Elastase-Induced Emphysema Does not Affect Atherosclerosis Development in *APOE*3-Leiden* Mice

Man C. Wong[1,2,3], P. Padmini S.J. Khedoe[1], Gerry T.M. Wagenaar[4], Jaap J. Plomp[5], Miranda van Eck[7], Louis M. Havekes[2,6,8], Patrick C.N. Rensen[2,3], Pieter S. Hiemstra[1], Jimmy F.P. Berbée[2,3]

Depts. of [1]Pulmonology, [2]Endocrinology and Metabolic Diseases, [3]Einthoven Laboratory for Experimental Vascular Medicine, [4]Pediatrics, [5]Neurology, and [6]Cardiology, Leiden University Medical Center, Leiden, the Netherlands; [7]Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden, The Netherlands; [8]Netherlands Organization for Applied Scientific Research – Metabolic Health Research, Gaubius Laboratory, Leiden, The Netherlands.

Submitted

Abstract

Chronic obstructive pulmonary disease (COPD) is a strong risk factor for cardiovascular diseases (CVD), independent of common risk factors such as smoking. The pathophysiological mechanism underlying the link between COPD and CVD, however, is incompletely understood. The aim of the present study was to investigate whether established emphysema in the absence of chronic pulmonary inflammation affects atherosclerosis development in mice. To address this, hypercholesterolemic *APOE*3-Leiden* transgenic mice were intratracheally instilled with 15 or 30 μg of porcine pancreatic elastase (PPE) to induce emphysema, or vehicle as control. After 3 weeks of recovery, mice were fed a diet with 0.4% (w/w) cholesterol to induce atherosclerosis. Blood was drawn to assess plasma lipid and inflammatory parameters. After 20 weeks of diet, mice were sacrificed to analyze the lungs, hearts, and atherosclerosis in the aortic root. Our results showed that intratracheal PPE instillation dose-dependently induced emphysema and right ventricular hypertrophy development. PPE instillation did not affect plasma levels of lipids and acute-phase proteins, but did result in a dose-dependent decrease in numbers of circulating monocytes and neutrophils. There was no difference in atherosclerotic lesion area, but the atherosclerotic lesion severity was dose-dependently decreased after PPE instillation. These data show that PPE-induced emphysema did not affect atherosclerotic lesion area and even reduced atherosclerosis severity in hypercholesterolemic *APOE*3-Leiden* transgenic mice. The findings suggest that emphysema, in the absence of (smoking-induced) inflammation, does not increase atherosclerotic CVD.

Introduction

Clinical epidemiological studies have demonstrated a strong positive correlation between chronic obstructive pulmonary disease (COPD) and cardiovascular disease (CVD).[1] This increased risk of CVD is not entirely explained by traditional shared risk factors, *e.g.* smoking, suggesting that COPD by itself may play a causal role in the pathophysiological mechanisms involved in CVD.[2] Markers of systemic inflammation are increased in many patients with COPD, especially in those with more severe disease and/or exacerbations.[3] This systemic inflammation, which may in part result from overspill of inflammatory mediators from the inflamed lung, could explain this increased risk for CVD.[2, 4]

Although this enhanced systemic inflammation appears to be a plausible link between CVD and COPD, little is known about the origin of these circulating inflammatory mediators. Moreover, the evidence for a prominent role of systemic inflammation in the association between COPD and extrapulmonary manifestations of COPD such as CVD is circumstantial.[3] Emphysema severity is associated with arterial stiffness independent of C-reactive protein (CRP).[5] Anti-inflammatory therapies in COPD, *e.g.* inhaled corticosteroids and tiotropium, have not been consistently shown to modify serum levels of CRP or IL-6, nor the long-term cardiovascular complications.[6,7] This suggests that while local delivery of anti-inflammatory drugs to the lungs does have a limited effect on lung inflammation, it does not reduce systemic inflammation. Furthermore, levels of inflammatory mediators (*i.e.* TNFα, IL-6 and IL-8) in induced sputum do not correlate with the values in blood in COPD patients.[8] Therefore, it is likely that other mechanisms than inflammation are also involved in the interaction between COPD and CVD.

Pulmonary emphysema is a major component of COPD and defined as abnormal airspace enlargement distal to the terminal bronchioles.[9] Recently, it was demonstrated in a study with current, ex- and never-smokers with or without COPD that the occurrence of atherosclerotic plaques in the internal carotid arteries is positively associated with two function parameters that are associated with pulmonary emphysema, *i.e.* a low diffusing capacity for carbon monoxide and a high residual volume.[10] These associations still remained significant after correction for established risk factors for atherosclerosis, such as older age, blood pressure, total cholesterol and smoking. Interestingly, after multivariate analysis, the extent of atherosclerosis was not associated with variables such as forced expiratory volume in one second (FEV_1) and CRP. These results suggest that in addition to airflow obstruction and low-grade systemic inflammation, other features of COPD (*i.e.* reduced diffusing capacity and increased residual volume) may contribute to the association between reduced lung function and CVD.

