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A novel SSAO inhibitor PXS-4728A suppresses inflammation and fibrosis and improves lung function in experimental chronic obstructive pulmonary disease

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Running title: A novel SSAO inhibitor improves experimental COPD

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BACKGROUND AND PURPOSE

Chronic obstructive pulmonary disease (COPD) is a major cause of illness and death that is often induced by cigarette smoking (CS). It is characterised by pulmonary inflammation and fibrosis that impairs lung function. Existing pharmaceuticals aim to control symptoms but have low efficacy, and there are no broadly effective treatments. A potential new therapeutic target is ectoenzyme semicarbazide-sensitive mono-amine oxidase (SSAO, or vascular adhesion protein-1, VAP-1). SSAO is elevated in smokers serum, and is a pro-inflammatory enzyme that facilitates the adhesion and transmigration of leukocytes from the vasculature to sites of inflammation.

EXPERIMENTAL APPROACH

PXS-4728A has been developed as a small molecule inhibitor of SSAO. We tested its ability to suppress SSAO activity and ameliorate inflammation and hallmark features of human disease in a mouse model of CS-induced experimental COPD. The model replicates key aspects of human COPD, including chronic airway inflammation, fibrosis and impaired lung function.

KEY RESULTS

PXS-4728A treatment completely inhibited lung and systemic SSAO activity induced by acute and chronic CS exposure. Daily oral treatment inhibited airway inflammation (immune cell influx and inflammatory factors) induced by acute CS exposure. Therapeutic treatment during chronic CS-exposure, when the key features of experimental COPD develop and progress, substantially suppressed inflammatory cell influx and fibrosis in the airways and improved lung function.

CONCLUSIONS AND IMPLICATIONS

Treatment with the SSAO small molecule inhibitor, PXS-4728A, suppresses airway inflammation and fibrosis and improves lung function in experimental COPD. This study demonstrates the therapeutic potential of PXS-4728A for this debilitating disease.

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Keywords

Chronic obstructive pulmonary disease; COPD; emphysema; fibrosis; inflammation; semicarbazide-sensitive amine oxidase; SSAO; vascular adhesion protein-1; VAP-1

Abbreviations

BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CXCL1, (C-X-C motif) ligand 1; EM, extracellular matrix; G-CSF, granulocyte-colony stimulating factor; MLI, mean linear intercept; MMP, matrix metalloproteinase; SSAO, semicarbazide-sensitive amine oxidase; TNFα, Tumour necrosis factor-alpha; VAP-1, vascular adhesion protein

Tables of Links

Hyperlinked Targets and Ligands

Targets
Enzymes
SSAO (VAP-1)

Ligands	
Collagen	Phosphodiesterase-4
CXCL1	Rolipram
G-CSF	TNFa
Hydrogen peroxide	VCAM
ICAM	

These tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to Pharmacology (Pawson *et al.*, 2013) and are permanently archived in the concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Chronic obstructive pulmonary disease (COPD) is the 3rd leading cause of chronic morbidity and death worldwide, and its prevalence is increasing (Lozano *et al.*, 2012). It is a heterogeneous disease variously comprised of debilitating and complex pathologic features including chronic pulmonary inflammation (bronchitis), fibrosis and emphysema (Keely *et al.*, 2011; Fricker *et al.*, 2014). These features combine to impair lung function and result in reduced oxygen transfer leading to breathlessness. Cigarette smoke (CS) exposure is the primary etiological agent in Western countries, accounting for more than 90% of cases. Once induced the patients' condition often continues to deteriorate, even after cessation of smoking. Wood and cooking smoke and pollution are also important risk factors particularly in developing nations (Fabbri *et al.*, 2004; Eisner *et al.*, 2010; KO and HUI, 2012).

There are no cures for COPD and current treatments have substantial limitations. High doses of corticosteroids, long acting β-agonists, anti-cholinergics, phosphodiesterase-4 inhibitors (e.g., Rolipram) and anti-muscarinics are used in an attempt to control acute inflammation and reduce exacerbations (Yang *et al.*, 2007; Calverley, 2014). However, these therapies have minimal effects on lung function, do not modify the underlying causes of disease, halt its progression or reverse pathological aspects. In addition, they are associated with deleterious side effects and can predispose patients to pneumonia, particularly when used long-term (Suissa *et al.*, 2013; Yang, 2015). Structural changes such as collagen deposition and fibrosis may also restrict the effectiveness of treatments. Vaccination protects against infections that cause exacerbations, but are less effective in COPD (Nath *et al.*, 2014). Thus, the development of novel more effective drugs that suppress symptoms and/or limit disease progression, would be of considerable clinical benefit in people with COPD.

The development of new therapies has been hampered by the lack of animal models that recapitulate the hallmark features of human COPD in a reasonable time frame (Vlahos and Bozinovski, 2014). To address this we have generated a model induced by direct inhalation of CS that develops the hallmark features of COPD, including inflammation, emphysema-like alveolar destruction, bronchitis, non-responsiveness to corticosteroid

treatment, in 8 weeks (Beckett *et al.*, 2013; Franklin *et al.*, 2014; Fricker *et al.*, 2014; Hansbro *et al.*, 2014; Chen-Yu Hsu *et al.*, 2015; Tay *et al.*, 2015). The levels of CS-exposure are representative of that of a pack-a-day human smoker (Fricker *et al.*, 2014).

Chronic CS exposure induces persistent inflammation dominated by macrophages, neutrophils and $CD8^+$ lymphocytes, which drive the pathology and development of disease features (Madeno *et al.*, 2007; Minematsu and Shapiro, 2007; Keely *et al.*, 2011; Duan *et al.*, 2012; Beckett *et al.*, 2013; Fricker *et al.*, 2014). We have recently shown that mast cells and the factors that they release may also play important pathogenetic roles by controlling macrophage responses (Beckett *et al.*, 2013; Hansbro *et al.*, 2014). The development, function and migration of these immune cells is controlled by stimulatory factors such as granulocyte-macrophage colony-stimulating factor (Vlahos *et al.*, 2010) and granulocyte-colony stimulating factor (G-CSF) (Adachi *et al.*, 2003; Yu *et al.*, 2006; Kiss *et al.*, 2008; Schilter *et al.*, 2015). When they reach the airways and lungs these cells release a range of inflammatory factors including cytokines (e.g., TNFα) and chemokines (e.g., CXCL1) (Keatings *et al.*, 1996; Beckett *et al.*, 2013). This leads to further inflammation and a feedback loop that results in chronic inflammatory responses and the further influx of cells to the site of injury. The repeated cycles of injury and repair results in collagen deposition and fibrosis of the airways, emphysema and reduced lung function (Keely *et al.*, 2011; Fricker *et al.*, 2014). However, the causal links between inflammation and fibrosis remain to be fully elucidated.

The development of fibrosis is a major problem in COPD with structural and compositional changes identified in most lung structures from the central airways down to the alveoli (Vignola *et al.*, 1997). It results from the sub-epithelial deposition of fibronectin, fibulin and tenascin and the accumulation of collagen (Dunsmore, 2008; Lau *et al.*, 2010; Jaffar *et al.*, 2014; Ge *et al.*, 2015). The development of fibrosis in the small airways strongly correlates with COPD severity, and results in narrowing of the airway lumen and airflow limitation (Bosken *et al.*, 1990; Hogg *et al.*, 2004). It has been suggested that fibrosis occurs initially in the small airways as a result of persistent inflammation induced by CS but becomes sustained as a consequence of reprogrammed immunity (Boorsma *et al.*, 2013).

Semicarbazide-sensitive amine oxidase (SSAO), also known as vascular adhesion protein-1 (VAP-1), is a copper dependent amine oxidase that is expressed in most tissues, particularly in the lung (Singh *et al.*, 2003). It has increased activity in numerous inflammation-associated diseases including Alzheimers (del Mar Hernandez *et al.*, 2005), diabetes (Boomsma *et al.*, 1995), stroke (Hernandez-Guillamon *et al.*, 2010), liver disease (Nemeth *et al.*, 2007), atherosclerosis (Karadi *et al.*, 2002) and multiple sclerosis (Airas *et al.*, 2006). A membrane bound form of the enzyme is predominantly expressed on the cell surface of adipocytes, smooth muscle and endothelial cells, and a soluble form occurs in the bloodstream (Precious *et al.*, 1988; Andres *et al.*, 2001). The link with COPD has not been widely explored but in smokers, serum SSAO activity is positively correlated with the number of pack years (2013i). SSAO predominantly catalyses the deamination of primary amines (e.g. methylamine, benzyamine, aminoacetone) into toxic byproducts (e.g. aldehydes, hydrogen peroxide (H_2O_2) and ammonia) (Lizcano *et al.*, 1994). Significantly, methylamine is a major component of CS and an end product of nicotine metabolism (Yu, 1998) XX Health services, 1982. It is metabolized almost exclusively by SSAO and the aldehyde produced, formaldehyde, is a well-recognized genotoxin and crosslinking agent (Boor *et al.*, 1992). The production of the reactive oxygen species H_2O_2 results in oxidative stress which contributes to disease through the development and maintenance of chronic inflammation and tissue damage. SSAO also facilitates both acute and chronic inflammation by promoting leukocyte trafficking from blood vessels through the endothelium and into inflamed tissues (Merinen *et al.*, 2005). Thus, treatments that inhibit both enzymatic and cell adhesive functions associated with SSAO activity may ameliorate inflammation, and subsequent pulmonary fibrosis and impaired lung function, and could provide a potential therapeutic approach in COPD.

Here we tested the ability of a selective, orally active small molecule inhibitor of SSAO, PXS-4728A (4-[(E)-2-(aminomethyl)-3-fluoro-allyloxy]-N-tert-butyl-benzamide hydro-chloride) (Schilter *et al.*, 2015) to inhibit hallmark features of experimental COPD in our mouse model that is representative of the human disease. In acute CS exposure we demonstrate that prophylactic and therapeutic PXS-4728A administration completely inhibits SSAO activity, and reduces inflammatory cell influx into the airways as well as cytokine and chemokine production. Importantly, therapeutic PXS-4728A administration in chronic CS

exposure when COPD features are established reduced the influx of inflammatory cells, in particular neutrophils and macrophages, suppressed collagen deposition around small airways and improved lung function. Thus, targeting SSAO with PXS-4728A may represent a viable therapeutic option in COPD.

