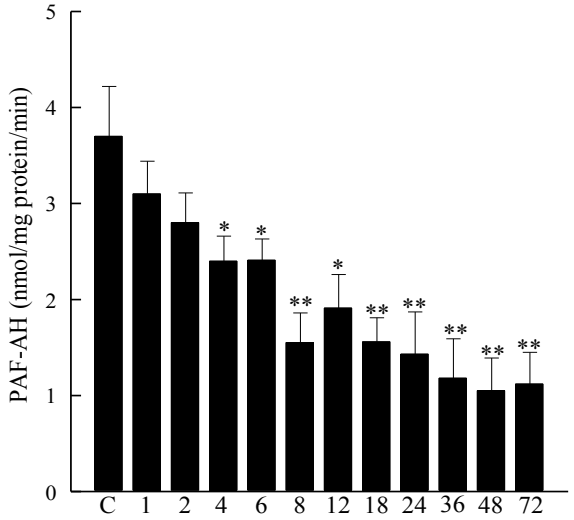

Figure 1. Platelet-activating factor acetylhydrolase (PAF-AH) activity in human small airways epithelial cells (HSAEC) exposed to cigarette smoke extract (CSE, 20 µg/ml) for up to 72 hours. *p<0.05, **p<0.01 when compared to untreated controls. Values are mean + SEM for 6 separate cell cultures.

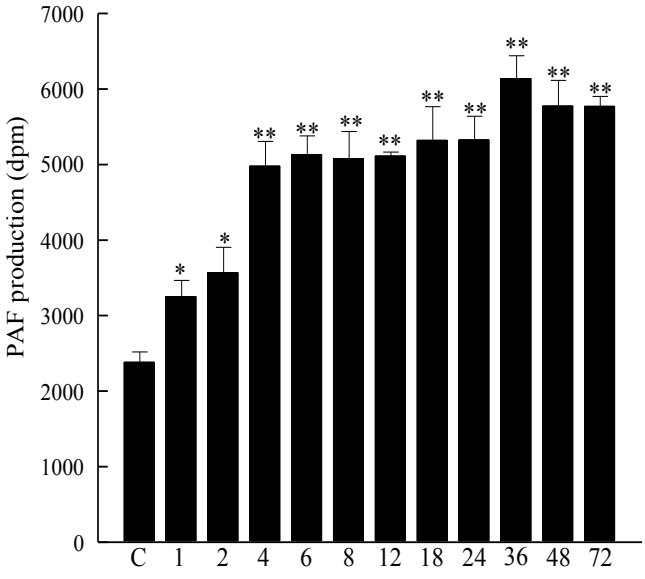

Figure 2. Platelet-activating factor (PAF) accumulation in human

small airways epithelial cells (HSAEC) exposed to cigarette smoke extract (CSE, 20 μg/ml) for up to 72 hours. *p<0.05, **p<0.01 when compared to untreated controls. Values are mean + SEM for 6 separate cell cultures.

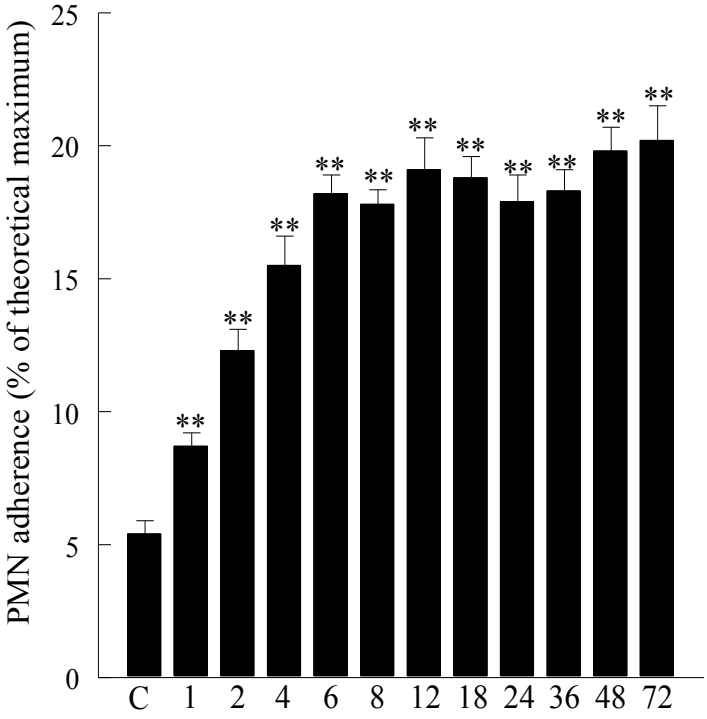

Figure 3. Polymorphonuclear leukocyte (PMN) adherence to human small airways epithelial cells (HSAEC) exposed to cigarette smoke extract (CSE, 20 μg/ml) for up to 72 hours. **p<0.01 when compared to untreated controls. Values are mean + SEM for 6 separate cell cultures.