Intratracheal administration of porcine pancreatic elastase (PPE) in animals reproduces key features of emphysema.[11] When compared to other emphysema models, *e.g.* chronic smoke exposure or intrapulmonary lipopolysaccharide instillation,[11] the PPE model is not characterized by chronic inflammation. This allows a separate analysis of the contribution of emphysema in absence of chronic inflammation alone to atherosclerosis development. This is important since it is unknown whether the main characteristic of emphysema, *i.e.* alveolar destruction,[12] in the absence of pulmonary inflammation can enhance atherosclerosis.[13] Intratracheal PPE

instillation induces a transient acute inflammatory response in the lung and, subsequently, alveolar destruction.[14] PPE-induced emphysema is thus a suitable model to investigate the role of alveolar destruction, in the absence of smoke-induced chronic inflammation, on atherosclerosis development.

The aim of this study was to assess whether emphysema *per se*, without concomitant pulmonary inflammation, enhances atherosclerosis development in atherosclerosis-prone *APOE*3-Leiden* (*E3L*) mice.[15] We found that mice with PPE-induced emphysema had similar plasma levels of lipids and inflammatory markers, but lower circulating monocyte and neutrophil levels. In line with these findings, their atherosclerotic lesion size was not different, while their atherosclerosis severity was reduced compared to the control mice without emphysema.

Materials and methods

Animals

Female *APOE*3-Leiden* (*E3L*) mice[15] of 10-12 weeks of age were housed under standard conditions with a 12-hour light/dark cycle and had free access to food and water. *E3L* mice represent a well-established model for human-like lipoprotein metabolism and develop atherosclerosis when fed a Western-type diet. All mice were fed a synthetic diet containing 15% (w/w) cacao butter (diet T, Hope Farms, Woerden, The Netherlands) for 3 weeks. Subsequently, mice were divided into 3 groups after matching for plasma cholesterol levels, age and body weight. To induce pulmonary emphysema, porcine pancreatic elastase (PPE) (E7885, Sigma-Aldrich, Schnelldorff, Germany) was administered intratracheally (15 μg or 30 μg in 40 μL PBS).[16, 17] Control mice received 40 μL sterile PBS (vehicle). After 3 weeks of recovery, all mice were fed a Western-type diet (*i.e.* diet T supplemented with 0.4% (w/w) cholesterol) to induce atherosclerosis development. All mice were weighed weekly and food intake was assessed for one week at 12 weeks after instillation of vehicle or PPE.

At baseline (before intratracheal PPE instillation) and every 4 weeks thereafter, blood was drawn in EDTA-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding after 4 hours of fasting, and plasma was isolated by centrifugation. All animal experiments were approved by the Institutional Ethical Committee on Animal Care and Experimentation of the Leiden University Medical Center (Leiden, The Netherlands).

Pulmonary function measurements

Total respiratory amplitude and respiratory rate were assessed 13 weeks after vehicle or PPE instillation with non-invasive whole body plethysmography (RM-80, Columbus Instruments, Columbus, OH, USA) as described previously.[18] The total respiratory amplitude was calculated from the measured peak-to-peak signals and reflects the tidal volume. Flow derived parameters of breath amplitude and frequency were collected for and averaged over 2 minutes per mouse. The signal was digitized using a Digidata 1440A interface (Axon Instruments/Molecular Devices, Union City, CA, USA) and analyzed with the event detection feature of Clampfit 9.2 (Axon Instruments/Molecular Devices).

Plasma lipids and inflammatory markers

Plasma total cholesterol, triglyceride and phospholipid levels were determined using enzymatic kits from Roche Molecular Biochemicals (Woerden, The Netherlands) according to the manufacturer's protocols. The cholesterol distribution over plasma lipoproteins was determined after size-fractionation of pooled plasma samples using an ÄKTA fast performance liquid chromatography (FPLC) system (Pharmacia, Roosendaal, The Netherlands).[19]

Plasma levels of serum amyloid A (SAA) and soluble E-selectin (sE-selectin) were determined at 24 weeks after intratracheal PPE instillation using the murine SAA assay kit (Tridelta, County Kildare, Ireland) and murine E-selectin ELISA kit (R&D, Minneapolis, MN), respectively, according to manufacturer's instructions.

Blood count analysis

Eight weeks after intratracheal vehicle or PPE instillation, unfasted blood was collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice. Complete blood cell count and hematological analysis was performed in whole blood using a Sysmex XT-2000iV veterinary hematology analyzer (Sysmex Corporation, Kobe, Japan), as described previously.[20] The XT-2000iV employs a fluorescent flow cytometry method using a fluorescent dye to stain cellular DNA and RNA and a semiconductor laser to detect forward-, side-scattered, and fluorescent light.