Methods

Mice

Female wild-type C57BL/6 mice were group housed under specific pathogen-free conditions at the Animal Services Unit, Hunter Medical Research Institute, Newcastle, NSW, Australia.

All experiments were conducted in accordance with approval from the animal ethics committee of the University of Newcastle, NSW, Australia. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

CS-exposure

Female C57BL/6 mice (6-8 weeks-old) were exposed to either CS from 12 research-grade cigarettes (3R4F, University of Kentucky, Lexington, KY, USA) or normal air for 75 min twice per day. Female mice were used as they have a 50% increase in COPD development compared to male mice. CS was delivered to mice using an in-house custom-designed and purpose-built nose-only, directed-flow inhalation and smoke-exposure system (CH Technologies, Westwood, NJ) housed in a fume and laminar flow hood. Mice were either acutely exposed each day for four days, or chronically for five days a week for 12 weeks (Beckett *et al.*, 2013; Franklin *et al.*, 2014; Hansbro *et al.*, 2014; Chen-Yu Hsu *et al.*, 2015).

Drug treatments

Mice were lightly anaesthetised by inhalation of 5% isoflorane. They were then treated with various doses of PXS-4728A or Rolipram (positive control, 3 $mg \cdot kg^{-1}$, Sigma Chemicals, St

Louis, MO, USA), resuspended in 0.5% w/v methylcellulose in sterile water (vehicle), or vehicle alone by oral gavage in a total volume of 200 µl immediately before CS-exposure. For treatment in the acute four-day exposure model, mice were treated before the first CS-exposure of each day. To test the therapeutic potential of PXS-4728A, mice were chronically exposed to CS for 12 weeks, with drug delivery prior to the first CS exposure of each day, commencing after six weeks, when features of COPD first emerge (Beckett *et al.*, 2013). Previous studies have shown that the vehicle control has no effect on immune responses compared to PBS-treated mice. Thus, in order to reduce animal use in these studies PBS groups were omitted.

SSAO enzyme activity

To measure SSAO activity, animals were euthanized and abdominal fat and lungs were collected and snap frozen. Tissue samples were weighed, homogenized in ice-cold HES buffer (20 mM HEPES, 1 mM EDTA, Sucrose 250 mM, 1x proteases and phosphatases inhibitor, pH 7.4) at a final concentration of 1 g·20 ml^{-1}. Homogenates were centrifuged at 2,000 x*g* for 5 min at 4^{o}C and the supernatants collected and diluted 1:5 in assay buffer (0.1 M sodium phosphate buffer) for the fluorometric assay (fat tissue) and left undiluted for radioactivity assay (lung tissue) of SSAO activity.

Fluorometric enzymatic activity measures were based on the production of H_2O_2 (Schilter *et al.*, 2015). Tissue samples were incubated with 0.5 mM of pargyline, to inhibit any potential endogenous monoamine oxidase A and B activity. Samples (25 µl) were then incubated for 30 min at 37 °C with or without the specific SSAO inhibitor Mofegiline (1 µM). Equal amounts of the reaction mixture containing amplex red (120 µM; Life Technologies, Australia), horseradish peroxidase (1.5 Uml; Sigma-Aldrich, Sydney, Australia) and benzylamine (80 µM) was prepared in 0.1 M sodium phosphate buffer and added 25 µl into each well after the 30 min incubation. The relative fluorescence units (RFU) were read every 2.5 min for 30 min at 37 °C, excitation 565 nm and emission 590 on a BMG FLUOstar OPTIMA Microplate Reader (Optima, BMG labtech), and the slope of the kinetic curve for each sample was calculated using MARS data analysis software (BMG labtech) in RFU·min^{-1}.

The signal obtained from the non-treated samples was considered to be 100%. The difference between the signals obtained in the samples not treated (high signal control) or in the presence of Mofegiline (low signal control) was considered to be the specific SSAO activity. The data from all treated samples were adjusted to yield a % response graph.

Radioactive enzymatic activity (REA) measures were based on the catalysis of ^{14}C-benzylamine to ^{14}C-benzaldehyde by SSAO (Devlin *et al.*, 1990). Assays were performed by a commercial service at Tetra-Q ADME, The University of Queensland, Brisbane, Australia. Tissue samples were incubated with 3μM Pargyline/Clorgyline to inhibit any potential endogenous monoamine oxidase A and B activity. Samples (150 μl) were incubated for 20 min at 37 °C with or without the specific SSAO inhibitor PXS-4728A (30 nM). ^{14}C-benzylamine (50 μl of 915.5 μM stock) was added to each sample, which were incubated for 30 min at 37 °C. Reactions were terminated by the addition of 50 μl of 2M citric acid and the products were extracted into 1 mL of toluene/ethyl acetate 1:1 (v/v) and assayed by liquid scintillation counting. The difference between the signals obtained in the absence of the inhibitor (high signal control) and in the presence of PXS-4728A (low signal control) was considered to be the specific SSAO activity in the sample. The data are presented as a % of the signal to the non-treated group (considered to be 100%).

In the REA assays, PXS-4728A was used to selectively and specifically inhibit the SSAO component in order to measure the background, i.e. non-SSAO mediated amine oxidase activity. As REA assays have a very high selectivity we used the most selective inhibitor known to us, which is PXS-4728A. Measurements in the presence of added PXS-4728A were only used to determine the "low" signal for normalisation while the "high" signal was that from non-drug treated animals not treated with a specific SSAO inhibitor. Thus, PXS-4728A was not added to samples from drug-treated animals and cannot interfere with the results in treatment groups. In contrast to REA in fluorometric measurements SSAO concentrations in tissues are substantial and selectivity and specificity was not critical. Therefore, the well-characterised inhibitor mofegiline was used in these studies.

Airway inflammation

Airway inflammation was assessed by enumerating total leukocyte cell numbers as well as specific inflammatory cell types in bronchoalveolar lavage fluid (BALF) (Beckett *et al.*, 2013; Hansbro *et al.*, 2014; Chen-Yu Hsu *et al.*, 2015). BALF was obtained by lavaging lungs with two 400 µl aliquots of PBS at room temperature. Total cell numbers were assessed using trypan blue exclusion. Cytospins were performed on the remaining BALF cells and differential cell counts were obtained based on morphology (Thorburn *et al.*, 2010; Asquith *et al.*, 2011; Beckett *et al.*, 2013; Essilfie *et al.*, 2015). Cytokine and chemokine levels were assessed in BALF. TNFα, CXCL1 and G-CSF were assessed using a BD™ Cytometric Bead Array kit (BD Biosciences, San Jose, CA, USA), according to the manufacturers instructions with a minimum detection limit of 0.274 pg/ml.

Small airway-associated fibrosis

Small airway-associated fibrosis was assessed in formalin-fixed lung sections by measuring the deposition of Masson's Trichrome Blue stained collagen around the small airways (Hansbro *et al.*, 2014). Stained lung sections on slides were photographed and the amount of collagen was calculated using Image J software (version 1.47) by assessing the area of deposition around small airways of less than 1mm diameter (W_{ct}/P_{bm}), where $W_{ct}=A_o-A_{i,}$ inner collagen area = A_i, outer collagen area = A_o and basement membrane length = P_{bm}.

Soluble lung collagen

Snap frozen sections of lung (~10 mg) were minced in ice-cold PBS. After washing with PBS, acid-soluble collagen was extracted by incubating overnight in 1 ml of 0.1 $mg{\cdot}kg^{-1}$ pepsin in 0.5 M acetic acid (Sigma Aldrich) at 4^oC. Soluble collagen was concentrated and measured using Sircol Collagen Assay Kits according to the manufacturer's instructions (Biocolor, Carrickfergus, UK) (Chow *et al.*, 2012).

Total lung collagen

Frozen lung tissues (10 mg) were weighed and incubated in HCl (6 N) for seven h at 130°C. Solutions were then placed in a 100°C drying oven to evaporate the liquid. The resulting pure collagen was resuspended in a 96 well plate in 100 µl ddH_20 per well. Plates were centrifuged (220 x*g* for 15 s) and supernatants removed. Chloramine T solution (100 µl total, 0.14:1:8 Chloramine T:n-Propanol:Citrate/acetate) was added to the samples and standards followed by incubation for 20 min at room temperature. Then Erhlich's solution (100 µl, Sigma Aldrich) was added and samples incubated for 18 min at 65°C. Total lung collagen was determined by measuring absorbance at 558 nm compared to hydroxyproline standards (Sigma-Aldrich, effecive range 0.625-40 $ug{\cdot}mL^{-1}$).

Emphysema

Sections (4 µm thick) of paraffin-embedded, formalin fixed inflated lung tissue (4 µm thick) mounted on microscope slides were stained with haematoxylin and eosin. Emphysema-like alveolar enlargement was assessed using the mean linear intercept (MLI) technique, which is a standard method for assessing alveolar size and emphysema in mice (Horvat *et al.*, 2010; Beckett *et al.*, 2013; Starkey *et al.*, 2013; Hansbro *et al.*, 2014; Chen-Yu Hsu *et al.*, 2015). A standardised template containing horizontal lines was overlayed over micrographs of lung sections. The number of alveolar intercepts were counted and the resulting average number of intercepts determined. Reduced numbers of intercepts is an indicator of increased alveolar size and of emphysema and tissue damage.

Lung function

Static lung compliance was obtained from quasistatic pressure-volume loops using Flexivent apparatus (Legacy System; SCIREQ, Montreal, Canada) as previously described (Harris, 2005). Mice were anaesthetised (50 µl/10 g i.p.) with a mixture of xylazine (2 mg/ml, Troy Laboratories) and ketamine (40 mg/ml, Ceva). Cannulae were inserted into mouse tracheas. Animals were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min, with increasing airway pressure from 2–30 cmH_2O into the lungs. The volume of air in the lungs at the end of maximal inspiration was determined. Static lung compliance was calculated as the

volume change divided by applied pressure change, while hysteresis was assessed by measuring the difference between inspiratory and expiratory pressure volume loops. Each manoeuvre was performed a minimum of three times, and the average calculated (Beckett *et al.*, 2013; Hansbro *et al.*, 2014; Chen-Yu Hsu *et al.*, 2015).

