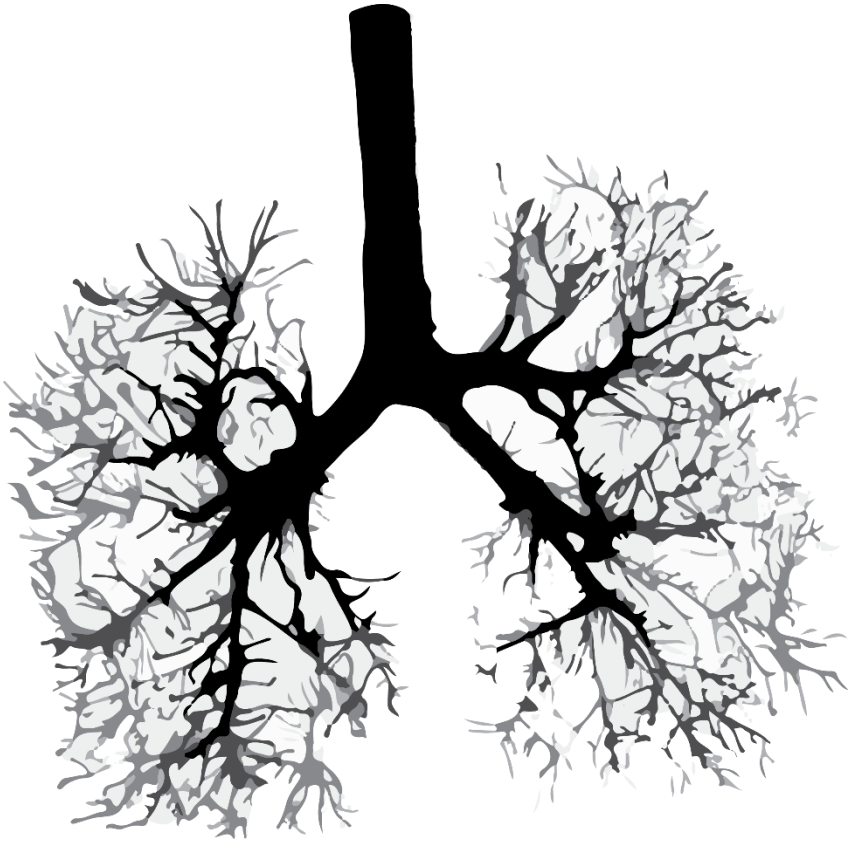
The Matrikine PGP in Lung Diseases

A Translational Study



Mojtaba Abdul Roda

2015

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بسمه تعالى

وَقُل رَّبِّ زِدْنِي عِلْمًا

صدق جلَّ و علا

“And say: O Lord, increase my knowledge” (Verse 114, chapter 20 of the holy Quran)

أودُّ أن أتوجَّه بكل الشكر والحب والتقدير والعرفان إلى والديَّ حفظهما ربّي، عارفاً بفضلهما مقراً بالتقصير في حقهما، فلولا فضل الخالق عزَّ وجل و تسديدهما و نصيحتهما، ما ظهرت هذه الأطروحة على اكمل وجه. اقدِّم بين يديكم جهدي هذا و ثمرة العمل المتواصل والتجارب المعقده الَّتي اجريتها راجياً قبولكم.

The Matrikine PGP in Lung Diseases: A Translational Study

De peptide PGP in longziekte: een translationele studie

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 23 september 2015 des middags te 4.15 uur

door

Mojtaba Abdul Roda

geboren op 25 maart 1985 te Najaf, Irak

Promotoren: Prof.dr. G. Folkerts

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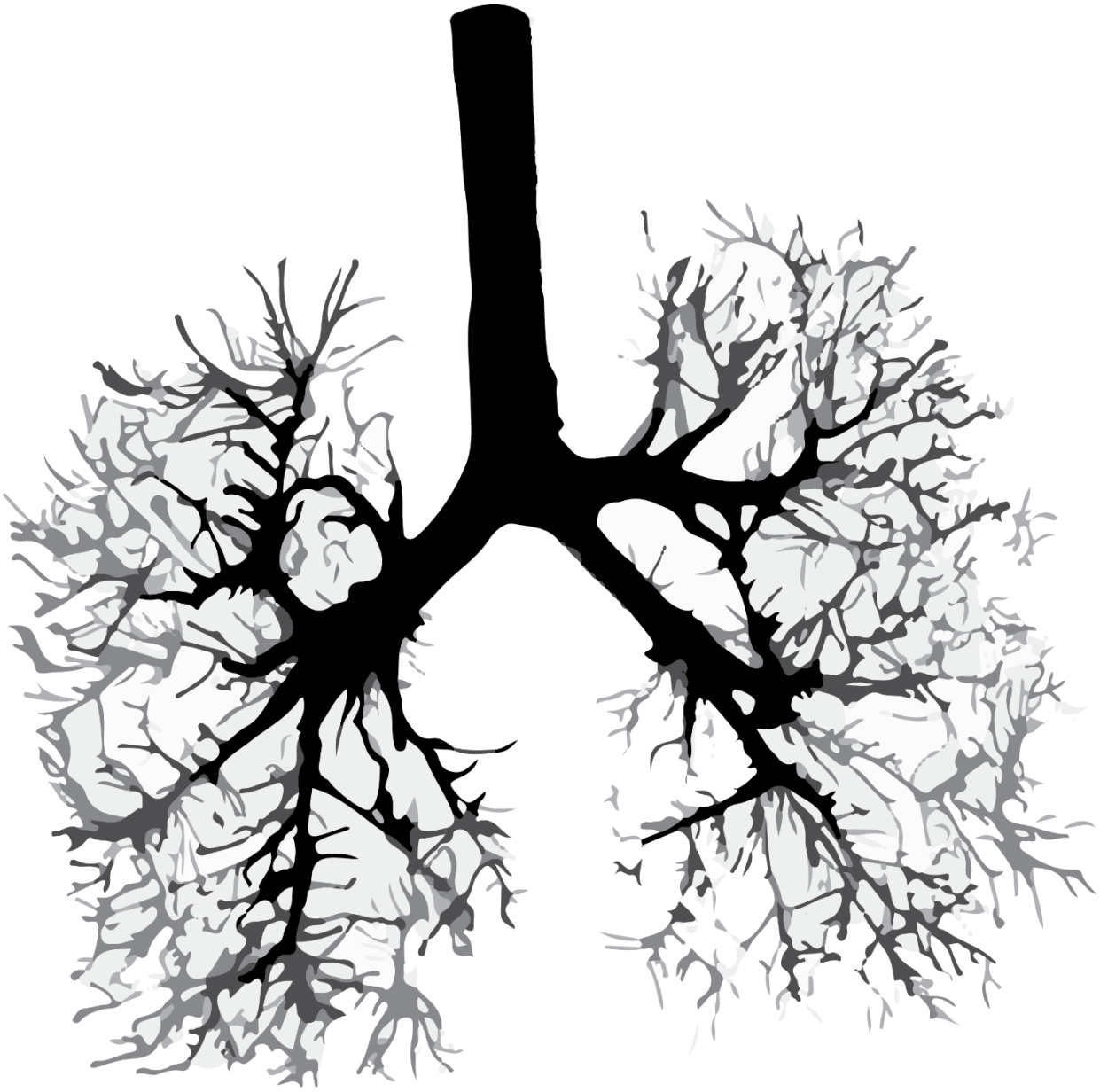
Chapter one



Thesis aims and outline

Chronic obstructive pulmonary disease (COPD) is a progressive disorder characterized by the development of airflow limitation. It encompasses chronic bronchitis (chronic inflammation and obstruction of small airways) and emphysema (parenchymal destruction), which lead to progressive narrowing of the airways and shortness of breath. The inflammation is resistant to corticosteroids and there are currently neither safe nor effective treatments available (1).

Acute respiratory distress syndrome (ARDS) is characterized by an increase in the permeability of the alveolar-capillary barrier leading to impaired gas exchange. This disease has a high mortality rate of 40% (2). In both COPD and in ARDS, neutrophils play a key role (1, 2). A better understanding of the underlying pathophysiology of these diseases is needed to eventually develop new therapies.

Several models are described in this thesis which comprise *in vitro* experiments in human cells, in *vivo* preclinical models for COPD and ARDS, and translational analysis in clinical samples such as blood and primary cells from patients and healthy individuals. Since cigarette smoking is the major risk factor for developing COPD, most of the *in vivo* models were tested in mice after cigarette smoke exposure (1). In all the models, the role of the neutrophil chemoattractant proline-glycine-proline (PGP) or the more potent form acetylated PGP (acPGP) was investigated (3). PGP peptides are derived from collagen after a sequential cleavage by matrix metalloproteinases and prolyl endopeptidase (PE) (3, 4). Furthermore, the role of PGP peptides in ARDS and cardiovascular diseases was investigated.

Aims

The main aim of this thesis is to get a better understanding on the role of PGP peptides during pathological conditions by using models for lung conditions such as COPD and ARDS. The following specific aims were explored:

1. What is the effect of a PE inhibitor on cigarette smoke-induced airway inflammation (chapter 3)

2. Is therapeutic neutralization of PGP effective in an emphysema model in mice (chapter 4)

3. Does acPGP play a role in the development of ARDS (chapter 5)

4. Does PGP play a role in the pathophysiological changes in heart and pulmonary artery in a murine model for subchronic cigarette smoke exposure (chapter 6).

In the following, the content of each chapter is described briefly:

Chapter 2, the general introduction, describes what is known until now about the PGP pathway. In particular, the biologic and clinical data related to the role of PGP in COPD are reviewed. The focus in this chapter is on the possible role of PGP as a clinical biomarker and potential therapeutic target in disease

In **chapter 3** valproic acid (VPA) is tested *in vitro* and *in vivo* on PGP generation in an acute model for cigarette smoke-induced pulmonary inflammation. VPA is an inhibitor of the enzyme prolyl endopeptidase (PE), which is responsible for the last cleavage step in the generation of PGP out of collagen (5, 6). Finally, the molecular interaction between VPA and PE is described.

In **chapter 4** a different approach was chosen to inhibit the PGP pathway. Instead of inhibiting PE as described in chapter three, a direct inhibitor for PGP was tested. The PGP neutralizing peptide L-arginine-threonine-arginine (RTR) was used in sub chronic and chronic models of cigarette

smoke-induced inflammation (7). Effects of PGP inhibition on the pathophysiology in lungs and heart were measured. In addition, effects of acPGP on activation of primary epithelia from COPD and healthy subjects were tested.

Chapter 5 describes the role of PGP in a different lung condition: ARDS. In this chapter the focus is not on epithelial cells like in the previous chapter, but rather on endothelial cells. Several *in vitro* and *in vivo* models are described together with the use of clinical samples. We describe a novel role for acPGP in regulating paracellular permeability during inflammatory disease and demonstrate the potential to target this ligand in various disorders characterized by excessive matrix turnover and vascular leak such as ARDS.

Chapter 6 focuses on the effect of PGP on the heart and pulmonary artery in a murine model for subchronic cigarette smoke exposure. In chapter four a protective effect was seen after targeting PGP on the heart. In this chapter a specific PE inhibitor was used as treatment. Techniques as echocardiography were used to better understand the effects of PGP depletion during inflammation after cigarette smoke exposure in mice.

Finally, **chapter 7** provides a summarizing discussion of the findings described in this thesis. Possible future research is described which is needed to better understand the PGP-induced pathology and may eventually lead to drug development.

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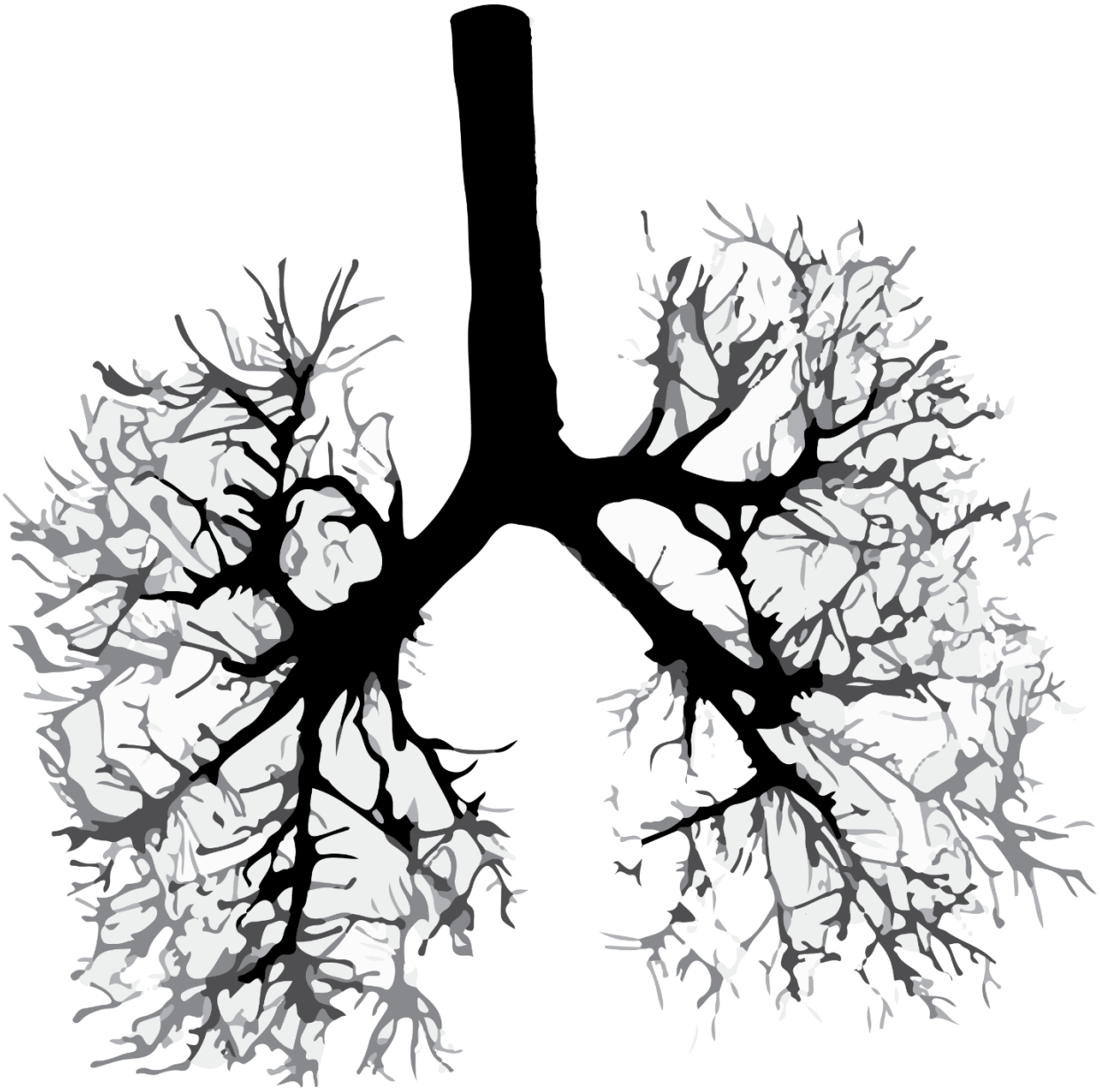
Chapter two



General introduction

This chapter is based on the article:

The matrikine PGP as a potential biomarker in COPD

Mojtaba Abdul Roda[1], Amanda M Fernstrand[1], Frank A Redegeld[1], J. Edwin Blalock[2], Amit Gaggar[2], Gert Folkerts[1].

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Abstract

The lack of a well-characterized biomarker for the diagnosis of chronic obstructive pulmonary disease (COPD) has increased interest towards finding one, as this would provide potential insight into disease pathogenesis and progression. Since persistent neutrophilia is an important hallmark in COPD Pro-Gly-Pro (PGP), an extracellular matrix derived neutrophil chemoattractant, has been suggested to be a potential biomarker in COPD. The purpose of this review is to critically examine both biologic and clinical data related to the role of PGP in COPD, with particular focus on its role as a clinical biomarker and potential therapeutic target in disease. The data provided in this review will offer insight into the potential use of PGP as endpoint for future clinical studies in COPD lung disease. Following PGP levels during disease might serve as a guide for the progression of lung disorders.

Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive disorder that causes major problems worldwide (1). The prevalence of COPD is high and it is associated with increased morbidity and mortality (2). It is now globally ranked as the third leading cause of mortality with more than 300 million people suffering from the disease and nearly 3 million deaths each year (3, 4). COPD affects people of all social classes all over the world, with a prevalent increase in developing countries. The lifetime risk of developing COPD has been estimated to be as high as ~25% (2).

COPD is characterized by the progressive development of airflow limitation. It encompasses chronic bronchitis (chronic inflammation and obstruction of small airways) and emphysema (parenchymal destruction), which lead to progressive narrowing of the airways and shortness of breath (5). The inflammation is resistant to corticosteroids and there are currently neither safe nor effective treatments available (1). COPD is directly related to the prevalence of tobacco smoking but air pollution and other inhalational exposures are also risk factors for COPD development. (6). Additionally, genetic risk factors may contribute to COPD development or progression. α1-antitrypsin deficiency is the best-documented hereditary condition associated with COPD although there is not complete genetic penetrance for emphysema development in this population. Additionally, the COPDGene study has identified susceptibility loci in genome-wide association studies which may contribute to disease progression or phenotypes (7).

Small airways disease and parenchymal destruction are two striking pathological features of COPD. In patients, the small airways are characterized by mucus accumulation in the lumen, peribronchiolar bronchitis, development of lymphoid follicles, and infiltration of both innate and

adaptive immune cells (4). COPD is characterized by chronic airway inflammation. Inhalation of noxious particles may cause pulmonary inflammation and, in individuals who develop COPD, the normal response to inflammation in the lungs appears to be augmented. This chronic inflammatory response modulates ongoing tissue destruction in the lungs leading to emphysema. Additionally, chronic inflammation could lead to disruption of the normal repair- and defense mechanisms, resulting in small airway fibrosis that lead to air trapping and progressive airflow limitation (8).

COPD is diagnosed when the post-bronchodilator FEC_1/FVC ratio is less than 0.70, which confirms the presence of persistent airflow limitation (8). The use of spirometry is required for diagnosis and to assess the severity of the disease, however FEV_1 is also commonly used as a biomarker in clinical trials. It is also the only marker widely accepted by regulatory agencies for new drug approval. Although FEV_1 represents a robust measure of lung function, it does not account for the pathogenic processes behind the disease progression and the poor reversibility observed in COPD. Measuring the degree of reversibility of airflow limitation (e.g. post-bronchodilator FEV_1/FVC) is therefore no longer recommended (4, 8). Additionally, in COPD, the lack of modifiable biomarkers represents a major barrier to drug discovery. Finding a biomarker that would permit therapeutic and perhaps preventative measures to be introduced earlier in the disease progression is therefore of utmost importance (9). In this review, we discuss the matrikine Pro-Gly-Pro (PGP) as a potential biomarker in COPD.

Inflammation in COPD

Tobacco smokers with a normal lung function display increased pulmonary inflammation. It is likely that COPD represents an enhanced response of the respiratory mucosa to inhaled irritants (1). In mice, exposure to cigarette smoke causes irreversible lung damage. The inflammatory

alterations in the airways are only partially reversed after smoking cessation, a phenomenon observed also in humans (10). Since the inflammation in COPD patients persist after smoking cessation, it is also likely that the inflammation is maintained by autonomous mechanisms (1).

The severity of the inflammation in COPD increases with disease progression. Most inflammation occurs in the peripheral airways and lung parenchyma, with increased numbers of neutrophils, macrophages and lymphocytes present in the lungs. The molecular mechanism behind COPD is complex and not yet fully understood. In the case of environmental factors, chronic inhalation of irritants initially activates pattern recognition receptors such as Toll-like receptors (TLRs), leading to the activation of innate immune reactions. The innate immune response is characterized by the influx of neutrophils and macrophages to the lungs as well as the activation of airway epithelial cells and mucus secretion. As the disease progresses, the adaptive immune system becomes activated with upregulation and activation of B- and T-lymphocytes (1). In COPD, neutrophils are the most abundant leukocytes present in the bronchial walls and lumen. Neutrophils have an important role in the host's immunological defense towards pathogens. They are the hallmark of acute inflammation and form the first line of host defense (11). Upon microbial invasion, neutrophils are rapidly recruited to the site of invasion where the invaders are being destroyed through phagocytosis (12). However, neutrophils also release toxic products and cessation of recruitment and clearance of neutrophils is therefore equally important in order to maintain homeostasis. Without active clearance, neutrophils are able to cause considerable bystander or collateral damage to the host tissue because of their toxicity. Chronic neutrophilic inflammation has been implicated in lung diseases such as COPD and cystic fibrosis (CF) (13). The increased accumulation of neutrophils in the COPD lung has suggested to be caused by a combination of enhanced neutrophilic recruitment and failure of clearance (12). Recruitment of neutrophils to the

site of inflammation is controlled and directed by the release of chemoattractant signals that can be both endogenous and pathogen-driven (14). The major chemoattractants for neutrophils are the Glu-Leu-Arg motif-containing ELR^+ CXC chemokines. Classical endogenous chemoattractants include (C-X-C motif) ligand 8 (CXCL8), and GRO-α, GRO-β and GRO-γ (CXCL1, CXL2, and CXCL3 respectively) in humans, and KC (CXCL1) and MIP-2 (CXCL2) in mice. Neutrophil migration is facilitated once the ELR^+ CXC chemokines bind to specific G-protein-coupled receptors on the surface of neutrophils (primarily CXCR1/2 (15-17). The mediators may be derived from macrophages and epithelial cells but also neutrophils are major sources of CXCL8 (18). Upon activation, neutrophils are recruited from the circulation to the pulmonary circulation, from which they adhere to the endothelial cells in the alveolar wall before they enter the alveolar space. At the site, neutrophils secrete proteases such as neutrophil elastase, cathepsin G, proteinase-3 and matrix metalloproteinases (MMPs). These contribute to the alveolar destruction, the tissue breakdown in the lung parenchyma causing emphysema, and the mucus hypersecretion from mucosal glands (19). Over the years, much attention has been given to neutrophil elastase (NE) and proteinase 3 in the breakdown of lung parenchyma. However increasing evidence has evolved, supporting MMPs derived from neutrophils to have a critical role in COPD due to their ability to generate chemotactic peptides promoting the recruitment of neutrophils to the lungs (5).

MMPs, PE, PGP and LTA_4H in chemotaxis of neutrophils

MMP-8 and MMP-9 are two matrix metalloproteinases implicated in the pathogenesis of tissue destruction in COPD. Although intrinsic lung cells are able to produce MMPs on their own, neutrophils are thought to be the primary source in chronic neutrophilic lung diseases. They are zinc-dependent proteases responsible for the breakdown of extracellular matrix (ECM) (20). It has been recognized for decades that fragments of matrix protein exert chemotactic activities on

neutrophils and monocytes, and that they possibly also exert additional proinflammatory actions (9). In 1995, Pfister *et al.* first discovered PGP in a rabbit model investigating alkali-induced damage to the eye. In the study, it was demonstrated that ulceration of the cornea was characterized by neutrophilic inflammation and led to the generation of two tripeptides: PGP and its acetylated form acPGP. The collagen-derived matrikines were established to be neutrophil chemoattractants. AcPGP was determined to have the highest chemotactic potency (20, 21). MMP-8 and MMP-9 are both able to digest collagen, an important component of ECM. A final reaction of the digestion is driven by prolyl endopeptidase (PE), leading to the generation of PGP (15, 22).

PE is a serine protease that cleaves to the carboxyl side of proline residues in oligopeptides. It is a proline-specific endopeptidase found in mammals. PE differs significantly from classical serine proteases (e.g. trypsin) both in structure and selectivity. It is selective for small peptide substrates only and is not able to cleave substrates larger than 30-100 amino acid (9, 23). Classical serine proteases are rarely involved in the cleaving of peptide bonds containing proline residues since they do not fit into the catalytic site of the enzymes. As many biologically active peptides contain proline, enzymes that cleave peptides at the proline residue may have an important biological effect (24). PE has been associated with the pathogenesis of neurological and cardiovascular conditions. It has also been implicated in respiratory disorders with chronic inflammation (9). Weathington et al. identified a novel pathway involved in the neutrophil influx signaling to the lung in which PE plays a critical role (15, 22). The most prominent chemokine in the COPD pathophysiology is CXCL8 (19). However, antagonizing CXCL8 using an α-CXCL8 antibody does not completely inhibit the neutrophil chemotaxis in COPD patients. The involvement of other chemoattractants has therefore been suggested (25). In recent years, the collagen derived tripeptide Pro-Gly-Pro (PGP) has been proposed as a plausible chemoattractant in COPD lungs.

Weathington *et al.* elegantly demonstrated the molecular mechanism behind PGP's chemotactic effects. In their study, it was demonstrated that PGP shares structural homology with ELR^+ chemokines such as CXCL8 and that activates CXCR1/2 upon binding, causing neutrophilic chemotaxis, which consequently augments the inflammatory cascade in COPD (15).

Generation of PGP from tissue breakdown was investigated both *in vitro* and *in vivo* and its chemotactic ability for neutrophils was displayed using a mouse model. Instillation of acPGP in murine models led to the recruitment of neutrophils to the airways and chronic administration of the tripeptide led to the development of COPD-like pathology in the lung (4, 15). Van Houwelingen et al. confirmed that PGP induces neutrophil migration in a dose-dependent manner *in vivo.* They also demonstrated that PGP induces emphysema-like changes in form of alveolar enlargement and right ventricular hypertrophy. The complementary peptide L-arginine-threonine-arginine (RTR) that binds PGP was found to impede both migration and activation of neutrophils induced by PGP *in vivo*. RTR also completely inhibited PGP-induced emphysema in the lungs of the mice. *In vitro,* RTR impeded both PGP- and CXCL8-induced chemotaxis (26).

Furthermore, recently we have shown that the PE inhibitor valproic acid (VPA) can diminish cigarette smoke mediated inflammation in an acute murine smoking model (27).

In 2010 however, Kruijf et al. published data suggesting that acPGP does not directly bind to human CXCR1/2. In their work they showed that acPGP was not able to displace the CXCR1/2 radioligand [I^{125}]CXCL8 from its receptors on either human neutrophils or the cell line HEK293T when incubated simultaneously. Also, using a G protein-dependent phospholipase C activation assay and a G protein-independent β-arrestin2 recruitment assay, acPGP did not directly activate CXCR2 (28). As these findings were in conflict with previous publications, other groups started investigating the receptor of acPGP. Almost a year later, Kim et al showed that acPGP does bind

to CXCR2 after all using a fluorescein isothiocyanate (FITC)–labeled PGP. The FITC-labeled PGP bound to murine CXCR2 positive RBL-2H3 transfected cells but not to empty vector controls (29). To better understand the findings of Kruijf et al., in 2012 Jackson et al. published new data describing four chiral isomers of acPGP. They showed that the isomer N-α-L-Pro-Gly-L-Pro (LL-acPGP) is chemotactic for neutrophils, whereas the isomer N-α-D-Pro-Gly-D-Pro (DD-NAc-PGP) acts like an antagonist (30). As Kruijf et al. did not report what isomer they have used, it could be speculated that this might be in part the reason for the conflicting findings. Jackson et al. added that acPGP should have been preincubated with neutrophils before adding CXCL8 to measure receptor binding as CXCL8 has a much higher binding affinity to CXCR2 compared to acPGP (28, 30).

The generation of PGP occurs after initial insult to the epithelial layer leading to exposure of collagen and subsequent collagen cleavage initiated by MMPs and completed by PE. Increased levels of PGP and PE have been observed in COPD and cystic fibrosis (CF). The generation of the increased amounts of PGP in the sputum of these patients was PE-dependent (22). Since MMP-8, MMP-9 and PE are present in neutrophils, and PE activity is increased in COPD serum and sputum, it is likely that the presence of these enzymes are responsible for the generation of PGP and the self-perpetuating cycle of neutrophilic inflammation in COPD (9). In neutrophils obtained from COPD patients, the intracellular basal PE activity was measured to be 25-fold higher compared to healthy donors. Additionally, PE proteins were expressed not only in neutrophils and macrophages but also in epithelial cells (31).

In physiological conditions, accumulation of PGP in the lungs is prevented by enzymatic degradation via leukotriene A4 hydrolase (LTA_4H). LTA_4H is a proinflammatory enzyme that generates the inflammatory mediator leukotriene B4 through hydrolase activity in the cytosol.

However, $LTA_4$4 also possesses aminopeptidase activity; and PGP is its physiological substrate. Although LTA_4H is able to degrade PGP, its more potent form AcPGP appears to be resistant to the enzymatic degradation. The exact molecular mechanism behind the acetylation of the N-terminal of PGP is still unknown but cigarette smoke condensate has the potential to do so. Also, cigarette smoke shifted the activity of LTA_4H toward a proinflammatory phenotype since it appears to inhibit the peptidase activity but not the hydrolase activity (4, 13). In a translational study by Wells *et al.* alterations to the LTA_4H-PGP pathways in a murine model of chronic cigarette smoke exposure was successfully translated into clinical disease. The research group demonstrated a strong association between acPGP and current cigarette smoking across all levels of COPD disease severity. Cigarette smoke selectively inhibits LTA_4H aminopeptidase activity that consequently initiates PGP accumulation and chronic neutrophilic inflammation. Interestingly, inactivation of LTA_4H aminopeptidase persists after smoking cessation. Endogenous generation of the reactive aldehyde acrolein at least partly causes the persistent inactivation of the aminopeptidase activity of the enzyme (32). In figure 1, the pathophysiology involving PGP is illustrated.

In this thesis, we have tried to look at several phenotypes as seen in COPD. Cardio vascular (CV) diseases in COPD for example are very common (5). This is why heart diseases like right ventricular hypertrophy (RVH) due to cigarette smoke exposure models are described in chapter 4 and 6. Targeting PGP in these models gives more insight about the role of this matrikine in a multi system disease such as COPD.

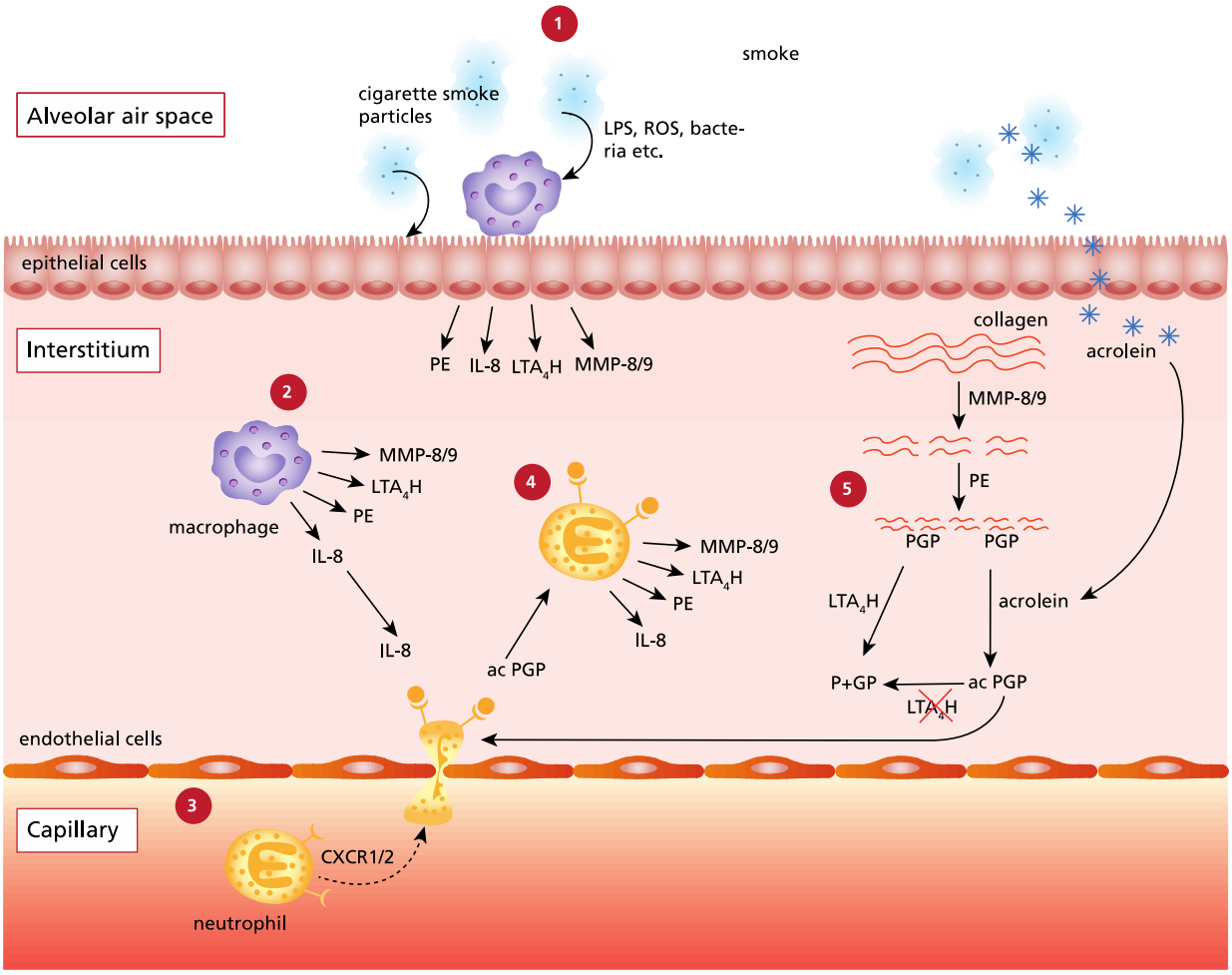

Figure 1. Illustration of the PGP pathology in neutrophilic inflammation. *(1) Cigarette smoke inhalation causes tissue resident cells, such as macrophages and epithelial cells to release several mediators, including PE, interleukin 8 (IL-8), LTA_4H and MMP8/9. (2+3) The neutrophilic chemokine IL-8 attracts neutrophils from the capillary via binding with CXCR1/2. (4+5) Neutrophils subsequently release MMPs and PE, which cleave collagen from the ECM to release the tripeptide and neutrophil chemoattractant PGP. LTA_4H can cleave and inactivate PGP. However, components of cigarette smoke, such as acrolein, inhibit LTA_4H and can acetylate the PGP to form the more potent acPGP. Moreover, acPGP cannot be cleaved by LTA_4H. PGP-generating enzymes can now be released by neutrophils after recruitment and activation by PGP and acPGP: a self-sustaining neutrophilic inflammation.*

During COPD exacerbations, patients suffer from pulmonary edema (5). The exact mechanism leading to vascular permeability is not clear. It could be speculated that the extra cellular matrix turnover during the inflammatory process can result in protein fragments that may or may not

modify vascular function. Therefore, in chapter 5 the effects of PGP on endothelial cells was further examined.