Arterial blood and tissue collection

Mice were anesthetized by intraperitoneal injection of 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.31 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands) 24 weeks after intratracheal PPE instillation. Blood was obtained by cardiac puncture. Subsequently, the mice were sacrificed by cervical dislocation and the pulmonary and systemic circulation was rinsed with ice-cold PBS. The lungs were fixated *in situ* by gentle infusion of fixative (phosphate-buffered 4% formaldehyde) by a continuous-release pump under constant pressure (12 mL/hour; 8 min) through a tracheal cannula. After excision, the lungs and heart were immersed in fresh fixative for a period of 24 hours at 4°C.

Histological analysis of the lungs

Lungs were processed for paraffin embedding and cut in 5 µm coronal sections. Tissue samples were stained with hematoxylin-eosin (HE). To assess air space enlargement, the mean linear intercept (MLI) and air/tissue ratio was quantified by one observer in a blinded fashion by superimposing a line grid with 21 lines and 42 points on the images of lung sections at a magnification of 200x as described previously.[21] To calculate the MLI, the number of intersections between the lines of the grid and the alveolar walls was quantified for each mouse in 10 non-overlapping fields. To determine the air/tissue ratio, the number of points in alveolar space was counted.

For immunohistochemical staining of macrophages and neutrophils in the lungs, rat anti-mouse MAC-3 antibody (1:50, BD Pharmingen, Breda, The Netherlands)[22] and anti-

myeloperoxidase (MPO) (1:1500, Thermo Fisher scientific, Runcorn, United Kingdom)[21] were used as described previously. Results of MAC-3- and MPO-positive cells are represented as the average count from 10 non-overlapping fields per mouse (400x magnification) corrected for tissue density by calculating the ratio between the number of cells and the average area of tissue per field. To determine the average area of tissue, the number of points superimposed on alveolar tissue was counted (400x magnification), using the same line grid for assessment of air space enlargement, as described above.

Histological analysis of the heart and aorta

Hearts were isolated and fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. A 5 μm transversal section of the heart halfway in the long axis was stained with HE. Thickness of the right and left ventricular free walls was assessed at a 40x magnification by averaging 6 measurements per structure with the NIH Image J program.

For quantification and classification of atherosclerosis, the hearts were cross-sectioned (5 μm) throughout the entire aortic root area. Per mouse, 4 sections with 40-μm intervals were used for quantification of atherosclerotic lesion area and characterization of lesion severity. Sections were stained with hematoxylin-phloxine-saffron. According to the guidelines of the American Heart Association, adapted for mice,[23, 24] atherosclerotic lesions were categorized for severity as follows:

- Type I: early fatty streak: per section up to 10 foam cells present in the intima.
- Type II: regular fatty streak: more than 10 foam cells present in the intima.
- Type III: mild plaque: extension of foam cells into the media and covered by a fibrotic cap.
- Type IV: moderate plaque: a more progressive lesion infiltrating into the media, fibrosis in the media, without loss of architecture.
- Type V: severe plaque: the media is severely damaged, elastic lamina are broken, presence of cholesterol clefts, mineralization and/or necrosis.

All segments were categorized into: 1) no lesions (undiseased) 2) mild (type I-III) and 3) severe (type IV-V) lesions. The percentage of lesion-free segments and the percentages of lesions belonging to the respective lesion categories were calculated.

AIA31240 rabbit antiserum (1:1000, Accurate Chemical and Scientific, Westbury, NY) was used for determination of adhering monocytes and macrophage- and mouse monoclonal antibody M0851 (1:800, Dako, Carpinteria, CA) for quantification of smooth muscle cell content in the lesions, described previously.[25] Sirius red (Chroma, Stuttgart, Germany) was used to stain for collagen in the lesions.

Total lesion area, macrophage -, smooth muscle cell - and collagen content were quantified using Cell^D image analysis software (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

Statistical differences were assessed with one-way ANOVA analysis, followed by post-hoc analysis using Fisher's LSD multiple comparison test. For lesion typing, differences were

assessed by the χ^2 test. SPSS 16.0 for Windows (SPSS, Chicago, Ill) was used for statistical analysis. Differences at $P<0.05$ were regarded as statistically significant. Data are presented as mean ± SEM.

Results

Intratracheal administration of PPE induces emphysema and increases the total respiratory amplitude

To investigate whether PPE-induced emphysema results in changes in respiration pattern, we used non-invasive whole body plethysmography. PPE instillation dose-dependently increased the total respiratory amplitude of the measured signal, reflecting an increased tidal volume (Fig. 1A). The respiration rate was not different between the groups (Fig. 1B).