Statistics

All datasets were determined to be normally distributed based on previous experiments, and were also tested using the Kolmogorov–Smirnov test (alpha = 0.05). Data were analysed using One-way ANOVA with Tukeys multiple comparison test using PRISM software (V6.0d, GraphPad, La Jolla, CA, USA). Data are means ± SEM (n = 6-8) with $p<0.05$ considered statistically significant.

Results

Oral delivery of PXS-4728A potently inhibits constitutive lung and peripheral SSAO activity

SSAO activity is found constitutively in the lungs, and at higher levels in fat, in particular adipocytes. Thus, the effectiveness of PXS-4728A treatment as an inhibitor was first assessed by measuring SSAO activity in the lungs and inguinal fat in an acute four day model of CS-exposure. Acute CS-exposure did not increase SSAO activity in the lungs when assessed 16 h after the last exposure compared to normal air-exposed controls (Figure 1). However, daily oral treatment with PXS-4728A at either 2, 6 or 20 $mg{\cdot}kg^{-1}$ doses inhibited constitutive SSAO activity compared to vehicle-treated CS-exposed mice. Additionally, SSAO activity in inguinal fat was completely inhibited with PXS-4728A treatment (Figure 1). This shows that orally delivered PXS-4728A is a potent *in vivo* inhibitor of SSAO activity both in the lung and peripheral tissue.

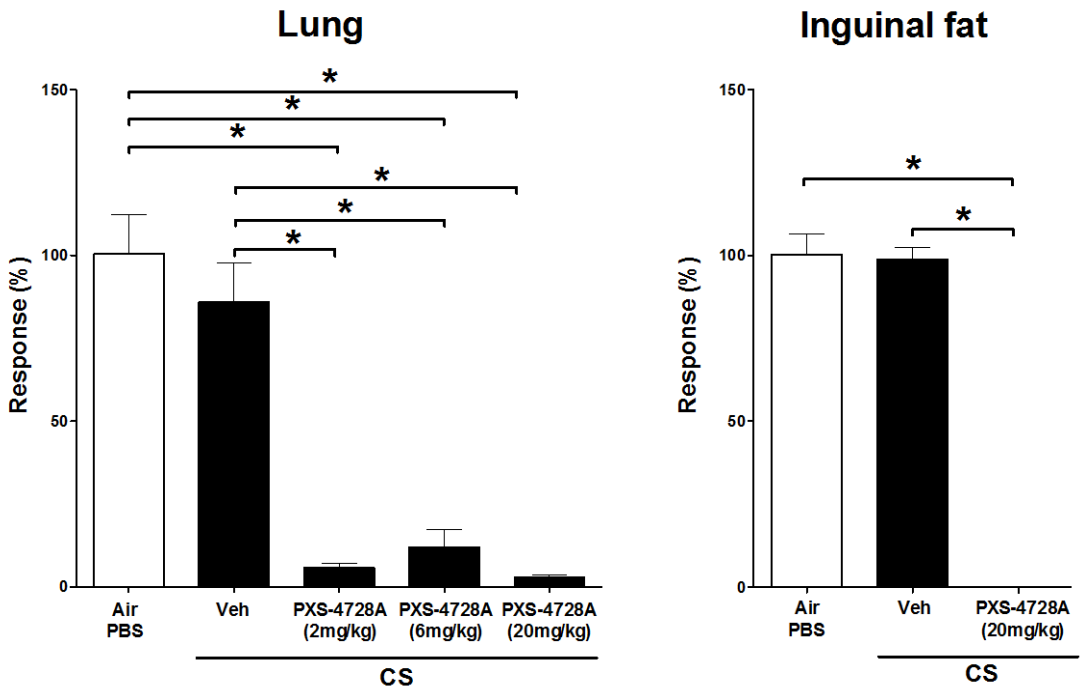


Figure 1: Oral treatment with PXS-4728A inhibits constitutive lung and peripheral SSAO enzymatic activity. Mice were exposed daily to CS or normal air for four days. Prior to each exposure, mice were treated by oral gavage with PXS-4728A (2, 6 or 20 $mg{\cdot}kg^{-1}$). Lungs and inguinal fat were collected and SSAO activity was measured using radiometric or fluorometric enzymatic assays, respectively. Responses are represented as a percentage of activity relative to the normal air-exposed group. Data represent the means ± SEM (6 mice/group). *P<0.05.

Oral delivery of PXS-4728A reduces inflammatory cell influx into the airways following acute CS exposure

SSAO promotes inflammation largely by facilitating inflammatory cell trafficking into inflamed tissues. Thus, we next assessed the effects of inhibiting SSAO with oral PXS-4728A treatment on the accumulation of total and differential leukocytes in the BALF of mice acutely exposed to CS for four days. Acute CS-exposure increased the numbers of total

leukocytes with significant increases in macrophages, neutrophils and lymphocytes compared to normal air-exposed controls (Figure 2). Daily oral treatment with 12 or 20 mg·kg^{-1} of PXS-4728A reduced the total number of leukocytes in the airways. With the lower dose the numbers of macrophages and lymphocytes were reduced whereas with the higher dose neutrophil numbers were significantly suppressed.

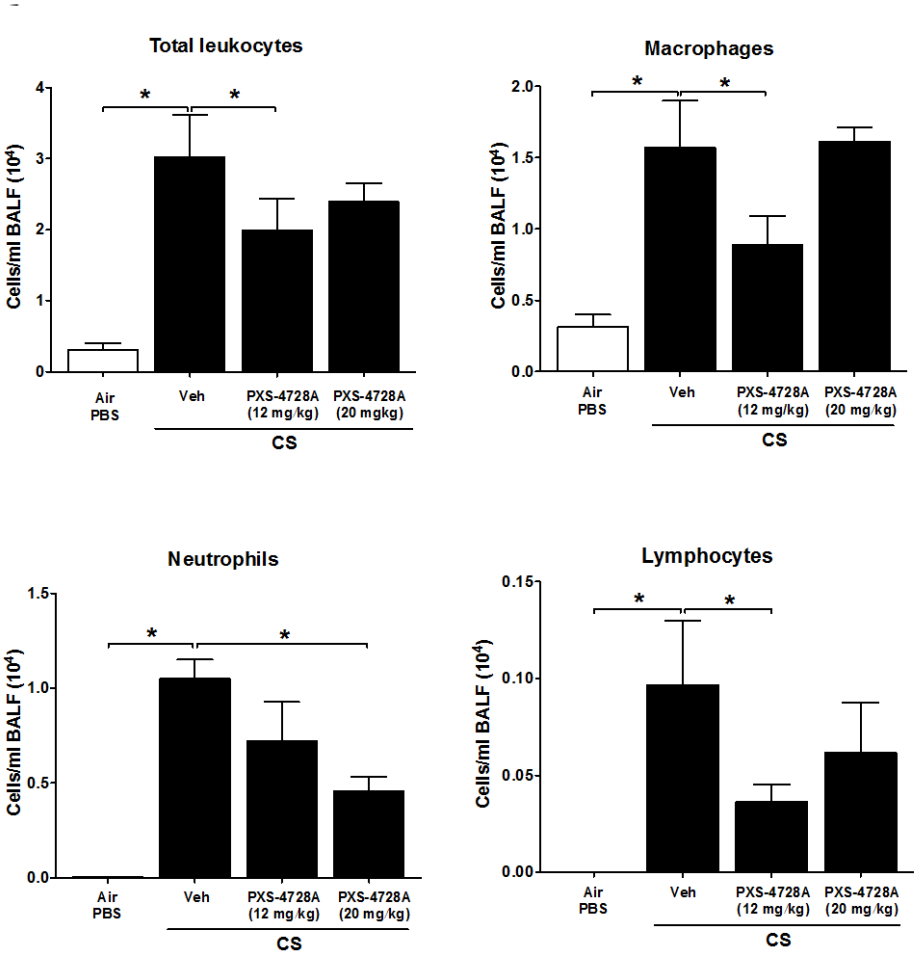


Figure 2: Oral treatment with PXS-4782A reduces the numbers of inflammatory cells in the airways in response to acute CS-exposure. Mice were exposed daily to CS for four days. Prior to each CS exposure, mice were treated by gavage with PXS-4728A (12 or 20 mg·kg^{-1}). BALF was collected and total leukocytes, macrophages, neutrophils and lymphocytes were enumerated in stained cytospin slides. Data represent the means ± SEM (6 mice/group),

*P<0.05, **P<0.05.

Oral delivery of PXS-4728A reduces pro-inflammatory cytokine and chemokine levels in the airways following acute CS exposure

We then assessed the effects of PXS-4728A treatment on pro-inflammatory cytokine and chemokine levels induced by CS-exposure. Acute CS-exposure induced increases in TNFα and CXCL1 protein levels in the BALF compared to normal air-exposed controls (Figure 3). Daily oral treatment with 12 or 20 mg·kg^{-1} of PXS-4728A reduced the levels of TNFα protein in a dose dependent manner, with the higher dose reducing levels back to baseline levels in normal air-exposed mice. CXCL1 levels were significantly reduced when treated with either dose.