Clinical investigation of PGP

The role of PGP in the vicious cycle leading to self-sustaining neutrophilic inflammation observed in chronic lung diseases such as COPD and CF has received more attention during the past years (4). The study of Weathington *et al.* measured PGP peptides in patients with COPD, although the sample size was limited, consisting of five COPD patients and 18 control subjects. Nevertheless, detectable levels of PGP was found in 3 out of 5 individuals in the patient group with an average of 363 pg/ml, and in 2 out of 18 control samples with an average of 22 pg/ml. The difference between the positive samples was pronounced and significant ($p<0.01$). Interestingly, two COPD patients negative for PGP were also tested negative for emphysema (15).

To further assess PGP as a potential biomarker, Gaggar *et al.* examined acPGP and PGP levels in sputum from CF patients with moderately sever lung disease ($n=10$) compared to healthy controls ($n=10$). 8 out of 10 CF patients displayed acPGP levels above detection threshold compared to 1 out of 10 in the control group with average concentrations of 3.78 ng/ml and 0.13 ng/ml respectively ($p<0.01$). For PGP, mean values were established to 204.8 ng/ml in CF samples and 16.2 ng/ml in sputum from healthy volunteers ($p<0.05$). Additionally, a correlation between acPGP and PGP levels was recognized, demonstrating a strong relationship between the presences of the collagen peptides in clinical samples. The initial results led to inquiries regarding the involvement of specific proteases involved in PGP generation in CF patients. PE was found to have a 5-fold increase in activity in sputum of CF patients compared to control and increased PE activity was confirmed with PGP generation (22).

As described previously, MMPs have been assigned an emerging role in the destruction of ECM in the pathogenesis of chronic neutrophilic lung diseases. Together with human neutrophil elastase (HNE), MMP isoforms have an increased proteolytic activity in sputum of CF patients. MMP-9 was the predominantly active MMP isoform (33). Its presence correlated with the decline in lung function in CF patients (34). Since both HNE and MMP, together with PE, have an increased activity in the sputum of CF patients, Gaggar *et al.* hypothesized that CF sputum contained the required components to generate PGP from collagen. By incubating CF and control sputum samples with either type I and type II collagen, it was demonstrated that CF sputum indeed did generate significantly more PGP from both collagen I and II compared to control. These findings led to consideration of whether this tripeptide could serve as a biomarker in chronic neutrophilic disease. Sputum samples from a CF inpatient cohort were therefore collected in the beginning of exacerbation (within 48 h of admission) and subsequently after ~14 days of standard inpatient therapy at the end of the hospitalization. Over the course of treatment, PGP levels were significantly reduced. Also, a trend towards significance was observed in the correlation between PGP decline and improvements in FEV1 and FVC. Upon discharge of the patients, PGP levels were still 5-fold higher than those of healthy controls. The elevated PGP levels after recovery were proposed to indicate ongoing inflammation and matrix degradation in the lungs of CF patients (22).

O'Reilly *et al.* further evaluated acPGP and PGP as a biomarker for COPD. PGP concentrations were determined in sputum samples of COPD patients (n=16), severe asthmatics (n=10) and controls (non-smokers with no history of lung disease; n=10). Sputum acPGP levels above the detection limit were identified in 13 out of 16 COPD subjects but not in the control- or the asthma cohort. Positive PGP levels were detected in all COPD samples and in a minority of the controls

(3 out of 10). The PGP levels in sputum from COPD patients was reported to be significantly higher than those of asthmatic and healthy controls, further supporting the appreciation of acPGP and PGP as potential biomarkers distinguishing COPD from other health states. In *ex vivo* experimentation, COPD sputum was incubated with collagen type I that was pre-dialyzed to remove any necessary enzymes to thrive PGP generation *de novo.* Since much smaller amounts of acPGP were generated, it was proposed that acetylation could be the rate-limiting reaction for acPGP formation. In further *ex vivo* experimentation, the same group established that inhibition of MMP-1, MMP-9 and PE but not HNE reduces PGP generation, supporting the role of MMPs and PE in PGP generation and consequently COPD pathogenesis. The macrolide antibiotic azithromycin was also able to reduce the PGP levels. Additionally, PGP levels were found to be twice as high in serum samples of COPD patients compared to control, with acceptable correlation ($r=0.71$; $p=0.11$) between sputum and serum levels considering the sample size ($n=6$): suggesting its potential usefulness as a serum biomarker as well a lung-based biomarker (9).

In a multicenter trial of azithromycin in addition to normal medication in stable COPD outpatients in 2013, an ancillary study was conducted to investigate whether sputum levels of PGP were altered by the treatment or associated with the frequency of exacerbations (35). Macrolide antibiotics accumulate in their host cells (e.g. macrophages and neutrophils) and exert anti-inflammatory effects via inhibition of inflammatory cytokines (e.g. CXCL8), reduction of neutrophil activation and induction of phagocytosis of apoptotic neutrophils (36). In a blinded fashion, O'Reilly et al. performed several *ex vivo* experiments to examine PGP, myeloperoxidase and MMPs and their ability to generate PGP, as well as the correlation with azithromycin use once the parent trial was unblinded (35). It should be noted that all patients were non-smokers or ceased smoking at least 6 months before the study since smoking is a known hypersensitivity to

macrolides (35). Treatment with azithromycin successfully lowered the levels of PGP in the sputum of COPD patients compared to placebo, particularly when taken during a longer period and as a result, the neutrophilic burden was reduced, measured as decreased levels of myeloperoxidase (MPO). Also, the clinical response was improved with a reduced exacerbation frequency. However, most striking was the indication of elevated PGP levels exceeding acute COPD exacerbation for as long as 35 days before the onset of symptoms observed in a few specimens. Although no conclusion could be drawn on a functional relationship, the data suggest the idea of PGP as a possible important player in COPD pathogenesis – particularly in exacerbations (35). In the parent study, add-on treatment with azithromycin demonstrated a trend for reduced number of exacerbations as well as reduced number of neutrophilic airway inflammation markers (36).

In the study by Wells *et al*., never smokers (n=18), control smokers (no airflow obstruction; n=18), current smoking COPD patients (n=13) and former smoking COPD patients (n=10) were investigated for alterations in acPGP, LTA_4H, and aminopeptidase levels. LTA_4H levels were increased in sputum of control smokers compared to never smokers, however a decrease of approximately 65% of the aminopeptidase activity was observed for the control-smoking group. Since the leukotriene B4 (LTB_4) concentrations were higher for smoking subjects, it could be concluded that cigarette smoke selectively inhibits the aminopeptidase activity of the LTA_4H enzyme without affecting the epoxide hydrolase function. Also the neutrophil marker MPO was significantly elevated in control smokers compared to never smokers and PGP levels were 3-fold as high in the smoking population. Furthermore, in a pilot study, sputum LTA_4H was significantly increased in COPD patients compared to smokers and never smokers. The LTA_4H levels did no differ between current and former smoking subjects. Although enzyme concentrations were

elevated for the COPD subjects, aminopeptidase activity was further inhibited compared to smokers and never smokers ($p=0.02$ and $p=0.015$ respectively). There was no difference between current and former smokers in the COPD groups. Sputum PGP levels were significantly higher among COPD subjects compared to never smokers; however no statistical significant difference was found between COPD subjects and control smokers. Nevertheless, significantly elevated acPGP levels were established in the sputum of all COPD patients when compared to control smokers. The acPGP concentrations remained elevated also in former smoking COPD subjects when compared to smoking controls. Additionally, sputum LTB_4 and MPO levels were elevated in COPD subjects compared to never smokers and similar to those observed in smoking controls. A correlation was found between acPGP and MPO levels. No correlation was observed between PGP/acPGP and LTB_4 amounts. To further investigate the PGP/LTA_4H pathway, sputum samples from current and former smoking COPD subjects enrolled in another study (ECLIPSE) were investigated for acPGP, LTA_4H, and aminopeptidase activity. In the ECLIPSE cohort, acPGP levels were elevated in current smokers compared with former smokers. No difference was observed between current and former COPD smokers in LTA_4H levels or aminopeptidase activity. The results were similar to those obtained in the pilot study. In further investigations, log acPGP levels were found to strongly correlate with cigarette smoking. The acPGP levels increased with disease stage (GOLD levels) for current smokers compared to former smokers. LTA_4H remained the same between the groups despite GOLD stage. This suggests that the increase in acPGP is a result of increased acPGP production and not due to alterations in the PGP/acPGP breakdown process. Moreover, acPGP levels were similar between current and former smoking COPD subject with emphysema alone and both emphysema and chronic bronchitis but trended towards elevated levels in smoking COPD subjects with chronic bronchitis alone. It can be concluded that smoke-

mediated loss of LTA4H aminopeptidase activity and elevations in PGP/acPGP amounts appear to have a role in chronic neutrophilic inflammation and in COPD pathophysiology (32).

It should be noted that all clinical studies reported in which PGP is measured have a small sample size. To better understand the role of PGP in human disease and the potential role as biomarker for COPD, more clinical studies are needed in which a larger population is investigated. These studies should point out if PGP could be used as a more specific biomarker for certain patients and perhaps as a predictor for exacerbations as was suggested by O'Reilly et al (35).

Also, several other diseases have been reported to show elevated PGP levels, such as CF, bronchiolitis obliterans syndrome, and inflammatory bowel disease (22, 37, 38). We speculate that PGP might be significantly elevated in any disease with a neutrophilic inflammation and a high cellular matrix turnover (22). Although O'Reilly et al have directly compared the PGP levels in COPD patients to patients with severe asthma, it could be useful to include other lung diseases as well. For example, the role of PGP in lung fibrosis as seen in in some types of interstitial lung disease (ILD) has not been investigated before. It would be interesting to see if PGP plays any role in ILD's to further examine the specificity of PGP.

Currently, further studies are needed to investigate if PGP could be used as (prognostic) biomarker reflecting the progression of the disease rather than biomarker to diagnose a disease. This will require larger well-phenotyped COPD patient cohorts, examining PGP peptide levels and clinical parameters to accurately assess the sensitivity of this biomarkers with changes in disease status. Fortunately, such large cohorts are now available and present a unique opportunity to prospectively study PGP in various COPD phenotypes (i.e. chronic bronchitis vs emphysema, frequent vs non-frequent exacerbators, etc).

Conclusion

COPD remains a prevalent pulmonary disorder with high attributable morbidity and mortality. The determination of a robust biomarker which is easily measured, highly reproducible, trends with clinical progression, and may serve as a surrogate marker for appropriate therapeutic intervention is critically needed in COPD lung disease (39). To date, PGP peptides have fulfilled all features of this paradigm of a biomarker in a COPD population and warrant ongoing evaluation in this deadly disorder. However, further clinical investigation with larger COPD patient populations and studies in additional lung disorders are needed to further examine the utility of PGP as potential biomarker.

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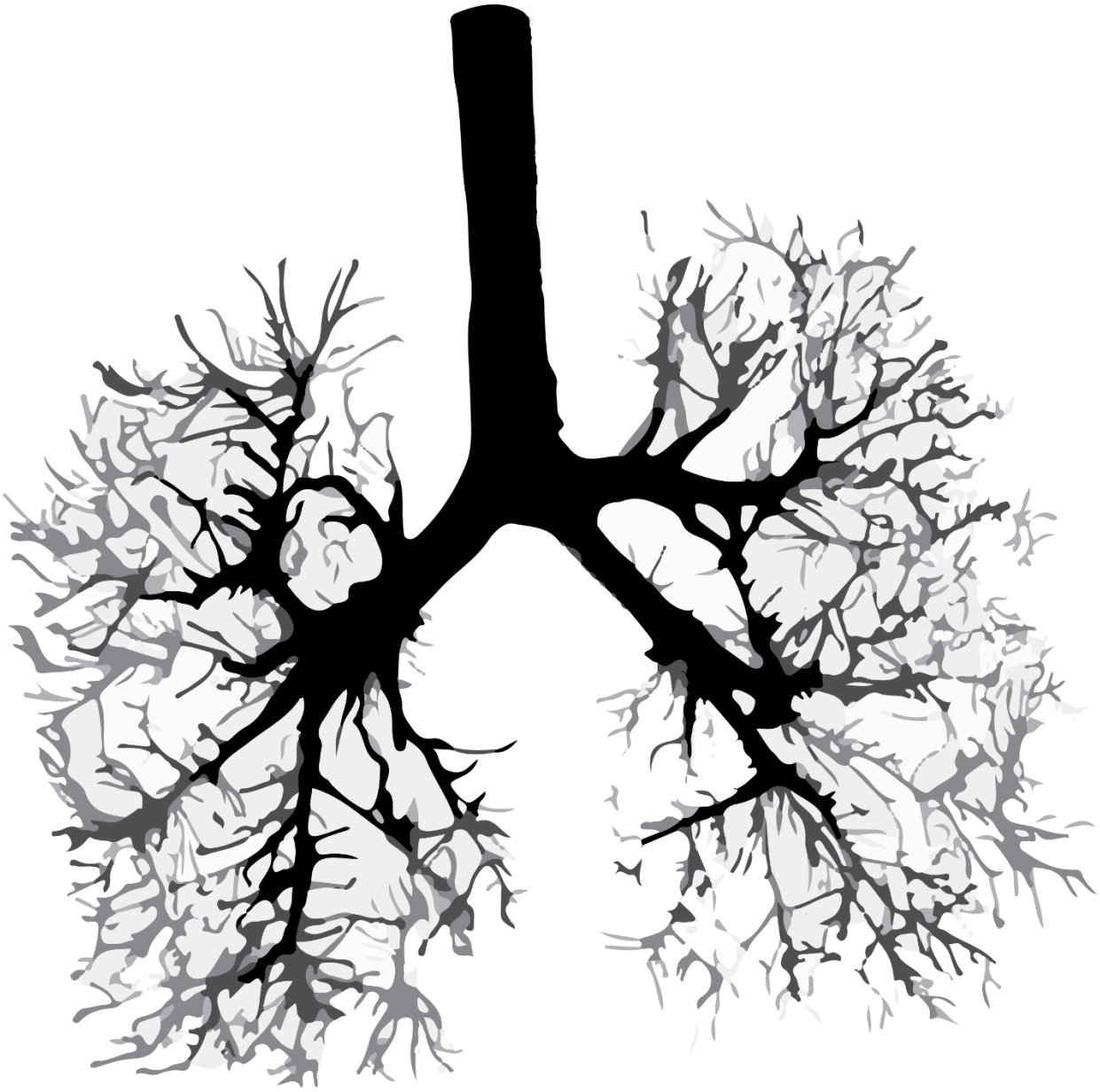
Chapter three



Targeting prolyl endopeptidase with valproic acid as a potential modulator of neutrophilic inflammation

This chapter is based on the article:

Targeting prolyl endopeptidase with valproic acid as a potential modulator of neutrophilic inflammation

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Abstract

A novel neutrophil chemoattractant derived from collagen, proline-glycine-proline (PGP), has been recently characterized in chronic obstructive pulmonary disease (COPD). This peptide is derived via the proteolytic activity of matrix metalloproteases (MMP's)-8/9 and PE, enzymes produced by neutrophils and present in COPD serum and sputum. Valproic acid (VPA) is an inhibitor of PE and could possibly have an effect on the severity of chronic inflammation. Here the interaction site of VPA to PE and the resulting effect on the secondary structure of PE is investigated. Also, the potential inhibition of PGP-generation by VPA was examined *in vitro* and *in vivo* to improve our understanding of the biological role of VPA.

UV- visible, fluorescence spectroscopy, CD and NMR were used to determine kinetic information and structural interactions between VPA and PE. *In vitro,* PGP generation was significantly inhibited by VPA. *In vivo,* VPA significantly reduced cigarette-smoke induced neutrophil influx.

Investigating the molecular interaction between VPA and PE showed that VPA modified the secondary structure of PE, making substrate binding at the catalytic side of PE impossible. Revealing the molecular interaction VPA to PE may lead to a better understanding of the involvement of PE and PGP in inflammatory conditions. In addition, the model of VPA interaction with PE suggests that PE inhibitors have a great potential to serve as therapeutics in inflammatory disorders.

Introduction

COPD is defined as a disease state characterized by airflow limitation that is not fully reversible. The chronic airflow limitation characteristic of COPD is caused by a mixture of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from person to person. The prevalence, morbidity, mortality and treatment costs of COPD are high and increasing. COPD is the third leading cause of death in the United States and the fourth worldwide (1, 2).

In COPD, multiple classes of proteases are released from neutrophils in the airway compartment, including endopeptidases, serine proteases, and matrix metalloproteinases (MMPs) (3). Recently, Weathington et al have characterized a novel neutrophil chemoattractant, proline-glycine-proline (PGP) which is derived from collagen (3, 4). An acetylated form of this peptide (acPGP) is also detected and demonstrates increased chemotactic properties compared to non-acetylated PGP. PGP acts as a neutrophil chemoattractant *in vitro* and induces neutrophilic inflammation when instilled into the airways of mice *in vivo*. PGP is known to act on CXC receptors 1 and 2 (CXCR1, CXCR2) on neutrophils due to a structural homology with ELR+ chemokines, such as interleukin-8 (IL-8). Chronic acPGP administration into murine airways for 12 weeks at biweekly intervals leads to the development of neutrophilic airway inflammation, alveolar enlargement, and right ventricular hypertrophy, all of which are features of COPD. The degree of alveolar enlargement is similar to that seen with mice exposed to cigarette smoke 6 times per week for 24 weeks (3, 4). Gaggar et al. have gone on to demonstrate a prominent role for this peptide in additional inflammatory neutrophilic lung conditions, such as cystic fibrosis (CF) and chronic allograft rejection after lung transplantation (3, 5).

Generation of PGP occurs via initial cleavage of collagen by matrix metalloproteases (MMP-8, MMP-9) and subsequently by prolyl endopeptidase (PE) (3). This occurs when there is some initial insult to the epithelial layer, which leads to an exposure of collagen. It has been shown that all three enzymes, MMP-8, 9 and PE, are found in neutrophils and are present in COPD serum and sputum (6, 7). PE is a protease that belongs to the serine protease family. This enzyme cleaves the carboxyl side of proline residues in oligopeptides (8).

Recently, PE has been described as part of the signaling pathways involved in phosphoinositides leading to neuronal cone growth in the brain (9). This work was done in an attempt to determine the pathophysiological mechanism of the mood stabilizer drug valproic acid (VPA). VPA is used clinically as a mood stabilizer in mania, bipolar disorder, epilepsy, attention-deficit hyperactivity disorder (ADHD), chorea, and for migraine headaches. The recommended therapeutic plasma level is 312-693 μM (10). In healthy volunteers, VPA has been shown to be highly protein bound (85–95%) after a single intravenous bolus dose (11).

Cheng et al. showed that VPA can directly inhibit recombinant human PE (rhPE) as well (12). It is surprising that VPA is a specific inhibitor of PE, as it does not resemble the normal peptide substrates of PE to act as a transition state analogue, nor does it fit a classical serine protease inhibitor family (13).

Many compounds are known to have an inhibitory effect on PE, such as ZPP (N-carbobenzoxy-proline-prolinal), S-17092 (2S,3aS,7aS)-1((R.R)-2-phenylcyclopropyl)carbonyl)-2-((thiazolidin-3-yl)car-bonyl)octahydro-1H-indole) and JTP-4819 ((S)-2-(((S)-2-(hydroxyacetyl)-1-pyrrolidinyl)carbonyl)-N- phenylmethyl)-1-pyrrolidinecarboxamide) (14-16). Yet, none of these compounds are registered as active drug compounds. VPA is the only drug compound we are aware of, that inhibits PE and is also approved to be given to patients (12, 17).

Thus, there is an increased need for knowledge regarding the structure of VPA bound to PE and the exact site or sites of binding on PE. To this end, we have undertaken studies to elicit data to further this knowledge through the use of circular dichroism (CD) and nuclear magnetic resonance (NMR). We have also shown a direct inhibitory effect of VPA on a system generating the matrikine PGP likely through inhibition of PE.

Methods

PE activity assays

The PE activity assay was performed with specific PE substrates (Bachem, Switzerland): N-succinyl-glycine-proline-para-nitroaniline (Suc-Gly-Pro-pNA) and N-succinyl-glycine-proline-7-amido-4-methyl-coumarin (Suc-Gly-Pro-AMC). Lithium (Sigma-Aldrich, USA) and VPA (Sigma-Aldrich, USA) were used as a competitor.

Recombinant human PE was expressed in E. coli using the plasmid pTrcHis PE, kindly provided by dr. A.W. Mudge, in Promega BLR1(DE3)pLys 3 E. coli competent cells as previously reported (12).

Activity assays were carried out in 100 mM phosphate buffer (pH 7.5). 1mM DL-Dithiothreitol (DTT) (Sigma-Aldrich, USA) and 10µM bovine serum albumin (BSA) (Sigma-Aldrich, USA) were added and left over night at 4 °C. The reactions were performed in a final volume of 100 µl with a final rhPE concentration of 10 nM.

PE-specific fluorogenic substrate Suc-Gly-Pro-AMC (0.2 mM) was used to do a lithium/VPA-dose response (0-10 mM), measured with a spectrofluorometer using excitation and emission wavelengths of 380 nm and 460 nm respectively, at 37 °C over 60 min.

PE-specific colorgenic substrate Suc-Gly-Pro-pNA (0-10 mM) was used to do a substrate dose response with three VPA concentrations: 0.8, 1.6 and 3.5 mM. PE-activity was measured with an Ultra violet- visible (UV/Vis) spectrometer at a wavelength of 405 at 37 °C during 60 min. Suc-Gly-Pro-pNA and Suc-Gly-Pro-AMC are both water soluble and were dissolved in phosphate buffer. In all cases, VPA was pre-incubated with the enzyme at 37 °C for 90 min.

Collagen digestion with PMN lysate

Polymorphonuclear neutrophils (PMNs) were isolated from a buffy coat of normal human blood donors (Research Blood Components). The buffy coat (30 ml) was diluted with PBS to 250 ml. PMNs were separated as described before (5). Briefly, the diluted buffy coat was separated by a Histopaque gradient (Sigma-Aldrich, St. Louis, MO). This step was repeated once more to remove the final inclusion of erythrocytes. PMNs were counted on a Hemacytometer slide after staining with Trypan blue (Sigma-Aldrich, USA).

Neutrophil lysate was obtained by two freeze–thaw cycles with 10 µg/ml aprotinin (Sigma-Aldrich) and 10 µM BSA, followed by centrifugation at 2000 x g at 4 °C for 10 min. Lysates were left on ice for one hour, and incubated hereafter with bestatin (Cayman Chemical, Ann Arbor,MI) and one of the following: PBS, 5, 10, 50 or 100 mM VPA at 37 °C for 30min. The lysate/VPA mixtures were then mixed with predialyzed Collagen Type I and II (Sigma-Aldrich). Final concentrations for both types were 0.11 mg/ml. From each sample 75µl was taken to measure PE activity. The samples were then left on a shaker for 20 hours at 37 °C. Every 5 hours, bestatin was re-added, to reach a final concentration of 0.9 mg/ml. Negative controls contained 100 mM VPA added to collagen alone and were treated the same as any the other sample (n=10 per group).

Animal work

Female A/J mice, 7-9 weeks (Jackson Labs) old were housed under controlled conditions in standard laboratory cages. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the UAB Institutional Animal Care and Use Committee

Cigarette smoke exposure

Mice were exposed in whole-body chambers (5L) to air (sham) or to 40 times diluted mainstream cigarette smoke during 5 consecutive days using a SIREQ (Tempe, AZ, USA) smoking device. Reference cigarettes 2R4F (University of Kentucky, Lexington, Kentucky) were puffed at 3L/min. Just before the exposure, filters were cut off from the cigarettes. Each cigarette was smoked in 10 minutes rounds (1 puff/min). Per round 2 cigarettes were smoked. Mice were exposed twice daily to cigarette smoke using 4 cigarettes per exposure on days 1-2, and 6 cigarettes on days 3-5. The mice administered either vehicle (70 µl PBS) or VPA (100 µg in 70µl sterile PBS) by oropharyngeal aspiration under light isoflurane anesthesia twice daily before each smoke exposure. The mice were sacrificed 16 hours after the last air or smoke exposure as described before (18).

Bronchoalveolar lavage

Immediately after i.p. injection with an overdose of ketamine/xylazine mix, the lungs of the mice were lavaged 4 times through a tracheal cannula with 1 ml PBS, pre-warmed at 37 °C. After centrifuging the bronchoalveolar lavage (BAL) fluid at 4°C (400 g, 5 min), the cell pellets of the

4 lavages were used for cell counts. The 4 cell pellets, kept on ice, were pooled per animal and resuspended in 150 µl cold saline. The supernatant of the first 1 ml lavage was used to measure PGP after mixing with 1mM bestatin. After staining with Türk solution, total cell counts per lung were made under light microscopy using a Burker-Turk chamber. Differential cell counts were performed on cytospin preparations stained by DiffQuick (Dade A.G., Düdingen, Switzerland). Cells were identified as macrophages, neutrophils and lymphocytes according to standard morphology. At least 200 cells were counted and the absolute number of each cell type was calculated (18).

CD spectroscopy

CD spectra were obtained of VPA (6, 12 and 24 mM) or ZPP (250, 500 and 1000 nM) titrated into 2.5 µM rhPE in PBS. Also, 24 mM VPA was added to PE:ZPP (2.5 µM : 1 µM) and 1 µM ZPP (Enzo) was added to PE:VPA (2.5 µM : 24 mM). PE was solubilized in PBS, VPA in H_2O and ZPP in 0.006% (v/v) dimethylsulfoxide (DMSO) (in H_2O). Samples were injected in a 0.5mm cell and measured with a Jasco J-815 CD-Spectrometer at wavelengths ranging from 260–194 nm (data pitch 0.5 nm). All samples were analyzed on the same day with the same sample stocks, in order to obtain reliable results.

NMR spectroscopy

^1H 1-D NMR was carried out on a Bruker Avance 700 MHz NMR spectrometer using a 5 mm cryoprobe operated at 20 °C. Samples of enzyme (PE) were run in a Shigemi microcell. The concentration of PE was approximately 10 µM in 250 µl PBS (90 % H_2O; 10 % D_2O). The residual water signal was suppressed using presaturation. ^1H NMR spectra were obtained for 10 mM VPA

(in PBS (95 % D_2O)) or 10 µM ZPP (<0.001 % (v/v) DMSO (PBS (95 % D_2O)) in the presence or absence of the enzyme. Also, 10 mM VPA was added to PE:ZPP (10 µM : 10 µM) and 10 µM ZPP was added to PE:VPA (10 µM : 10 mM).

PGP measurement. Neutrophil lysate with collagen samples were 10 kDa filtered and analyzed by electrospray ionization-liquid chromatography-mass spec/mass spec (ESI-LC-MS/MS) for PGP, as described before (5). BAL fluid was analyzed utilizing the same method without a filtration step.

During all experiments the pH of all solutions was measured before and after adding the PE inhibitors to PE in PBS. The pH of all solutions did not drop under 7.0 after adding the inhibitors.

Results

PE activity

VPA inhibited PE in a dose dependent way, with a K_i of approximately 1-2 mM, as seen in figure 1A and B. Lithium, a drug used in bipolar disease as well, showed no effect on the activity of PE at the same dose range as VPA (0-10 mM), similar to the findings of Cheng et al (12). Increasing VPA concentrations was shown to decrease the V_{max} of the enzyme, in a non-competitive way. 1.6 mM VPA lowered the V_{max} of the enzyme to 0.21 mOD * min^{-1} * μl^{-1} compared to a V_{max} of 0.40 mOD * min^{-1} * μl^{-1} in the control group. However, fitting the data in a Lineweaver-Burk plot, a modified form of the Lineweaver-Burk form was obtained repeatedly, as shown in figure 1C. This confirms that VPA inhibits PE in a mixed non-competitive way, as published before (12).

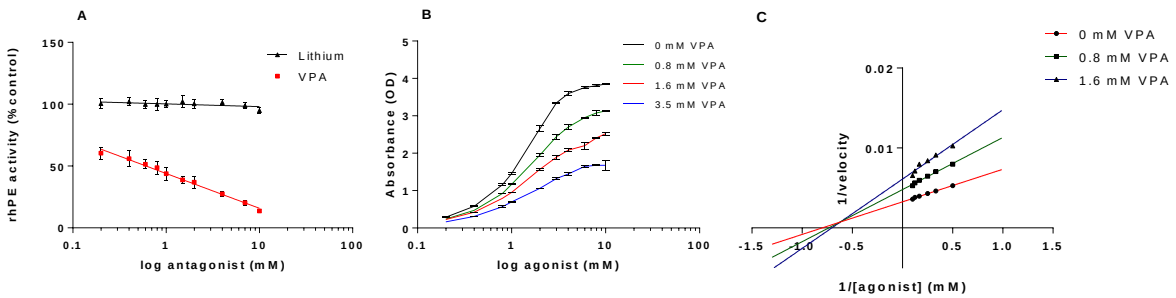

Figure 1. Effect of VPA on the activity of rhPE. (A) *Activity of 10nM purified rhPE was measured in presence of ten doses of VPA or lithium ranging from 0.2 – 10 mM. Relative activity of rhPE in the presence of VPA or lithium is shown as a percentage of activity in the absence of VPA and lithium. VPA showed a K_i of approximately 1mM lithium showed no effect on PE activity.* ***(B)*** *Inhibition curves of three VPA concentrations (0.8, 1.6 and 3.5 mM) were obtained by incubating VPA with 10nM rhPE during 90 min at 37 ^{0}0.* ***(C)*** *A Lineweaver-Burk plot was made based on the rhPE activity assays with 0.8 and 1.6mM VPA as inhibitor. Enzyme activity was measured with increasing substrate (Suc-Gly-Pro-pNA) concentrations, ranging from 0.2 – 10 mM. The velocity was calculated as mM * min^{-1} * ml^{-1}. Data are shown as the mean ± S.E.M. (n=3 per group).*

PGP generation

Using the lysates of PMNs, collagen was digested to generate PGP in the presence or absence of VPA, as seen in figure 2A. In the absence of a PE inhibitor, PGP levels of up to 2.1 ng/ml were obtained with negative controls providing a baseline measurement. A total VPA concentration of 50 and 100 mM inhibited the PGP generation from collagen Type I and II significantly ($p<0.0001$). 5 and 10 mM total VPA showed inhibition though not reaching significance, $p=0.14$ and $p=0.052$ respectively. The PE activity showed a significant decline with 10, 50 and 100mM VPA (figure 2B).

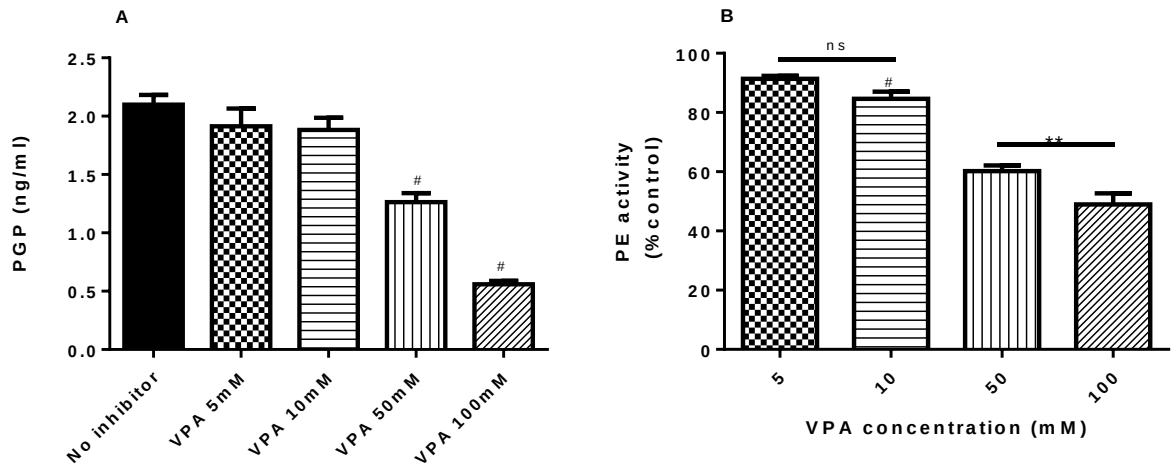

Figure 2. Inhibition of PGP generation by VPA. (A) *Dialyzed Collagen Type I and II were incubated with the lysate of 4.6 * 10^6 PMN at 37°C for 20 hours to generate PGP.* ***(B)*** *PE activity was measured 30 minutes after incubation of lysate/collagen/VPA mixture and compared to control (no VPA). Data are shown as the mean ± S.E.M. (n=5-10 per group). Representative of 4 experiments. # p<0.0001 compared to control, $ p<0.01 compared to control, ** p<0.01, ns p>0.05.*

Cigarette smoke exposure

A significant increase in BAL fluid neutrophils and macrophages was observed after 5 days cigarette smoke exposure compared to the air-exposed mice (figure 3A-C). Unlike the macrophages, cigarette smoke-induced neutrophil influx in the BAL fluid was significantly decreased after VPA administration. Accordingly, the acPGP levels and the PE activity were the highest in the BAL fluid of the PBS treated smoked mice. The acPGP levels and the PE activity of the other groups was not significantly different from each other (Figure 3D-E).