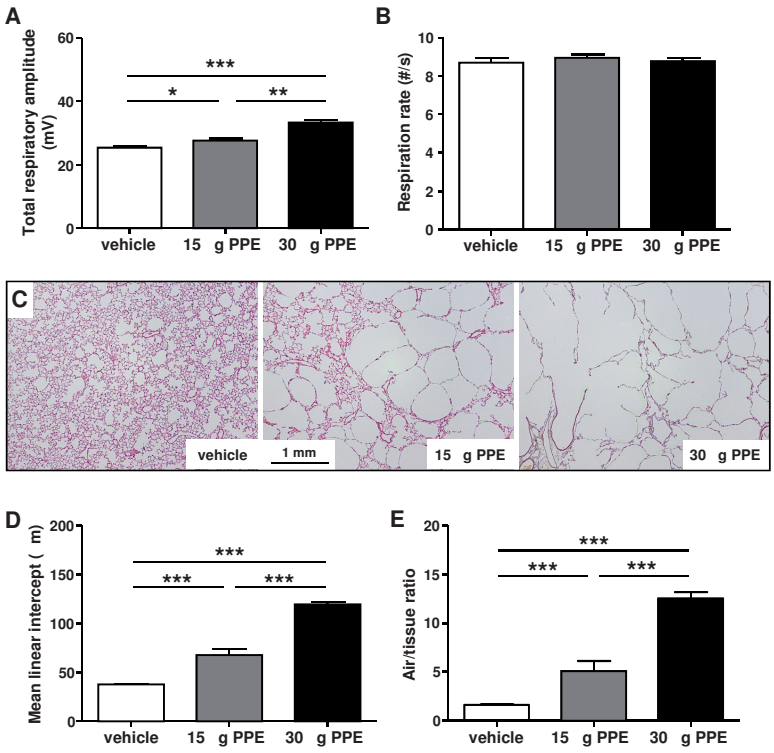

Fig. 1. Intratracheal PPE instillation dose-dependently increases total respiratory amplitude, mean linear intercept (MLI) and air/tissue ratio. Thirteen weeks after instillation of vehicle or PPE, total respiratory amplitude (A) and respiration rate (B) were analyzed by non-invasive whole body plethysmography. Twenty-four weeks after instillation *E3L* mice were sacrificed. The lungs were isolated and sections were HE-stained (C) to determine MLI (D) and air/tissue ratio (E). Values are means ± SEM; n=13-18; **P*<0.05, ***P*<0.01, ****P*<0.001.

To determine the extent of emphysema development after intratracheal PPE instillation, the lungs were perfused under continuous pressure at 24 weeks after PPE instillation and analyzed by morphometry (Fig. 1C). PPE instillation caused a significant dose-dependent increase in mean linear intercept (MLI) (Fig. 1D) and air/tissue ratio (Fig. 1E) as compared to instillation of vehicle, indicating destruction and enlargement of alveolar space. To assess whether PPE instillation generates prolonged inflammatory cell influx into the lungs, we also quantified the number of macrophages and neutrophils by immunohistochemistry. No differences in the number of macrophages and neutrophils in lung tissue were observed (Fig. 2A-B).

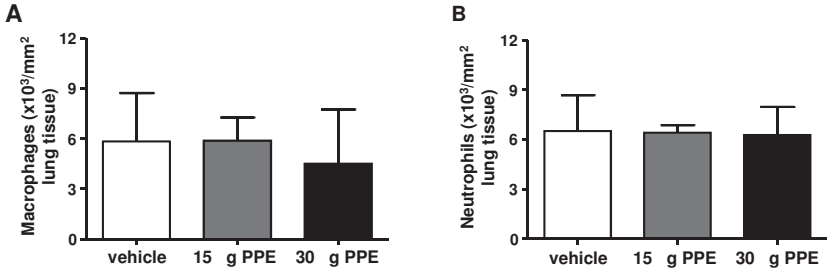

Fig. 2. Intratracheal instillation of PPE does not affect number of macrophages and neutrophils in the lungs. Twenty-four weeks after instillation of vehicle or PPE, *E3L* mice were sacrificed and the lungs were isolated to determine the number of macrophages (A) and neutrophils (B) by immunohistochemistry. Values are means ± SEM; n=13-16.

Intratracheal administration of PPE induces right ventricular hypertrophy

In a previous study in which mice were treated repetitively with PPE, right ventricular hypertrophy was observed.[17] Therefore, we determined whether the development of emphysema also affected the pulmonary circulation and the heart in this study. We found a dose-dependent increase in right ventricular hypertrophy with a single dose of PPE (Table 1 and Fig. 3A-B), which was also reflected by a significantly higher total heart weight/body weight ratio in the mice treated with the highest dose of PPE (Table 1).

Table 1. Intratracheal PPE instillation increases heart weight and induces right ventricular hypertrophy.

Group	RV free wall thickness (mm)	LV free wall thickness (mm)	Heart weight / body weight ($x10^{-3}$)
Vehicle	0.21 ± 0.009	0.96 ± 0.02	4.9 ± 0.1
15 µg PPE	0.25 ± 0.020**	0.97 ± 0.04	4.8 ± 0.1
30 µg PPE	0.30 ± 0.008***$^{\Delta\Delta}$	1.00 ± 0.03	5.1 ± 0.1$^{\Delta}$

Twenty-four weeks after intratracheal vehicle or PPE instillation, mice were weighed and sacrificed. The hearts were isolated, weighed and processed for histological analysis. Values are means ± SEM; n=11-18; **P<0.01, ***P<0.001 compared to vehicle, $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 compared to 15 µg PPE.