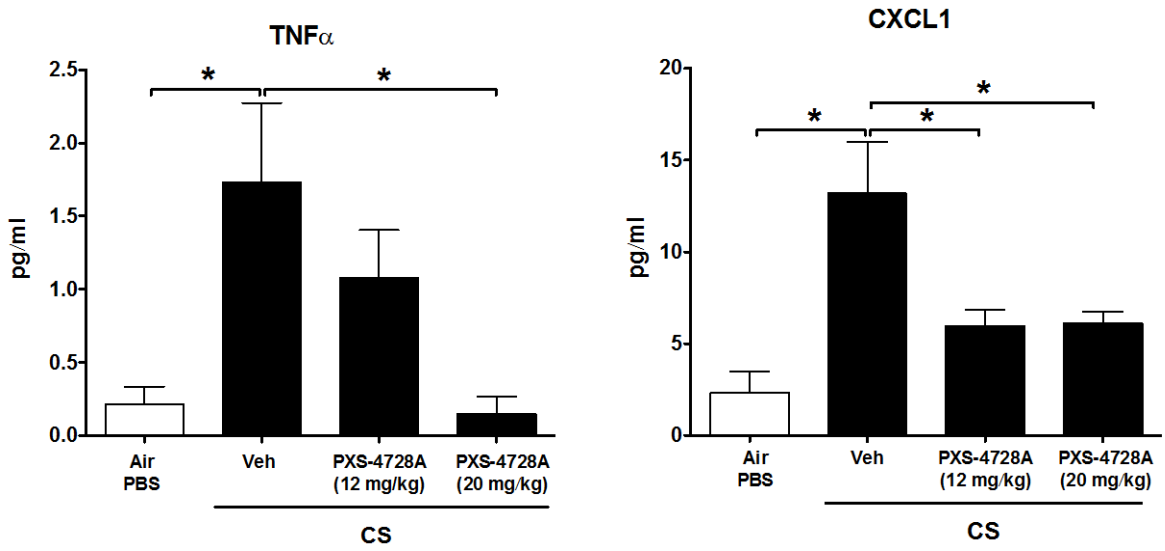


Figure 3: Oral treatment with PXS-4728A reduces pro-inflammatory cytokine and chemokine levels in the airways in response to acute CS-exposure. Mice were exposed daily

to CS or normal air for four days. Prior to each CS-exposure, mice were treated by gavage with PXS-4728A (12 or 20 mg·kg^{-1}) or vehicle. BALF was collected and the levels of the TNFα and CXCL1 measured by ELISA. Data represent the means ± SEM (6 mice/group), **P*<0.05.

Therapeutic treatment with PXS-4728A potently inhibits SSAO activity in the lungs in experimental COPD

PXS-4728A treatment reduced SSAO activity and inflammatory parameters during acute CS-exposure. Thus, we determined whether therapeutic delivery would reduce disease features in a chronic model of COPD, which replicates the hallmarks features of the human disease. Our established model of experimental COPD is characterised by the development of the first signs of disease after six weeks of nose-only CS exposure, which then progresses after 12 weeks of exposure. Disease features include chronic inflammation, fibrosis, emphysema and reduced lung function (Beckett *et al.*, 2013; Franklin *et al.*, 2014; Hansbro *et al.*, 2014; Hsu *et al.*, 2013; Tay *et al.*, 2015). As the higher dose had greater effects in the acute model, mice were treated with PXS-4728A (20 mg·kg^{-1}), or with a positive control phosphodiesterase-4 inhibitor Rolipram (3 mg·kg^{-1}) from 6 weeks of CS-exposure. Rolipram is used here as pharmacological comparator potential control for inhibition of COPD and comparator to the effects of inhibiting SSAO activity, as PDE4 inhibitors have been shown to be beneficial in other models. Rolipram has no known direct inhibitory activity on SSAO. We first assessed the impact of treatment on SSAO activity in the lung. The development and progression of experimental COPD was accompanied by a substantial increase in lung SSAO activity 16 h after the last exposure compared to normal air-exposed controls (Figure 4). Daily oral treatment with PXS-4728A completely inhibited SSAO activity induced by CS-exposure. In comparison treatment with Rolipram reduced SSAO activity to levels found in normal air-exposed controls. This is likely due to indirect effects that result from the suppression of inflammation.

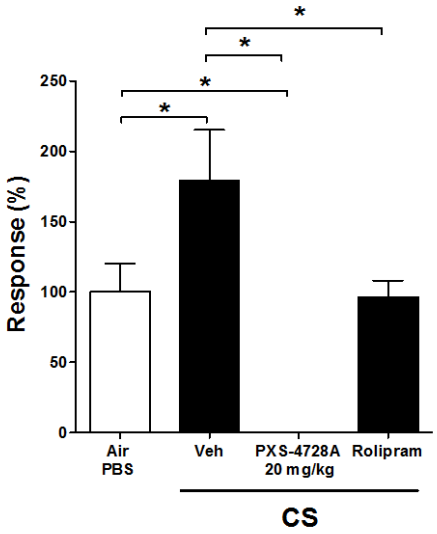


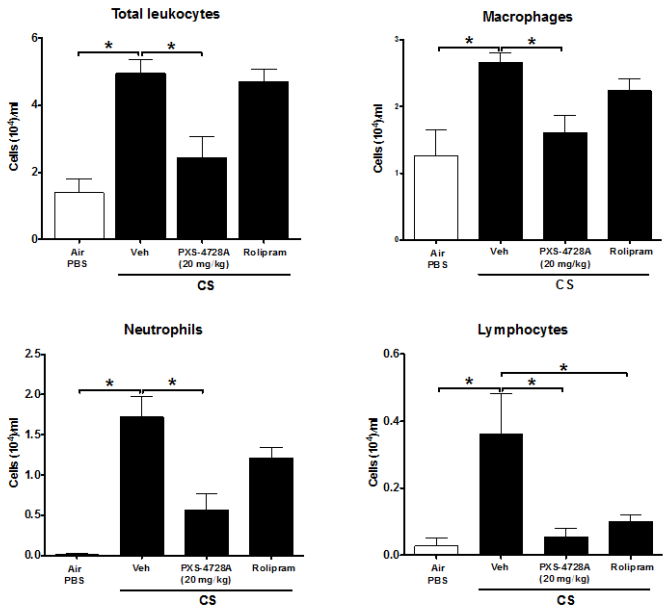
Figure 4: Therapeutic oral treatment with PXS-4728A completely inhibits SSAO activity in the lung in experimental COPD. Mice were exposed to CS or normal air five days per week for 12 weeks. For the last six weeks, mice were treated by daily oral gavage, one h prior to CS-exposure with PXS-4728A, Rolipram or vehicle. Lung tissue was collected and SSAO activity was measured using REA. Activity is presented as the percentage of activity relative to the normal air-exposed group. Data represent the means ± SEM (6-8 mice/group), **P*<0.05.

Therapeutic treatment with PXS-4728A reduces inflammatory cell influx into the airways in experimental COPD

We next assessed the effects of treatment on pulmonary inflammation in experimental COPD. Chronic CS-exposure increased the numbers of total leukocytes and macrophages, neutrophils and lymphocytes in the BALF of mice with experimental COPD compared to normal air-exposed controls (Figure 5). Treatment with PXS-4728A significantly reduced the total numbers of leukocytes in the BALF. The numbers of macrophages and lymphocytes were reduced to those found in normal air-exposed mice and neutrophil numbers were also significantly decreased. Rolipram treatment had no effect on total leukocyte numbers. It did induce a shift in the proportion of the type of leukocytes and increased neutrophils but

reduced lymphocytes. Interestingly, the reduction in leukocytes induced by PXS-4728A treatment was not associated with changes in chemoattractants or growth factors associated with the influx into the airways and proliferation of inflammatory cells (i.e. TNFα, CXCL1 and G-CSF) at this time-point.

A



B

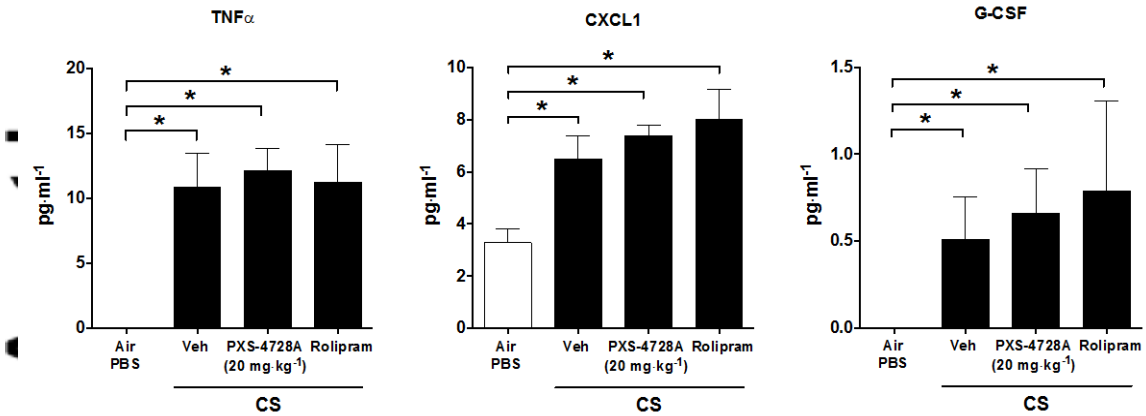
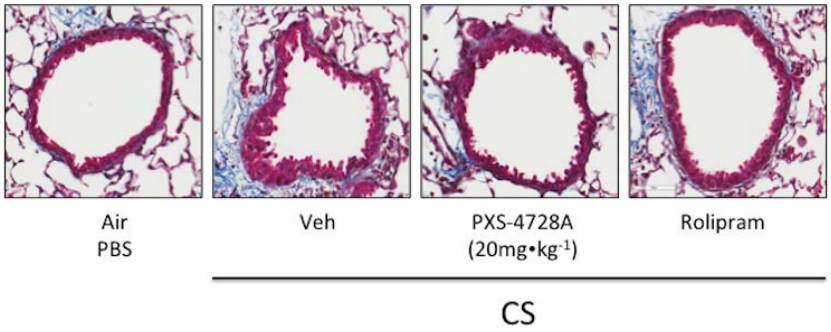


Figure 5:



Therapeutic oral treatment with PXS-4728A reduces the numbers of inflammatory cells in the airways in experimental COPD. Mice were exposed to CS or normal air for five days per week for 12 weeks. For the last six weeks, mice were treated by daily oral gavage, one hour prior to CS exposure with PXS-4728A, Rolipram or vehicle. **A.** BALF was collected and total leukocytes, macrophages, neutrophils and lymphocytes were enumerated in stained cytospin slides. **B.** The levels of TNFα, CXCL1 and G-CSF were measured by ELISA. Data represent the means + SEM (6-8 mice/group), **P*<0.05.

Therapeutic treatment with PXS-4728A reduces small airway fibrosis in experimental COPD

We then assessed the effects of treatment on airway fibrosis and remodelling in experimental COPD. In our model 12 weeks of CS-exposure resulted in significantly increased peribronchiolar collagen deposition around the small airways compared to normal air-exposed controls (Figure 6). Treatment with PXS-4728A completely inhibited excess collagen deposition around the small airways with levels reduced to those in normal air-exposed mice. In contrast, Rolipram did not have any significant effects. Total lung collagen measured by hydroxyproline content was not altered by any treatment.