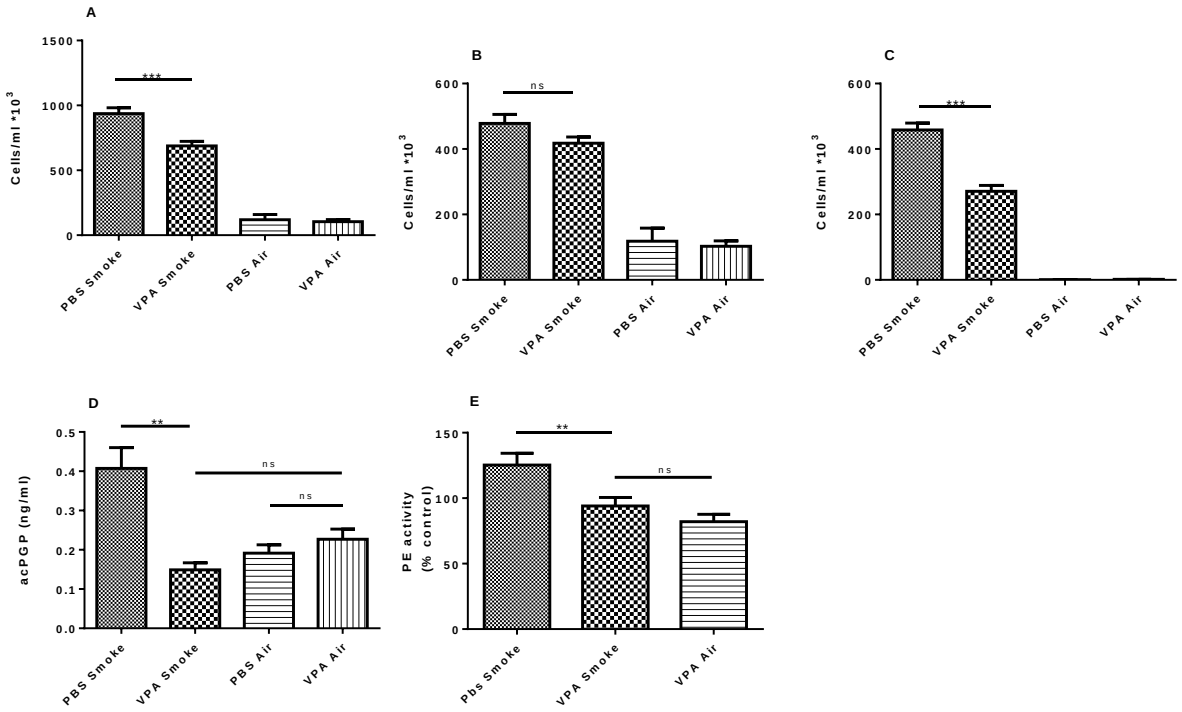

Figure 3. VPA decreases cigarette smoke-induced neutrophil influx in BAL fluid of mice. ***(A)****Total cell numbers,* ***(B)*** *macrophages and* ***(C)*** *neutrophils in the BAL fluid of mice exposed to air or whole body cigarette smoke twice daily during 5 days. The mice received vehicle (PBS) or VPA (100µg/70µl PBS) by oropharyngeal aspiration 15 minutes prior to air/smoke exposure.* ***(D)*** *AcPGP was measure in the BAL fluid.* ***(E)*** *PE activity was measured in the BAL fluid and compared to control (PBS treated/air exposed mice). N = 5-10 animals per group. Values are expressed as mean +/- S.E.M. **P ≤ 0.01, ***P ≤ 0.001, ns p> 0.05.*

CD spectroscopy. Titrating VPA into PE resulted in a loss in signal compared with the same dilution with PBS added to 2.5 µM PE (figure 4A). Adding up to 1 µM ZPP (1000 times K_i) on top of the PE:VPA mixture, did not give any additional change in the secondary structure of the enzyme (figure 4B). When adding 1 µM ZPP to PE, no change was observed to the PE secondary structure compared to the same dilution with PBS. Adding up to 24 mM VPA on top of the PE:ZPP mixture did not show any change in signal. These results suggest that VPA changes the secondary structure of rhPE by binding at or near the binding pocket of rhPE.

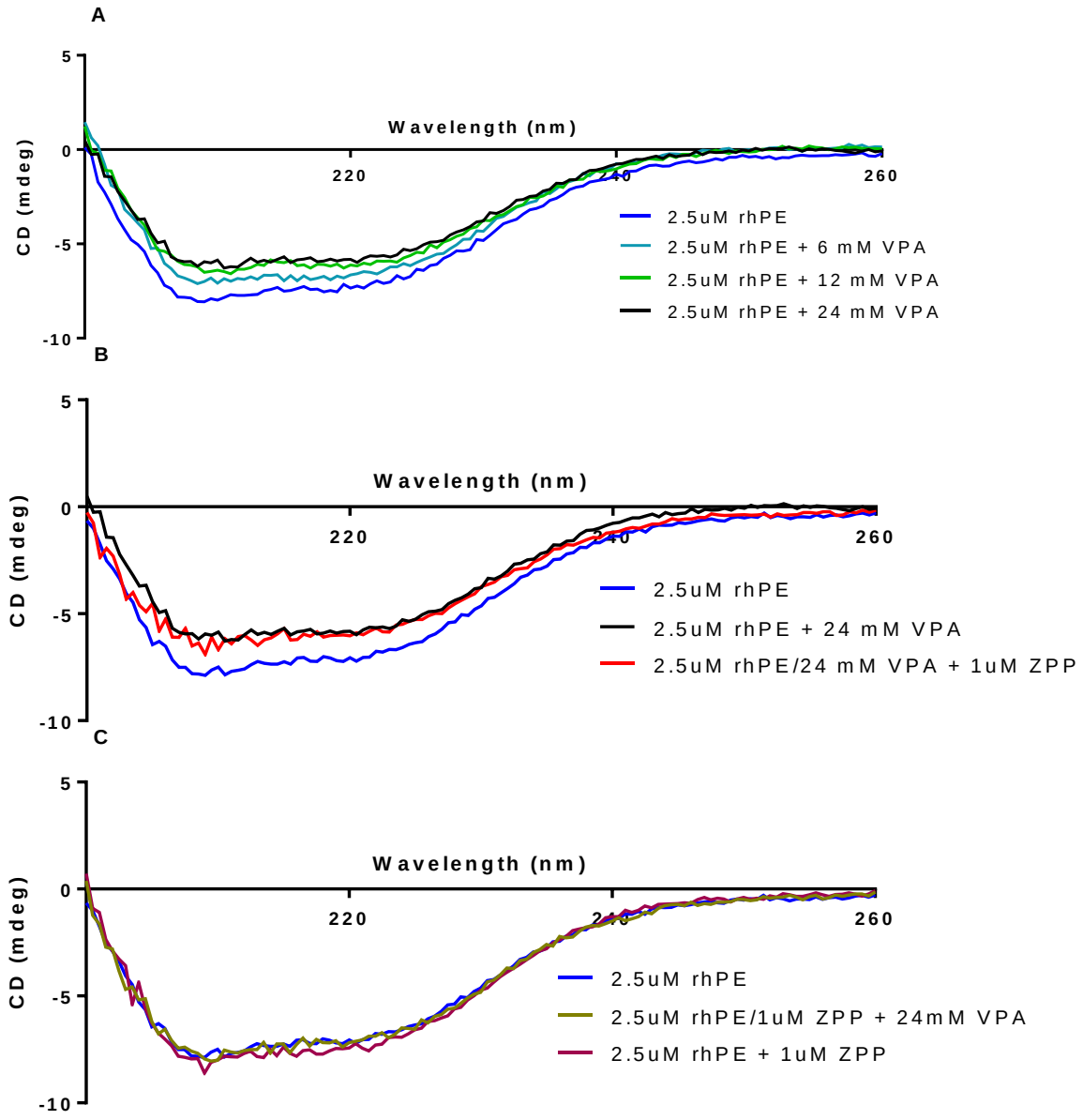

Figure 4. Effect of VPA on the secondary structure of rhPE. (A)*Three VPA concentrations (6, 12 and 24 mM) were titrated into 2.5µM rhPE. VPA caused a significant change in the secondary structure of the enzyme.* ***(B)*** *A high concentration of ZPP (1µM) was added to the 2.5µM rhPE / 24mM VPA mixture. ZPP did not cause any change in the secondary structure of the enzyme.* ***(C)*** *Adding 1µM ZPP to 2.5µM rhPE does not cause any secondary protein structure change. Adding 24mM VPA on top of that mixture doesn't cause any structure change.*

NMR spectroscopy

NMR showed that when ZPP bound to PE (figure 5B), the carbobenzoxy peaks, 7.20 and 7.28 ppm seen with free-ZPP (figure 5C), decreased in intensity with a concurrent appearance of new peaks at 7.24 and 7.35 ppm respectively. Adding 10 mM VPA on top of PE:ZPP (10 µM:10 µM) did not change the carbobenzoxy peaks (figure 5A). However, there was no appearance of these new peaks at 7.24 and 7.35 ppm when 10 mM VPA was added to the enzyme first with subsequent addition of ZPP (figure 5D).

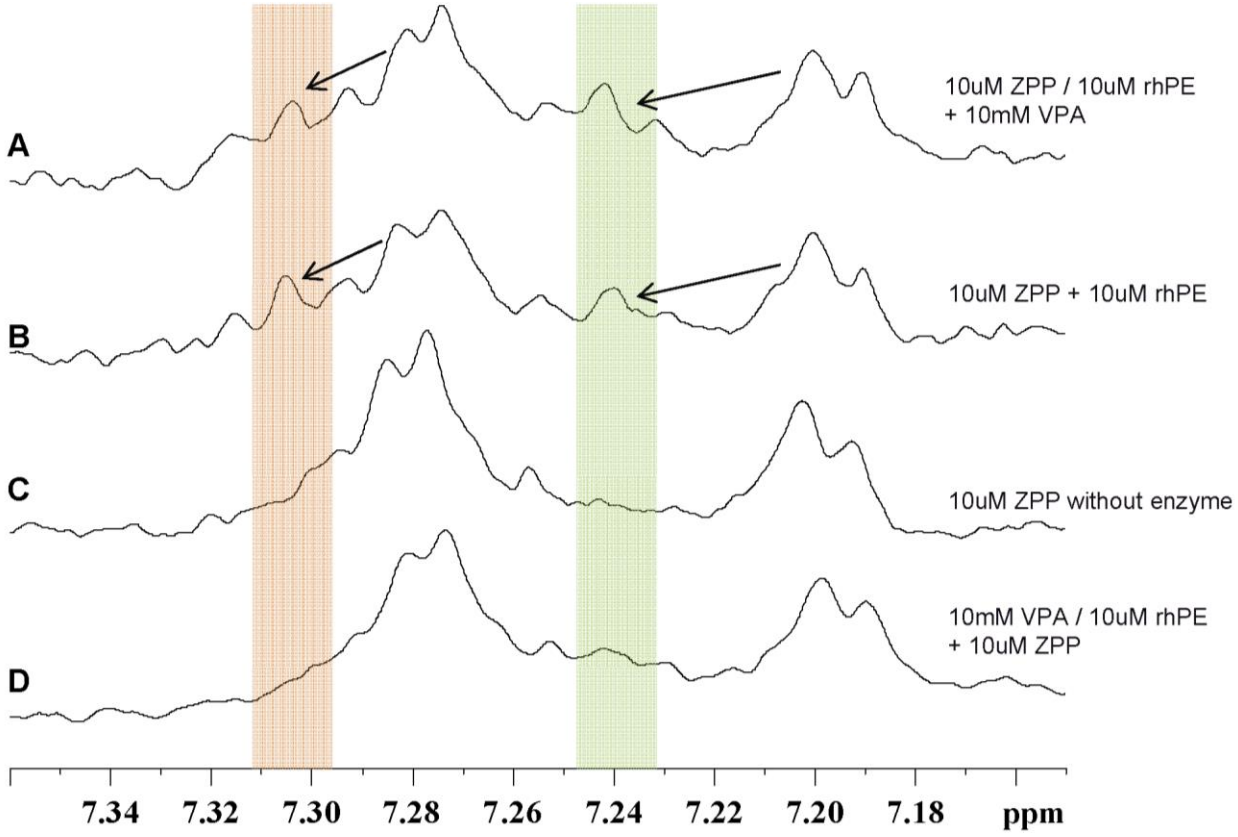

Figure 5. NMR spectra of the carbobenzoxy-group of ZPP (A) *10µM ZPP was added to 10µM rhPE, 10mM VPA was added to that mixture. A shift of the free ZPP peaks at 7.28 and 7.20 ppm to the left is seen (indicated in the red and green bars respectively).* ***(B)*** *10µM ZPP was added to 10µM rhPE. A shift of the free ZPP peaks at 7.28 and 7.20 ppm to the left is seen.* ***(C)*** *10µM ZPP was measured without enzyme. The peaks at 7.28 and 7.20 ppm are the free ZPP fractions.* ***(D)*** *10mM VPA was added to 10µM rhPE, 10µM ZPP was added to that mixture. Note that the free ZPP peaks at 7.28 and 7.20 ppm do not show a shift to the left; there are no peaks in the highlighted areas.*

Discussion

In this report, we identified the manner of binding of VPA to PE and demonstrate an effect on the secondary structure of PE by VPA. Also, we demonstrate a novel aspect of VPA as a potential modulator of inflammation through its actions as an inhibitor of PE and PGP generation. The latter indicates that VPA has an anti-inflammatory effect and may serve as a lead compound to design PE inhibitors. One concern is the high concentrations VPA needed to inhibit PE activity in this study might lead to toxic side effects. Though the mechanism of VPA teratogenicity is currently unknown, this toxicity maybe altered through combination with compounds such as resveratrol, vitamin E and N-acetylcysteine (NAC) (19, 20). The latter is a particularly appealing combination as NAC at high concentrations has also been shown effective in treatment of COPD (21).

VPA is known to be a highly protein-bound drug (85-95%) in serum (11). Since cell lysate contains a high quantity of proteins, other than PE, it is therefore uncertain how much unbound VPA is available to inhibit the enzyme. Maes M et al. investigated PE activity before and after a sub-chronic VPA treatment of 14 days (range 12-23 days) in manic patients who did not take any drugs seven days prior to treatment. A mean dose of 1400 mg (± 592) VPA/day reduced the PE-activity significantly ($p=0.02$) (17). This demonstrates that despite the high protein binding of VPA sufficient drug can be available to effectively inhibit enzyme activity.

Recently, VPA has shown a remarkable effect on MMPs by their action on histone deacetylase. 2mM of VPA decreases MMP-2 and MMP-9 expression levels and thus its activity significantly. Tissue-specific inhibitor 1, the natural inhibitor of MMPs, showed enhanced expression levels ($p<0.05$) when G361, SKNMC or U87MG cells were treated with 1 mM VPA during 24 h (22). At this time there is no evidence to suggest that VPA has a direct inhibitory action on MMPs thus

the inhibitory effects of VPA in neutrophil lysates is likely due to its direct action on PE as was demonstrated in figure 2B.

We had our concerns that VPA being an acid, might influence PE by lowering the pH. That is why the pH of all solutions was measured before and after adding VPA. Little to no effect on the pH was observed caused by VPA: the pH never dropped under 7.0.

In vivo a clear inhibitory effect of VPA administration on cigarette smoke-induced neutrophil influx into the BAL fluid is shown in this study. Those results were backed up by decreased acPGP levels and reduced PE activity in the BAL fluid of VPA treated mice which were exposed to cigarette smoke. These results are in agreement with the importance of PE in the PGP generation. As shown before, PGP induces neutrophil chemotaxis, and decreasing PGP levels results in lower neutrophil infiltration and less alveolar enlargement (3, 4).

With CD spectrometry, VPA clearly demonstrated an effect on the secondary structure of rhPE. This effect results in a decrease in PE signal at 220 nm. The effect of VPA on PE secondary structure could not be explained by dilution of the enzyme, nor did we observe any indication of precipitation of PE that could account for this signal change. ZPP, a competitive slow-binding PE inhibitor, is known to act on the active site of the enzyme (23, 24). Thus, VPA was compared to ZPP both singularly and in an additive relationship to elucidate whether the VPA effects were at the active site. Adding ZPP to the enzyme prior to adding VPA to the ZPP:PE mixture did not affect secondary structure of the enzyme as compared to VPA alone (Fig. 4). This clearly suggests that VPA is interacting at or near the binding pocket of PE and not through non-specific binding to the exterior of the molecule.

In order to further examine the exact mechanism underlying the effects of VPA on the secondary structure and activity of PE, we carried out NMR studies. PE, with a molecular weight of almost

80 kDa is too large for direct measurements by 1H or 13C NMR and would require labeling with 15N,13C isotopes. Thus we carried out studies to look at the 1H shifts of ZPP and VPA in the presence of PE. We compared the interaction of each inhibitor with PE in presence or absence of the other inhibitor. When ZPP is added to PE, the protons of the carbobenzoxy group on ZPP are detected at 7.28 and 7.20 ppm (figure 5B). Compared with the free ZPP peaks (figure 5C), the bound ZPP peaks show a decreased signal, with an increasing growth of new peaks at 7.34 and 7.25 ppm respectively (figure 5B). This was somewhat surprising but suggests that a slow exchange between free ZPP and the bound ZPP. When VPA was added to the PE prior to the addition of ZPP, the only peaks observed are for the unbound ZPP. These results suggest that ZPP was unable to reach the active site of the enzyme, due to the effect of VPA on the enzyme. VPA peaks (methyl-groups) were seen between 0.68-0.70 ppm (data not shown) and did not interfere with the carbobenzoxy peaks of the ZPP. There is a significant overlap of VPA peaks and the residual non exchanging peaks of PE in this region therefore we were unable to determine the effects of addition of ZPP on the VPA peaks.

Recent descriptions of the anti-cancer properties of VPA especially in cigarette smoke induced lung cancers are leading to the development new pro-drugs based upon VPA (25). Our data suggest that a bound structure of VPA and its derivatives could benefit this new drug development. Given the worldwide burden of morbidity and mortality associated with inflammatory airway disorders, the use of VPA or its derivatives to target extracellular PE activity and subsequent acPGP generation may have notable changes in disease phenotypes. More importantly, the successful targeting of this protease in the COPD airway may improve disease related clinical outcomes such as exacerbation rates, improvement in lung function decline over time, airway mucociliary transport, and quality of life indices.

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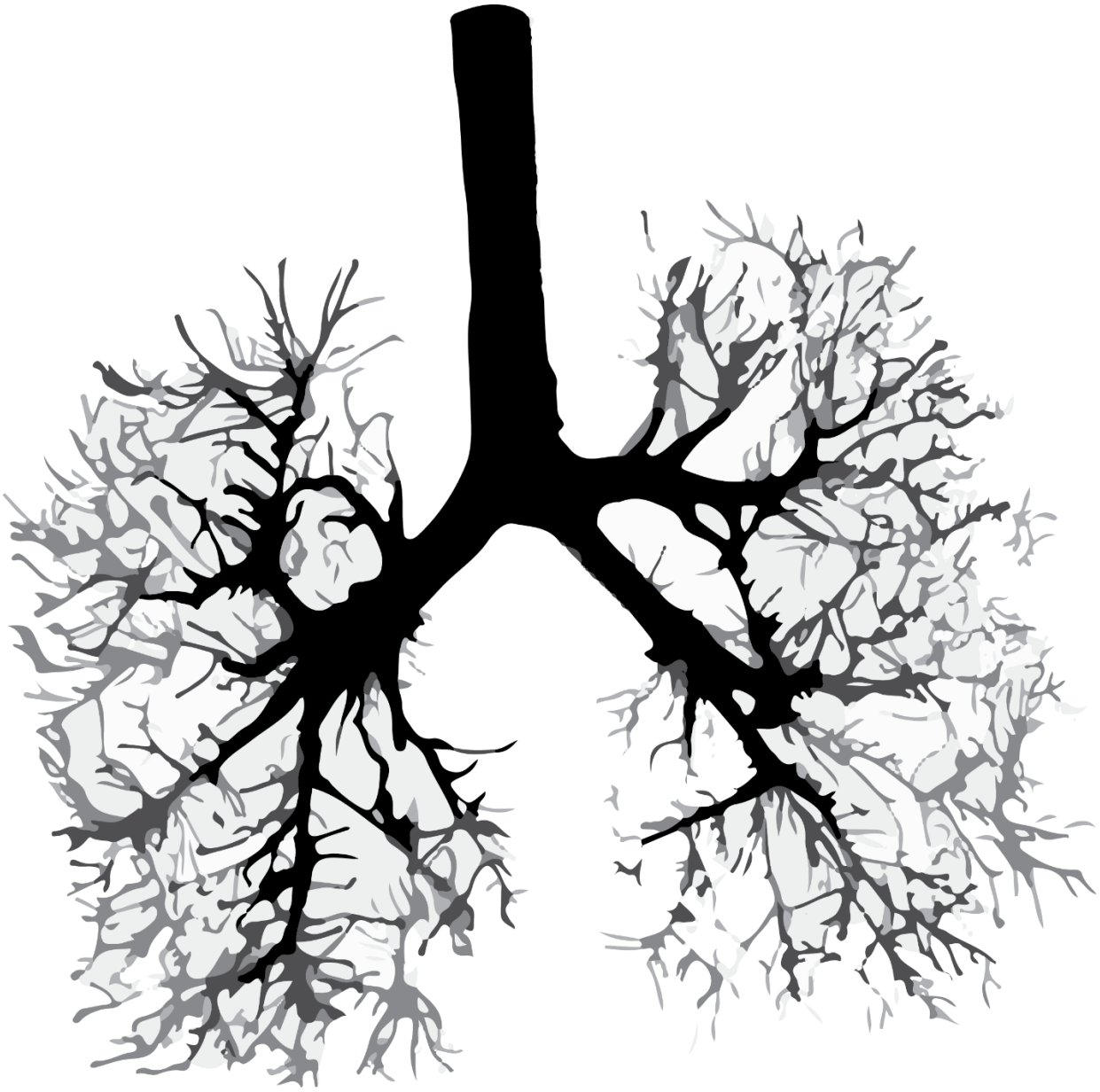
Chapter four



Therapeutic neutralization of the matrikine PGP suppresses the development of lung emphysema in cigarette smoke exposed mice

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Abstract

Chronic obstructive pulmonary disease (COPD) is a one of the major causes of mortality worldwide which is mainly caused by cigarette smoking. The inflammation in COPD is characterized by an excessive neutrophilic response. The matrikine proline-glycine-proline (PGP) and the more potent acetylated form acPGP is derived from collagen and is chemotactic to neutrophils by acting on CXC receptor 2 (CXCR2). The enzymes matrix metalloproteinase 8 and 9 (MMP-8/9) followed by prolyl endopeptidase (PE) are responsible for this cleavage. Some studies have suggested a link between PGP peptides and an emphysema phenotype as seen in COPD. In this report we investigate the impact of directly neutralizing PGP peptides in an emphysema model in mice. The PGP neutralizing peptide L-arginine-threonine-arginine (RTR) was used in a 6 weeks and 23 weeks cigarette smoke exposure murine model. In the chronic model, mice started the RTR treatment after 10 weeks of cigarette smoke exposure and continued the treatment together with the smoke exposure during the last 13 weeks. RTR significantly inhibited neutrophil and macrophage influx into the lungs as seen in the bronchoalveolar lavage (BAL) fluid as well as the development of right ventricular hypertrophy (RVH). Furthermore, in the chronic model, mice receiving RTR during the last 13 weeks did not develop an emphysema phenotype, compared to controls. Finally, *in vitro* experiments using primary human bronchial epithelial (HBE) cells from healthy individuals or COPD patients were carried out. Pretreatment of the cells with cigarette smoke extract and stimulated with acPGP resulted in a higher CXCR2 protein signal in the cells with COPD background. Also, acPGP facilitated the release of MMP-9 and interleukine-8 from primary HBE cells, thereby highlighting a new mechanism for acPGP to augment neutrophilic inflammation. Our data strongly underscore an important role for PGP in the development of the emphysema and epithelial cell dysfunction in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is both progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases (1). According to the WHO, COPD has become one of the major causes of mortality worldwide and is predicted to be ranked third in 2030 as the most common causes of death (2). The main risk factor for COPD is cigarette smoking, which initiates a neutrophilic inflammatory response (3).

In the last decade, several studies have characterized the collagen-derived neutrophil chemoattractant PGP (proline-glycine-proline) and PE (prolyl endopeptidase) as possible novel biomarkers in COPD and cystic fibrosis. PGP is a neutrophil chemoattractant and is generated from collagen cleavage instigated by matrix metalloproteases (MMP-8 and MMP-9) and finalized by PE (4-6). Acetylation of PGP (acPGP) increases the chemotactic activity even more (7). The enzyme leukotriene A_4 hydrolase (LTA_4H) aminopeptidase degrades the N terminus of the unacetylated PGP to inactivate the peptide, but acetylation of PGP protects the peptide from this enzymatic degradation (8).

Various studies have linked production PGP or acPGP with emphysema. For instance, mice have elevated PGP levels after chronic smoke exposure with an emphysema phenotype (9, 10). Paige et al have shown that smoke exposed mice treated with a pharmaceutical agent 4-methoxydiphenylmethane (4MDM) that selectively augments the LTA4H aminopeptidase without affecting the bioproduction of leukotriene B4 during 6 months prevents the development of emphysema (11). Furthermore, in clinical studies significantly higher PGP levels are detected in

samples taken from patients with COPD compared to non COPD patients or healthy volunteers (10, 12, 13).

Although the proximal mechanisms leading to this increased proteolytic degradation of collagen are unknown, stimulation of airway epithelial cells by cigarette smoke or noxious particles are thought to be significant contributors (9). In a previous study, we showed that by inhibiting the generation of PGP by valproic acid in an acute 5 days smoking model, that the cigarette smoke induced neutrophil influx is significantly reduced (14). However, to date, the impact of directly neutralizing PGP peptides in an emphysema model has not clearly been delineated. Here, we utilize the PGP neutralizing peptide L-arginine-threonine-arginine (RTR) to inhibit both inflammation and heart remodeling in a 6 week model of smoke exposure (15). Further, we show the potential to utilize RTR as a therapeutic in a 23 week model of smoke exposure. Finally, *in vitro* experiments suggest that PGP can signal the release of both MMP-9 and IL-8 from airway epithelia, thereby highlighting a new mechanism for PGP to augment neutrophilic inflammation.

Methods

Murine smoking models

All the animal work conducted in this study was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of UAB (Animal Protocol #120709133) and conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (16). Two murine smoke models were conducted. In a sub-chronic model, female BALB/c mice (8-10 weeks old) were randomly assigned to 5 days/week for 6 consecutive weeks to either cigarette smoke or air exposure (n= 4-6 mice per group). Mice were exposed 60 min/day

to mainstream cigarette smoke with standard University of Kentucky 3R4F research cigarettes (9.4mg tar/0.726mg nicotine, University of Kentucky). Cigarette smoke was generated and delivered by the SCIREQ "InExpose" smoking system (SCIREQ, Montreal, QB, Canada) using the same protocol as published before (17). Prior to each exposure, mice received either vehicle (saline), RTR or the control peptide alanine-serine-alanine (ASA). Therapeutic agents were administered by oropharyngeal aspiration under light isoflurane anesthesia.

In a chronic murine model with mice of the same gender, strain and age as described above were smoked and treated during 23 weeks. In this experiment, 70 mice were randomly divided in three groups: a cigarette smoke exposed group treated with vehicle (n=40) or air exposed groups receiving either vehicle (n=15) or RTR (n=15). After 10 weeks of smoke exposure, the 40 smoke exposed and vehicle treated mice were then divided into two groups: a smoke exposed group still receiving a vehicle (n=20) or a smoke exposed group receiving RTR rescue (n=20). Before sacrificing the mice, lung volume of 7-8 mice per group was measured using the flexiVent (SCIREQ, Montreal, QB, Canada).

Sixteen hours after the last smoke exposure mice were sacrificed to collect bronchoalveolar lavage (BAL) fluid and blood.

Histology and morphometric analysis

After sacrificing the mice, the lungs of 6-8 mice per group were fixed with a 10% formalin infusion through a tracheal cannula at a constant pressure of 25 cm H2O (18). The whole lung was stored in 10% formalin afterwards for at least 24h and embedded in paraffin. Five μm sections were cut and stained with hematoxylin/eosin (H&E) according to standard methods. Using light

microscopy, morphometric assessment of emphysema was done by determination of the average inter-alveolar distance (the mean linear intercept (Lm)). The Lm was calculated at a total magnification of 200x of 6 random photomicroscopic images per slide and 10 slides per lung. The lung tissue sections were analyzed with a reference grid using the program Image-Pro MC Plus 7.0 (19).

Right ventricular heart hypertrophy measurement

To estimate right ventricular hypertrophy, post mortem mice hearts were collected. The hearts were dissected under 10x microscopic magnification into the right ventricle and the left ventricle plus septum. Connecter atria were gently removed. The right ventricle and the left ventricle plus septum were weighed and the ratio of the weights was calculated as follows: (right ventricle)/(left ventricle + septum) (13).

Prolyl endopeptidase activity assay

PE activity in BAL fluid of chronically cigarette smoke exposed mice was measured using a specific PE substrate N-succinyl-glycine-proline-7-amido-4-methyl-coumarin (Suc-Gly-Pro-AMC) ((Bachem, Switzerland). Activity assays were carried out in 100 mM phosphate buffer (pH 7.5) with 1mM DL-Dithiothreitol (DTT) (Sigma-Aldrich, USA) and 10µM bovine serum albumin (BSA) (Sigma-Aldrich, USA) using 20µl sample and 80µl reaction buffer.

Cleavage of the PE-specific fluorogenic substrate Suc-Gly-Pro-AMC (0.2 mM) measured with a spectrofluorometer using excitation and emission wavelengths of 380 nm and 460 nm respectively, at 37 °C during 90 min.

Electrospray ionization-liquid chromatrography-mass spec/mass spec (ESI-LC-MS/MS)

PGP peptides in BAL fluid are measured using a MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 spectrometer equipped with a Shimadzu HPLC (Columbia, MD). HPLC is conducted using a 2.0 x 150 mm Jupiter 4u Proteo column (Phenomenex, Torrance, CA) with A: 0.1% HCOOH and B: MeCN + 0.1% HCOOH: 0 min-0.5 min 5% buffer B/95% buffer A, then increased over 0.5-2.5 min to 100% buffer B/0% buffer A. Background is removed by flushing with 100% isopropanol / 0.1% formic acid. Positive electrospray mass transitions were at 312–112,312–140 M/z for acPGP and 270-70, 270-116, 270-173 for PGP. Area under the curve is measured, and PGP peptide concentration calculated using a relative standard curve method as previously described (13).

Cigarette smoke extract

Cigarette smoke extract (CSE) was prepared by using 3R4F research cigarettes (University of Kentucky, USA). Cigarette smoke was bubbled at a rate of 10ml/puff, three times a minute through PBS and filtered through a 0.2µm filter. A working concentration of 2% CSE was used to prime epithelial cells.

In vitro activation of primary human bronchial epithelial cells

Use of human cells and tissues was approved by the UAB Institutional Review Board. Primary human bronchial epithelial (HBE) cells were derived from lung explants after written informed consent was obtained from COPD and non-COPD individuals. During 4 weeks, the cells were grown and terminally differentiated in polarizing conditions in inserts.

The inserts with primary airway epithelia from COPD and non-lung disease controls were pretreated with 2% CSE. Hereafter, the primary HBE cells were stimulated with 0.5 mg/ml acPGP or PBS alone for 24 hours and then lysed in cold (4°C) RIPA lysis buffer (Santa Cruz Biotechnology, Inc.**,** Dallas, Texas, USA). The lysates were centrifuged to remove the debris, and the supernatants were then boiled for 5 min in Laemmli buffer. Both COPD and non-COPD lysate samples (20 μg total protein/lane) were electrophoresed through 10% SDS-polyacrylamide gels and were transferred onto nitrocellular membranes, which were blocked in TBST (pH 7.4) containing 5% BSA for 1 h and probed with 1:500 anti-CXCR2 (1:500, Abcam, Cambridge, MA, USA) overnight, followed by the appropriate secondary antibody coupled to horseradish peroxidase (HRP) goat-anti-rabbit (1:10,000; Promega, Madison, WI, USA). Immunoblots were then developed using ChemiDoc^{TM} XRS+ Imager.