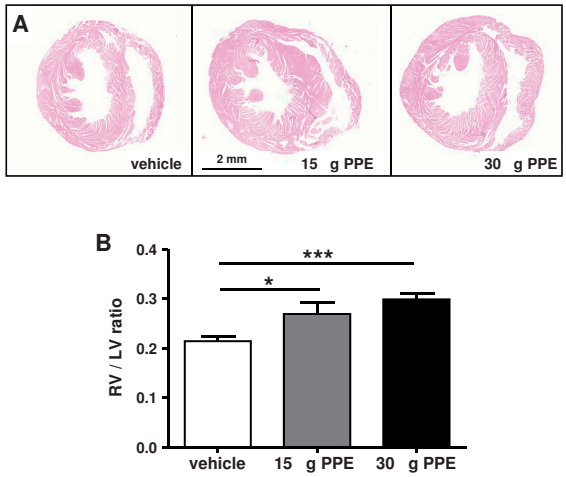

Fig. 3. Intratracheal PPE instillation dose-dependently induces right ventricular hypertrophy. Twenty-four weeks after intratracheal instillation of vehicle or PPE, *E3L* mice were sacrificed and the hearts were isolated. Transversal sections of the hearts were HE-stained (A). Right ventricular (RV) and left ventricular (LV) wall thickness was determined and the RV/LV was calculated (B). Values are means ± SEM; n=11-18; **P*<0.05, ****P*<0.001.

Intratracheal administration of PPE reduces circulating monocytes and neutrophils

To evaluate whether PPE instillation affected systemic inflammation, we measured leukocytes concentration in whole blood and plasma levels of the acute-phase proteins SAA and sE-selectin. Circulating levels of monocytes and of neutrophils were dose-dependently decreased in the PPE-treated groups. Despite this decrease, the concentration of total leukocytes was similar between the groups, since the majority (86%) of leukocytes consisted of lymphocytes, which was unaffected by PPE treatment (Fig. 4A-D). Plasma levels of SAA and sE-selectin were not different between groups (not shown).

As a measure for chronic hypoxia, erythrocyte count, hemoglobin and hematocrit were determined. The total number of erythrocytes, the hemoglobin concentration and hematocrit value in blood were increased in the 30 µg PPE group (Table 2).

Table 2. Intratracheal PPE instillation increases number of erythrocytes, hemoglobin concentration and hematocrit.

Group	Erythrocytes (x10^6/µL)	Hemoglobin (mmol/L)	Hematocrit (vol%)
Vehicle	8.7 ± 0.5	8.2 ± 0.4	40.1 ± 2.1
15 µg PPE	8.5 ± 0.6	8.4 ± 0.4	39.4 ± 2.5
30 µg PPE	9.9 ± 0.3$^{\Delta}$	9.2 ± 0.1*	45.2 ± 1.0*$^{\Delta}$

Eight weeks after intratracheal vehicle or PPE instillation, blood was collected to determine the number of erythrocytes, hemoglobin concentration and hematocrit. Values are means ± SEM; n=13-18; **P*<0.05 compared to vehicle, $^{\Delta}$*P*<0.05 compared to 15 µg PPE.

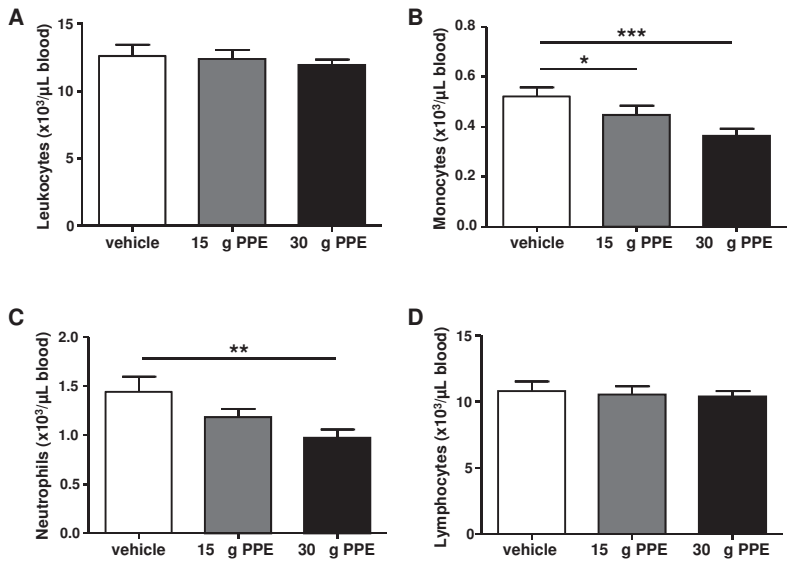

Fig. 4. PPE-induced emphysema dose-dependently reduces circulating monocytes and neutrophils. Eight weeks after intratracheal instillation of vehicle or PPE, blood was drawn to determine total leukocyte count (A), monocytes (B), neutrophils (C) and lymphocytes (D) using an automated veterinary hematology analyzer. Values are means ± SEM; n=13-16; **P*<0.05, ***P*<0.01, ****P*<0.001.