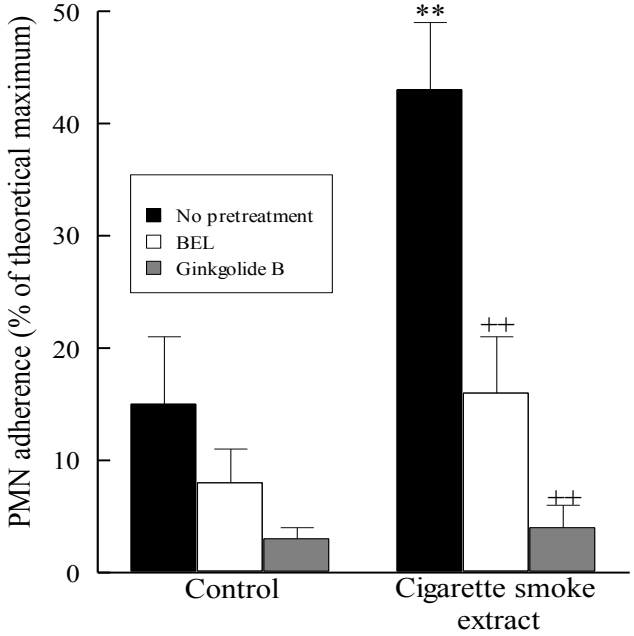

Figure 4. Polymorphonuclear leukocyte (PMN) adherence to human small airways epithelial cells (HSAEC) exposed to cigarette smoke extract (CSE, 20 μg/ml, 18 hours) . Adherence was inhibited following pretreatment of HSAEC with bromoenol lactone (BEL, 5 μM, 10 mins prior to CSE addition) or pretreatment of PMN with ginkgolide B (10 μM, 10 mins prior to addition to HSAEC). **p<0.01 when compared to untreated controls. ++p<0.01 when comparing values in the presence or absence of inhibitor. Values are mean + SEM for 6 separate cell cultures.

To gain access to the alveolar spaces, neutrophils must cross the epithelial cell barrier in a basolateral-to-apical direction, which necessitates modification of most commonly used methods to study transmigration. In separate experiments, we examined whether CSE incubation induced PMN transmigration across HSAEC monolayers grown on Transwell inserts. Once cells had achieved a monolayer on the insert, Transwells were inverted and PMN applied in order to measure transmigration in a basolateral to apical direction. When CSE was applied to the apical side of HSAEC, PMN transmigration increased almost 3-fold (Figure 5). The transmigration was inhibited by pretreatment of HSAEC with BEL to block PAF production or pretreatment of PMN with ginkgolide B (Figure 5). Taken together, these data suggest that targeting PAF production or the PAF receptor may be a viable therapeutic option to manage inflammation mediated via PAF.

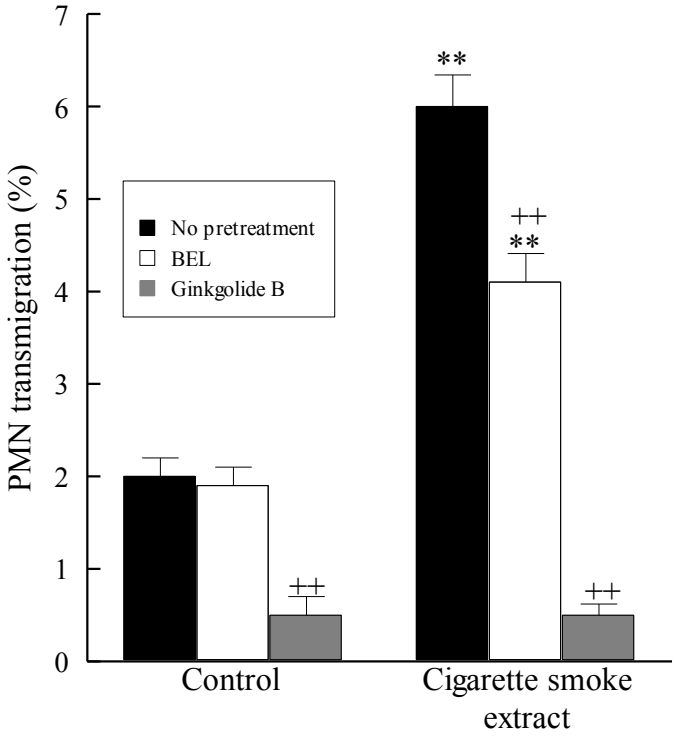

Figure 5. Polymorphonuclear leukocyte (PMN) transmigration across human small airways epithelial cells (HSAEC) monolayers exposed to cigarette smoke extract (CSE, 20 μg/ml, 18 hours) . Transmigration was inhibited following pretreatment of HSAEC with bromoenol lactone (BEL, 5 μM, 10 mins prior to CSE addition) or pretreatment of PMN with ginkgolide B (10 μM, 10 mins prior to addition to Tran-

swell). **p<0.01 when compared to untreated controls. ++p<0.01 when comparing values in the presence or absence of inhibitor. Values are mean + SEM for 6 separate cell cultures.