In a different set of experiments, the primary airway epithelia from COPD and non-lung disease controls in inserts were treated with 1 of the 4 treatments in the basolateral compartment: 1). MEM alone, 2). 2 mM RTR in MEM, 3). 2 mM acPGP in MEM or 4). 2 mM of both acPGP and RTR in MEM. After 8 hours of incubation at 37 ^{0}C and 5% CO_2 the basolateral supernatant was aliquoted and frozen until analysis. Total MMP-9 ELISA (R&D systems, UK), lactate dehydrogenase (LDH) cytotoxicity detection assay (Roche diagnostics, Netherlands), IL-8 ELISA (R&D systems, UK) was performed on the supernatants according to the manufacturer's assay procedures.

Statistics

When appropriate, an unpaired t-test was used or analysis of variance followed by Newman-Keuls multiple comparisons test were carried out using GraphPad prism 6. $p \leq 0.05$ was considered significant.

Results

Murine smoking models

In order to establish a potential link between the early development of emphysema and inflammation with PGP peptides, mice were exposed to cigarette smoke for six weeks. At the end of the experiments, BAL fluid was collected and the hearts were analyzed for RVH development. As expected, exposure to smoke led to increased leukocyte influx into the lungs.

Treatment with RTR decreased the total cell influx measured in the BAL fluid with 63% compared to treatment with control peptide (ASA) and 66% compared to vehicle (Figure 1A). The cell influx after smoke exposure was due to an increased number of neutrophils and macrophages, which was significantly attenuated after RTR treatment but not after treatment with control peptide (Figure 1B and 1C). In addition, RTR treatment attenuated right ventricular hypertrophy (RVH) when compared to ASA-treated mice, highlighting the improvement of another clinical parameter observed with emphysema phenotype. These findings are associated with acPGP peptide levels measured in BAL fluid (Figure 1E).

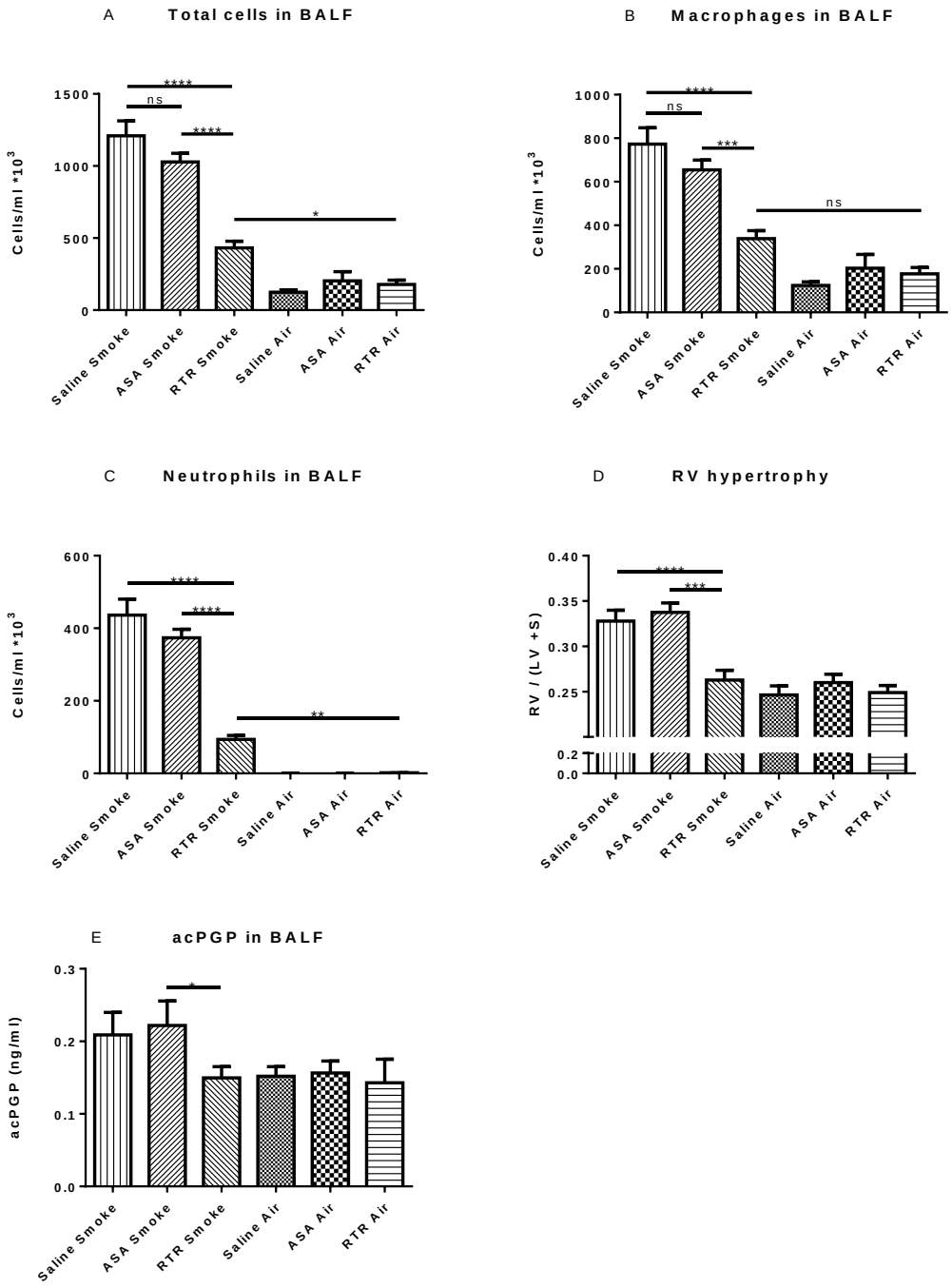

Figure 1. RTR tested in a 6 weeks murine smoke model. *RTR was administered intratracheally five days a week during 6 weeks directly prior to smoke exposure in mice. The tripeptide ASA was used as a control. RTR significantly inhibited leukocyte influx into the lungs (A). More specifically, macrophage (B) and neutrophil (C) influx was significantly decreased. Right ventricular hypertrophy caused by cigarette smoke exposure was also diminished due to RTR treatment (D). The tripeptide acPGP was significantly increased in the BAL fluid of smoked mice and back to*

*baseline levels after RTR treatment (E). In all cases the ASA treated/smoke exposed mice did not significantly differ from the smoke control group. Data are shown as the mean ± S.E.M (n=5-6 mice per group). Statistical analysis was by one-way analysis of variance followed by Newman-Keuls multiple comparison test. ns= not significant, * p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.*

We next sought to study the potential for targeting PGP peptides in an emphysema murine model. Mice were exposed to cigarette smoke for 23 weeks after which BAL and airways were collected for histology. Mice exposed to smoke had elevated total cell influx in the BAL fluid (Figure 2A). As observed in the 6 weeks model, this increased number was due to infiltration of macrophages and neutrophils (Figures 2B and 2C). The RTR treatment caused a significant attenuation of both total cell counts, neutrophils and macrophages, comparable to levels observed in air-treated mice (Figures 2A-2C). As observed in the short-term smoke group, the acPGP levels were increased in smoke exposure and were significantly reduced by RTR treatment (Figure 2D). This decrease also associated with a loss of PE activity, suggesting a potential mechanism by which reducing peptide burden may have an impact on the inflammatory cascade (Figure 2E).

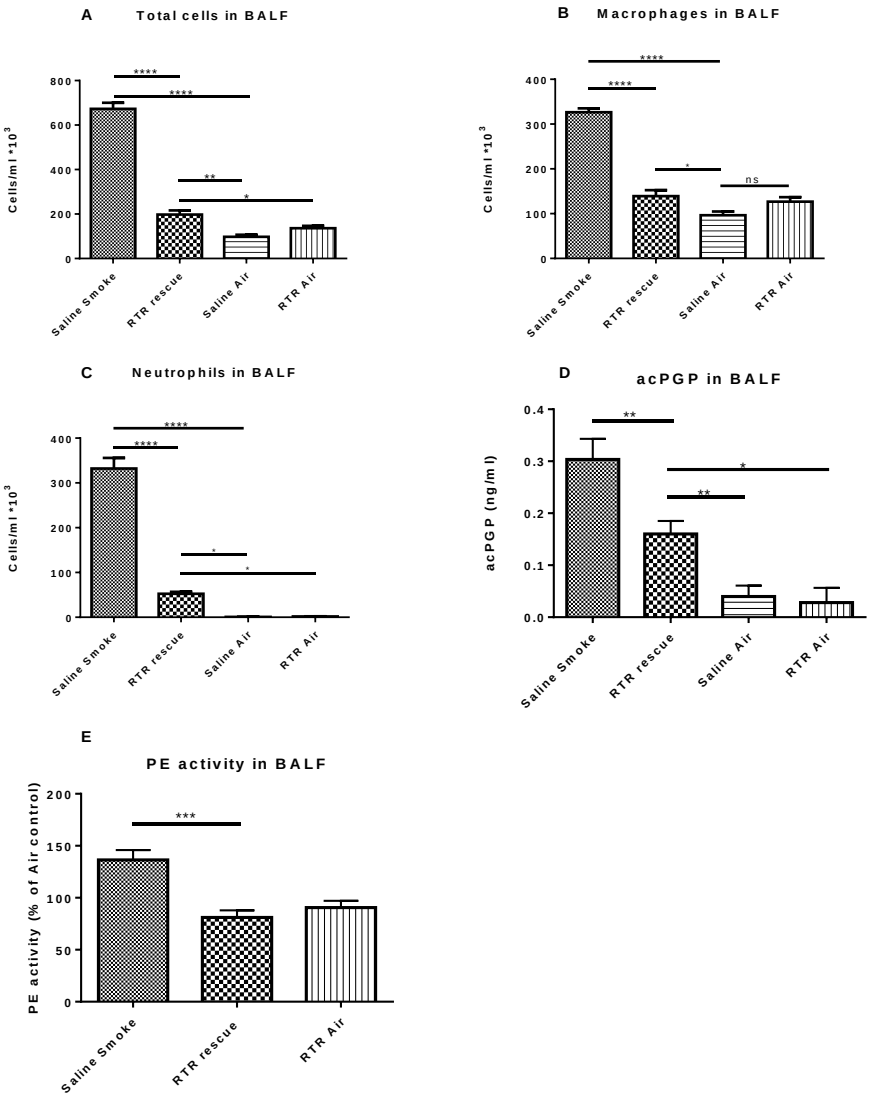

Figure 2. RTR tested in a 23 weeks murine smoke model. *RTR was administered intratracheally five days a week during 13 weeks directly prior to smoke exposure in mice after exposing those mice to cigarette smoke during 10 weeks and treated with vehicle (saline) to develop a rescue phenotype (RTR rescue). RTR treatment caused a significant drop in total cell influx into the lungs (A). This was caused by an influx inhibition of macrophages (B) and neutrophils (C) (data are shown as the mean ± S.E.M (n=8-12 mice per group). Statistical analysis was by one-way analysis of variance followed by Newman-Keuls multiple comparison test. ns= not significant, * p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001.*

Representative histology from these groups is shown in Figure 3A, highlighting increased alveolar enlargement after smoke exposure which is reduced with RTR rescue. An increased Lm was

brought down to levels of the air control with RTR treatment (Figure 3B). This was also reflected in an increased inspiratory capacity of smoked mice receiving vehicle control (Figure 3C). These effects were associated with development of RVH which is significantly improved with RTR rescue as seen in Figure 3D. Overall, these results strongly indicate a central role for PGP peptides in experimental emphysema.

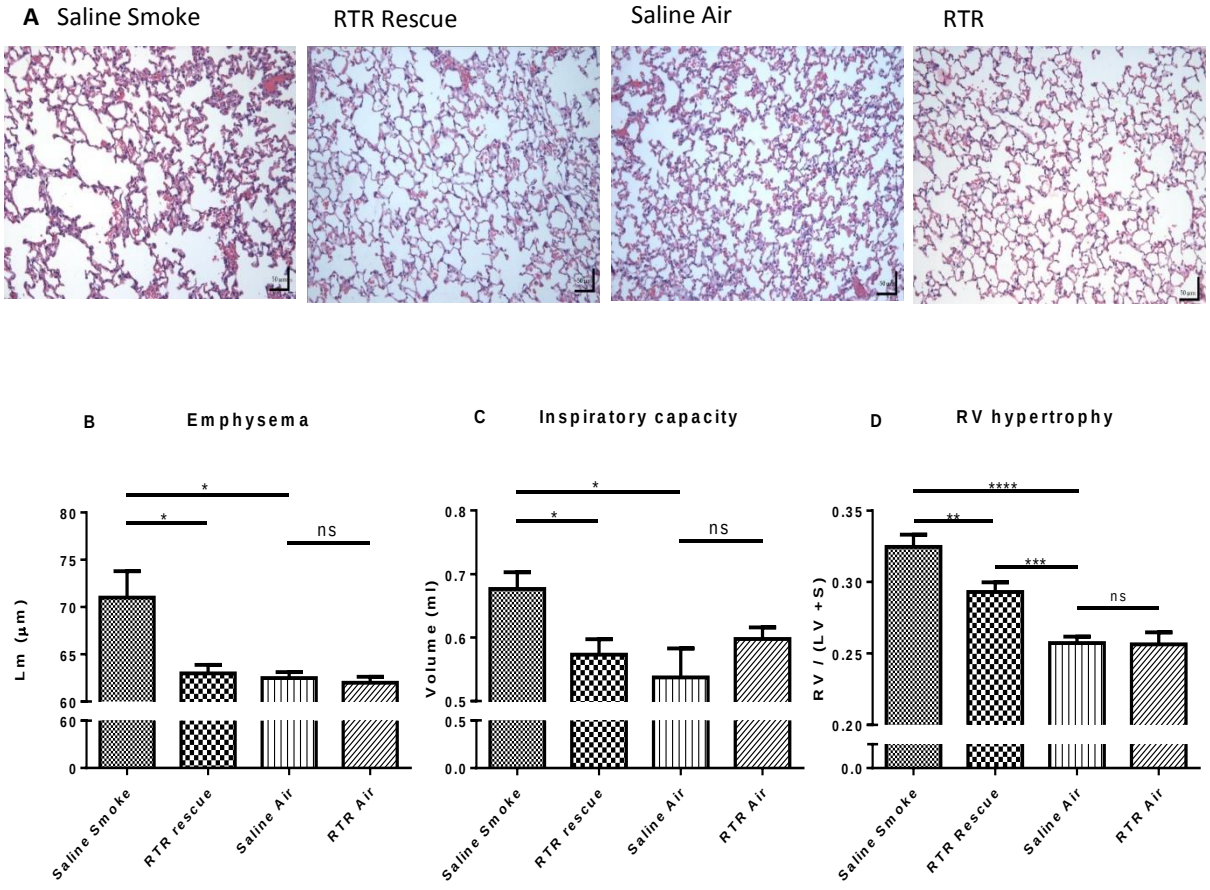

Figure 3. RTR inhibits the development of emphysema in mice. *Mice exposed to cigarette smoke and treated with vehicle (saline) during 23 weeks developed emphysema, measured as an increase in mean linear intercept (Lm) (A) and lung volume (B). Mice receiving RTR intratracheally prior to smoke exposure during the last 13 weeks (RTR rescue group) did not show any sign of emphysema development. Representative hematoxylin and eosin stained lung sections of all groups are shown in figure 3D. Right ventricular hypertrophy caused by cigarette smoke exposure was significantly inhibited due to RTR treatment (C) although not reaching baseline levels as seen during the 6 weeks smoke exposure model. Data are shown as the mean ± S.E.M (n=6-12 mice per group). Statistical analysis was by one-way analysis of variance followed by Newman-Keuls multiple comparison test. ns= not significant, * $p<0.05$, ** $p<0.01$.*

Effects of acPGP on Primary Human Bronchial Epithelial cells

The attenuation of PE with PGP peptide neutralization indicates a regulatory role this peptide has in its ongoing generation.

When primary airway epithelia from COPD and non-lung disease controls are exposed to 2% CSE and treated with acPGP, it is interesting to see that there is more CXCR2 protein to be found in the cell lysis (Figure 4A).

After exposure of a new set of primary HBE cells to acPGP alone, we observe increased release of IL-8 and MMP-9 (Figure 4A and 4B). Preincubation of acPGP with RTR nullifies the effects seen of acPGP. It was notable that primary airway epithelia from COPD patients secreted almost two times more IL-8 compared to the primary airway epithelia from the non-lung disease background. MMP-9 could only be detected in the supernatant of the acPGP-stimulated primary airway epithelia from COPD patients.

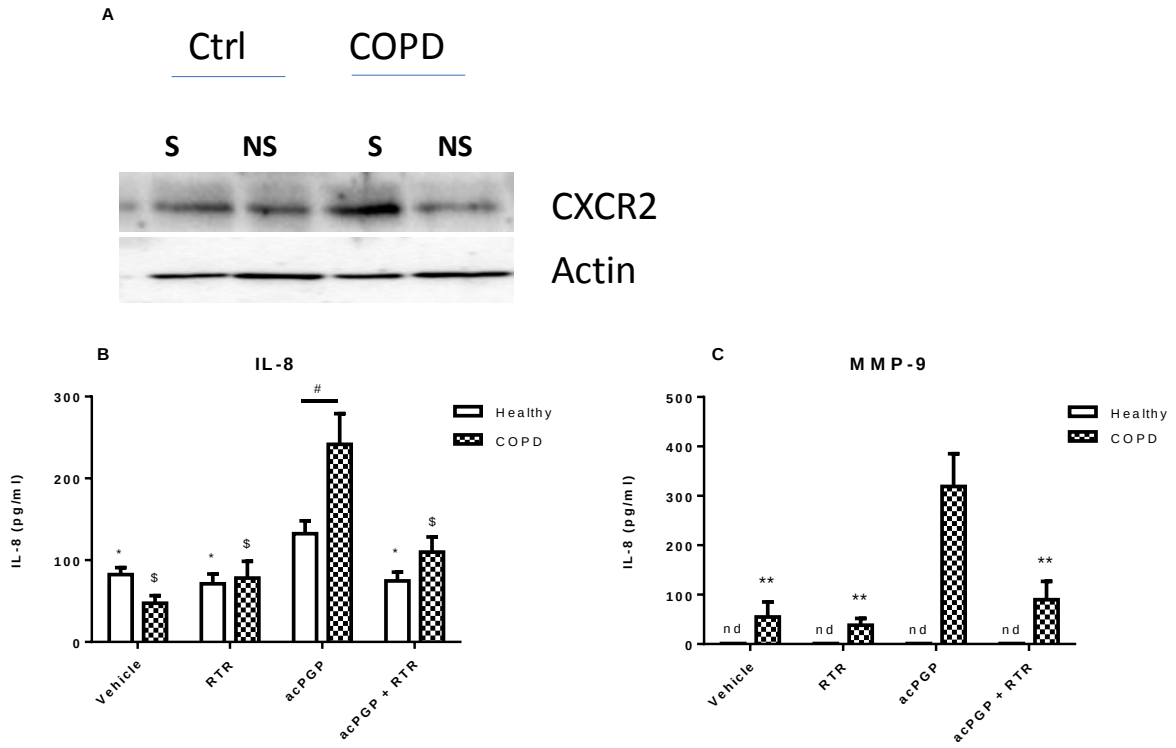

Figure 4. AcPGP stimulates epithelial cells to release IL-8 and MMP-9. *Inserts with primary epithelia taken from a COPD patient or a healthy volunteer were stimulated with acPGP or PBS after pretreatment with 2% CSE. COPD epithelia pretreated with CSE showed the highest CXCR2 protein signal, as seen by western blot (A). In a different experiment, epithelia taken from a COPD patient or a healthy volunteer were treated with acPGP with or without RTR and appropriate controls. IL-8 levels (A) and total MMP-9 (B) were measured via ELIAS in the supernatant after 8 hours of incubation. Statistical analysis was by unpaired t-test: # p<0.05; and one-way analysis of variance followed by Newman-Keuls multiple comparison test: * p<0.05 compared to the acPGP group, $ p<0.05 compared to the acPGP group, ** p<0.01 compared to the acPGP group. S, stimulated with acPGP. NS, stimulated with PBS. Nd, non-detectable.*

Discussion

In this report we describe the role of PGP after subchronic and chronic exposure of mice to cigarette smoke. Neutralizing PGP using RTR resulted in milder inflammatory outcomes. We further provide *in vitro* evidence that epithelial cells from COPD patients respond more strongly to stimulation by PGP.

In the subchronic smoking murine model, RTR was superior compared to vehicle or the control peptide ASA in reducing the inflammatory response to cigarette smoke exposure. In contrast to RTR, ASA did not reduce the cigarette smoke related inflammatory response in mice, indicating the specificity of the RTR effect. In the 6 weeks model, RTR treatment completely prevented development of RVH. These results underscore the potential for the development of RTR as a biologic inhibitor of the PMN inflammation and ongoing matrix remodeling observed in cigarette-smoke injury. In the chronic model, smoke-exposed mice were treated therapeutically with RTR at 10 weeks after start of the cigarette smoke exposure and onset of inflammation. Although, the RVH development was still higher in the RTR-treated group compared to the air control groups, the further development of RVH was halted. The studies demonstrate a potential for utilizing RTR as a therapeutic in the progression of experimental model of emphysema.

In previous work we showed that RTR may have a dual effect, besides neutralizing PGP, it also binds and inhibits IL-8 in a neutrophil chemotaxis assay (15). However, to what extend RTR effects the murine IL-8 homologues KC and MIP-2 is not clearly delineated.

In total, these results demonstrate a critical role of CXCR signaling and bioactive matrix fragments as mediators of emphysema and the potential for targeting these fragments in COPD lung disease.

In addition, we now present data demonstrating the presence of CXCR2 in primary airway epithelial cells which highlights increased CXCR2 expression in COPD airway epithelia compared to non-lung disease controls after smoke exposure. We also show the potential of acPGP to amplify the inflammatory response and extracellular degradation. AcPGP stimulated IL-8 and MMP-9 release from airway epithelia cells. Interestingly, airway epithelial cells from COPD showed an enhanced response to PGP, which could indicate a general propagation for CXCR2 signaling in these cells during COPD. In total, these results suggest that the epithelial cells from COPD patients

used in our study may be more primed to respond to acPGP, due to an increased CXCR2 expression.

To our knowledge, this is the first report which describes that a therapeutic intervention directed to PGP peptides in a murine emphysema model reduces the airway inflammation and development of morphological and physiological signs of emphysema. These results are also the first to demonstrate the effect of acPGP on CXCR2 signaling from airway epithelial cells. Our data strongly underscore an important role for PGP in the development of emphysema and epithelial cell dysfunction in COPD.

Grants

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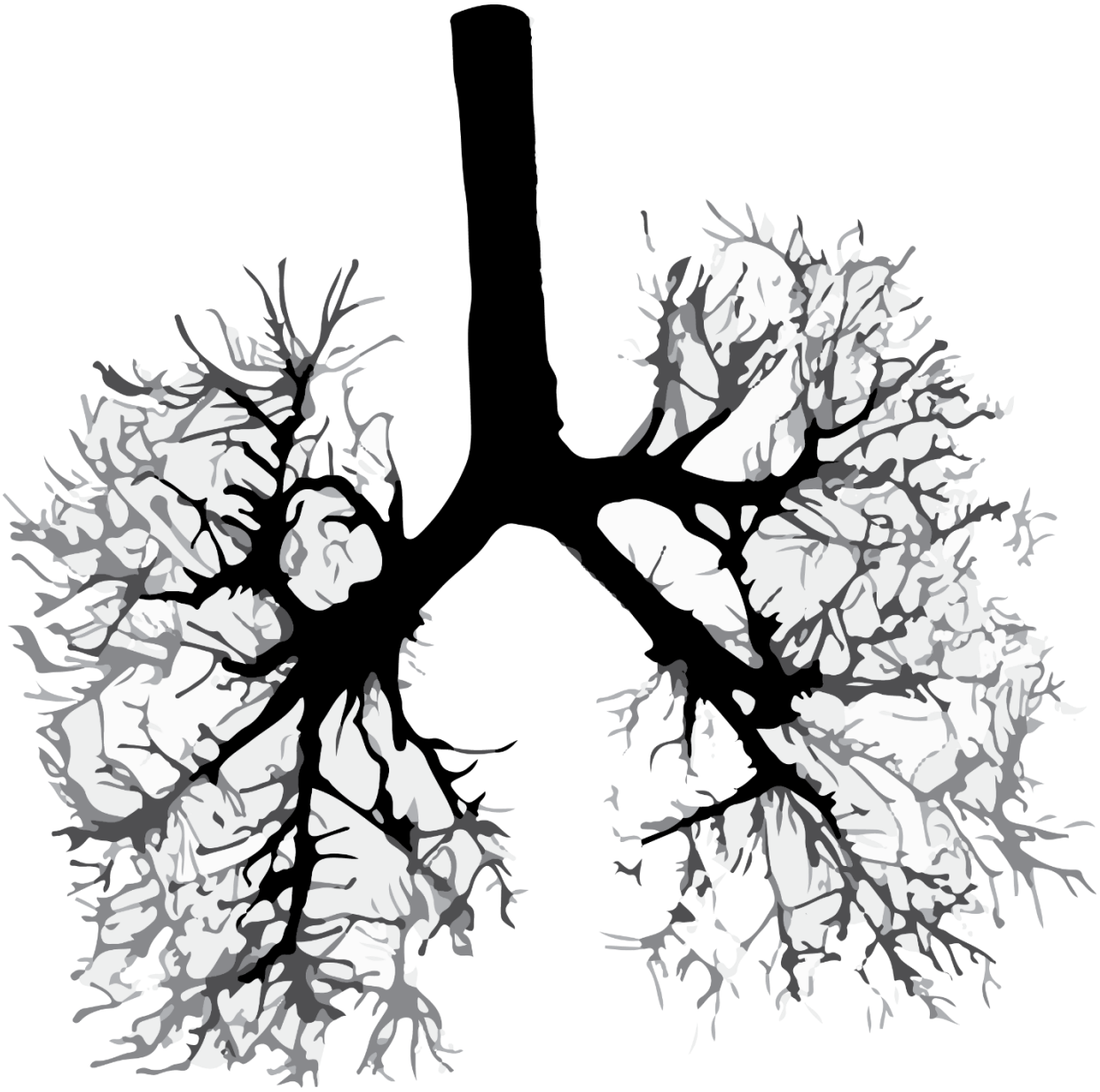
Chapter five



The matrikine acPGP couples extracellular matrix fragmentation to endothelial permeability

This chapter is based on the article:

The Matrikine AcPGP Couples Extracellular Matrix Fragmentation to Endothelial Permeability

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Abstract

The compartmentalization and transport of proteins and solutes across the endothelium is a critical biologic function altered during inflammation and disease, leading to pathology in multiple disorders. The impact of tissue damage and subsequent extracellular matrix (ECM) fragmentation in regulating this process is unknown. Herein, we demonstrate that the collagen-derived matrikine acetylated proline-glycine-proline (acPGP) serves as a critical regulator of endothelial permeability. AcPGP activates human endothelial cells via CXCR2, triggering monolayer permeability through a discrete intracellular signaling pathway. *In vivo*, acPGP induces local vascular leak after subcutaneous administration and pulmonary vascular permeability after systemic administration. Further, neutralization of acPGP attenuates LPS- induced lung leak. Finally, we demonstrate that plasma from patients with acute respiratory distress syndrome (ARDS) induces vascular endothelial – cadherin (VE-cadherin) phosphorylation in human endothelial cells and this activation is attenuated by acPGP blockade with a concomitant improvement in endothelial monolayer impedance. These results identify acPGP as a novel ECM-derived matrikine regulating paracellular permeability during inflammatory disease and demonstrate the potential to target this ligand in various disorders characterized by excessive matrix turnover and vascular leak such as ARDS.

Introduction

The endothelium is a monolayer of cells lining the luminal surface of blood vessels, serving as a barrier between the circulation and underlying tissue. The ability of the endothelium to regulate paracellular transport of proteins and fluid across this monolayer is thought to serve as a critical developmental feature delineating the increasingly complex cellular functions of higher order organisms. Although selective endothelial permeability is considered critical to the maintenance of health in humans, many diseases are characterized by excessive vascular leak. Often, these conditions feature considerable extracellular matrix (ECM) turnover and inflammation, suggesting a potential role for specific ECM fragments as regulators of vascular endothelial function.

Previous research has suggested that fragmentation of ECM can lead to the formation of small peptides (termed matrikines) which have the capacity to regulate inflammatory cell phenotype both *in vitro* and *in vivo* (*1*). For example, we and others have demonstrated that alkali hydrolysis or proteolytic digestion of the extracellular matrix protein collagen results in production of the tripeptide matrikine proline-glycine-proline (PGP) which can be N-terminal acetylated (acPGP) through direct chemical modification (*2*); both are bioactive but due to different stabilities *in vivo*, acPGP was utilized to model total bioactive PGP peptides. (*3-5*). AcPGP can activate cell signaling via the CXC-chemokine receptor 2 (CXCR2) (*6, 7*). To date, the focus of studies on the cellular effects of acPGP have centered on its role as a neutrophil chemoattractant (*8, 9*), while the potential effects on other CXCR2 expressing cell populations have not been evaluated.

AcPGP is the first matrikine reported in the airway and plasma of patients with chronic inflammatory lung disease and in the intestines of patients with inflammatory bowel disease (*10-13*). These conditions are characterized by increased protease activity and extracellular matrix

turnover and, in a variety of animal models of disease, the blockade of acPGP or its generating proteases has led to amelioration of disease (*3, 14*).

Our group recently described the increased presence of critical proteases required for acPGP generation in the airway secretions from patients with the acute respiratory distress syndrome (ARDS) (*15*). ARDS is an acute inflammatory lung disorder characterized initially by a neutrophil-rich inflammatory response and dysregulated vascular permeability, leading to impaired gas exchange. While various stimuli can trigger ARDS, common features of the condition are ongoing matrix remodeling and dysregulated vascular permeability, leading to disease progression and increased morbidity and mortality. Whether matrix remodeling and vascular leak are mechanistically integrated or not in ARDS remains unclear. We hypothesized that acPGP is a critical link in the development of disease-mediated tissue damage and paracellular endothelial cell permeability. In this report, we show that acPGP can selectively activate primary human endothelial cells to induce monolayer permeability, in the absence of broad inflammatory signaling, in a CXCR2-dependent manner. Further, we demonstrate that acPGP induces both local and systemic vascular leak when directly administrated and functions as a critical component of lipopolysaccharide (LPS)-mediated vascular permeability. Finally, we highlight acPGP as an important mediator of endothelial permeability in clinical specimens from patients with ARDS.

Methods

Reagents

Primary human umbilical vein endothelial cells (HUVECs), human coronary arterial endothelial cells (HCoAEC), human aortic endothelial cells (HAEC), human pulmonary microvascular endothelial cells (HPmvEC) were from Lonza (Rockville, MD) and THP-1 cells were from ATCC (Rockville, MD). MCDB-131, heat-inactivated fetal bovine serum (HI-FBS), L-glutamine, and penicillin/streptomycin were from Invitrogen (Carlsbad, CA). AcPGP, proline-glycine-glycine (PGG), and L-arginine-threonine-arginine (RTR) were from Bachem (Torrance, CA). All other reagents were from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Patient Populations and Plasma Collection

ARDS patients secondary to gram negative sepsis (n=6) and non-lung disease patients (n=6), who were also intubated and mechanically ventilated, were recruited from the University of Alabama at Birmingham (UAB) hospital. All subjects carried the diagnosis of ARDS based on accepted diagnostic criteria (*37*). Samples and health information were labeled using unique identifiers to protect subject confidentiality. Blood samples were collected in sodium citrate tube, centrifuged at 3500 rpm for 15 min, and plasma stored at -80°C for later analysis. All human studies were approved by the UAB Institutional Review Board (Protocol #: F081016007).

Cell Culture

ECs were maintained as previously described (*38*). For treatment, cells at one day post-confluence were serum starved for two hours before stimulation as described for each experiment. THP-1 cells were maintained in RPMI 1640 containing 10% FBS, penicillin (100U/ml)/streptomycin (100

μg/ml), and 2 μM beta-mercaptoethanol. For adhesion experiments, THP-1 monocytes were labeled with Cell-Tracker Green (1 μM) for 15 min at 37°C.

Transwell permeability assay

Permeability assays were conducted as previously described (*26*). HUVECs were plated onto gelatin coated 3.0 micron polycarbonate transwell membranes (Corning Costar) and cultured for 4 days with media changes every day. Cells were serum starved for two hours in phenol red free DMEM/F12 and treated as described for experiments. Horseradish peroxidase (HRP) was added to the upper chambers at a final concentration of 1.5μg/ml and after 30 min the filters were removed and medium was harvested from the lower chamber. HRP leak was determined by absorbance at 450 nm after incubation with 0.5 mM guaiacol, 50 mM Na_2HPO_4, and 0.6 mM H_2O_2 for 15-30 min.

xPERT permeability assay

Experiments were performed with a modification of the previously reported protocol (*20*). Briefly, 12 mm glass coverslips were incubated with 2% 3-aminopropyltriehoxysilane isopropanol for 30 seconds, washed in isopropanol followed by washing with 100 mM sodium phosphate pH 6.8, and then incubated with 5% glutaraldehyde in 100 mM sodium phosphate pH 6.8 for 5 min. After washing in 100 mM sodium biocarbonate pH 8.3, coverslips were coated with 0.25 mg/ml biotinylated collagen in the aforementioned buffer for two hours at room temperature. Coverslips were then washed in PBS and free aldehydes were quenched by incubation with complete growth media. Cells were plated at $2x10^5$ per well and after 48 hours were treated as described. To visualize permeability, 0.5 ml of 1:2000 dilution of streptavidin Alexa488 was added to wells for

3 min. Wells were then quickly washed with 3.7% paraformaldehyde and then incubated in 3.7% paraformaldehyde for 20 min at room temperature. After exhaustive washing with PBS, coverslips were mounted for viewing. Three random fields from three independent coverslips per condition were captured using a 20x objective and the process was repeated at least three times. Data are represented as percent Alexa488 positive area per field of view as assessed using NIH ImageJ.