A

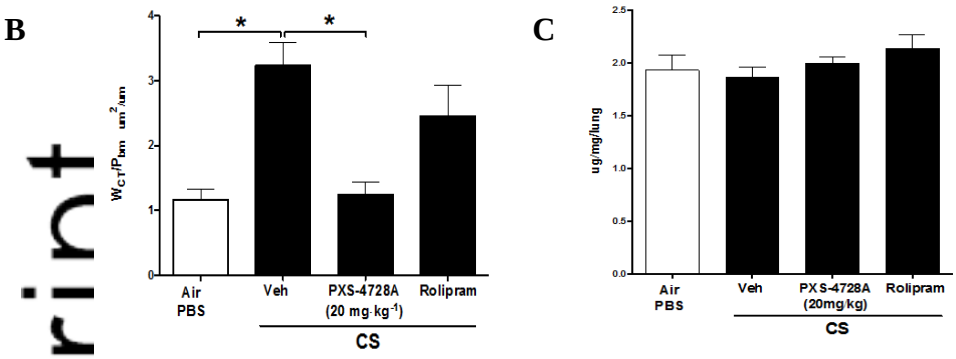


Figure 6: Therapeutic oral treatment with PXS-4728A reduces airway fibrosis and remodelling in experimental COPD. Mice were exposed to CS or normal air for five days per week for 12 weeks. For the last six weeks, mice were treated daily by oral gavage prior to CS-exposure with PXS-4728A, Rolipram or vehicle. Lungs were perfused and inflated and airway associated fibrosis and remodelling was assessed by measuring the deposition of collagen around small airways in stained sections. **A.** Representative micrographs of sections stained with Masson's Trichrome Blue. **B.** Area of fibrosis around the small airways. **C.** Hydroxyproline detected in lung lobes. Data represent the means ± SEM (6-8 mice/group), **P*<0.05.

Therapeutic treatment with PXS-4728A does not reverse emphysema-like alveolar enlargement in experimental COPD

We then assessed the effects of treatment on emphysema-like alveolar enlargement in experimental COPD. In our model 12 weeks of CS-exposure results in alveolar destruction with increases in MLI measurements that is representative of emphysema in human COPD compared to normal air-exposed controls (Figure 7). No treatment had any significant effect on alveolar enlargement.

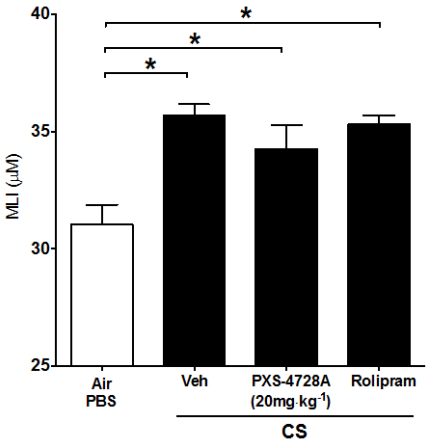


Figure 7: Therapeutic oral treatment with PXS-4728A does not reduce emphysema. Mice were exposed to CS or normal air for five days per week for 12 weeks. For the last six weeks, mice were treated by daily oral gavage prior to CS-exposure with PXS-4728A, Rolipram or vehicle. Lungs were perfused and emphysema-like alveolar enlargement assessed by determining alveolar wall MLI in stained sections. Data represent the means ± SEM (6-8 mice/group), **P*<0.05.

Therapeutic treatment with PXS-4728A improves lung function in experimental COPD

As chronic CS-exposure induced pathological changes that result in impaired lung function, mouse lung function was assessed and analysis performed on pressure-volume (PV) loops, which represent the relationship between changes in pressure and lung volumes. Associated lung compliance (lungs ability to stretch under a given pressure) and hysteresis (difference between compliance on inspiration vs expiration) were also assessed. After 12 weeks of CS-exposure there was an increase in PV loops, lung compliance and hysteresis compared to normal air-exposed controls (Figure 8). Treatment with either PXS-4728A or Rolipram improved lung function and completely inhibited changes in PV loops and lung compliance with levels equivalent to those in normal air-exposed controls.

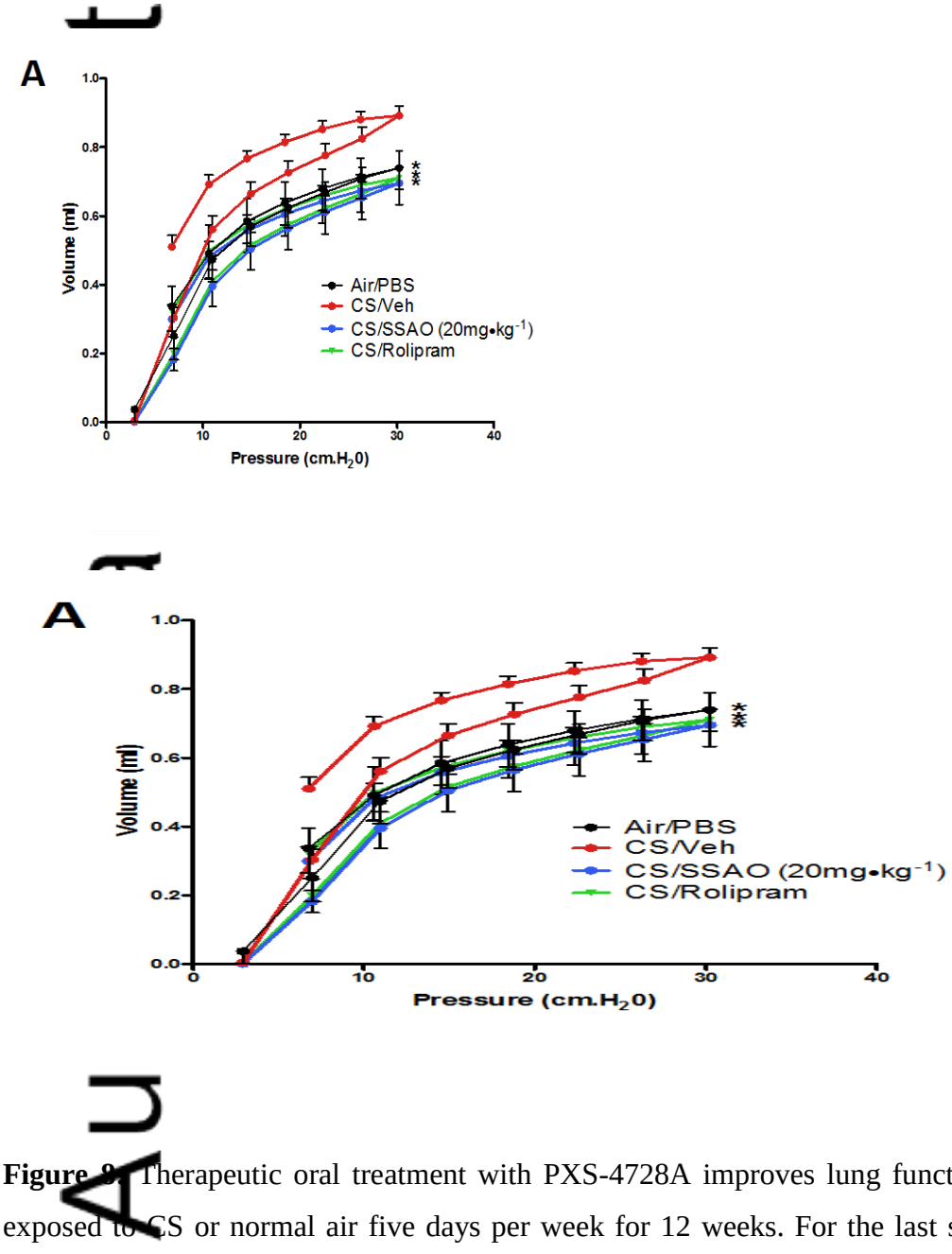


Figure 8: Therapeutic oral treatment with PXS-4728A improves lung function. Mice were exposed to CS or normal air five days per week for 12 weeks. For the last six weeks, mice

were treated by daily oral gavage prior to CS-exposure with PXS-4728A, Rolipram or vehicle. Mice were anaesthetised and cannulated and **A.** Pressure volume loops, **B.** lung static compliance and **C.** hysteresis were measured using flexiVent apparatus. Data represent the means ± SEM (6-8 mice/group), *p<0.05

Discussion

COPD is a heterogeneous disease that results from a set of diverse pathologies and processes that can occur simultaneously, such as inflammation and fibrosis, resulting in poorer lung function, morbidity and mortality. There are no effective treatments that suppress the major disease features of COPD. SSAO levels are increased in the serum in smokers (Wang *et al.*, 2013) and it may play an important role in COPD pathogenesis. Its enzymic activity results in the conversion of CS components such as methylamines into toxic byproducts, while its non-enzymic activities induce pulmonary inflammation through facilitating leukocyte influx (Yu *et al.*, 2006). When it becomes chronic, inflammation leads to the development of fibrosis and impaired lung function (Wong *et al.*, 2014). Here we demonstrate that a small molecular inhibitor of SSAO, PXS-4728A, ameliorates important features of experimental COPD.

Initial assessment of lung SSAO demonstrated that its activity was not increased with acute CS exposure, but required chronic exposure to induce significantly increased activity. Analysing activity in the lung has proven extremely difficult, as the Amplex Red/Horse Radish Peroxidase assay available is not sufficiently sensitivity to determine lung SSAO activity. To overcome this, a more sensitive radioactivity based assay was successfully employed, which for the first time enabled the demonstration of increased SSAO activity in lungs in experimental COPD.

In both acute and chronic CS exposures, daily oral treatment of mice with PXS-4728A

was effective in completely inhibiting SSAO activity in the lung. The PXS-4728A doses used were initially based on previous studies which defined the pharmaco-dynamic and -kinetic properties of the inhibitor. In particular its specificity in targeting SSAO, its bioavailability, and lack of off-target effects (Foot *et al.*, 2013; Schilter *et al.*, 2015). Oral treatment also inhibited systemic SSAO activity, as determined in inguinal fat after acute CS-exposure. The adipose tissue was chosen as it contains high quantities of SSAO, allowing a large assay window to detect enzyme inhibition.