Intratracheal administration of PPE does not affect plasma lipids

PPE treatment did not affect body weight gain (Table 3) and food intake (2.8 ± 0.15 g/mouse/day for vehicle; 2.7 ± 0.11 for 15 µg PPE; 2.3 ± 0.07 for 30 µg PPE). Since dyslipidemia is a major contributor to atherosclerosis development, we next assessed the possibility that PPE-induced emphysema affects plasma lipid levels. After PPE or vehicle instillation, mice had a recovery period of 3 weeks before the mice were fed the Western-type diet (containing 0.4% cholesterol, w/w). By using this design, PPE activity and the accompanying inflammatory response had resolved[26,27] before atherosclerosis development was initiated. Plasma total cholesterol levels (Fig. 5A), as well as triglycerides and phospholipids levels (not shown) were similar in all groups throughout the study. In addition, the plasma lipoprotein distribution was not different (not shown).

Table 3. Intratracheal PPE instillation does not affect body weight.

	Body weight		
Group	**Before instillation (t=0 wks)**	**Before start of diet (t=4 wks)**	**At end of study (t=24 wks)**
Vehicle	20.1 ± 0.3	21.3 ± 0.28	36.7 ± 0.44
15 µg PPE	20.2 ± 0.4	21.5 ± 0.40	37.3 ± 0.58
30 µg PPE	20.3 ± 0.3	21.6 ± 0.23	37.2 ± 0.52

Values are means ± SEM; n=13-18.

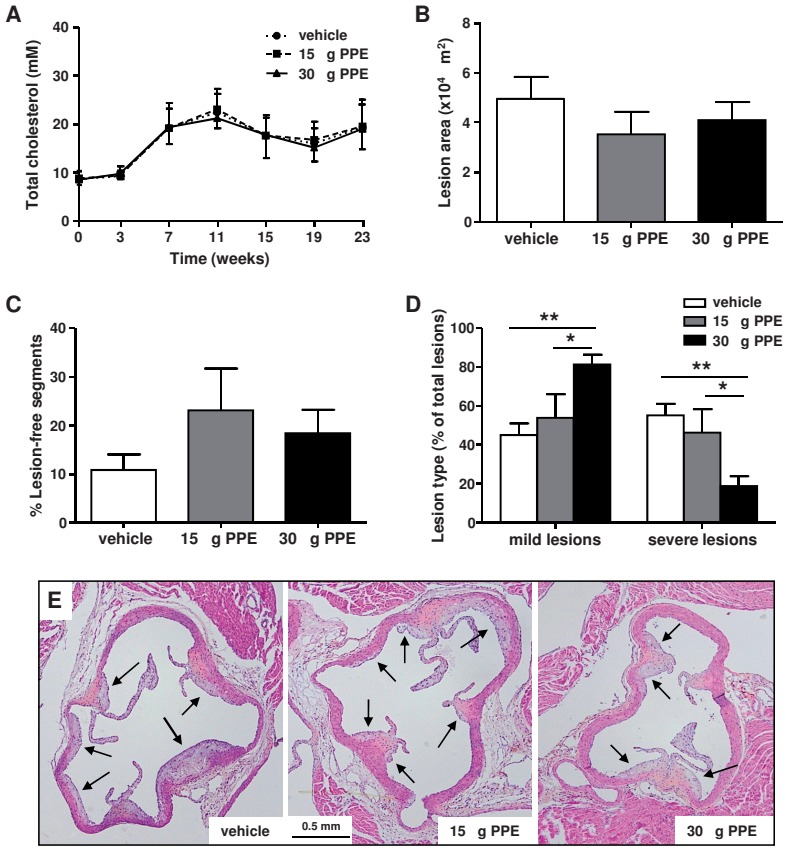

Fig. 5. Intratracheal PPE instillation does not affect atherosclerosis lesion area, but reduces lesion severity. Three weeks after intratracheal vehicle or PPE instillation, mice were fed a Western-type diet. Every 4 weeks thereafter, blood was drawn to assess plasma cholesterol levels (A). After 20 weeks of diet, the mice were sacrificed and the total lesion area was determined in the segments between the three aortic valves (B). In addition, the percentages of lesion-free segments (C), and with mild and severe lesions (D) were assessed. Representative pictures of each group are shown (E). Arrows indicate lesions. Values are means ± SEM, n=12-18; **P*<0.05, ***P*<0.01.