Real-Time Cell Analysis (RTCA) experiments

Barrier integrity of HUVEC was analyzed using the xCELLigence *R*eal-*T*ime *C*ell *A*nalyzer [RTCA] (Acea Biosciences/Roche Applied Science, Basel, Switzerland). This technology measures electrical impedance as a readout for the barrier integrity in cells grown on microelectrodes to provide a constant assessment of barrier function. Decreases in cellular impedance (presented as area under curve) reflect disruption in barrier integrity and increased paracellular permeability. $5x10^4$ cells were plated in each well of an E-Plate 16 (Roche Applied Science) and allowed to grow for 48 hours. Impedance readings were recorded at 10 minutes intervals for one hour to confirm that stable junctions were present. Cells were then changed to serum free media and monitored until junctions had again stabilized (approximately 2.5 hours) and treated as described. Area under the curve was calculated as the net difference in relative cell index for each treatment group as compared to control over selected time points using Microsoft Excel and plotted using GraphPad.

GTPase activity assay

Rac1 activity was determined by pull-downs using GST-PBD and GST-RBD (generous gift of A. Wayne Orr, LSUHSC-Shreveport). Cells were serum starved for two hours before being

stimulated as described, were washed twice with ice cold PBS and lysed (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM NaF, 1 mM Na_3Vao_4, 1 mM PMSF) for 10 min on ice before clarification by centrifugation at 14,000 x *g* for 5 min. Lysates were incubated with 25 µg GST-PBD or GST-RBD and 25 µl packed GST-sepharose (GE Life Sciences) for 45 min at 4^{o}C with gentle shaking (10% of the lysates was reserved as input control). Beads were washed extensively with lysis buffer and bound proteins were released by boiling in SDS-PAGE sample buffer. Reserved whole cell lysates and bound proteins were resolved by Western blot analysis as described below.

Western blot

Samples resolved on 4-15% TGX gels (BioRad, Hercules, CA) and were transferred to PVDF membranes. Blots were blocked with 5% milk in TBS + 0.1% Tween-20 (TBST) (except for pVE-Cadherin which was blocked in 5% BSA-TBST) and incubated overnight at 4^{o}C with antibodies against pERK (T202, Y204) (4370), ERK (4695) and ICAM-1 (4915) (Cell Signaling Technology-Danvers, MA) pVE-Cadherin (Y658) (441144G)), pPAK (S141) (44940G) (Invitrogen), Rac1 (610650) (BD Transduction Labs-San Jose, CA), VE-cadherin (sc-28644), γPAK (sc-1872) and VCAM-1 (sc8304) (Santa Cruz Biotechnology-Santa Cruz, CA) in 2% heat denatured BSA-TBST. Blots were washed in TBST, incubated with species appropriate HRP-conjugated secondary antibody, washed again in TBST, and signals were detected using enhanced chemiluminescence and x-ray film.

THP-1 adhesion assay

Assays were performed as previously described (*39*). Briefly, HUVEC were grown in 48-well plates and treated as described for each experiment. Cells were washed with warm PBS and incubated with 6 x 10^4 Cell-Tracker Green labeled THP-1 monocytes for 30 min at 37°C. Plates were gently washed with PBS and fluorescence was measured on a Victor^2 Perkin-Elmer Fluorescent plate reader (Excitation wave= 485nm, Emission wave= 535nm).

Mice

C57/Bl6 female mice (Jackson Laboratory, Bar Harbor, USA) were utilized in these studies. All animal studies were approved by the UAB Institutional Animal Care and Use Committee (Animal Protocol Number 130709133).

Miles assay

In vivo skin permeability was determined as previously described (*17*). Six-to-eight week old C57BL/6 mice had their abdominal fur removed by depilatory agents, were injected via the tail vein with 200µl of 1% Evans blue, and were injected subcutaneously on the abdomen with 200 µl of PBS or 250 µg acPGP, 250 µg PGG, or 50 ng vascular endothelial growth factor (VEGF). One hour after injections, mice were sacrificed and abdominal skin was removed and weighed. Evans blue was extracted from skin tissue by overnight incubation in formamide at 56ºC. Absorbance was measured at 620 nm and Evans blue concentration was determined from a standard curve and presented compared to tissue mass.

Murine model of Lipopolysaccharide (LPS)-induced pulmonary microvascular permeability

Six-to-eight week old C57BL/6 mice were injected with either saline vehicle or 50 µg of RTR daily for four days via tail vein. 30 minutes after each dose, mice were administered 75 µg of LPS from *E Coli* (Sigma-Aldrich) intraperitoneally to induce pulmonary permeability changes as previously described (*40, 41*). Control animals received saline instead of LPS in the same manner. Twenty four hours after last treatment, pulmonary microvascular permeability was assessed using the Evans blue dye extravasation technique as described previously (*23*). Briefly, Evans blue (20 mg/kg; Sigma-Aldrich) was injected intravenously one hour prior to isoflurane euthanasia. Lungs were perfused, removed, and Evans blue was extracted. Absorbance was measured at 620 nm and Evan's blue concentration was determined from a standard curve.

Murine model of acPGP-induced lung injury

Saline (50 µL) containing 50 µg of acPGP was intraperitoneally administered daily for four days to six-to-eight week old C57BL/6 mice. Saline only and saline containing 50 µg of PGG and 75 µg of LPS served as controls. Twenty four hours after the last dose, mice were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (8 mg/kg). Through a 1-cm abdominal midline incision, blood was collected by intracardiac puncture before euthanasia. Blood samples were centrifuged at 3500 rpm for 15 minutes and plasma was aspirated and stored at -80C^0. Bronchoalveolar lavage (BAL) was collected using three 1.0-mL injections of saline through a tracheal cannula. To estimate pulmonary vascular permeability, BAL supernatant was analyzed for immunoglobulin M (IgM) using an enzyme immunoassay (ICL, Portland, OR).

Electrospray ionization-liquid chromatrography-mass spec/mass spec (ESI-LC-MS/MS)

Plasma samples were processed by filtering through a Millipore 10,000 Molecular Weight cutoff centrifugal filter, then washing with 40 µl of 1 mM HCl and BAL samples were not filtered. PGP peptides in plasma and BAL were quantified using two different devices: for BAL, an MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 triple quadrupole mass spectrometer equipped with a Shimadzu HPLC (Columbia, MD) and a 2.0 x 150 mm Jupiter 4µ Proteo column (Phenomenex, Torrance, CA) and for plasma, a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer was used with electrospray ionization (Thermo Fisher Scientific, San Jose, California, USA) on an Atlantis dC18 column (100 mm×2.1 mm, d_p=3 µm; Waters Chromatography, Milford, Massachusetts, USA) with an Atlantis dC18 pre-column (10×2.1 mm, d_p=3 µm; Waters), also coupled to a Shimadzu LC system. The mobile phase consisted in both sample types out of (A) 0.1% (v/v) formic acid in water and (B) 0.1% formic acid in acetonitrile using gradient elution: 0 min-0.5 min 5% B/95% A, then increased linearly over 0.5-2.5 min to 100% B/0% A. During the run, samples were kept at 4 ^{0}C and the column at 30 ^{0}C. Positive electrospray mass transitions were at 312–140,312–112 and 312-70 M/z for acPGP and 270-70, 270-116, 270-173 for PGP. Peak area were measured, and PGP peptide concentrations were calculated using a relative standard curve method as previously described (*6, 25*).

Murine tissue preparation for Western blot analysis

All murine tissues assessed for Western blot were removed immediately after sacrifice and snap frozen in liquid nitrogen. The tissue was lysed (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na_3Vao_4, and 1 mM PMSF) and homogenized. Lysates were clarified by centrifugation at 14,000 x g for 10 min and protein concentration was

determined by BCA assay (BioRad). Equal amounts of protein were analyzed by Western blot analysis as described above.

Statistics

For multiple comparisons, analysis of variance (ANOVA) and post-hoc analysis (Tukey for one-way and Bonferroni for two-way) for multiple comparison tests were applied. The Student's t-test was used for comparisons of the mean values of two different samples. $p \leq 0.05$ was considered significant.

Results

AcPGP activates endothelial permeability signaling via CXCR2

We first determined if different tissue-specific endothelial cell lines displayed signaling through CXCR2. Human coronary artery, umbilical vein, pulmonary microvascular, and aortic endothelial cells were stimulated with the CXCR2 ligand IL-8 and the phosphorylation of extracellular signal-regulated kinase (ERK), a known downstream effector of IL-8 stimulated CXCR2 signaling (*16*), was determined. IL-8 treatment induced ERK phosphorylation in all cell lines tested indicating that they could all support CXCR2 mediated cell signaling (Fig. S1). To determine if the novel CXCR2 agonist acPGP could activate permeability signaling in

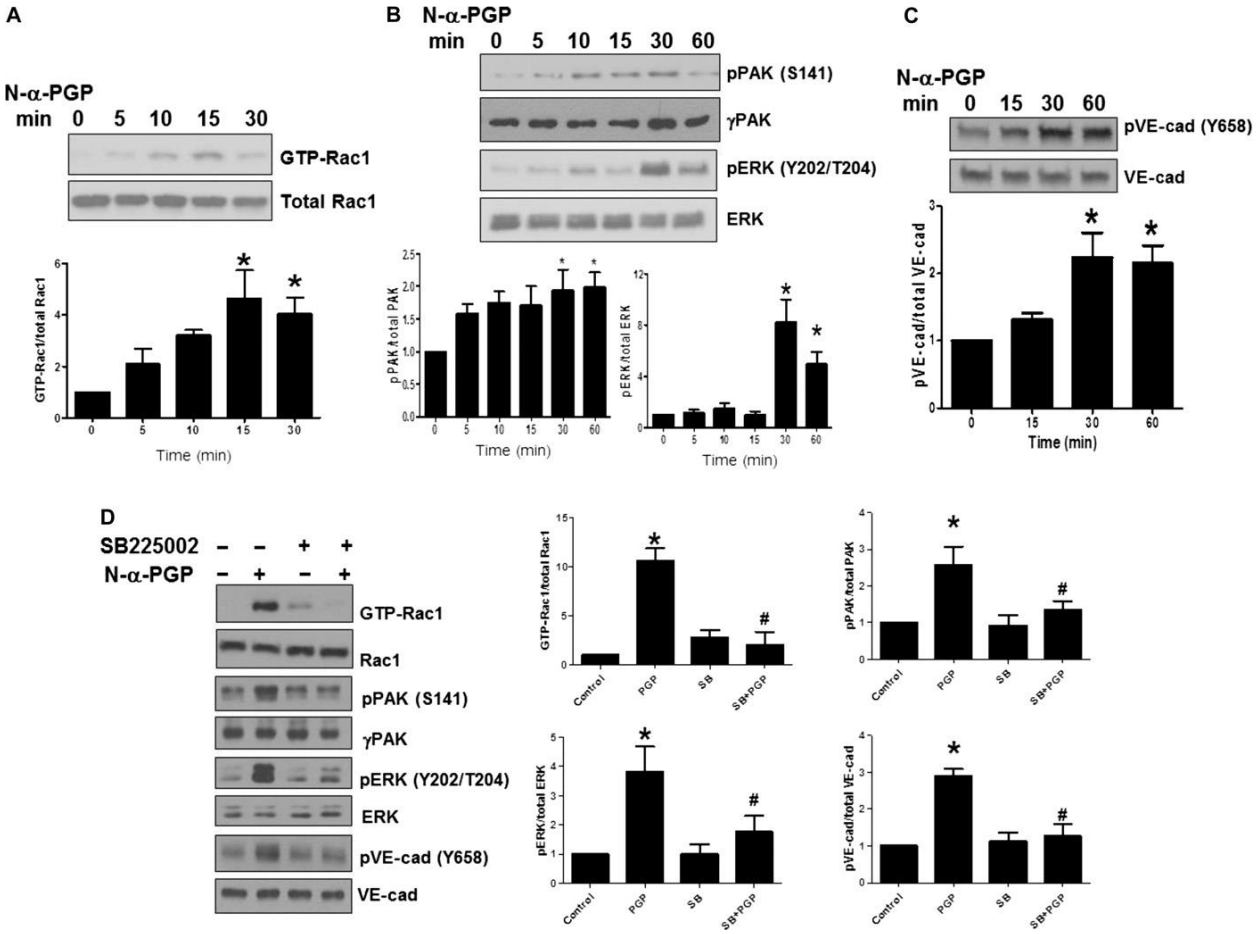

Figure 1. AcPGP activates endothelial cell signaling through CXCR2. *HUVECs were serum starved for two hours before stimulation with 0.5 mg/ml acPGP for indicated times and activation of Rac1 (**A**) and phosphorylation of PAK and ERK (**B**), and VE-Cadherin **(C)** were determined by Western blot. Shown are representative Western blots together with quantification. Bar graphs show mean ± SEM, n = 3 *P<0.05 relative to time 0 by 1-way ANOVA with Tukey post-test.* ***(D)*** *HUVEC were untreated or treated with 0.5 mg/ml AcPGP (30mins) alone or after pretreatment with 200 nM SB225002, and Rac1 activity and phosphorylation of ERK, PAK and VE-cadherin was determined by Western blot. Shown are representative Western blots together with quantification. Bar graphs show mean ± SEM, n = 3 *P<0.05 relative to time 0, #P<0.05 relative to PGP by 1-way ANOVA with Tukey post-test. N-α-PGP = acPGP.*

endothelial cells, human umbilical vein endothelial cells (HUVECs) were stimulated with acPGP for 0-60 min and Rac1 activation and p21 activated kinase (PAK), ERK, and VE-cadherin phosphorylation were assessed as each has been implicated in IL-8 induced, CXCR2 mediated, endothelial permeability (*17*). Figure 1A demonstrates that acPGP rapidly activated Rac1 as

determined by GST-PBD pull down assays. AcPGP increased phosphorylation of ERK and PAK (Fig. 1B), and subsequent phosphorylation of VE-cadhedrin (Fig. 1C). The latter is a major component of adherens junctions found specifically in endothelial cells and phosphorylation of which is associated with paracellular permeability both *in vivo* and *in vitro* (*18*). Stimulation of HUVEC with a control peptide, PGG, did not result in similar increases in Rac1 activation or ERK phosphorylation (Fig. S2) thus demonstrating the specificity of acPGP in activating endothelial cells.

AcPGP induces signaling through CXCR2. We, therefore, tested to see if a CXCR2 inhibitor, SB225002 (*19*), could abolish these signaling effects in endothelial cells. SB225002 inhibited acPGP induced Rac activation and similarly prevented PAK, ERK, and VE-cadherin phosphorylation (Fig. 1D). The ERK-related dependency of this pathway was further confirmed by the use of U0126, a highly selective inhibitor of both MEK1/2, to block ERK-activation (data not shown) and downstream phosphorylation of VE-cadherin after acPGP administration (Fig. S3). AcPGP-dependent stimulation of ERK, VE-cadherin and PAK phosphorylation was confirmed in pulmonary microvascular endothelial cells (PMVEC) also confirming a potential role of this signaling pathway in ARDS permeability mechanisms (Fig S4A-E)

AcPGP induces paracellular permeability in endothelial cells

To test whether AcPGP could induce monolayer permeability, primary HUVECs were left untreated or treated with acPGP, PGG, or VEGF as a positive control. As shown in Figure 2A, acPGP induced a significant increase in HRP leak across HUVEC monolayers whereas the control peptide, PGG had no effect compared to control. AcPGP-induced HRP leak was dependent on CXCR2 as demonstrated by the preservation of monolayer integrity with the addition of the

CXCR2-specific inhibitor SB225002 (Fig. 2B). This pro-permeability effect of acPGP was independently confirmed using the xPERT permeability assay (*20*). As seen in Figure 2C, acPGP induced a significant increase in the Alexa488 positive area demonstrating a loss of endothelial barrier integrity as compared to untreated cells; this permeability was prevented by SB225002 (Fig. 2D).

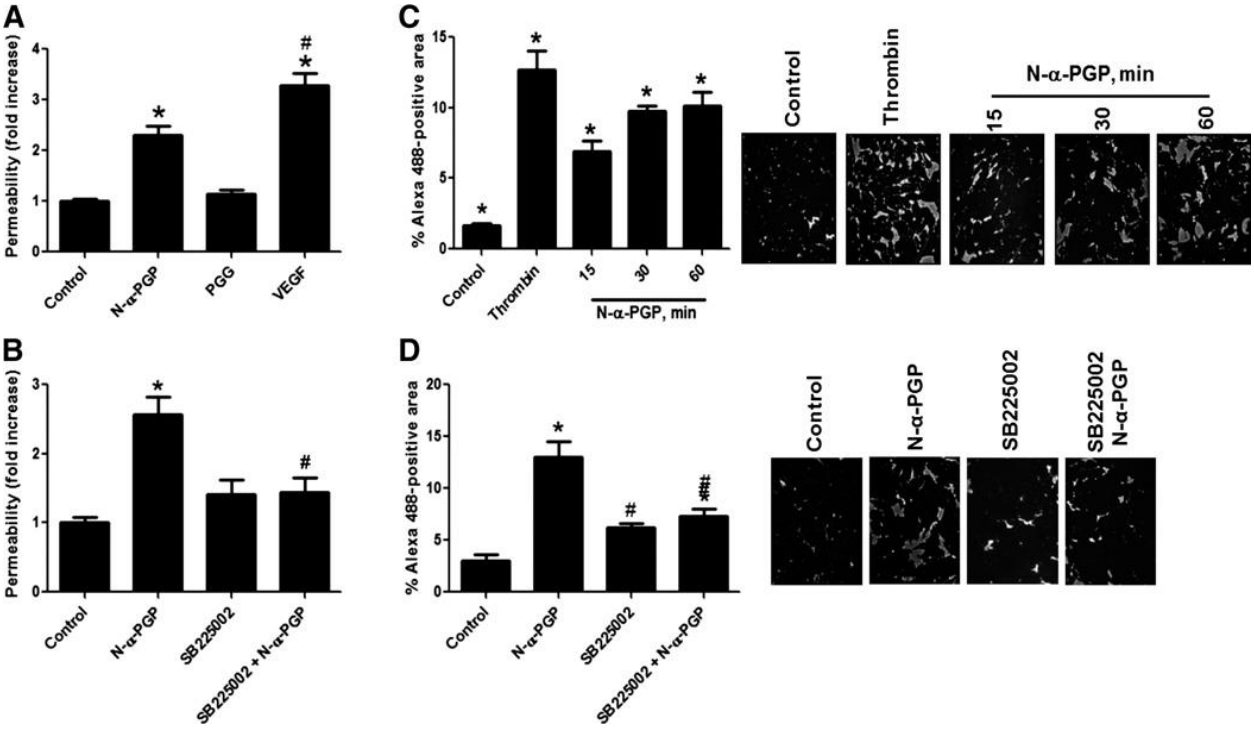

Figure 2. AcPGP induces endothelial permeability through CXCR2. ***(A)*** *HUVECs were grown on 3.0 micron transwell membranes and left untreated or stimulated with 0.5 mg/ml AcPGP , 0.5 mg/ml PGG, or 50 ng/ml VEGF for 30 min, and* ***(B)*** *HUVECs were cultured as before and some cells were pretreated with 200 nM SB225002 for 30 min prior to stimulation with 0.5 mg/ml acPGP. HRP leak from the upper chamber to the lower chamber was measured as described in methods.* ***(C)*** *As a separate readout of permeability, HUVECs were grown on biotinylated collagen and stimulated with 0.5 mg/ml acPGP for 0-60 min or with 0.2 U/ml thrombin for 10 min and monolayer leak was determined by staining with streptavidin Alexa488.* ***(D)*** *HUVECs were treated with 0.5 mg/ml acPGP with or without 200 nM SB225002 for 30 min with streptavidin Alexa488 staining *p<0.05 vs. control; # p<0.05 vs. acPGP. Data are mean ± SEM, n=6 for panels A-B, and n=3 for panel C-D. N-α-PGP = acPGP.*

Similarly, acPGP increased permeability in PMVEC in a dose-dependent manner and was inhibited by SB225002 (Fig S4 F-G). These results mechanistically link acPGP-mediated permeability signaling with a notable phenotype of paracellular permeability.

Interestingly, acPGP treatment did not increase phosphorylation of the p65 subunit of NFκB, ICAM-1/VCAM-1 expression, or monocyte adhesion whereas Tumor Necrosis Factor alpha (TNF-α) induced potent increases in all of these readouts (Fig. S5). Moreover, acPGP had no effect on pro-inflammatory cytokine release from HUVEC or PMVEC (Fig S5). These data indicate the effects of acPGP on endothelial cells are selective for permeability-related pathways and are not of a broad inflammatory nature.

AcPGP induces permeability in vivo

To determine if acPGP induced local permeability *in vivo,* the Miles assay was utilized as a model (*17*). C57BL/6 mice were injected via the tail vein with Evans blue and followed by abdominal subcutaneous injection with either PBS alone or PBS containing acPGP, PGG, or VEGF. As seen by the representative images in Figure 3A, both acPGP and VEGF significantly increased Evans blue leak to skin tissue whereas PGG had no effect above PBS control. Total Evans blue per gram of tissue was quantified and confirmed that acPGP treatment induced increased leak (Fig. 3B). Next we wanted to determine if *in vivo* administration of acPGP could induce similar protein phosphorylation as seen in HUVEC. Mice were again administered with PBS alone or with acPGP, PGG, or VEGF. Western blot analysis of skin tissue lysates for VE-Cadherin phosphorylation (as an endothelial cell specific readout) indicated both acPGP and VEGF increased phosphorylation above levels of PBS or control peptide (Fig. 3C). These data confirm that acPGP can induce local vascular permeability and endothelial cell activation *in vivo*.

While local administration of acPGP induces the endothelial changes outlined above, the effects of short-term systemic administration of acPGP on lung vascular permeability are unknown. We addressed this question by administering saline, acPGP, PGG, or LPS for 4 days intraperitoneally (i.p.) to mice and analyzing bronchoalveolar lavage (BAL) fluid. Utilizing measurement of IgM levels, a sensitive marker of vascular leak in the lung (*21*), acPGP significantly increased IgM levels in BAL relative to saline or PGG control and comparable to that observed after treatment with LPS, a known regulator of pulmonary vascular leak (*22*) (Fig. 3D).

AcPGP had no effect on BAL cell count suggesting no pro-inflammatory response, consistent with our observations described above (Fig. S5). Moreover, the ratio of phosphorylated VE-Cadherin to total VE-Cadherin in lung homogenates was increased over control in mice treated with acPGP, highlighting lung endothelial cell activation *in vivo* (Fig. 3F). Overall, these data demonstrate that acPGP activates pro-permeability pathways and endothelial leak independent of its role as a neutrophil chemoattractant *in vivo*.

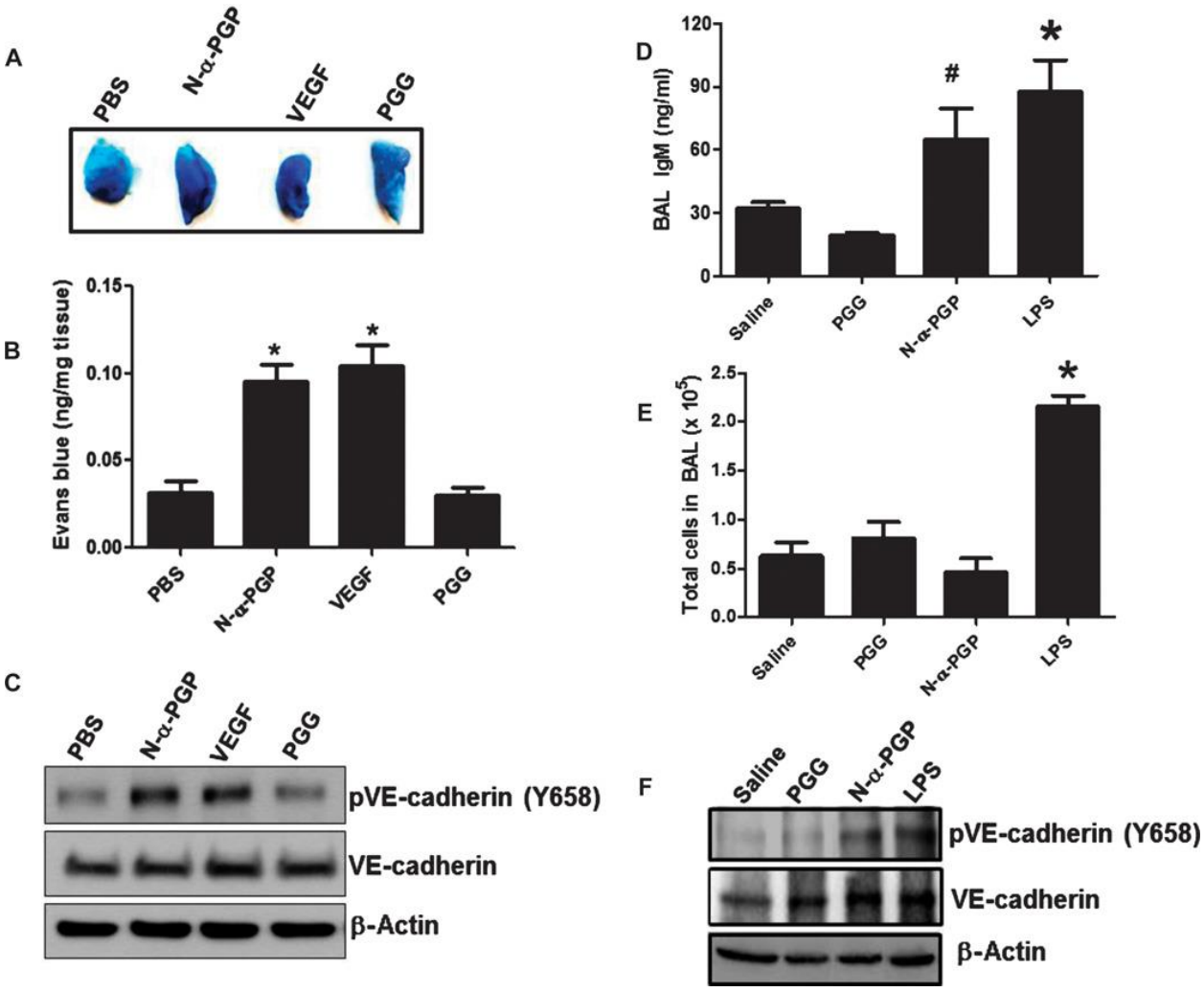

Figure 3. AcPGP induces skin and pulmonary microvascular permeability. ***(A-C)*** *Mice (n=4-5 per group) were injected via the tail vein with Evans blue and then received abdominal subcutaneous injection of PBS alone, acPGP (250 μg), PGG (250 μg), or VEGF (50 ng). Evans blue leak to the skin tissue was visually assessed (**A**) and quantified (**B**) and VE-Cadherin phosphorylation after treatment was determined by Western blot, representative image (**C**) *p<0.05 vs. PBS control by 1-way ANOVA with Tukey post-test.* ***(D-F)*** *Mice (n=4-5 per group) were intraperitoneally injected with saline alone or containing acPGP (250 μg), PGG (250 μg), LPS (75 μg) once a day for four days and then the total IgM in BAL fluid was measured by immunoassay (**D**), total BAL cell (**E**), and VE-Cadherin phosphorylation in lung lysate measured by Western blot (**F**) *p<0.05 vs. saline or PGG, $^{\#}$p<0.05 vs. PGG by 1-way ANOVA with Tukey's multiple comparison post-test. All values represent mean ± SEM. N-α-PGP = acPGP.*

AcPGP acts as a critical regulator in LPS-mediated lung injury

Recent data has demonstrated that endothelial cell expression of CXCR2 is required for vascular leak in an LPS-induced model of acute lung injury (*23*). CXCR2 does not act as a ligand for LPS, suggesting other mediators are involved in the mechanism of CXCR2-dependent vascular permeability in this model. To determine if acPGP is a critical regulator for LPS-mediated endothelial permeability, mice were administered LPS intraperitoneally every day for four days with or without RTR (arginine-threonine-arginine) peptide, which sequesters and inhibits PGP-containing peptides (*14*). Figure 4A shows that LPS treatment increased serum PGP peptides, which is ameliorated by RTR. Mice were injected with Evans blue and lungs were isolated to assess for vascular leak. Mice that were concomitantly treated with RTR and LPS demonstrated gross attenuation of Evans blue in the lungs as compared to LPS alone (Fig. 4B) which was found to be comparable to that observed in saline treated mice (Fig. 4B). RTR treatment also inhibited IgM accumulation in the BAL and decreased VE-cadherin phosphorylation compared to lungs from mice treated with LPS alone (Fig. 4C-D). Overall, these studies demonstrate the capability of acPGP to act as a regulator of endothelial permeability *in vivo* and its relative importance in LPS-mediated lung injury.

PGP peptides participate in endothelial cell dysfunction observed in ARDS

ARDS is a complex disease characterized by increased lung vascular permeability and altered systemic inflammatory response (*24*). We sought to recapitulate our *in vitro* and *in vivo* animal findings in human disease. Plasma was collected from intubated subjects with ARDS secondary to gram negative sepsis and intubated subjects without lung disease. Plasma samples were collected within 24 hours of intubation and PGP peptide values were measured. Patient

demographics for the two groups are shown in Table 1 with differences noted between groups for gender, arterial O_2 to inspired oxygen (PaO_2/FIO_2) ratio, Acute Physiology and Chronic Health Evaluation (APACHE) II score, and 30-day all-cause mortality. Figure 5A shows that total PGP peptide levels were higher in ARDS specimens compared to non-lung disease controls (0.53 ± 0.14 and 0.14 ± 0.08 ng/ml respectively), with acPGP comprising between 5-10% of total PGP peptide levels. Fig 5B shows that the acPGP dose dependent stimulation of PMVEC permeability; with 1ng/ml causing a ~2-fold increase relative to control. Fig 5B inset shows that both acPGP and PGP (both at 1ng/ml) elicit similar increases in PMVEC permeability. ARDS vs. non-lung disease plasma samples (n=3 per group) were then pooled, added to HUVECs, and induction of phospho-VE-Cadherin was assessed. As observed in Figure 5C, ARDS plasma induces a notable increase in the ratio of phosphorylated VE-Cadherin to total VE-Cadherin compared to non-lung disease plasma as early as 15 minutes after exposure, consistent with previous *in vitro* stimulation experiments. Utilizing this time point, we next compared the phosphorylated VE-Cadherin/total VE-Cadherin ratio between individual plasma samples incubated with RTR to the same plasma samples treated with RTR vehicle. Figure 5D and 5E highlight a significant reduction in the phosphorylation of VE-Cadherin with RTR co-incubation with ARDS plasma, with levels comparable to non-stimulated endothelial cells. Of note, non-lung disease plasma demonstrated no notable increase in phosphorylated VE-Cadherin/total VE-Cadherin ratio compared to control (Fig. 5C).

We next wanted to determine whether RTR could reduce permeability associated with inflammatory factors, principally PGP peptides, in plasma of ARDS patients. To better understand the dynamics of how this may occur, we used a technique to measure cellular impedance, a measurement of junctional integrity, for these assays.