In addition to reducing SSAO activity, PXS-4728A treatment also suppressed the airway leukocyte populations that dominate in the airways after acute and chronic CS exposure. Treatment reduced the numbers of macrophages, neutrophils and lymphocytes in the airways following acute CS exposure back to baseline levels (macrophages and lymphocytes). Suppression of these cell types was greatest with long-term treatment in the chronic model, suggesting that longer treatments may be needed in humans. As there was no increase activity after exposure to acute CS, reduced activity to below control levels and the subsequent reduction in numbers of leukocyte subpopulations suggests a role for SSAO in cellular trafficking. We and others have previously demonstrated that macrophages play important roles in CS-induced pathogenesis (Duan *et al.*, 2012; Lenzo *et al.*, 2012; Beckett *et al.*, 2013) and their depletion prevented the development of experimental COPD (Beckett *et al.*, 2013). While its effects on neutrophils has been well documented (Yu *et al.*, 2006; Kiss *et al.*, 2008; Foot *et al.*, 2013), there has been no previously reported direct effect of alterations in SSAO activity on macrophages. The acute reduction in inflammatory cells was accompanied by significant decreases in the protein levels of the pro-inflammatory cytokine TNFα. TNFα amongst its other roles contributes to the recruitment of inflammatory cells by increasing the expression of adhesion molecules (e. g. ICAM, VCAM and E-selectin) on endothelial cells (Stangl *et al.*, 2001; Mako *et al.*, 2010; Kolaczkowska and Kubes, 2013; Schilter *et al.*, 2015). SSAO activity can also induce the production of ROS products which can increase P-selectin (Stangl *et al.*, 2001; Mako *et al.*, 2010). Leukocytes are able to tether to these molecules, which enables cellular attachment to endothelial surfaces and transmigration into sites of inflammation. Thus, PXS-4728A treatment may reduce

inflammatory cell influx into the lung by affecting adhesion molecules and endothelial tethering.

We previously showed that PXS-4728A treatment reduced the number of airway neutrophils that occurred in response to challenge with bacteria, viruses and allergens (Schilter *et al.*, 2015), while others have demonstrated the effects of SSAO on neutrophils in pulmonary inflammation and ischemic reperfusion injury (Yu *et al.*, 2006; Kiss *et al.*, 2008; Foot *et al.*, 2013). Here we used our models of CS exposure and experimental COPD to demonstrate for the first time that inhibition of SSAO reduces neutrophilic lung inflammation. TNFα can have pleiotropic effects on neutrophils depending on the environment. As alveolar macrophages are the major source of TNFα and other chemokines in the lung, even a partial suppression in the numbers of these cells would result in the reduction in surface molecule expression, a restricted immune response and subsequent cellular inflammation. TNFα contributes to the recruitment of these cells to the site of inflammation by inducing increases in the levels of surface molecules on neutrophils (e.g. CD44) and endothelial cells (e. g. ICAM, VCAM and E-selectin) (Mako *et al.*, 2010; Kolaczkowska and Kubes, 2013). It can also induce neutrophil apoptosis (Cross *et al.*, 2008). PXS-4728A treatment also reduced the levels of the neutrophil chemokine CXCL1, which can be induced by TNFα, and is produced by macrophages, neutrophils, epithelial and endothelial cells (Miyake *et al.*, 2013; Hallstrand *et al.*, 2014; Lo *et al.*, 2014; Shieh *et al.*, 2014). Phosphodiesterase-4 inhibitors have also been shown to reduce CXCL1-associated migration of neutrophils from COPD patients (Turner *et al.*, 2011). Thus, PXS-4728A blocking of SSAO may reduce the number of neutrophils in the lung by impairing transmigration and reducing the levels of TNFα that suppresses endothelial CXCL1 production, and further restricts neutrophil chemoattraction to the lung. In COPD oxidative stress and inflammation become self-sustaining even after the cessation of smoking. We considered that it would be more difficult to suppress disease with ongoing CS exposure. Thus, we used a more stringent test of the inhibitor by using it therapeutically during continued smoke exposure. Nevertheless, treatment is likely to be relevant to ex-smokers also. Although we found that therapeutic treatment suppressed inflammatory cell influx into the airways in both the acute and chronic models, this was not associated with the suppression of

TNFα or CXCL1, or of the granulocyte stimulatory factor G-CSF in the chronic model. Thus, the attenuation of inflammatory cell numbers is likely to occur through the reduction in endothelial markers or other inflammatory factors described above, which requires further study. Nevertheless collectively our data show that the inhibition of SSAO can suppress the cascade of inflammatory events and results in the reduction of acute CS-induced inflammatory cell influx into the airways. Treatment had little effect on emphysema. PXS-4728A was administered therapeutically after six weeks of CS-exposure. At this time conditions for the development of emphysema are likely to have already occurred. While PXS-4728A treatment diminishes several important features of COPD such as inflammation and fibrosis, the treatment regime does not affect all the drivers of emphysema, such as the apoptosis of endothelial/epithelial cells. The inhibition of inflammation over time however is likely to prevent the progression of emphysema although this has not been tested.

We demonstrate here for the first time that inhibition of SSAO inhibits fibrosis of the small airways in experimental COPD. Importantly, this was achieved by treating mice therapeutically when the first signs of COPD develop (Beckett *et al.*, 2013). Other studies in different organs have examined the role of SSAO in the formation of fibrosis. SSAO activity positively correlated with hepatic liver disease including the influx of leukocytes, induction of oxidative stress through the production of toxic metabolites and the expression of fibrosis-associated genes (Weston *et al.*, 2014). Inhibition of SSAO with PXS-4728A in an acute model of renal fibrosis reduced liver remodelling also through the suppression of leukocyte accumulation, oxidative stress and pro-inflammatory and pro-fibrotic gene expression (Wong *et al.*, 2014). In COPD, the extracellular matrix and collagen that form structural integrity of alveoli is often destroyed, whereas conversely these factors and thus fibrosis, are increased around the small airways, causing stiffening and reducing lung function. Here no change was observed total lung tissue collagen levels, which is consistent with our emphysema data, which showed that there was no difference in alveolar enlargement after PXS-4728A treatment. Others have shown that chronic CS exposure can simultaneously increase peribronchiolar fibrosis, resulting in restricted airflow, while at the same time causing the loss

of parenchymal collagen, resulting in tissue destruction and emphysema (Chung and Adcock, 2008).

PXS-4728A treatment, and its associated protection against pathological changes, including reduced inflammation and fibrosis, resulted in a significant improvement in lung function. This involved reduction of experimental COPD-associated increases in pressure-volume loops, and improved static compliance and hysteresis. Many studies show that inflammation and airway remodeling are linked to decreased lung function. Indeed, persistent inflammation induces the development of fibrosis that particularly affects the function of the bronchioles and small airways (Wallace *et al.*, 2006). Increased inflammation correlates with poorer lung function in patients with COPD (Turato *et al.*, 2002; Donaldson *et al.*, 2005; Aronson *et al.*, 2006; Hancox *et al.*, 2007), and small airway fibrosis contributes to airflow restriction through exaggerated tissue contraction, perhaps through the altered balance between fibrotic matrix metaloproteinases and their inhibitors (Hogg *et al.*, 2004; Rennard, 2006; Salazar and Herrera, 2011; Churg *et al.*, 2012; Jankowich and Rounds, 2012). Increases in the levels of alveolar macrophage-associated matrix metallo-proteinases (MMPs) negatively correlate with forced expiratory volume in 1 s/forced vital capacity in COPD patients (Ishii *et al.*, 2013). We have shown that clodronate depletion of macrophages improves transpulmonary and airway resistance as well as dynamic compliance in experimental COPD (Beckett *et al.*, 2013), which possibly occurs through the reduction in MMPs. Thus, PXS-4728A associated reductions in inflammation, particularly the number of immune cells, and potentially their phenotypic features, as well as attenuated airway remodeling reverses the decreases in lung function.

In summary, here we show for the first time we show that SSAO activity is significantly increased in the lungs in experimental COPD and that the inhibition of SSAO activity with PX4728A results in suppression of inflammation and small airway remodelling and consequently improved lung function. These data in conjunction with other observations demonstrating the beneficial effect of inhibiting SSAO in liver and kidney associated pathologies, provides strong evidence that PXS-4728A may have broad efficacy in inflammatory and fibrotic diseases, with potential therapeutic benefits.

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Conflicts of interest

AGJ is a former employee of Pharmaxis P/L.

Author Contributions

AGJ wrote the manuscript, planned and performed experiments and analysed data, HS planned and performed experiments and analysed data, TTY performed experiments and analysed data, AET, GL, KW performed experiments, JSF, WJ and PMH designed the study, planned the experiments, analysed data, and revised the manuscript.