Intratracheal administration of PPE reduces atherosclerotic lesion severity

To study whether PPE-induced emphysema enhanced atherosclerosis development in the absence of chronic pulmonal inflammation, we determined the atherosclerotic plaque area and plaque severity in the aortic root area of the heart. Total lesion area, lesion severity and lesion composition were scored in the segments between the three aortic valves. PPE instillation did not affect atherosclerotic lesion size and percentage of lesion-free segments as compared to vehicle-treated control mice (Fig. 5B-C). However, classification of the segments that contained lesions showed a dose-dependent decrease in severity in the PPE-treated groups, as is demonstrated by an increased percentage of mild lesions and a decreased percentage of

severe lesions (Fig. 5D-E). With respect to the lesion composition, no differences in adhering monocytes to the vessel wall and lesion content of macrophages and smooth muscle actin was observed (Fig. 6A-C). The group treated with 15 µg of PPE had a significantly higher collagen content in the lesions (Fig. 6D) compared to the control group, but no difference was observed between the 30 µg PPE group and the control or 15 µg PPE groups.

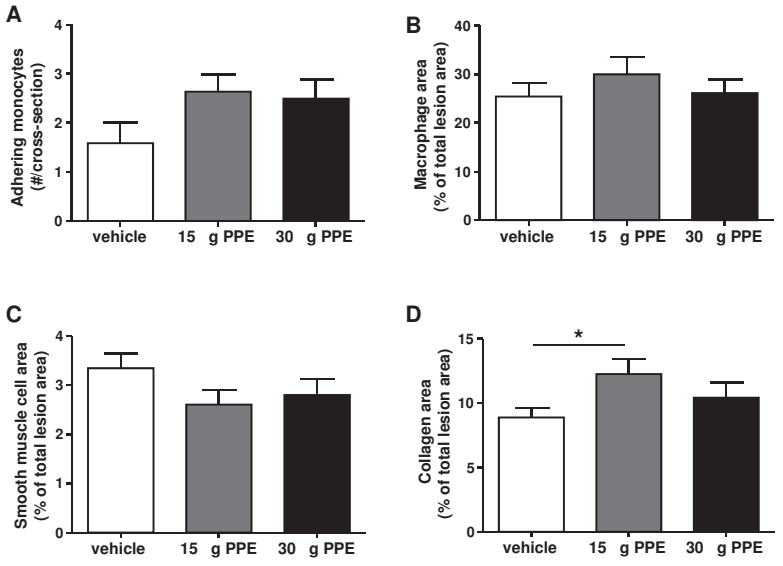

Fig. 6. Intratracheal PPE instillation does not affect adhering monocytes and tends to increase collagen content. Twenty-four weeks after intratracheal vehicle or PPE instillation, mice were sacrificed and the number of adhering monocytes (A), macrophage content (B), smooth muscle content (C) and collagen content (D) in the atherosclerotic lesions in the aortic root was determined. Values are means ± SEM; n=12-18; *P<0.05.

Discussion

There is ample clinical evidence that a poor lung function, most commonly caused by COPD, is an independent predictor of CVD.[28,29,30] Since smoking is the leading cause of COPD, smoke models have been utilized to investigate the relationship between pulmonary emphysema and atherosclerosis.[31, 32] Smoking itself, however, has a direct effect on atherogenesis, *i.e.* through induction of a systemic inflammatory response and dyslipidemia, and therefore interferes with the elucidation of the role of the different aspects of COPD in atherosclerosis development. Furthermore, although the majority of COPD patients are current or former smokers (~90%), the disease also occurs in patients who have never smoked.[33] Emphysema, characterized by alveolar tissue destruction, is a common manifestation of COPD, but its role in atherosclerosis development independent of systemic inflammation has never been studied. Therefore, in the present study we investigated whether emphysema in the absence of smoke-induced pulmonary inflammation would affect atherosclerosis development.

For the first time we show that PPE-induced emphysema *per se* does not worsen atherosclerosis development. Atherosclerotic lesion size and composition were not different between the groups. Thus, our results indicate that alveolar destruction alone cannot explain the increased incidence of CVD in COPD patients. Systemic inflammation has been regarded as a possible mediator between COPD and CVD,[4,34] although the evidence for this link is circumstantial.[3] Nevertheless, because of the known role of systemic inflammation in aggravating CVD, the absence of enhanced systemic inflammation in the current study may explain why atherosclerosis was not affected by the presence of emphysema in our study. Our results are in line with a study showing that allergic airways inflammation induced by *Aspergillus fumigatus*, resulted in marked increase in pulmonary cytokine levels and inflammatory cell influx and mild increased plasma SAA levels, but did not affect atherosclerosis development in *apoe*$^{-/-}$ mice.[35] This indicates that an increase in pulmonary (and subsequent systemic) inflammation does not necessarily affect atherosclerosis development. In our study, emphysema was developed before the induction of atherosclerosis, whereas in humans these diseases often develop concomitantly. The reason for this study design is that we aimed to study the effect of alveolar destruction separate from the effect of lung inflammation and spill over in the systemic circulation. Therefore, the study was designed to make sure that during development of atherosclerosis, which was started 3 weeks after PPE instillation to induce emphysema, systemic inflammation due to a spillover from the lung is no longer a contributing factor. Future studies using a different study design and other experimental COPD models should therefore focus on whether pulmonary inflammation by itself, or synergistically with alveolar destruction affects atherosclerosis development.