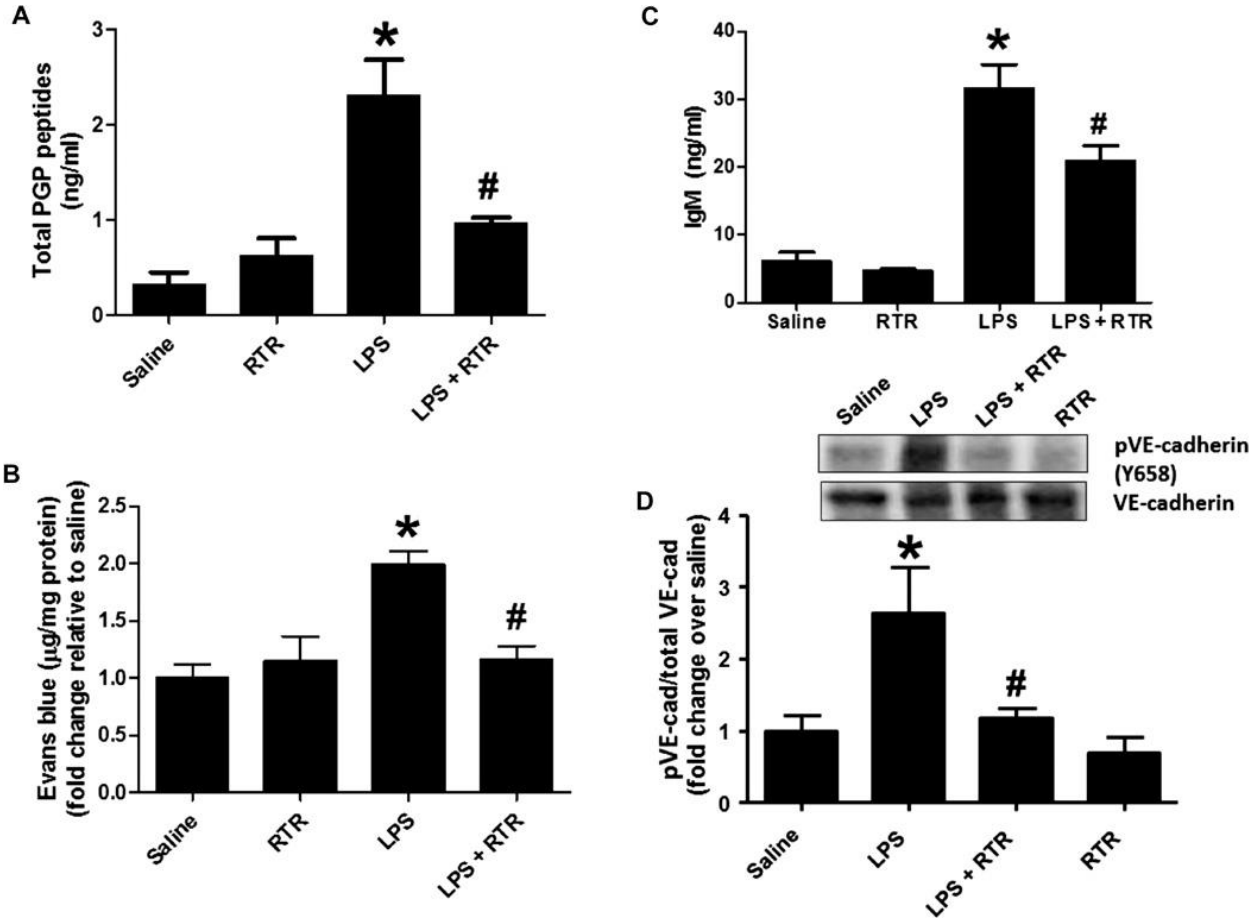

Figure 4. RTR attenuates LPS-induced pulmonary microvascular permeability. *Mice were injected via the tail vein with 50 µl PBS alone or containing 50 µg RTR and then intraperitoneally administered with 75 µg LPS (in 100µl PBS) once a day. After four days treatment, mice were sacrificed for serum measurements of PGP (n=6)* ***(A)*** *or injected via the tail vein with Evans blue. Evans blue leak to the lung was quantified and normalized to protein; data show fold change relative to saline (n=7-11) (**B**). IgM levels were measured in the BAL, n=4-6 (**C**). VE-Cadherin phosphorylation in lung homogenates was assessed by Western blot, representative image (**D**). *p<0.05 vs. saline control or #p<0.05 relative to LPS for panels A-C by 1-way ANOVA with Tukey post-test and #p<0.05 relative to LPS by t-test for Panel D. All values represent means ± SEM.*

HUVECs were serum starved before being treated with RTR, plasma from ARDS patients, or plasma that had been incubated for 1 hour with RTR prior to addition to the cells for 30 minutes. Plasma from ARDS subjects significantly reduced impedance (presented as relative cell index) relative to cells alone indicating an increase in permeability (Fig 5F).

Table 1. Demographics for ARDS and non-lung disease ICU patients

Group	**Age**	**Sex**	**Race**	**PaO_2/FlO_2**	**APACHE II score**	**30-Day Mortality**
ARDS (n=6)	59.5 (6.8)	50% Male 50% Female	66% White 34% Black	149.5 (60)	25.5 (2.6)	66%
Non-lung disease (n=6)	46.1 (9.7)	66% Male 34% Female	66% White 34% Black	469.2 (80)	11.0 (4.9)	0%
p-value (between groups)	<0.05	ns	ns	<0.05	<0.05	<0.05

Legend: *Values represent mean (± SD). All ARDS subjects were secondary to documented gram negative sepsis and intubated due to respiratory failure. Non lung disease control subjects were intubated for various reasons unrelated to respiratory failure (one for airway protection secondary to GI bleed, two for metabolic acidosis secondary to DKA, two for overdose, one for airway protection s/p CVA)* ***Key****:* ***ARDS****, Acute respiratory distress syndrome;* ***$PaO_2/FI0_2$****, partial pressure arterial oxygen/fractional inspired oxygen****;* ***APACHE:*** *acute physiology and chronic health evaluation;* ***ns****, no significant difference.*

When the ARDS plasma had been preincubated with RTR to inactivate PGP peptides, there was a significant attenuation of impedance reduction (Fig 5G), which was sustained for over an hour. Overall, these results highlight the differences in the capacity of ARDS plasma vs. non-lung disease plasma in activating permeability-related signaling in endothelial cells and the ability of RTR to block this activation and modulate endothelial permeability observed in ARDS.

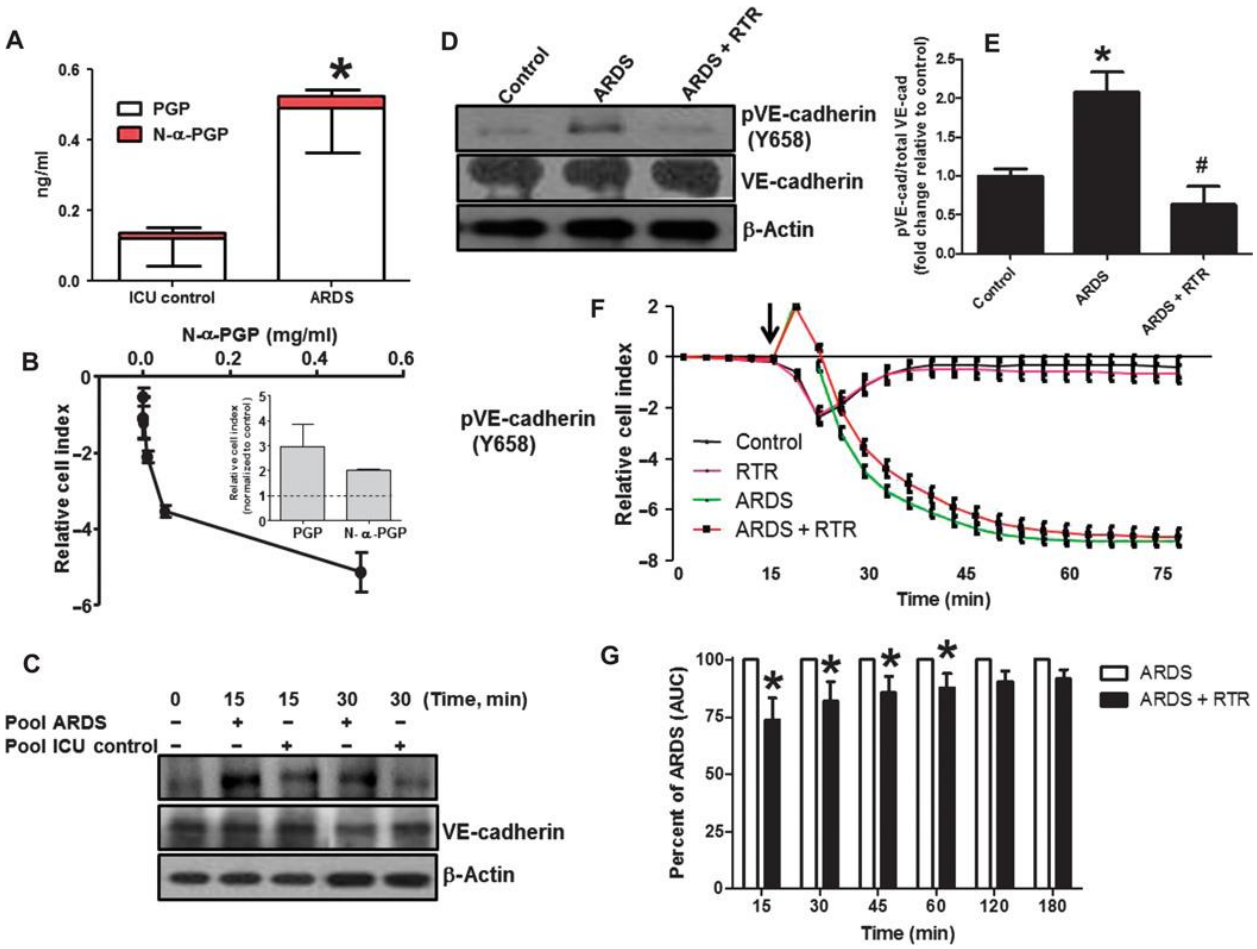

Figure 5. ARDS plasma induces endothelial activation which is attenuated by RTR. *Plasma was collected from patients with ARDS and normal control, and plasma PGP and acPGP levels measured via ESI-LC-MS/MS* ***(A)*** **p<0.05 by t-test between ARDS and normal for all PGP peptides, N=6. PMVEC were treated with acPGP and permeability assessed (see supplementary Fig 4F).* ***(B)*** *shows maximal changes in relative cell index as a function of NαPGP concentrations. Inset compares the effects of acPGP and PGP (both at 1ng/ml).* ***(C)*** *After two hours serum starvation, HUVEC were treated with pooled ARDS (from 3 patients) or non-lung disease ICU patients (n=3) plasma, at two different time points and VE-Cadherin phosphorylation measured by Western blot. Shown is a representative image* ***(C).*** *HUVEC were treated as above for 15min with plasma collected from individual ARDS with or without RTR (30µg/ml) and changes in VE-Cadherin phosphorylation assessed. Shown is a representative Western blot* ***(D)*** *and quantitation* ***(E)*** *******P<0.05 relative to control, #P<0.05 relative to ARDS by 1-way ANOVA with Tukey post-test, N=3. Measurements of cellular impedance were made in HUVECs over 75 min with ARDS plasma vs. ARDS plasma + RTR. Panel shows representative traces from 1 patient with 4 intraexperimental replicates. Arrow denotes addition of plasma* ***(F).*** *Percent attenuation by RTR on ARDS plasma time-dependent permeability changes* ***(G).*** **p<0.05 by 2-way RM-ANOVA with Bonferroni post-test. All values represent means ± SEM, N=3. N-α-PGP = acPGP.*

Discussion

The present data demonstrate that the CXCR2 agonist matrikine acPGP induces endothelial permeability. Multiple reports have now demonstrated that acPGP is increased in a variety of inflammatory conditions (*10, 11, 18, 25*). These studies have focused solely on the role of acPGP as a neutrophil chemoattractant and have not addressed other potential physiological functions. Importantly, each disease model studied is associated not only with neutrophilic injury but also increased endothelial permeability, suggesting the possibility that acPGP also directly regulates vascular function. Our findings provide the first evidence for circulating matrix fragments as biologic effectors of vascular endothelial function and, more specifically, demonstrate that acPGP selectively increases vascular permeability via CXCR2 dependent signaling. Interestingly, the downstream signaling induced by acPGP is similar to other pro-angiogenic factors that are associated with disruption of monolayer permeability that also fail to induce a broad inflammatory response (*26*). We focused on the Rac1-PAK-ERK signaling axis here, which is well-established in mediating permeability (*17, 26*); the potential for acPGP to mediate effects via additional signaling mediators downstream of CXCR2 (e.g. p38 MAPK) remain to be tested.

Published *in vitro* studies have suggested that CXCR2 signaling may play important roles in endothelial function including activation of small GTPase (*17, 27*), increase proliferation and migration (*28, 29*), promotion of angiogenesis (*16, 30*), and induction of monolayer permeability (*17, 31, 32*). However, to our knowledge, the current findings are the first to link a single CXCR2 ligand to endothelial permeability in cell culture and in animal models of disease. Moreover, data suggesting pro-permeability functions of PGP peptides in human plasma from ARDS patients highlights the potential clinical impact of this peptide in human disease as a therapeutic target.

Our *in vivo* studies clearly demonstrate that local administration of acPGP induces vascular leak and that systemic administration can lead to increased lung permeability similar to that observed with LPS administration. The importance of this signaling is highlighted by the prevention of LPS-mediated endothelial permeability through acPGP inhibition and suggests a role for this peptide as a novel effector of endotoxin induced injury. LPS has been previously shown to induce the extracellular activity and expression of numerous proteases implicated in PGP peptide generation (*3*, *6*), strongly suggesting a feed-forward inflammatory system which may serve as a critical target in sepsis and sepsis-related sequelae such as ARDS.

We also demonstrate increased PGP peptides in ARDS patient plasma. AcPGP was lower compared to PGP, which may reflect the relative activity of pathways leading to peptide acetylation versus PGP breakdown. We note that the ratio between acPGP and PGP varies in different acute and chronic disease states (*3, 13, 33*); understanding this balance in sepsis and ARDS is the subject of ongoing studies. We note the limited sample size for these measurements and that it is possible that additional confounding parameters due to the heterogeneity of ARDS pathogenesis could impact these data. However, these data do provide initial evidence as proof-of-concept that bioactive PGP-peptides serve as potential targets in treating disease-related vascular permeability. The implication of acPGP as a regulator of endothelial dysfunction in the plasma of patients with ARDS is of particular importance as recent evidence has coupled the amount of extravascular lung water as an independent predictor of mortality in the condition (*34*). Although previous interest in reducing alveolar lung water has focused on mobilizing fluid from the alveolar space after leak is established (*35*), there remains a need to target the dysfunctional

endothelial paracellular leak from the vasculature. RTR is a tetramerized hydropathic peptide targeting both PGP and acPGP. It has been tested in animal models in which PGP is operative with inhibition of neutrophilic influx demonstrated (*14*). Our results present the first example of RTR targeting endothelial permeability *in vivo*, which suggests a role for endogenous bioactive PGP-containing peptides in mediating lung fluid accumulation in ARDS. Since RTR inhibits all PGP-containing peptides, future studies are needed to determine the relative contribution of PGP versus acPGP in endothelial permeability, and the potential effect of microenvironment in regulating PGP-peptide bioactivity. However, we note that both PGP and acPGP at levels similar to that observed *in vivo*, stimulated endothelial permeability (Fig 5B). Combined with RTR-dependent inhibition of permeability *in vivo* and of human disease samples *ex vivo*, these data underscore the potential that targeting PGP-peptides may inhibit two key facets in the pathogenesis of ARDS: ongoing neutrophilic inflammation (*6*) and endothelial permeability.

Bioactive PGP peptides represent the first matrix-derived breakdown product found to be increased systemically in human inflammatory diseases that can modify vascular function. This finding is particularly interesting as these peptides represents a unique CXCR2 agonist which is regulated by direct tissue damage and not by transcriptional regulation (such as ELR^+ CXC chemokines) and may therefore induce a feed-forward mechanism of ongoing vascular leak (*36*). It will be necessary to examine acPGP levels as a potential regulator of progression in other diseases associated with matrix turnover and vascular dysfunction such as tissue fibrosis, ischemia-reperfusion injury, pulmonary vascular disease, kidney injury, and myocardial infarction. To conclude, we have identified a novel role for acPGP as an activator of endothelial cell signaling which actively couples vascular permeability and tissue injury. This peptide seems to be critically

important in the endothelial activation associated with ARDS and may serve as a novel therapeutic target in this disease.

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Grants

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Supplementary figures

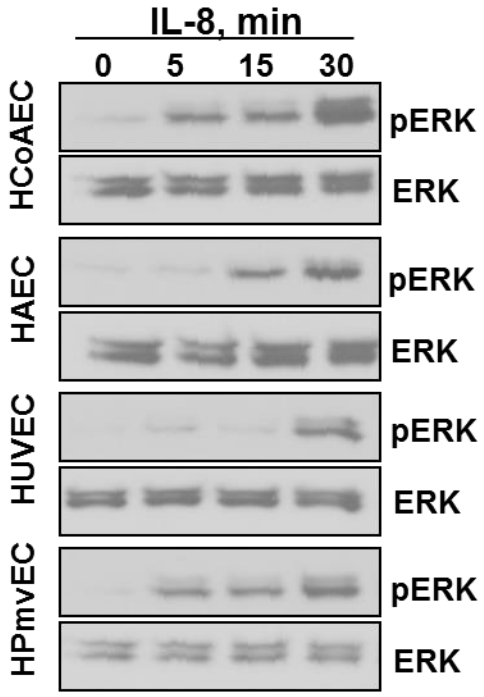

Figure S1. Multiple endothelial cells demonstrate ERK phosphorylation with IL-8 stimulation. *HUVEC, pulmonary microvascular (HPmvEC), coronary artery (HCoAEC), and aortic (HAEC) endothelial cells were grown to confluence, serum starved for two hours, and then stimulated with100 ng/ml IL-8 for 0-30 min. pERK was assessed by western blot analysis. Representative results from three independent experiments are shown.*

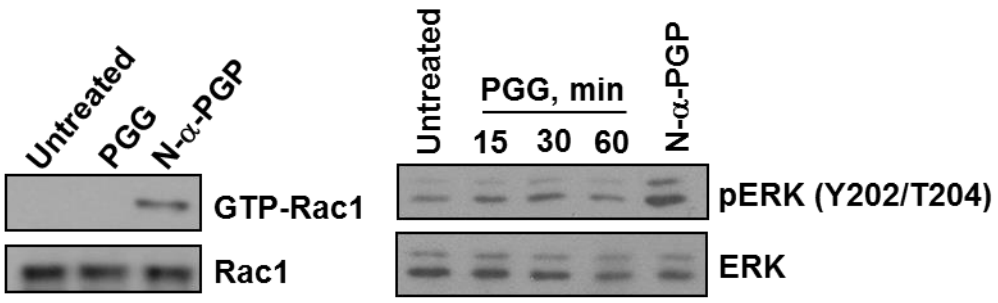

Figure S2. PGG stimulation does not activate HUVEC cells. *HUVECs were serum starved for two hours before stimulation with 0.5 mg/ml PGG for 0-60 min or with 0.5 mg/ml acPGP for 60 min and activation of Rac1(GTP-Rac1)* ***(A)*** *and phosphorylation of ERK* ***(B)*** *were determined by Western blot. N-α-PGP = acPGP.*

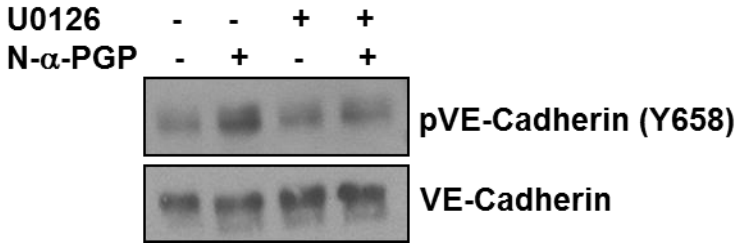

Figure S3. Blockade of ERK mitigates AcPGP-mediated VE-Cadherin activation in endothelial cells. *HUVECs were untreated or treated with 0.5 mg/ml acPGP alone or after pretreatment with 10 μM U0126 and phosphorylation of VE-Cadherin were determined by Western blot. N-α-PGP = acPGP.*

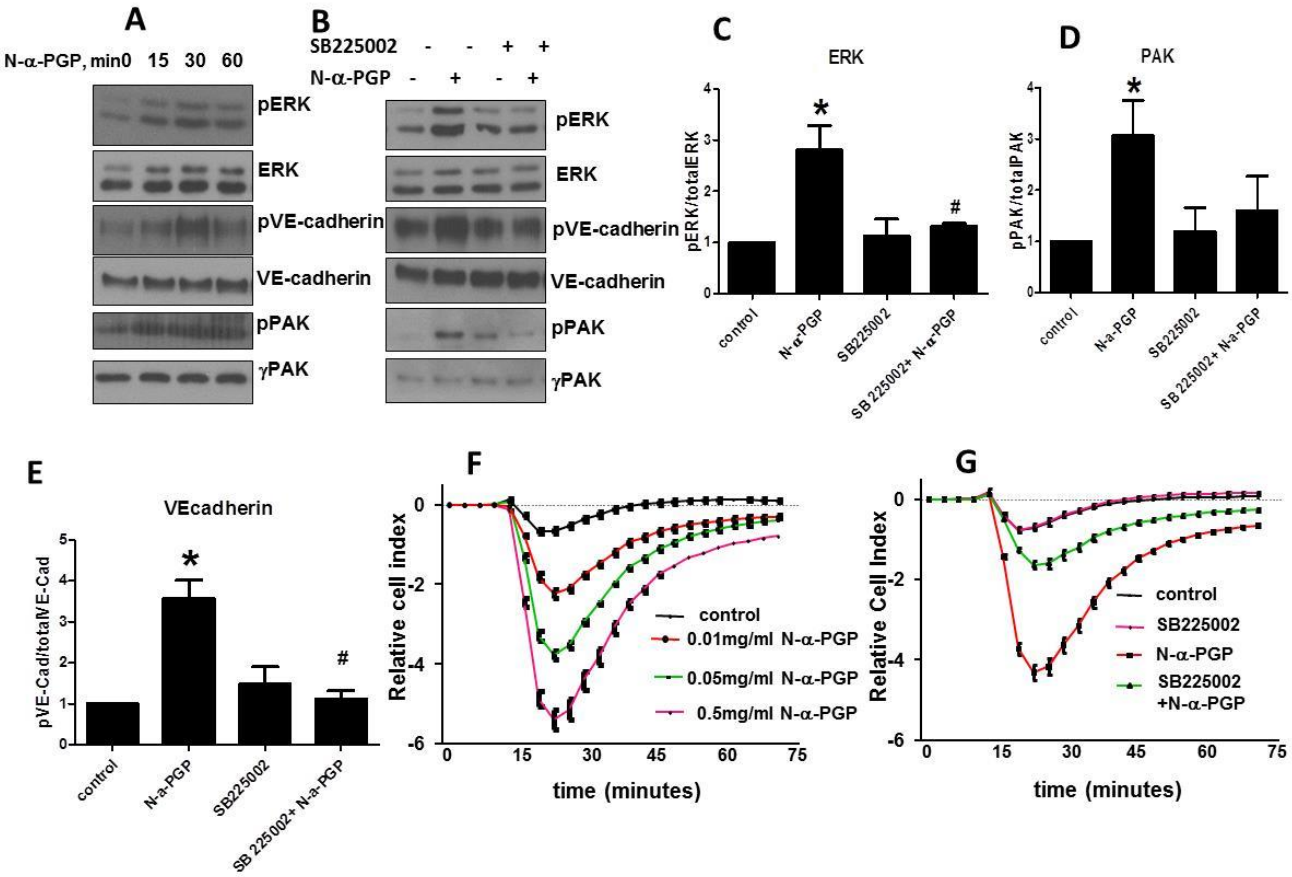

Figure S4: Effects of acPGP on PMVEC permeability. ***(A)*** *PMVECs were serum starved for two hours before stimulation with 0.05mg/ml acPGP and time-dependent activation of ERK, VE-cadherin and PAK determined.* ***(B-E)*** *AcPGP-dependent activation of ERK, VE-cadherin and PAK was determined at 15min in the presence of absence of SB225002. Shown are representative Western blots together with quantification. Bar graphs show mean ± SEM, n = 3 *P<0.05 relative to time 0 by 1-way ANOVA with Tukey post-test. Shown are representative Western blots together with quantification. Bar graphs show mean ± SEM, n = 3 *P<0.05 relative to control, #P<0.05 relative to acPGP by 1-way ANOVA with Tukey post-test. Changes in permeability in response to different doses of acPGP peptide* ***(F)*** *and in the presence or absence of SB225002* ***(G)****. Graphs present mean ± SEM from representative experiments. N-α-PGP = acPGP.*

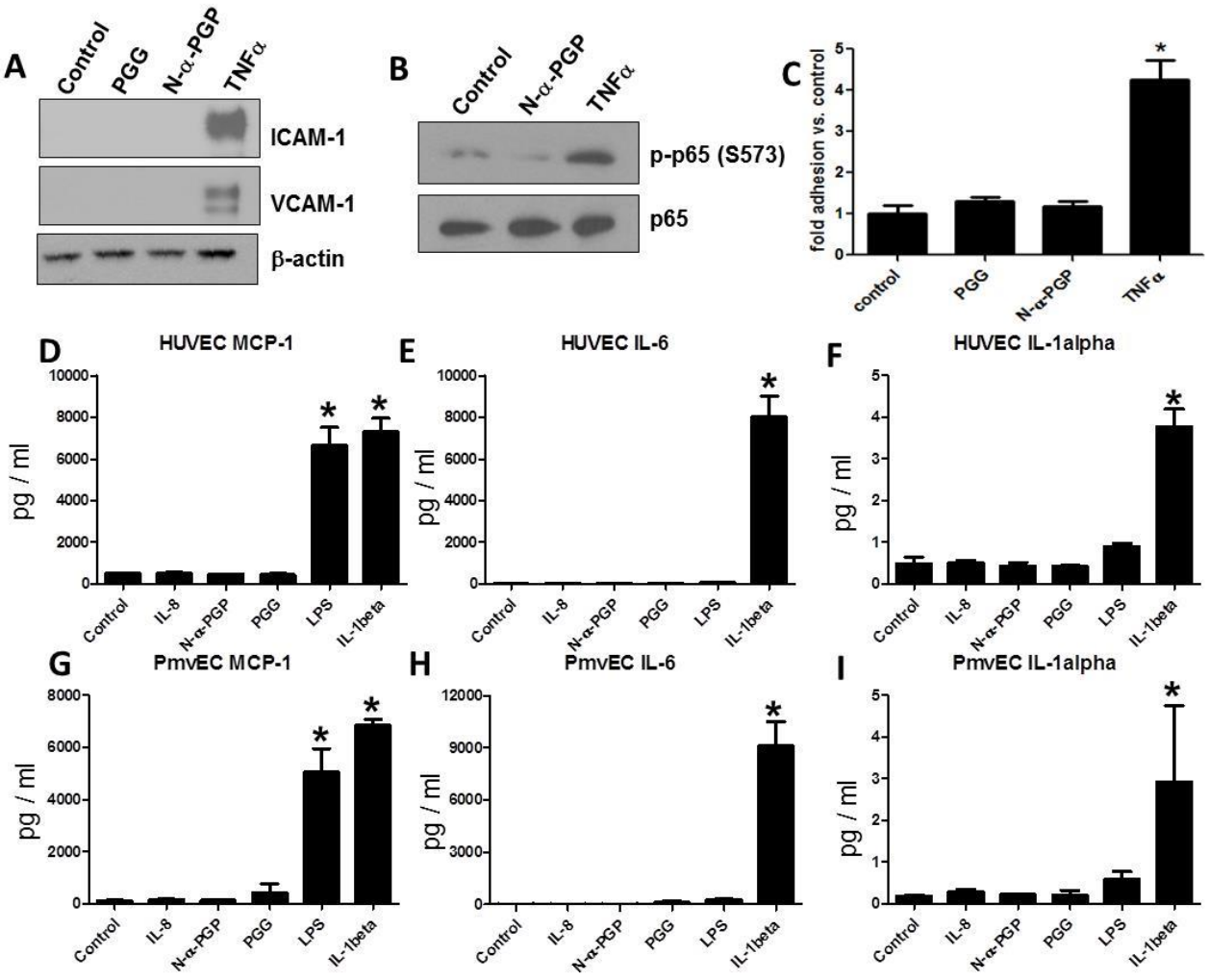

Figure S5. AcPGP does not induce pro-inflammatory signaling in endothelial cells. *HUVEC were untreated or treated with 0.5 mg/ml acPGP, 0.5 mg/ml PGG control peptide, or 10 ng/ml TNF**α** for six hours. Lysates were collected and Western blot analysis was conducted for ICAM-1, VCAM-1 and b-actin **(A)** and for phosphorylation of p65 **(B)**. Cells were treated as described above and their ability to support THP-1 monocyte adhesion was determined **(C)**. Blots are representative of three separate experiments and THP-1 adhesion is representative of two separate experiments with 4-6 replicates per condition in each experiment. HUVEC **(D-F)** or PMVEC **(G-I)** were treated with IL8 (100ng/ml), acPGP (0.05mg/ml), PGG (0.1mg/ml), LPS (100ng/ml) or IL1β (1ng/ml) for 12h and indicated cytokines measured. Data shown mean ± SEM (n=3), *P<0.05 relative to control by 1-way ANOVA with Tukey post-test. N-α-PGP = acPGP.*

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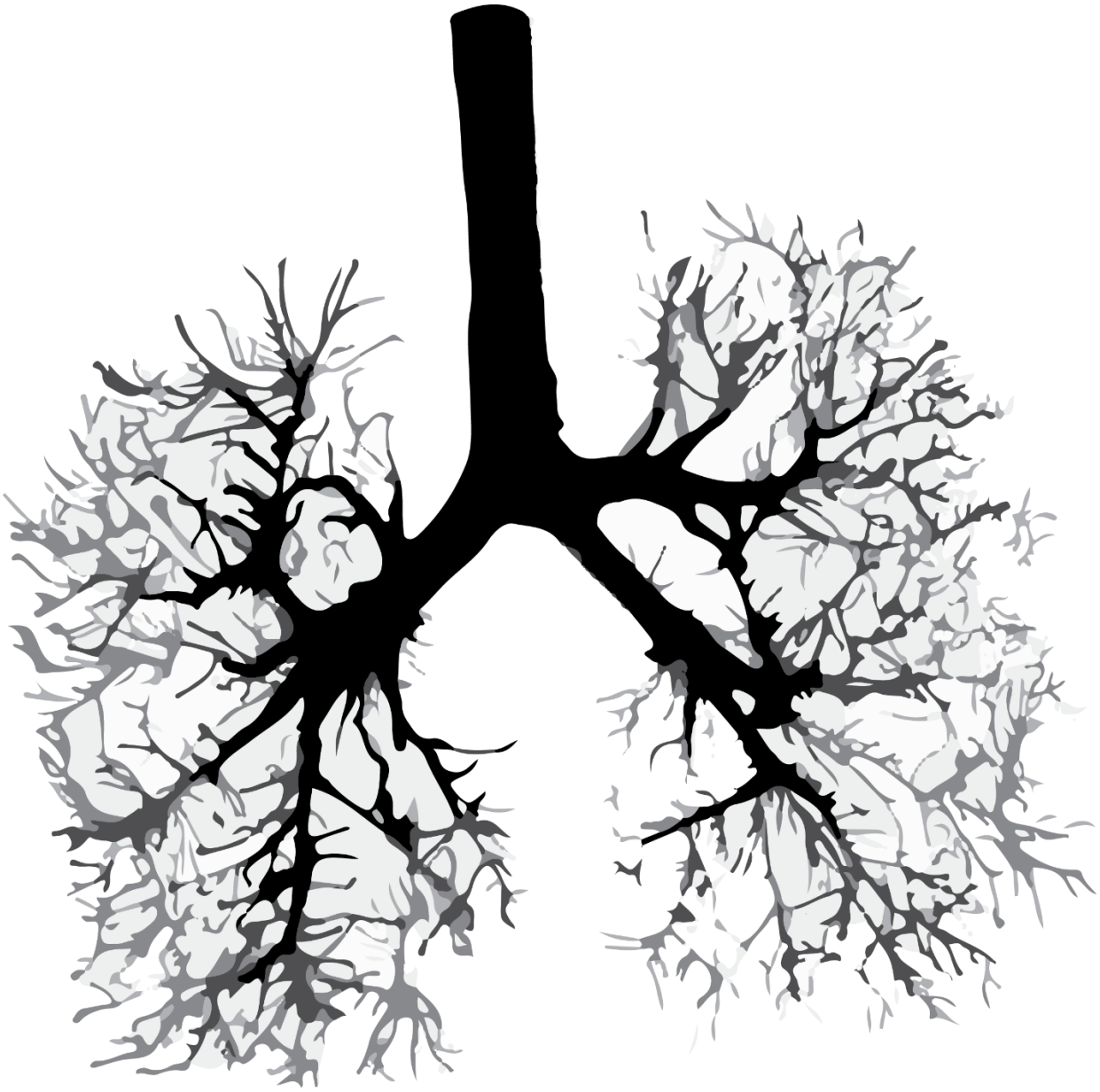
Chapter six



Targeting the matrikine PGP prevents right ventricular hypertrophy development in a cigarette smoke murine model

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Abstract

Patients with chronic obstructive pulmonary disease (COPD) often develop cardiovascular diseases such as pulmonary hypertension and right ventricular hypertrophy (RVH). Ongoing neutrophilic recruitment is a major driver of the inflammatory process in COPD. The matrikine proline-glycine-proline (PGP) is generated in a sequential fashion through the activities of matrix metalloproteinases and the serine protease, prolyl endopeptidase (PE), on collagen and plays an important role in neutrophilic influx. In this study, we investigate the development of RVH in a subchronic murine smoking model using the PE inhibitor benzyloxycarbonyl-proline-prolinal (ZPP) as treatment. Mice exposed to cigarette smoke for 6 weeks developed inflammation in the lungs and RVH. Via echocardiography, a significant increase of pulmonary artery pressure and right ventricular systolic pressure (RVSP) was calculated using the pulmonary artery acceleration time/pulmonary artery ejection time ratio. In blood, the more potent form of PGP, acetylated PGP (acPGP) was significantly increased after smoke exposure. Nitrite and nitrate levels in blood were measured and the calculated ratio was elevated after smoke exposure as well. Treating the mice with ZPP intratracheally prior to cigarette smoke exposure significantly decreased the inflammatory process in the lungs and prevented the mice from developing RVH or an increase in pulmonary artery pressure and RVSP. Furthermore, the nitrite/nitrate ratio maintained basal levels, suggesting less oxidative stress. This manuscript demonstrates that targeting of PGP peptides in a smoke model impacts the development of RVH. These results highlight the pleotropic effects of these bioactive collagen fragment in the development of a COPD phenotype.