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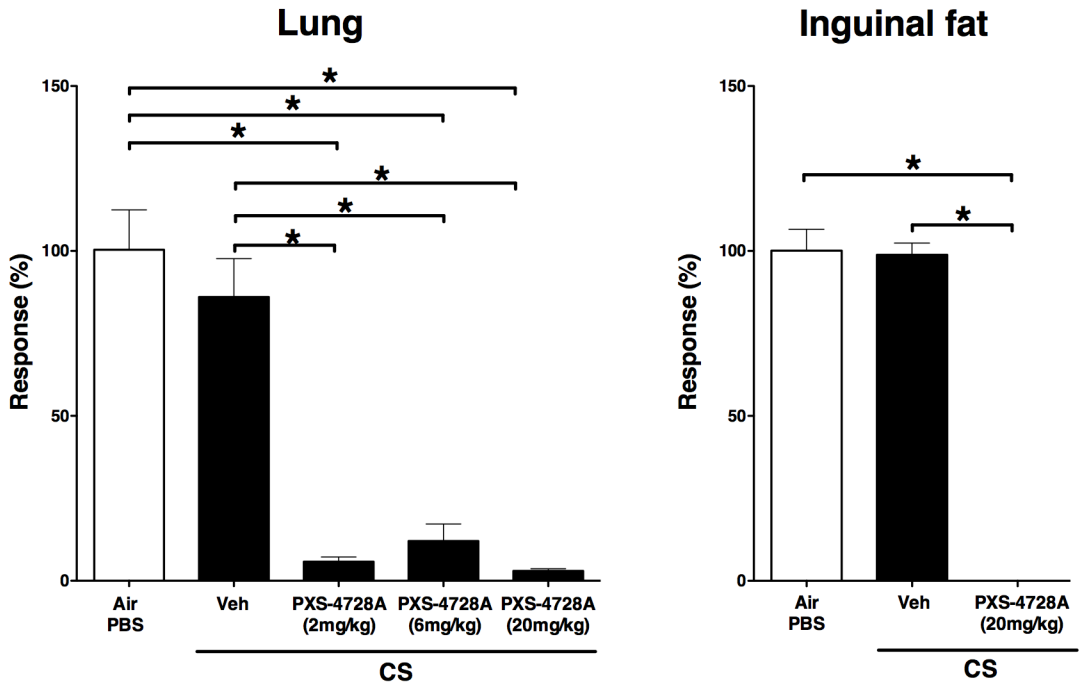

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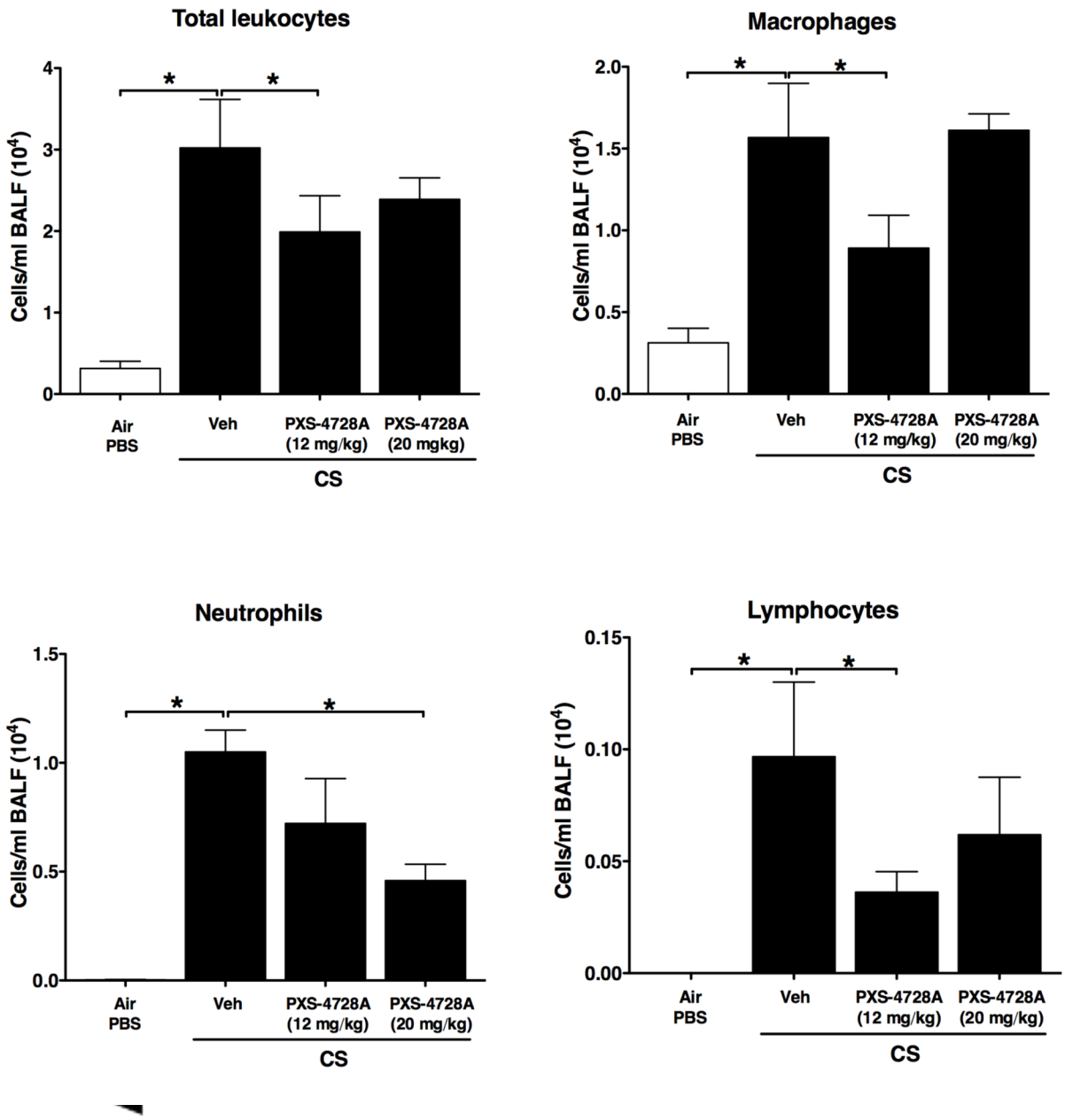

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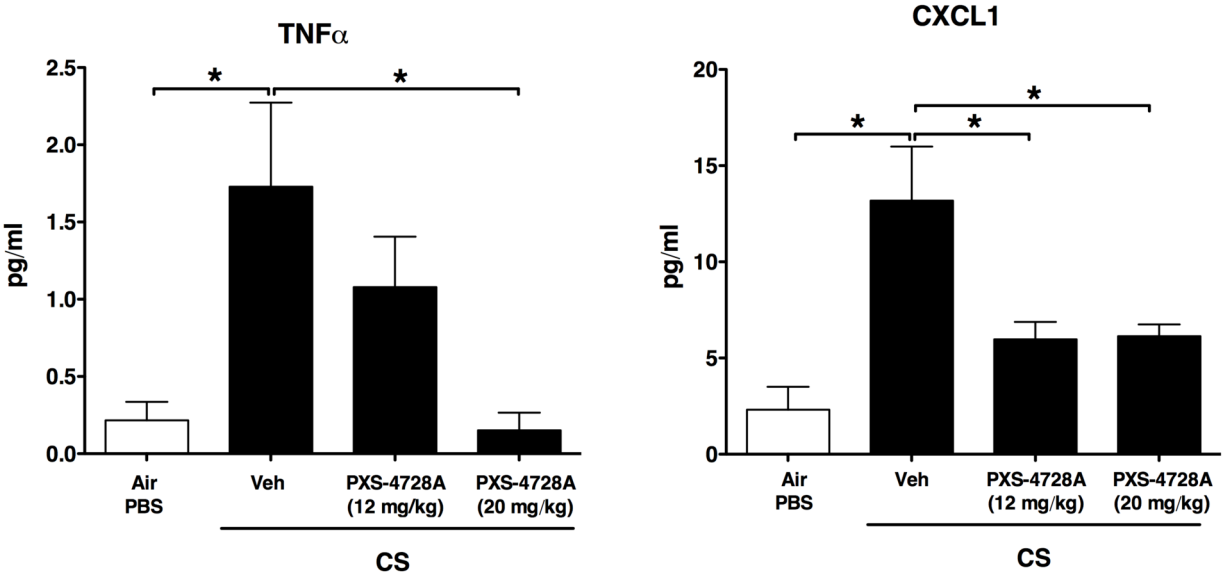

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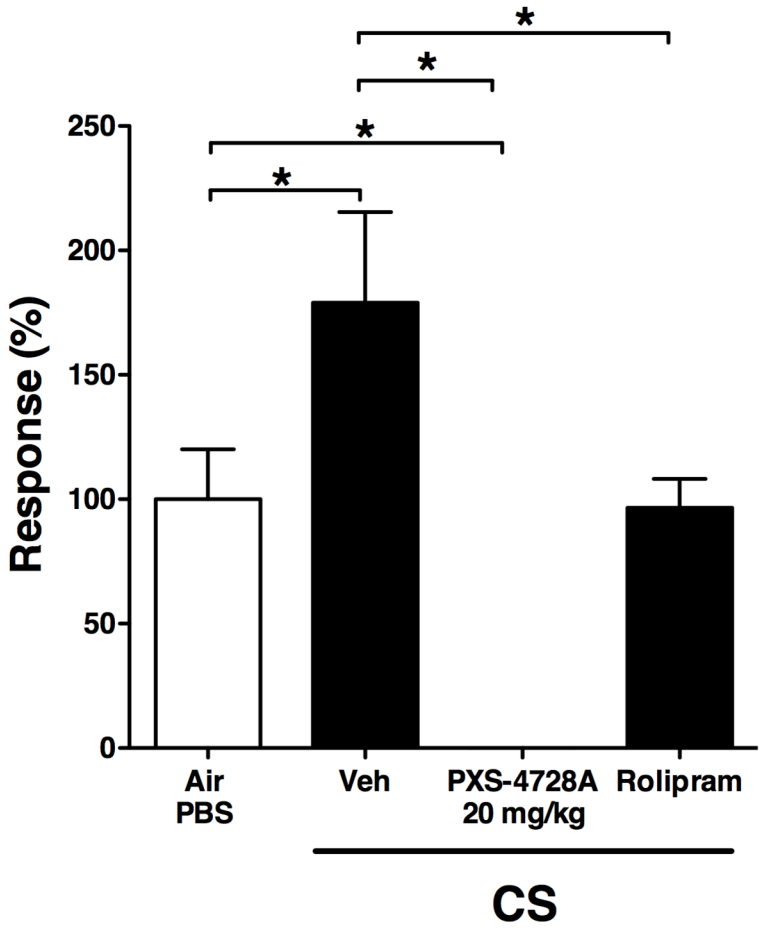