We have previously determined that inhibition of iPLA$_2$β blocks PMN adherence to lung endothelial cells exposed to CSE [3]. Here, we have demonstrated that iPLA$_2$ inhibition is associated with decreased PMN adherence and migration across the HSAEC monolayer. To determine whether the absence of iPLA$_2$β was associated with decreased inflammatory cells in the lungs of an animal model of smoking, we exposed wild type (WT) and iPLA$_2$β knockout (iPLA$_2$β-KO) mice to cigarette smoke (48 min/day, 5 days/week) for 2 or 4 weeks. After cigarette smoke exposure, the bronchoalveolar lavage (BAL) demonstrated increased inflammatory cell number when compared to room air in the WT mice (Figure 6). The number of inflammatory cells in the BAL taken from iPLA$_2$β-KO mice was also increased in mice exposed to CSE when compared to room air (figure 6). However, there was a significantly lower number of cells present in the BAL from iPLA$_2$β-KO mice compared to WT at each experimental time point (Figure 6). These data suggest that the absence of iPLA$_2$β contributes to a decrease in inflammatory cells in the alveolar spaces of mice exposed to cigarette smoke.

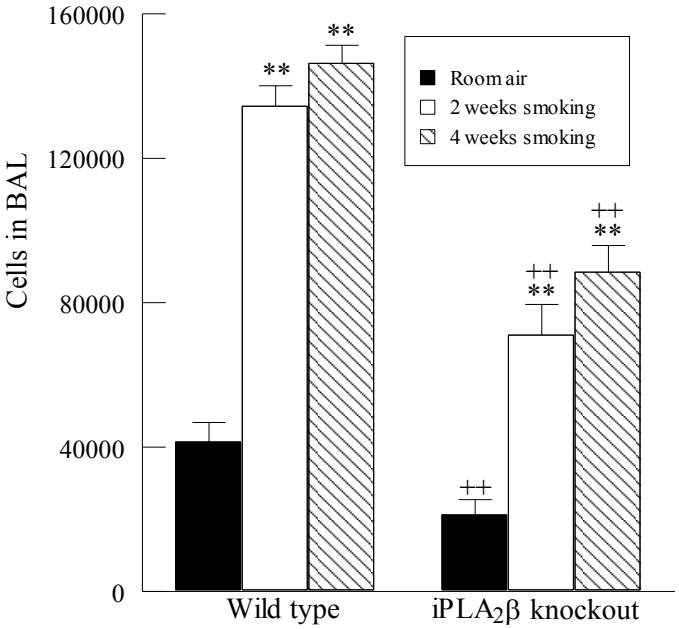

Figure 6. Number of inflammatory cells in the bronchoalveolar lavage (BAL) fluid from the lungs of wild type (WT) and calcium-independent phospholipase A$_2$β knockout (iPLA$_2$β-KO) mice exposed to cigarette smoke (48 min/day, 5 days/week) for 2 or 4 weeks. **p<0.01 when compared to room air controls. ++p<0.01 when comparing values between WT and iPLA$_2$β-KO mice. Values are mean + SEM for 6 separate cell cultures.

Cigarette smoking causes small airway inflammation and recruitment of inflammatory cells resulting in widespread damage and may eventually lead to cancer [31-39]. Cell recruitment requires migration of circulating inflammatory cells across the endothelial and epithelial cell barriers. Since PAF is a potent inflammatory cell recruiter that promotes transendothelial and transepithelial migration, we hypothesized that increased PAF accumulation in the airways of smokers enhances inflammatory cell recruitment and exacerbates inflammation. We have shown previously that the absence of iPLA$_2$β results in a lack of endothelial cell PAF accumulation and PMN adherence in response to cigarette smoke extract. In this study, we show that the lack of iPLA$_2$β is also associated with decreased PAF accumulation and PMN adherence to HSAEC and that there are significantly fewer inflammatory cells in the lungs of iPLA$_2$β-KO mice exposed to cigarette smoke when compared to WT. These data indicate that iPLA$_2$β is involved in PAF accumulation and inflammatory cell recruitment, particularly in response to smoking. The development of specific iPLA$_2$β inhibitors may be of therapeutic benefit to manage inflammation in the lungs of smokers by managing PAF accumulation in lung epithelial and endothelial cells.

Conclusion

Our data presented here suggest that inflammatory cell accumulation in the lungs of smokers is mediated, at least in part, by PAF accumulation as a result of decreased catabolism by PAF-AH. Targeting PAF synthesis or the PAF receptor represent therapeutic options to manage inflammation in the lungs of smokers.

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