Introduction

Chronic obstructive pulmonary disease (COPD) is a lung condition characterized by progressive and not fully reversible airflow limitation. Moreover, the lungs show an abnormal inflammatory response to the noxious particles or gases such as cigarette smoke (1). The peripheral and central airways undergo cellular and structural changes as a result of ongoing inflammation that may spread to the lung parenchyma, pulmonary arteries and peripheral tissues (2). Over time, patients with COPD also suffer from systemic inflammation which results in the development of comorbidities such as osteoporosis, diabetes and cardiovascular (CV) disease (3). Although CV disease is frequently associated with COPD, the exact mechanisms connecting COPD to CV diseases are not fully known (1). In addition to atherosclerotic disease, there is also associated right-sided heart failure. This is associated with increased blood pressure in the pulmonary arteries (pulmonary hypertension) due to narrowing of the blood vessels. This leads to excess strain on the right ventricle and the development of right ventricle hypertrophy (RVH) over time (4).

Ongoing neutrophilic recruitment is a major driver of the inflammatory process in COPD. Previously we reported that the matrikine proline-glycine-proline (PGP) plays an important role in neutrophilic chemotaxis through CXC receptor (CXCR) 1 and 2. This is due to structural homology with ELR^+ CXC chemokines such as interleukin-8 (IL-8) (5, 6). The acetylated form of PGP (acPGP) is the more potent form of the peptide. PGP is generated in a sequential fashion through the activities of matrix metalloproteinases and the serine protease prolyl endopeptidase (PE) on collagen (5).

In this study, we investigate the development of RVH in a subchronic murine cigarette smoke-induced inflammation model. We use benzyloxycarbonyl-proline-prolinal (ZPP), a highly specific

noncompetitive inhibitor of PE, as treatment (7). A possible link between CV disease and acPGP is described in this report.

Methods

Murine smoking model

The Institutional Laboratory Animal Care and Use Committee of UAB (Animal Protocol #120709133) has reviewed and approved the animal work conducted in this study. The murine work was conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (8).

Female BALB/c mice (8-10 weeks old) were exposed to room air or cigarette smoke 5 days/week for 6 consecutive weeks (n= 8-10 mice per group). Using the SCIREQ "InExpose" smoking system (SCIREQ, Montreal, QB, Canada), mice were whole body exposed to cigarette smoke using the same protocol as published before (9). In short, mice were exposed 60 min/day to mainstream cigarette smoke with standard University of Kentucky 3R4F research cigarettes (9.4mg tar/0.726mg nicotine, University of Kentucky). Via oropharyngeal aspiration under light isoflurane anesthesia, mice received either 2µg of ZPP (Enzo Life Sciences, USA) in 0.1% dimethylsulfoxide (DMSO) or vehicle (0.1% DMSO) 15 minutes prior to smoke or sham exposure.

Bronchoalveolar lavage

Sixteen hours after the last smoke exposure, mice were sacrificed by an intraperitoneal injection with an overdose of ketamine/xylazine mix. Using a tracheal cannula, the lungs of the mice were lavaged 4 times 1 ml PBS, pre-warmed at 37 °C. The bronchoalveolar lavage (BAL) fluid was

pooled and centrifuged at 4°C (400 g, 5 min) to collect the cells. Cells were counted and differentiated as described before (10, 11).

Right ventricular heart hypertrophy measurement

Murine hearts were collected and inspected for right ventricular hypertrophy development under 10x microscopic magnification. The right ventricle and the left ventricle plus septum were dissected and weighed after removal of the connected atria. The ratio of the weights was calculated as follows: (right ventricle)/(left ventricle + septum) (12).

Nitrate and nitrite in blood

Whole blood was collected from mice through cardiac puncture and aliquoted for several analysis techniques. Fifty microliter was added to 100ul nitrate (NO_3^-) and nitrite (NO_2^-) free ice cold methanol, vortexed for 7 seconds and immediately stored in liquid nitrogen. After thawing, samples were centrifuged at 800 *g* at 4°C for 10 min, and the supernatant was then assayed for nitrite and nitrate. The nitrite and nitrate ion concentrations in solution were measured by the combination of a diazocoupling method and high-performance liquid chromatography (ENO-20 NOx Analyser; EiCom, Kyoto, Japan). The method for NOx analysis has been previously described in detail (13).

Electrospray ionization-liquid chromatrography-mass spec/mass spec (ESI-LC-MS/MS)

AcPGP was measured in serum after a filtration step using 10kDa cut off filters (Millipore using a MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 spectrometer equipped with a Shimadzu HPLC (Columbia, MD). HPLC is conducted using a 2.0 x 150 mm Jupiter 4u Proteo

column (Phenomenex, Torrance, CA) with A: 0.1% HCOOH and B: MeCN + 0.1% HCOOH: 0 min-0.5 min 5% buffer B/95% buffer A, then increased over 0.5-2.5 min to 100% buffer B/0% buffer A. Background is removed by flushing with 100% isopropanol / 0.1% formic acid. Positive electrospray mass transitions were at 312–112,312–140 M/z for Ac-PGP and 270-70, 270-116, 270-173 for PGP. Area under the curve is measured, and PGP peptide concentration calculated using a relative standard curve method as previously described (14).

Echocardiography

After 6 weeks of exposure, mice were anesthetized using isofluorane (1–2%) and their body temperature was monitored and kept at 37 ^{0}C to maintain a constant heart rate of approximately 500 beats per minute. Analysis of Pulsed-Wave Doppler mode echocardiography was performed non-invasively using an echocardiography high resolution imaging system VEVO 770 equipped with a 30 MHz transducer (Visual Sonics, Toronto, Canada). The pulmonary artery was imaged to measure the pulmonary artery acceleration time (PAAT) and the pulmonary artery ejection time (PAET). The ratio of PAAT/PAET was calculated to estimate differences in pulmonary artery pressure between the different treatment and exposure groups.

The right ventricular systolic pressure (RVSP) was calculated using the equation RVSP mmHg = -83.7*(PAAT/PAET) + 64.5 as reported before (15).

Statistics

When appropriate, an unpaired t-test was used or analysis of variance followed by Newman-Keuls multiple comparisons test was carried out using GraphPad prism 6. $p \leq 0.05$ was considered significant.

Results

Leukocytes in the lung

Mice were exposed to cigarette smoke during a six week period. To investigate whether using the antagonist ZPP in a cigarette smoke exposure murine model affects the inflammatory process in the lungs, mice underwent BAL and the fluid was analyzed by cytospins. Mice developed an inflammatory reaction to cigarette smoke exposure, which was reflected by increased leukocytes numbers (Figure 1A). Mice treated with ZPP had significantly lower leukocyte infiltration. Macrophage and neutrophils infiltration was significantly decreased after ZPP treatment with a 52% and 69% reduction respectively in smoke-exposed mice (Figure 1B and 1C). Mice treated with ZPP and only exposed to air did not show any sign of inflammation in the lungs.

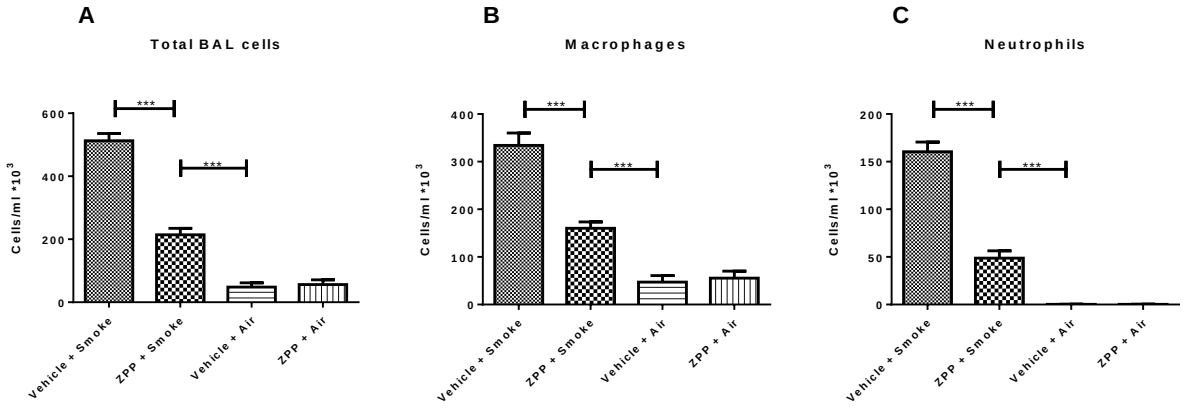

Figure 1. ZPP attenuates leukocytes influx into the lungs after smoke exposure. *Mice exposed to mainstream cigarette smoke during 6 weeks 5 days/week have increase leukocytes influx in their BAL fluid (A). This is due to increased macrophages (B) and neutrophils (C). Mice treated with ZPP showed significant lower cell numbers. N = 8-10 animals per group. Data shown as mean +/- S.E.M. ***P ≤ 0.001.*

Cardiovascular effects

Using ESI-LC-MS/MS, acPGP was quantified in serum. Mice exposed to cigarette smoke had a mean acPGP serum level of approximately 200 pg/ml. ZPP treated, cigarette smoke exposed mice had significantly lower acPGP levels in serum to 74pg/ml (Figure 2A). Furthermore, cigarette smoke exposed mice developed RVH after 6 weeks of smoke exposure, reflected in an increased mass ratio of right ventricle to the left ventricle and septum. Treatment with ZPP completely diminished this effect (Figure 2B).

Lastly, whole blood was analyzed for nitrite and nitrate to indirectly determine the effect of the treatment on the free radical nitric oxide. Nitrite and nitrate levels were measured and the ratio of nitrite/nitrate was calculated (Figure 2C). Cigarette smoke exposure resulted in a high nitrite/nitrate ratio, which was reduced to basal level after ZPP treatment.

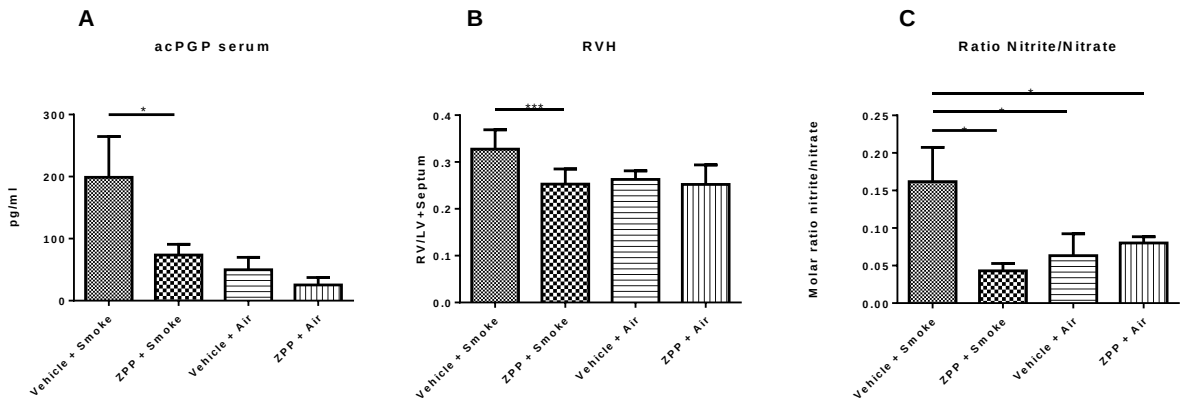

Figure 2. AcPGP, RVH and NOx are affected by ZPP. *Mice exposed to cigarette smoke during 6 weeks have increase acPGP levels in serum (A), which is in trend with the development of RVH (B) and an increased molar ratio of nitrite/nitrate in whole blood (C). ZPP treatment prevented all of the above. N = 8-10 animals per group. Data shown as mean +/- S.E.M. * P ≤ 0.5, *** P≤ 0.001. RVH, right ventricular hypertrophy.*

Using echocardiography, blood flow of the pulmonary artery was imaged and the average PAAT and PAET after three heartbeats was calculated (Figure 3A). A ratio of PAAT/PAET was calculated as an inverse indicator of the pulmonary artery pressure (16). Control mice exposed to cigarette smoke had a significantly lower PAAT/PAET ratio compared to the air exposed control group, indicating an increased blood pressure in the pulmonary artery (Figure 3B). ZPP treatment to cigarette exposed mice protected the mice from an increase in blood pressure in the pulmonary artery. In addition, ZPP treatment protected cigarette smoke exposed mice from development of increased RVSP (Figure 3C).

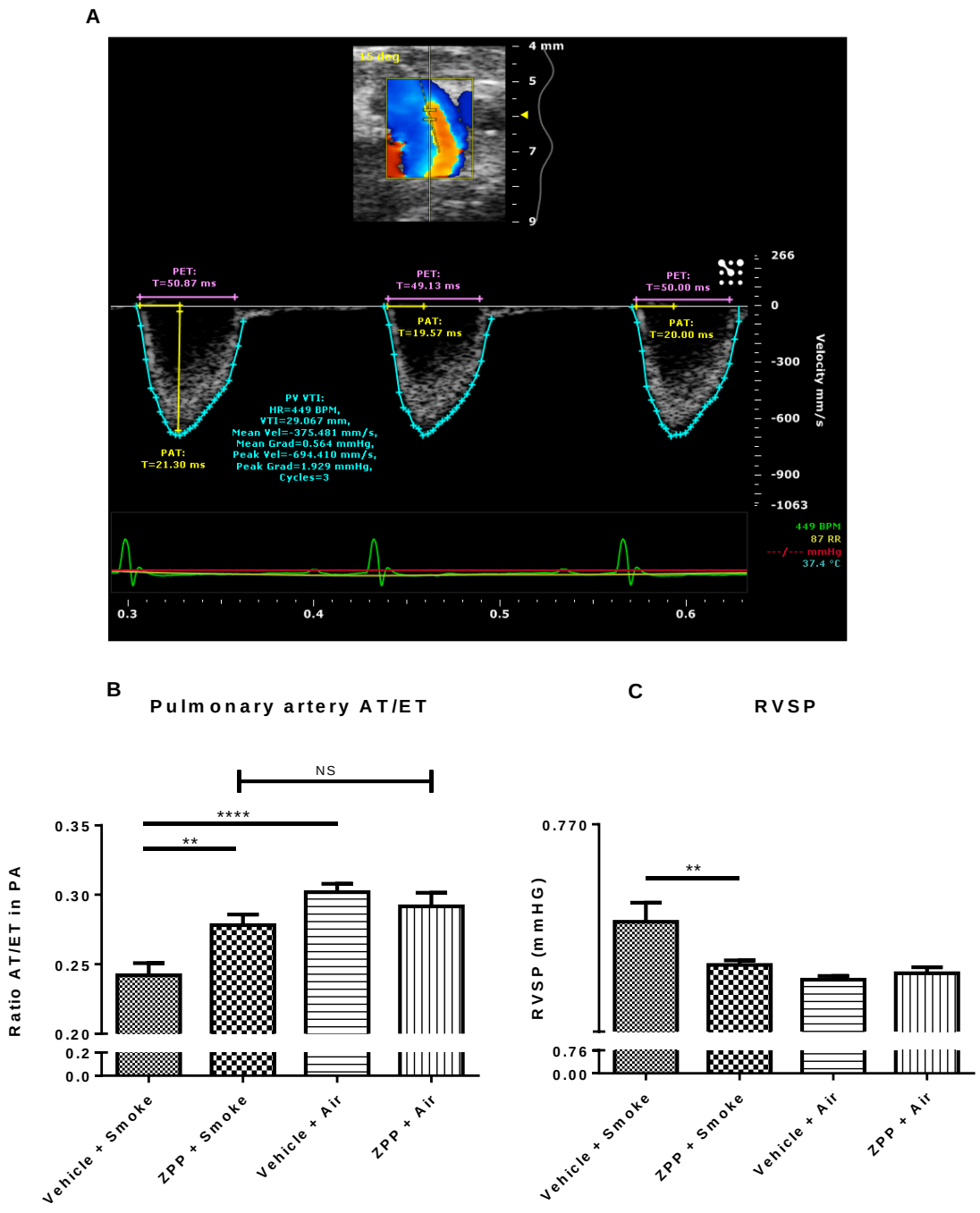

Figure 3. ZPP prevents smoke exposed mice to develop increased pressure in the pulmonary artery and in the right ventricle during systole. *Using echocardiography, the PAAT and PAET were measured (A) and the ratio calculated (B) which is inversely correlated to pulmonary artery pressure (16). The RVSP was calculated as described before (C) (15). Inhibiting PGP generation by ZPP resulted into normal pressures unlike seen in the vehicle treated, cigarette smoke exposed mice. Data shown as mean +/- S.E.M. ** P ≤ 0.01, **** P≤ 0.0001. AT/ET, acceleration time/ejection time. RVSP, right ventricular systolic pressure.*

Discussion

In this report we describe a potential new role for PGP peptides in CV diseases due to cigarette smoke. Using the inhibitor ZPP as treatment, mice exposed to cigarette smoke were protected from deterioration effects of inflammation in lung and cardiovascular system. These results demonstrate a new mechanism of biological action of PGP peptides linking the development of lung disease with the development of right heart failure. Blocking these bioactive matrikines affect not only pulmonary inflammation but presumably endothelial dysfunction. Indeed, recent evidence from our group highlights the capability of PGP peptides to induce endothelial signaling independent of inflammation (17).

It is interesting to see that ZPP effected the PAAT/PAET ratio after cigarette smoke exposure in mice. This PAAT/PAET ratio is a robust measurement that has been shown to be strongly inversely correlated with mean pulmonary artery pressure that is independent of heart rate (16, 18). Pulmonary hypertension is a common disease and may result in a number of disorders, including left heart disease, lung disease, and chronic thromboembolic disease. The ongoing inflammatory process together with the high arterial blood pressure leads into progressive remodeling of the distal pulmonary arteries resulting in elevated pulmonary vascular resistance and, eventually, in right ventricular hypertrophy and failure (19). Therefore, an adequate therapy targeting the underlying mechanism of pulmonary hypertension could be very beneficial to patients with CV diseases. As ZPP also prevented the cigarette smoke exposed mice from developing a high RVSP, it seems that PGP peptides are interesting targets for drug development in CV diseases.

Furthermore, a significantly lower molar ratio of nitrite/nitrate was measured in blood of the ZPP treated, cigarette smoke exposed mice. Nitrite and nitrate are stable end products of the nitric oxide (NO) metabolism. NO is known to be a potent endogenous vasodilator and inhibitor of key

processes of atherosclerosis like monocyte adhesion and vascular smooth muscle cell proliferation. Impaired endothelial NO production and/or NO bioavailability due to enhanced oxidative stress such as caused by cigarette smoke however, is a main feature of endothelial dysfunction and signifies an early step in the course of atherosclerotic vascular disease (20). A closer look at the data shows that although nitrite levels are different between the groups, nitrate levels are unaffected. High nitrite levels are consistent with endothelial nitric-oxide synthase (eNOS) and inducible NOS (iNOS) activity (21). However, a lack of change in nitrate levels suggests no systemic activation of iNOS. Alternatively, high nitrite levels in blood might be the result of cigarette smoke exposure, leading to increased NOx from nitrogen oxides present in the smoke itself (22). However, as the mice in the different treatment groups were exposed to the same cigarette smoke simultaneously and for the same duration of time, it is more likely that there is an increased eNOS activity. In fact, recent evidence from our group has demonstrated that direct PGP peptide administration to primary endothelial cells can attenuate eNOS activity (data not shown). In conclusion, this manuscript demonstrates the targeting of PGP peptides in a smoke model impacts the development of RVH. These results highlight the pleotropic effects of these bioactive collagen fragments in the development of a COPD phenotype and suggest a new therapeutic agent and target for the development of pulmonary hypertension related to COPD.

Grants

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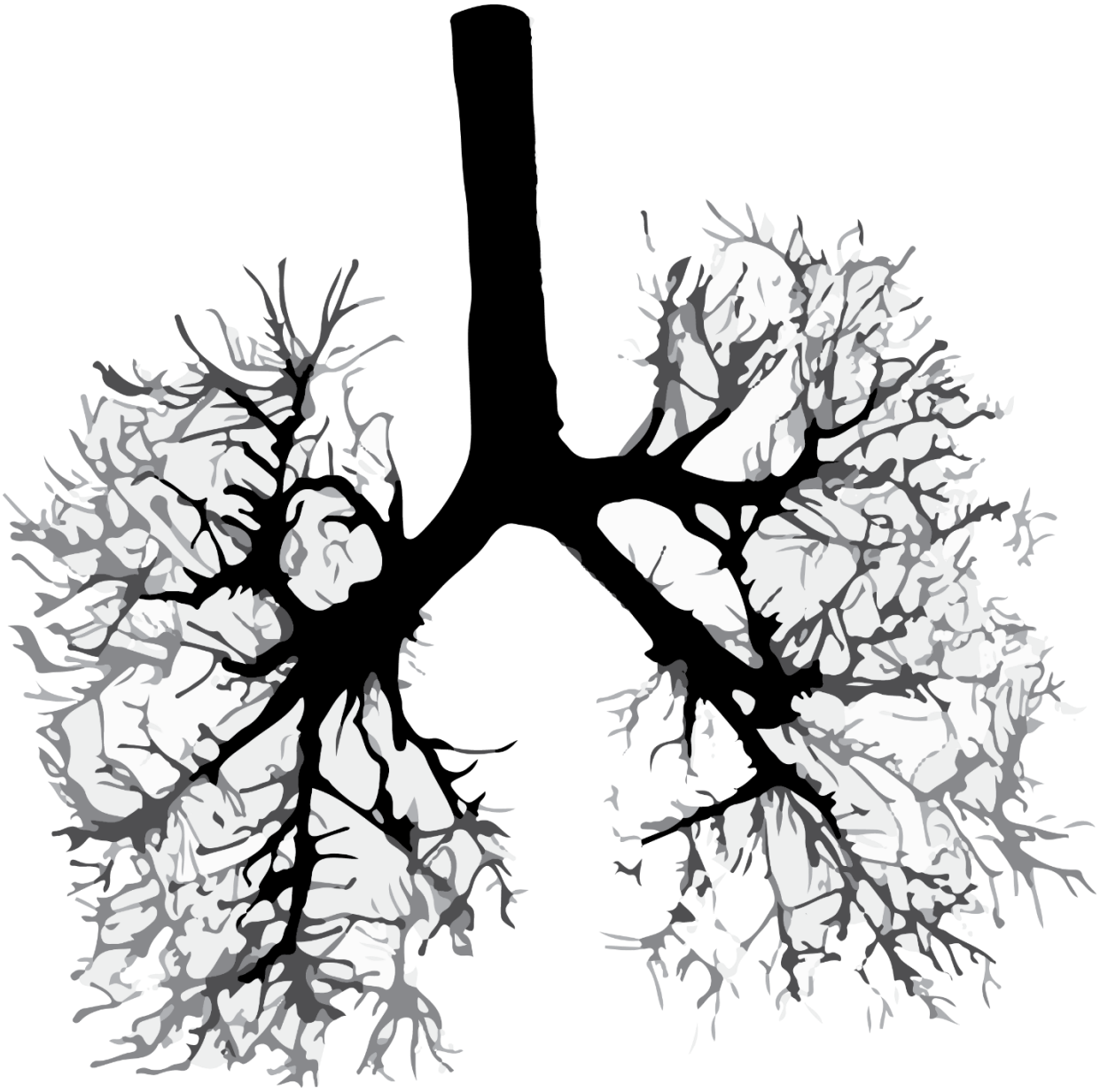
Chapter seven



Summarizing discussion

The main aim of this thesis was to gain more knowledge and understanding of the role of the matrikine proline-glycine-proline (PGP) during pathological conditions such as chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS).

In chapter 3, we focused on the mode of action of valproic acid on the PGP generating enzyme prolyl endopeptidase (PE). Although it was described that VPA is a poor PE inhibitor, we were intrigued by its molecular structure (1). Unlike other PE inhibitors, VPA does not resemble the normal peptide substrates of PE to act as a transition state analogue, nor does it fit a classical serine protease inhibitor (2). Our study showed that VPA is interacting at or near the binding pocket of PE, causing a change in the secondary structure of the enzyme. It would be interesting to synthetize new molecules, based on the molecular structure of VPA which have a higher affinity for PE. Although VPA is a weak inhibitor of PE, in an acute murine cigarette smoke-induced inflammation model, VPA showed to significantly reduce PE activity, resulting in lower PGP peptide concentration in the bronchoalveolar lavage fluid (3). Using these data as proof of concept, we continued on targeting PGP peptides in smoke-induced lung inflammation in mice.

In chapter 4, we utilized two murine models to test the PGP neutralizing peptide L-arginine-threonine-arginine (RTR) in a short term (6 weeks) and long term (23 weeks) cigarette smoke exposure murine model. Targeting the PGP pathway in those models was very effective. A notable reduced inflammation in the lungs was observed in both models, including no emphysema development in the long-term model. Furthermore, in both models a protective effect was seen of RTR treatment on the heart physiology. In *in vitro* experiments, using primary epithelia from

COPD patients and healthy individuals, we showed that acPGP triggers the release of the neutrophil chemoattractant interleukine-8 (IL-8) and the enzyme matrix metallaprotease-9 (MMP-9). MMP-9 initially cleaves collagen in small fragments, where after PE further cleaves the collagen fragments into other peptides, such as PGP. It is interesting to note that unlike the COPD primary epithelia, the healthy primary epithelia did not release MMP-9 in measurable levels after incubation with acPGP. We hypothesized that epithelia from COPD patients might be more primed to PGP peptides. As CXCR2 is a known receptor for PGP, we looked into CXCR2 levels on the primary epithelia, pretreated with cigarette smoke extract and stimulated with acPGP (4, 5). An interesting finding was that CXCR2 protein levels were increased after acPGP stimulation in the COPD primary epithelial cells and not in those from healthy individuals. These data together show the potential of acPGP to amplify the inflammatory response and extracellular degradation in COPD.

We then took a different approach to gain more knowledge in the PGP pathophysiology. In the past, several studies have been published describing the effects of PGP peptides on neutrophils, such as chemotactic and activating effects (6, 7). In chapter four, the effects of PGP on epithelial cells were investigated. However, the effects of PGP peptides on endothelial cells had not been investigated yet. In chapter five we link extracellular fragmentation to vascular permeability. In a series of experiments we show that acPGP activates endothelial cells via CXCR2 to induce vascular permeability. This was further substantiated by preclinical studies and measurement in clinical samples from patients with ARDS. An interesting finding was that although PGP peptides have proinflammatory effects on neutrophils and epithelial cells, the effects on endothelial cells were quite different. We showed that indeed PGP peptides do facilitate the inflammatory response

by enhancing the vascular permeability and thus facilitating the migration of leukocytes to the site of inflammation. However, we did not observe a direct proinflammatory effect of PGP on endothelial cells. Proinflammatory cytokines such as IL-6, IL-1alpha and monocyte chemoattractant protein-1 were not secreted by endothelial cells after incubation with acPGP. It could be speculated that if PGP would have a proinflammatory effect on endothelial cells, patients with high PGP blood values would have a more severe inflammatory reaction.

Considering the effects of PGP peptides on the heart in chapter four and the endothelial cells in chapter five, we decided to look more into cardiovascular effects of PGP. In chapter six we utilized a subchronic cigarette smoke-induced neutrophilia murine model with a specific PE inhibitor as treatment: benzyloxycarbonyl-proline-prolinal (ZPP). Unlike VPA, ZPP is more specific to PE and has a higher affinity for the enzyme. Inhibiting the PGP generating enzyme resulted into protective effects in the murine model. The inflammatory process in the lungs was significantly lower as well as no development of right ventricular hypertrophy (RVH) was detected. Together with a significantly lower right ventricular systolic pressure and less oxidative stress (measured as nitrite/nitrate ratio) compared to controls, these data suggest an important role for PGP in cardiovascular diseases. In future studies, it would be interesting to further elucidate the exact effects are of PGP peptides on the heart and the related mechanism. In figure 1 the effects of PGP in different organs are illustrated.

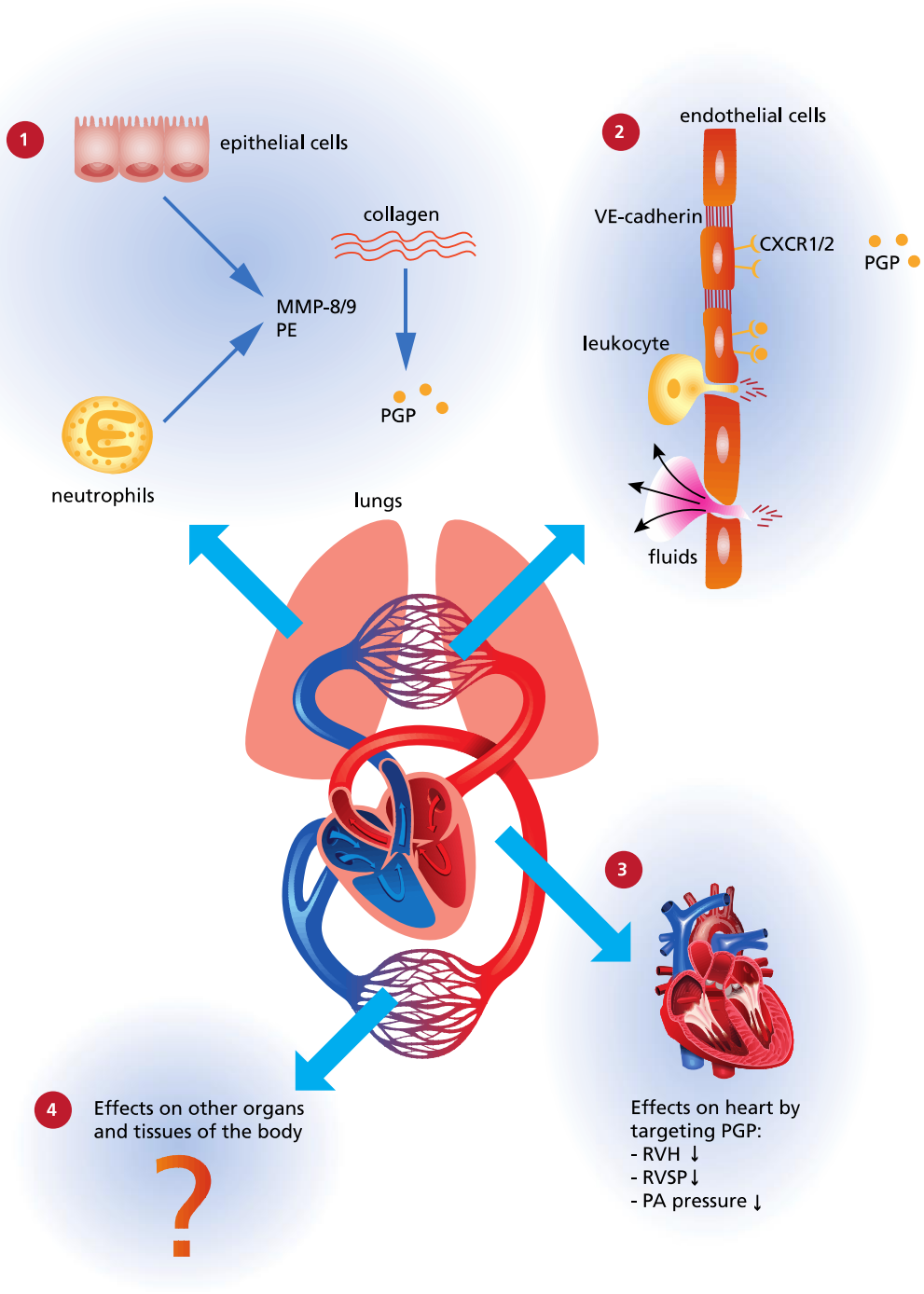


Figure 1. Overview illustration of the PGP biology in different organs. *During inflammation, PGP is cleaved out of collagen in the lungs (1). Not shown: PGP attracts neutrophils into the lungs and has a pro-inflammatory effect on epithelial cells. PGP peptides leak into the blood and modify vascular permeability (2). By inactivating PGP in cigarette smoke exposed mice, less RVH is developed together with a lower RVSP and PA pressure compared to control mice (3). PGP may play a role in other organs during inflammation (4). RVH: right ventricular hypertrophy. RVSP: right ventricular systolic pressure. PA pressure: pulmonary artery pressure.*

The PGP antagonist RTR was utilized in various experimental models throughout the thesis. Although it has been shown to have potent neutralization effects on PGP-peptides, it has been shown to neutralize IL-8 (8, 9). However, specific studies have not been carried out to examine whether RTR would antagonize KC/MIP-2 or not. It is quite possible that such neutralization may occur and provide some of the biologic changes observed in these studies.

In our studies, the PGP pathway was targeted either indirectly by inhibiting the PGP generating enzyme or directly by binding and inactivating PGP. Recently, Paige et al. took an interesting different approach. They targeted the enzyme leukotriene A4 hydrolase (LTA4H) which is responsible for degrading the unacetylated PGP (10, 11). They selectively augmented the LTA4H aminopeptidase activity in a cigarette smoke induced emphysema murine model. This lead to low levels of PGP in the bronchoalveolar lavage fluid and less infiltration of neutrophils into the lungs resulting in a protection of emphysematous alveolar remodeling (10). These findings are in line with our findings as described in chapter four.

Several studies have suggested using PGP peptides as biomarkers for COPD (12, 13). Although the data look very promising, only small patient cohorts have been studied. To better understand the role of PGP in human disease and the potential role as biomarker for COPD, more clinical studies are needed in which a larger population is investigated. These studies should point out if PGP could be used as a more specific biomarker for certain patients and perhaps as a predictor for exacerbations. The ARDS patient cohort studied in chapter five existed out of 6 ARDS patients and 6 controls. For a better understanding of the role of PGP peptides in ARDS, more clinical studies are needed with larger patient cohorts.