Unexpectedly, we found a dose-dependent decrease in lesion severity in the PPE-treated groups. We therefore considered the possibility that PPE treatment affected mechanisms involved in atherosclerosis development. First, dyslipidemia is one of the main contributors to atherosclerosis development. We found however, that intratracheal PPE instillation did not affect plasma lipid levels nor the lipoprotein profile. Therefore, the moderating effect of PPE-induced emphysema on atherosclerosis severity in our mouse model cannot be explained by a reduction in lipid levels. Second, inflammation is another well-known player in atherogenesis and may be decreased in experimental emphysema. A reduction of inflammation in the lungs and accompanying decrease in systemic inflammation in the PPE-treated mice may explain the observed decrease in atherosclerosis severity. However, at the same time point of determination of atherosclerosis development, *i.e.* 24 weeks after intratracheal PPE instillation, no differences in the number of macrophages and neutrophils in the lungs were found, making this mechanism unlikely. On the other hand, we found a reduction in circulating monocytes and neutrophils after intratracheal PPE instillation, which may have contributed to the reduced atherosclerotic lesion severity in these mice.

How do we explain the observed decrease in the number of circulating monocytes and neutrophils? Recruitment of monocytes and neutrophils towards the lung during the acute phase of inflammation due to PPE-induced damage could be a mechanism.[36] This is however an unlikely explanation, since the acute inflammatory reaction after intratracheal administration of PPE to rodents is resolved within 3 weeks,[37,38] and we found that the number of macrophages

and neutrophils in lung tissue at the end of the study was not affected by PPE treatment. A second potential mechanism for the decrease in circulating monocytes and neutrophils in the PPE-treated animals is that it is part of a resolution process. Resolution after inflammation is programmed within the normal inflammatory response itself after an acute injury to minimize tissue and organ damage.[39] Possibly, this resolution of inflammation reduces systemic inflammation involved in atherosclerosis. Failure of effective resolution has been suggested to be a contributor to the dysregulated inflammatory process in atherosclerosis development.[40] In addition, the decrease in circulating monocytes and neutrophils may be the result of a progressive negative feedback mechanism for the production of inflammatory cells, after the acute inflammation induced by PPE instillation. In view of the time span of 8 weeks between the intratracheal PPE instillation and the observed reduction in circulating monocytes and neutrophils, this possibility is not presumable. It is likely that there are other causes for this reduction which are currently unknown to us.

Another potential mechanism linking COPD to enhanced CVD is chronic hypoxia.[2] PPE-treated mice also showed signs of chronic hypoxia, indicated by a significantly higher number of circulating erythrocytes, hemoglobin and hematocrit levels. As it is known that hypoxia can promote atherogenesis,[41] it seems contradictory that the mice treated with PPE in our study showed less severe atherosclerotic lesions. However, the magnitude of hypoxia in the PPE-treated mice was likely compensated by several mechanisms. First, the respiration amplitude was increased, probably as a compensation mechanism for the reduction in diffusion area caused by PPE instillation. This compensating mechanism may have sufficed to prevent hypoxia to some degree, since the respiratory rate did not differ between the groups. In addition, hypoxia was probably at least partly compensated by the increase in the number of circulating erythrocytes. Moreover, the development of right ventricular hypertrophy as observed in the present study, and reported in COPD patients by others[42] may also have acted as a compensation mechanism. Therefore, in the PPE-treated mice several compensatory mechanisms are likely to have developed in order to minimize the level of hypoxia. Finally, the possibility that hypoxia does increase atherosclerosis only in presence of nicotine needs to be considered. Previous studies have shown that nicotine increases growth of atherosclerotic lesions in mice.[43] Since this effect was subsequently shown to be mediated by the α7-non-neuronal nicotinic acetylcholine receptor, and because the endothelial expression of this receptor is increased by hypoxia,[44] which may have resulted from emphysema in the present study, it is possible that nicotine-mediated effects on atherosclerotic plaque growth are increased in PPE-treated mice.

Finally, physical inactivity and obesity are risk factors for the development of atherosclerosis.[45] In the present study, PPE treatment did not affect body weight gain and food intake. Therefore, it is unlikely that these factors have influenced atherosclerosis development. Although we did not measure the physical activity of the mice, there was no evident difference in appearance and behavior between the groups, but we cannot formally exclude this.

In summary, our results indicate that in atherosclerosis-prone *E3L* mice, emphysema *per se*, in the absence of inflammation, does not aggravate the development of atherosclerosis size and even diminishes its severity. Thereby, this study has provided more mechanistic insight into the development of the major comorbidity CVD in COPD patients.

Acknowledgements

The authors thank Vera E. van Rijn, Ebru Sucu and Lianne C.J.A. van der Wee-Pals for excellent technical assistance.

This study was supported by grants from the F.R. Nieuwenkamp stichting (LUMC); Netherlands Asthma Foundation (grant 3.2.10.048) and Netherlands Heart Foundation (grant 2009T038).

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