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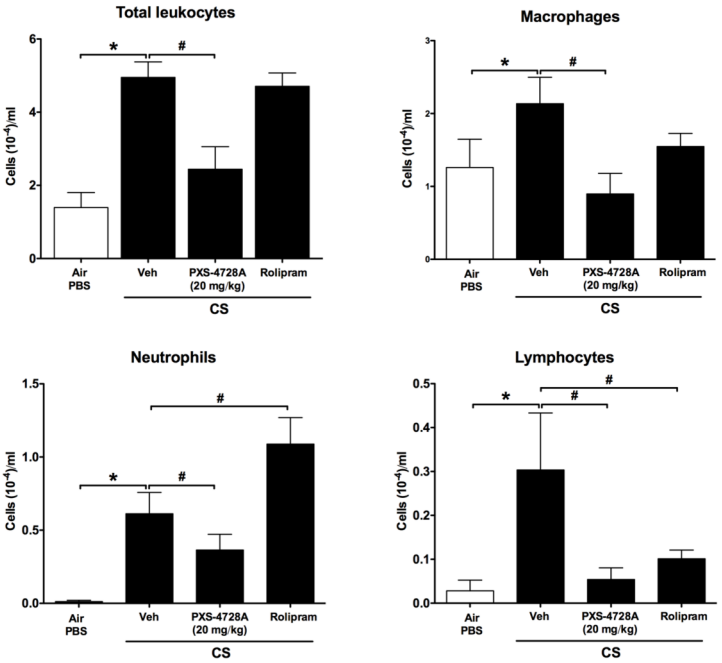

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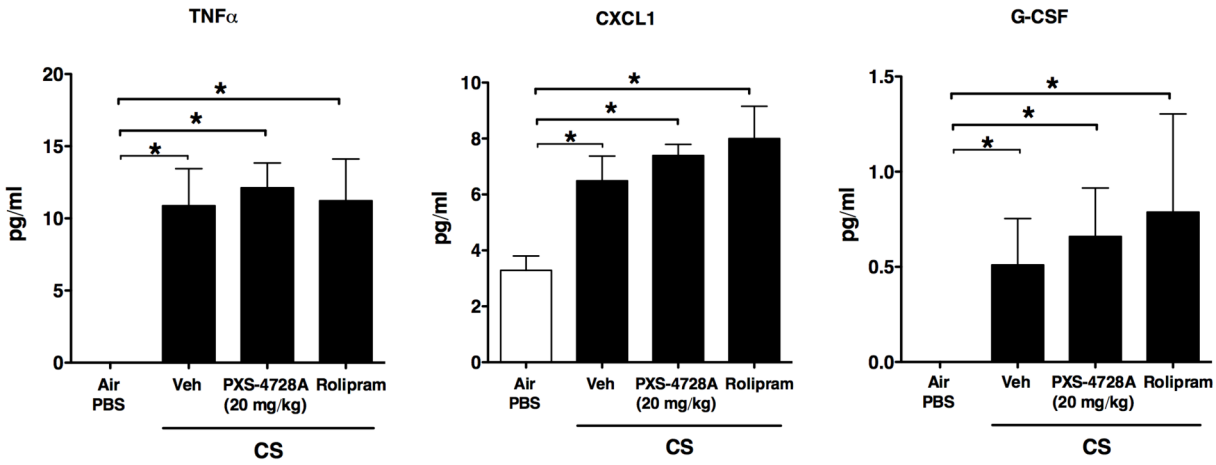

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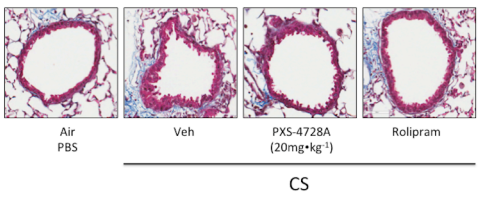

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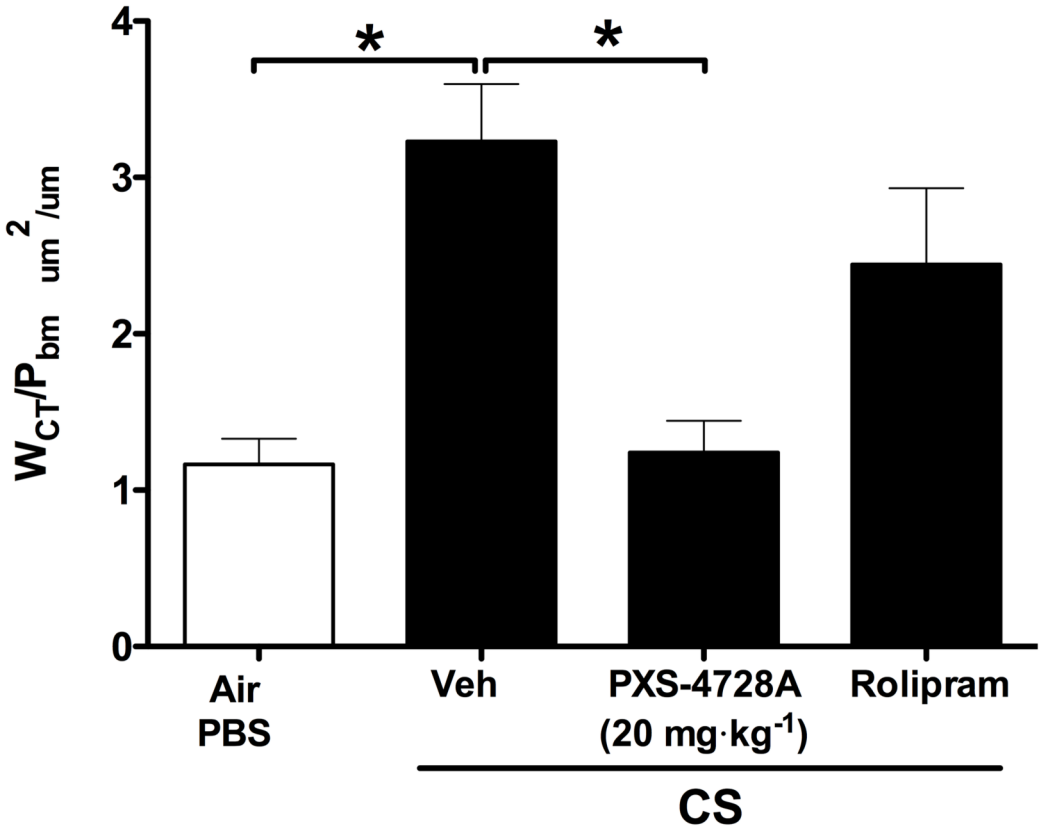

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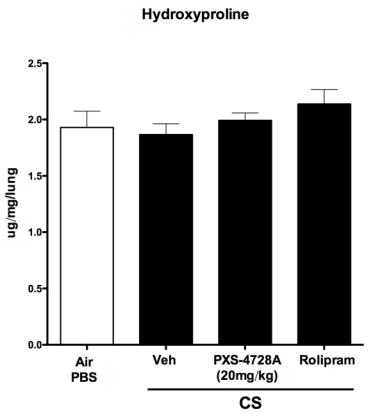

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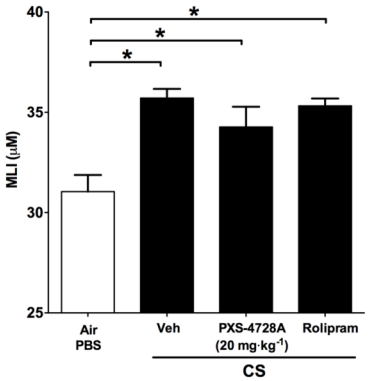

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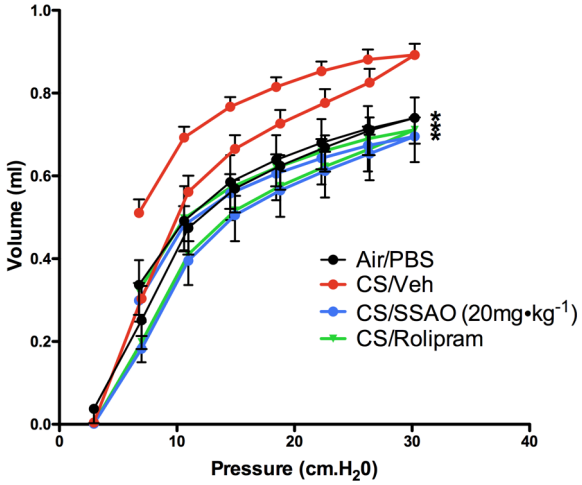

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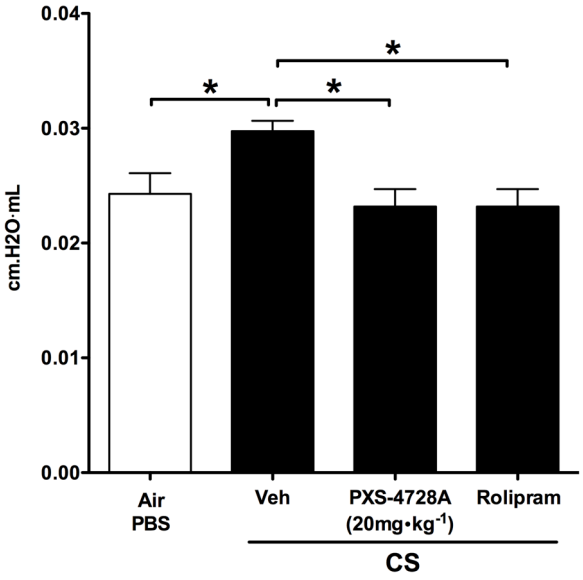

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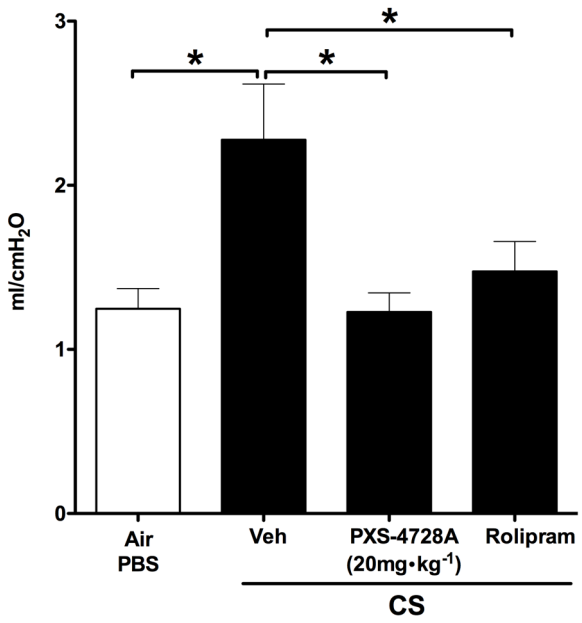

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