Lastly, we want to raise the following question: why does the PGP system exist and persist throughout the evolution? Based on the current literature, it seems that the PGP system serves as an important danger signal for innate immune response when invading bacteria breech all defenses and begin to damage the matrix. From a global perspective, the degradation of matrix may also prime the endothelium to become more permeable, not only for fluid but also to allow immune cells to come from the circulation to sites of injury. And finally, PGP may also prime endothelia to induce ongoing proteolytic generation by priming ongoing IL-8 and MMP-9 release, thereby inducing more PGP to be released in settings of overwhelming inflammation as a protection mechanism.

In conclusion, the findings in this thesis strongly underscore an important role for PGP in the development of emphysema and RVH in COPD. Furthermore, we have identified a novel role for acPGP as an activator of endothelial cell signaling which actively couples vascular permeability and tissue injury. This study indicates that PGP-induced inflammation may be an interesting therapeutic target.

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Appendices

Nederlandse samenvatting

Acknowledgements

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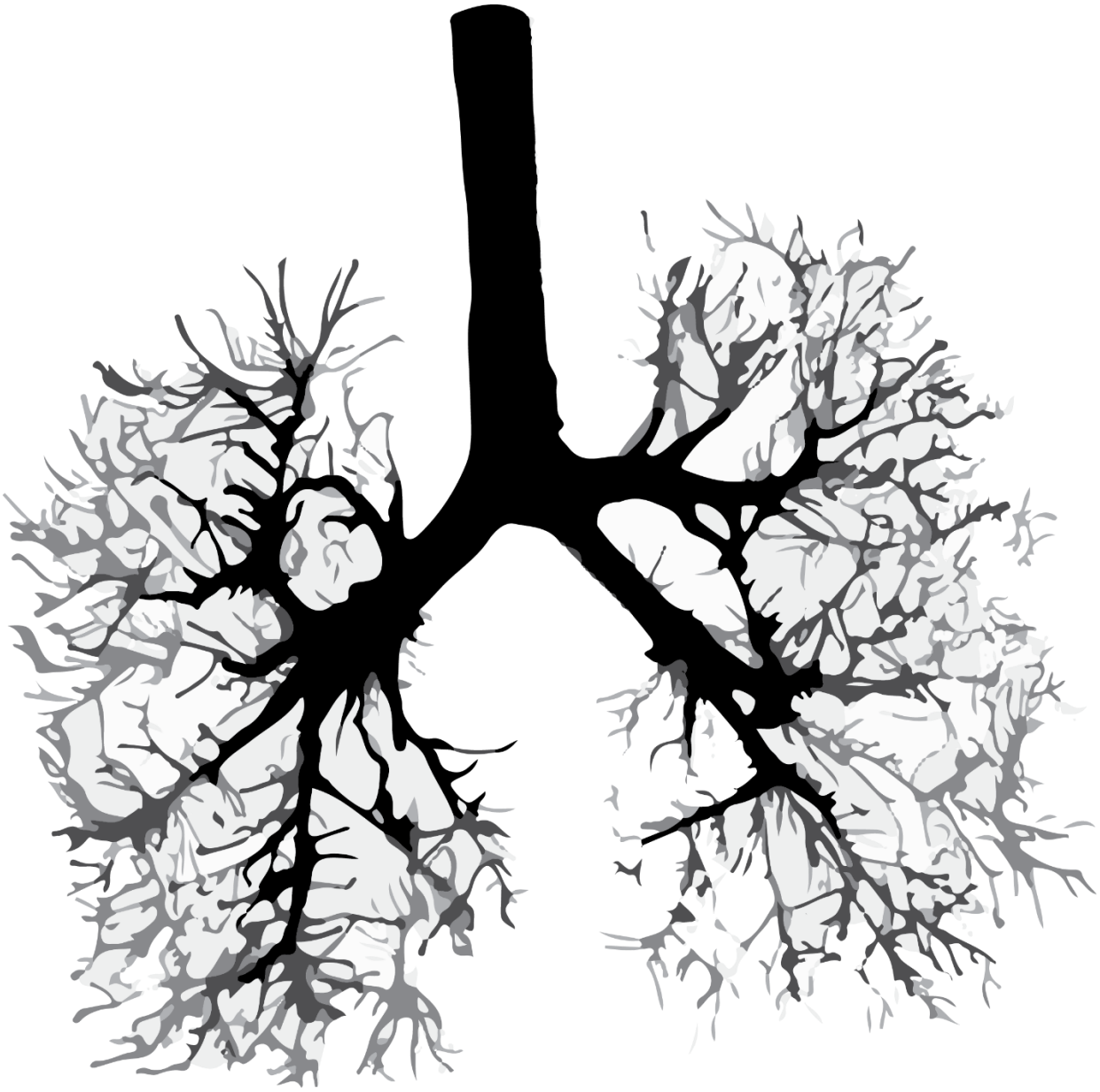
Nederlandse samenvatting



Nederlandse samenvatting

Door middel van het onderzoek dat beschreven is in dit proefschrift wordt getracht een beter beeld te verkrijgen van de achterliggende mechanismen in een aantal ontstekingsziekten. De ziekten die bestudeerd zijn, zijn voornamelijk luchtwegaandoeningen zoals chronisch obstructieve longziekte (COPD).

Inleiding

COPD is de afkorting voor de engelse benaming “chronic obstructive pulmonary disease” en is een verzamelnaam voor een aantal luchtwegaandoeningen. De belangrijkste hiervan zijn longemfyseem en chronische bronchitis. In dit werk is met name de ontwikkeling van longemfyseem onderzocht. Longemfyseem is vaak het gevolg van het roken van sigaretten, waarbij de longblaasjes in de longen verloren gaan. Longblaasjes zijn van belang bij het uitwisselen van zuurstof door de longen met het bloed. In 90% van de gevallen hebben patiënten met COPD in het verleden gerookt. Daarom wordt in de diermodellen die in dit proefschrift worden beschreven, sigarettenrook gebruikt om de ziekte ontwikkeling na te bootsen.

De focus in dit proefschrift ligt rond het peptide proline-glycine-proline (PGP). PGP is een peptide dat vrijkomt wanneer het eiwit collageen wordt afgebroken. Uit eerdere studies is gebleken dat PGP in staat is om witte bloedcellen zoals neutrofielen aan te trekken naar de ontstekingsplek en deze vervolgens te activeren. Hierdoor kan bij een onsteking een overvloed aan neutrofielen aangetrokken worden naar de ontstekingsplek waardoor een buiten proportioneel effect van de inflammatie ontstaat en het omliggende weefsel beschadigd kan raken. Het remmen van dit proces kan dus baat bieden bij patiënten met dergelijke ontstekingen.

Bevindingen

Eerst hebben we geprobeerd het enzym te remmen dat PGP aanmaakt uit collageen. Hiervoor hebben we valproïnezuur (VPA) gebruikt. VPA is een geneesmiddel dat al jaren gebruikt wordt als behandeling bij onder andere epilepsie en manie. In een 5 daags rookmodel in muizen bleek de behandeling met VPA inderdaad de aanvoer van wittebloedcellen te remmen. Dit effect was met name zichtbaar bij neutrofielen. Op basis van deze bevindingen hebben we besloten een langere blootstelling van sigarettenrook bij muizen uit te proberen waarbij PGP geneutraliseerd wordt. Hiervoor hebben we het peptide arginine-threonine-arginine (RTR) gebruikt welke in staat is PGP te binden en daarmee te neutraliseren. In een 6 weeks rookmodel met muizen bleken de ontstekingsparameters omlaag te gaan, evenals in een model van 23 weken. Verder bleek het remmen van PGP de ontwikkeling van longemfyseem door blootstelling aan sigarettenrook in muizen tegen te gaan. Een interessante bevinding was dat de -met RTR behandelde- muizen geen rechter ventrikel hypertrofie (RVH) ontwikkelden door toedoen van sigarettenrookblootstelling in het 6 weekse model. De niet-behandelde muizen ontwikkelden wel RVH. Soortgelijke bevindingen waren ook zichtbaar in het 23 weekse model.

Tenslotte wilden we meer weten over de invloed van PGP op het cardiovasculaire stelsel. Hiervoor hebben we eerst gekeken wat het effect van PGP is op endotheelcellen, die deel uitmaken van bloedvaten. Het bleek dat de geacetyleerde vorm van PGP, acPGP, endotheelcellen kan activeren, dat vervolgens kan leiden tot een verhoogde permeabiliteit van de bloedvaten. Hierdoor kan plasma uit de bloedvaten lekken. Gebruikmakend van diermodellen bleek dat het neutraliseren van acPGP de permeabiliteit significant verlaagt.

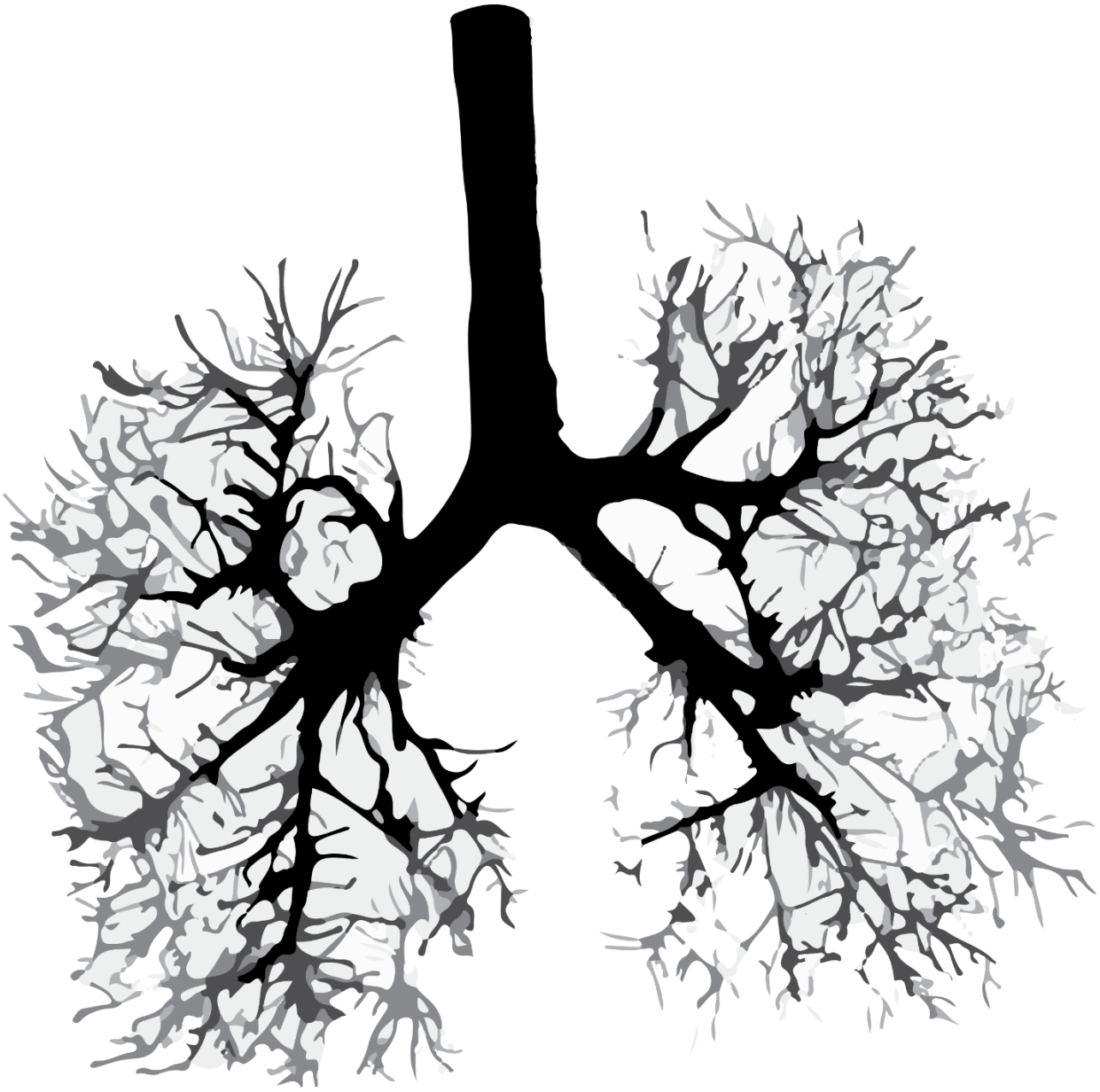
Wanneer in de longen bloedvaten lekken, kan er longoedeem ontstaan. Dit is een verschijnsel dat onder andere optreedt bij de luchtwegaandoening ARDS. Acute Respiratory Distress Syndrome wordt gekenmerkt door een plotseling falen van de longfunctie door vochtophoping in de longen. Patiënten belanden op de intensive care unit en gemiddeld overleeft slechts de helft van hen na behandeling. We besloten dan ook om te kijken of patiënten met ARDS wellicht verhoogde PGP peptides hebben in hun plasma. Wanneer we de PGP peptides concentratie in het plasma van patiënten uit de intensive care unit met en zonder ARDS met elkaar vergelijken, blijkt dat de ARDS patiënten inderdaad verhoogde PGP waarden hebben in hun plasma. Dit plasma is in staat endotheelcellen te activeren en tot een verhoogde permeabiliteit te zorgen. Dit kon door PGP inhibitie worden tegen gegaan.

Om nog meer te begrijpen over de rol van PGP op het cardiovasculaire stelsel, besloten we opnieuw muizen aan sigaretterook bloot te stellen en deze te behandelen met een zeer potente remmer van het enzym dat PGP aanmaakt. Door middel van een echoscopie van het hart van de muizen konden we achterhalen dat muizen door toedoen van de rook blootstelling verhoogde druk hadden in de pulmonaire arterie. Ook de druk in rechter venrtricle tijdens de systole was verhoogd. Echter, de met RTR-behandelde muizen vertoonden in beide gevallen normale waarden. Ook ontwikkelde de RTR-behandelde muizen in tegenstelling tot de controle muizen geen RVH.

Conclusie

Tot slot, de bevindingen van dit proefschrift benadrukken een belangrijke rol van de peptide PGP in de ontwikkeling van longemfyseem en RVH in COPD. Verder beschrijven we voor het eerst de rol van PGP peptides bij het activeren van endotheelcellen leidend tot een verhoogde doorlaatbaarheid van bloedvaten.

Acknowledgements



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First and above all, I thank God the almighty for providing this opportunity and granting me the capability to put this thesis together. Then I would like to thank my parents who have always believed in me and encouraged and supported me.

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PhD, where we got to travel to many conferences. Do you remember our trip to Estoril in Portugal? That was the first time I had to give an oral presentation in front of a big audience during a scientific conference. Because this was on the first of April, you suggested some last minute changes to my presentation (and by changes I mean inserting a joke) and therefore fooling the audience into thinking that the beamer was defect. We had a blast!

During all these years it has always been easy to come to your office and discuss with you any problems I was facing. Your positive attitude always left me feeling very confident and happy about my work. Thank you for these great years! Although in the past few months you have been going through a very tough period health wise, you still kept helping in every single way, and for that I cannot express how grateful I am. Thank you, for all the great memories and your guidance and I hope that we keep on going strong in the coming years.

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Ed, do you remember the first time we met? It was in Utrecht when you were drinking Coca Cola the "Amarican" way. The conversation we had then led to me coming to your lab for a 6 months internship. I felt very welcome in the lab and have always enjoyed working there with everyone. I learned a lot from the way you analyze data and how you always come up with brilliant ideas to set up a new experiment. Because of you I got interested in Amarican college football. Go Crimson Tide! Thanks for the advice and the guidance that you have given me for the past few years. You and Shawn are always welcome to visit us whenever you are in Holland.

Amit, for some reason I never saw you as my supervisor. I always saw you as a good friend with whom I could discuss lab/research problems with. You have always put faith in me which I really appreciate. Once I got to know you during my internship, I decided that I wanted to do a PhD preferably in collaboration with you and Ed. We successfully wrote the Mosaic grant together and this has resulted in me coming back to Birmingham for another year. This was probably the best decision I have made during my PhD. Your help didn't end just in the lab. It continued to helping me during my daily life in Birmingham. For example, I really appreciated all the times that you gave me a ride to the grocery store when I still didn't have a car. You have also helped me appreciate Indian food through Sitar and the great dishes your wife made at home. Thank you for your friendliness and kindness Amit, and just like Ed, please come visit us when you are in Utrecht. Bring Shilpa and the kids with you!

And of course Frank, you have also been there for me during the difficult times. Especially these last four months you have been a great support and help. In the absence of Gert, you have stepped up and offered your guidance and mentorship even more than before. I really appreciate all your hard work and fast replies to my emails. All this made it possible for me finish my thesis on time. I couldn't have done it without you.

Pat, although officially you were not my supervisor, you always have guided and supported me as your own student. My internship was probably going to fail if you had not helped me with the valproic acid project. Of course the same goes for my PhD. I could come to you with questions at any time asking about things such as cigarette smoke and chemistry. Thanks for your great help.

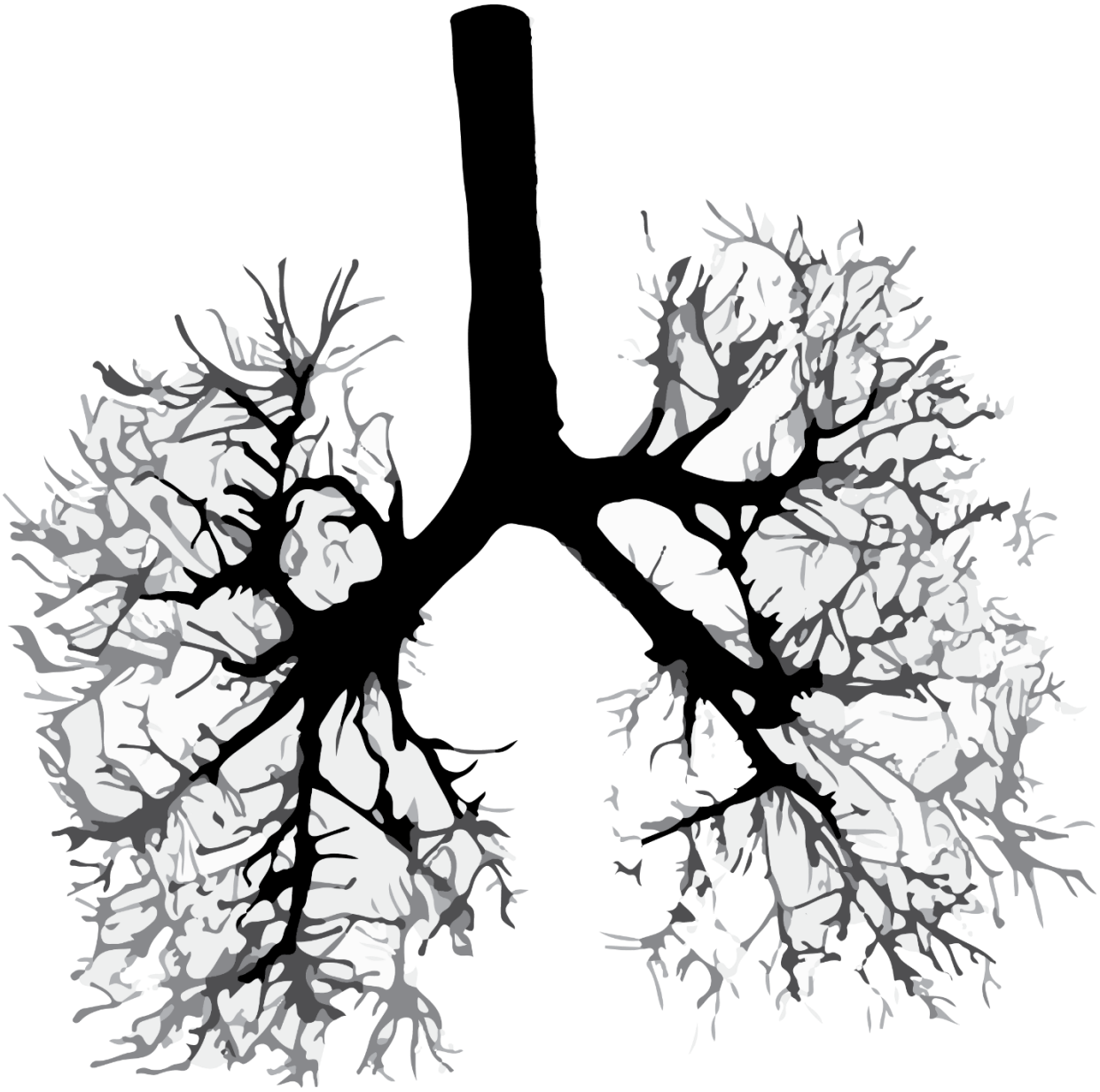
Xin Xu, during my stay in Birmingham you have always offered your help inside and outside the lab. I have learned a lot from you and I always have appreciated your wisdom. Thanks for the

many rides you have given me when I did not have my own car. Especially when you risked your life by driven me to the hood (3 times) so I could get my own car.

There are many other colleagues and friends from UAB that I want to send my sincere thanks to. You have made working in the lab easy and fun. Thanks to: Mike, Tarek, Lili, Preston, Carmel, Daniel, Shawn, Diane, Brynn, Tomasz and Philip.

In Utrecht I learned a lot of the techniques that I was able to apply in the US. For that I want to thank Ingrid, Thea, Rolf, Bart, Marije, Kim, Henk, Saskia and Saskia. And last but not least, a big thanks to all my colleagues from Utrecht University such as Johan, Aletta, Gerard, Gemma, Marlotte, Mara, Caroline, Lidija, Atanaska, Suzan, Paula, Betty, Paul, Jiang-bo, Payman and all the others that I may have failed to mention. Thanks for the wonderful time.

List of abbreviations

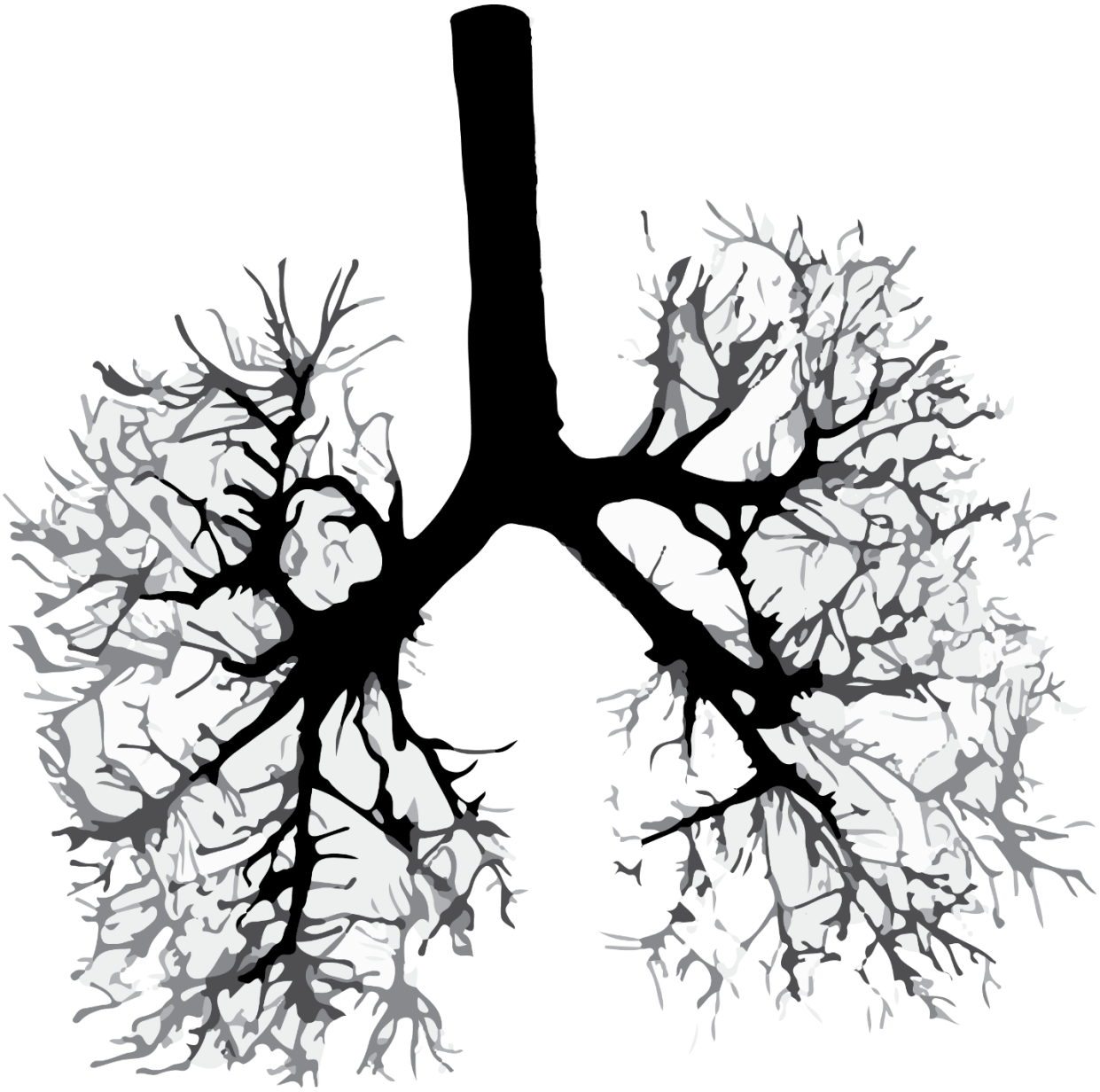


List of abbreviations

4MDM	4-methoxydiphenylmethane
acPGP	acetylated proline-glycine-proline
ADHD	attention-deficit hyperactivity disorder
ANOVA	analysis of variance
APACHE	acute physiology and chronic health evaluation
ARDS	acute respiratory distress syndrome
ASA	alanine-serine-alanine
BAL	bronchoalveolar lavage
CD	circular dichroism
CF	cystic fibrosis
COPD	chronic obstructive pulmonary disease
CSE	cigarette smoke extract
CV	cardiovascular
CXCL	(C-X-C motif) ligand
CXCR	(C-X-C motif) receptor
DMSO	dimethylsulfoxide
DTT	DL-dithiothreitol
ECM	extracellular matrix
eNOS	endothelial nitric-oxide synthase
ERK	extracellular signal-regulated kinase
ESI-LC-MS/MS	electrospray ionization-liquid chromatography-mass spec/mass spec
FBS	fetal bovine serum
H&E	hematoxylin/eosin
HAEC	human aortic endothelial cells
HBE	human bronchial epithelial
HCoAEC	human coronary arterial endothelial cells
HNE	human neutrophil elastase
HPmvEC	human pulmonary microvascular endothelial cells
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells

IgM	immunoglobulin M
IL-8	interleukin 8
iNOS	inducible nitric-oxide synthase
Lm	mean linear intercept
LPS	lipopolysaccharide
LTA4H	leukotriene A4 hydrolase
LTB4	leukotriene B4
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NMR	nuclear magnetic resonance
PAAT	pulmonary artery acceleration time
PAET	pulmonary artery ejection time
PAK	p21 activated kinase
PE	prolyl endopeptidase
PGG	proline-glycine-glycine
PGP	proline-glycine-proline
PMNs	polymorphonuclear neutrophils
rhPE	recombinant human PE
RTR	L-arginine-threonine-arginine
RVH	right ventricular hypertrophy
RVSP	right ventricular systolic pressure
Suc-Gly-Pro-AMC	N-succinyl-glycine-proline-7-amido-4-methyl-coumarin
Suc-Gly-Pro-pNA	N-succinyl-glycine-proline-para-nitroaniline
TLRs	toll-like receptors
TNF-α	tumor Necrosis Factor alpha
UV/Vis	ultra violet- visible
VE-cadherin	vascular endothelial - cadherin
VEGF	vascular endothelial growth factor
VPA	valproic acid
ZPP	benzyloxycarbonyl-proline-prolinal

Curriculum vitae



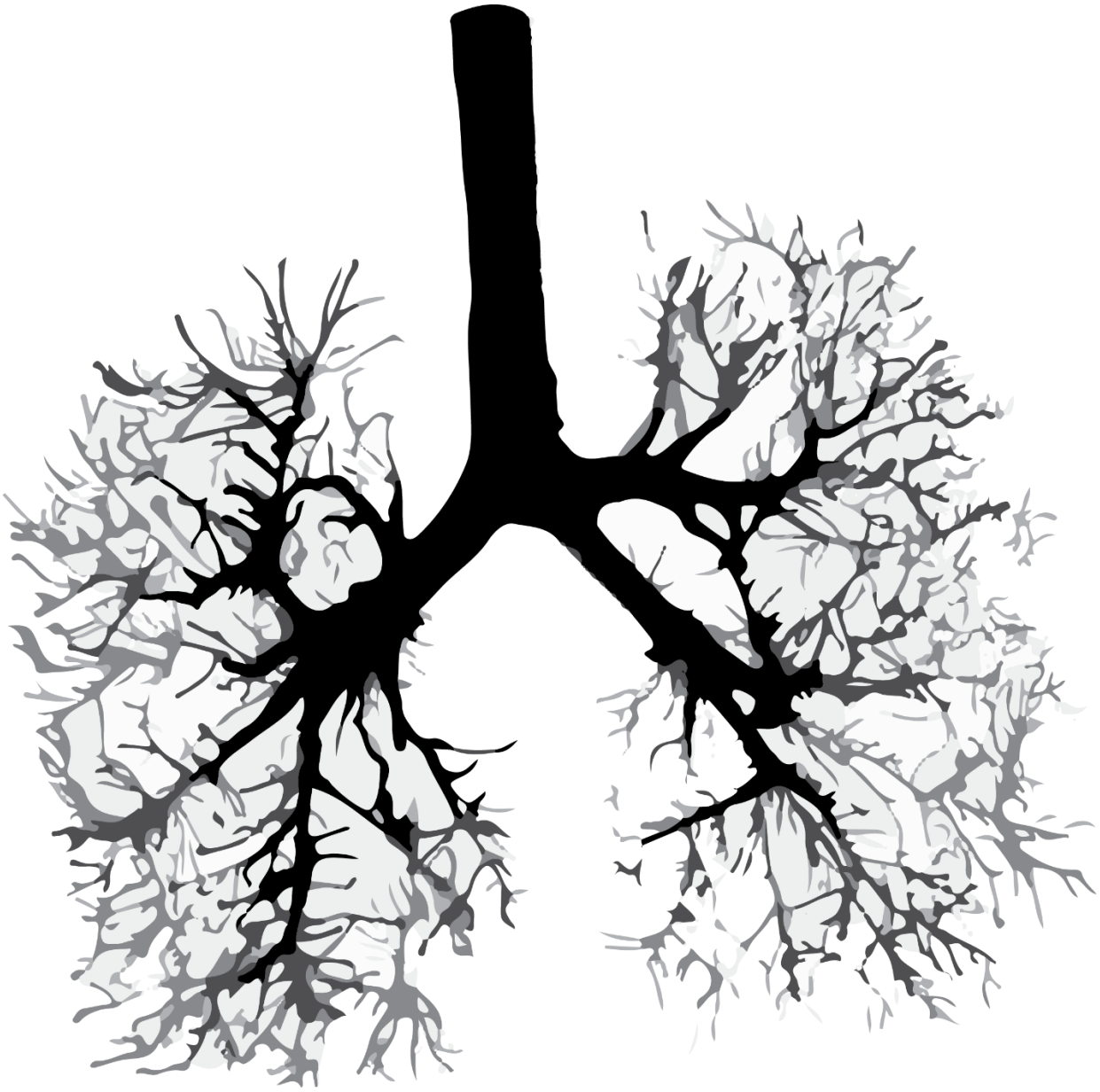
Curriculum vitae

Mojtaba Abdul Roda was born on March 25th, 1985 in Najaf, Iraq. In 1993 he immigrated together with his family to The Netherlands. After graduating from grammar school (st. Willibrord Gymnasium, Deurne) in 2005, he started studying Pharmacy at Utrecht University. In 2011 he obtained his Master of Science in Pharmacy in addition to completing the Honours Programme in Pharmacy.

In 2011, Mojtaba was awarded the NWO Mosaic grant which led to a 4 years PhD. The Mosaic grant is a subsidy of €200.000 awarded to talented young scientists. After starting his PhD at the Pharmacology division in Utrecht University, he was invited to continue his research at the University of Alabama at Birmingham, USA. After a year of extensive research abroad, Mojtaba returned to Utrecht University to finalize his PhD.

In June 2015 Mojtaba won the PhD Competition of the Utrecht Institute for Pharmaceutical Sciences and will be competing during the national PhD competition in October 2015. During his post-doc, Mojtaba will continue researching the role of PGP under pathophysiological conditions in collaboration with the pharmaceutical company Bayer. The focus will be on translating the findings of this thesis into a new therapeutic for COPD.

List of publications



List of publications

• Hahn CS*, Scott DW*, Xu X*, **Abdul Roda M**, Payne GA, Wells JM, Viera L, Winstead CJ, Bratcher P, Sparidans RW, Redegeld FA, Jackson PL, Folkerts G, Blalock JE, Patel RP*, Gaggar A*. The matrikine N-α-PGP couples extracellular matrix fragmentation to endothelial permeability. Science Advances 2015;1:e1500175

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• Xu X, Jackson PL, Tanner S, Hardison MT, **Abdul Roda M**, Blalock JE, Gaggar A. A Self-Propagating Matrix Metalloprotease-9 (MMP-9) Dependent Cycle of Chronic Neutrophilic Inflammation. PLoS One, Jan 2011.