Pioglitazone attenuates endotoxin induced acute lung injury by reducing neutrophil recruitment

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Abbreviations: ALI, acute lung injury, LPS, lipopolysaccharide, ROS, reactive oxygen species, PPAR, Peroxisome proliferator-activated receptors, BALF, bronchioalveolar lavage fluid
Abstract

Background: Treatment of acute lung injury (ALI) remains an unsolved problem in intensive care medicine. Activation and recruitment of neutrophils are regarded as key mechanisms in progression of ALI. As pioglitazone (pio) holds potent pleiotropic anti-inflammatory effects, we here explored its effects during ALI.

Methods: C57Bl/6 mice were exposed to aerosolized LPS (500 μg/ml) and alveolar, interstitial, and intravascular neutrophils were assessed 4 h later. Lung permeability changes were evaluated by FITC-dextran clearance and protein content in the BAL fluid. In vitro, human isolated neutrophils were pre-treated with pio (1 or 3 hours, 10μM) and then activated with fMLP. Neutrophil activation, adhesion, release and formation of reactive oxygen species and phagocytosis were measured thereafter.

Results: Pio treatment before or after induction of ALI significantly diminished alveolar (pio before LPS: reduction by 73 %; pio after LPS: reduction by 67 %) and interstitial neutrophil influx (pio before LPS: reduction by 55 %; pio after LPS: reduction by 63 %) and reduced lung permeability changes (pio before LPS: reduction by 64 %; pio after LPS: reduction by 58 %) indicating a protective role of pio in ALI. Moreover, pio significantly reduced degranulation and adhesion of neutrophils without affecting reactive oxygen formation and release or bacterial phagocytosis.

Conclusion: Pio reduces recruitment and activation of neutrophils hereby protecting from LPS-induced ALI. Our results imply a potential role for pio in the management of ALI.

Keywords: acute lung injury, neutrophils, pioglitazone
Introduction

Acute lung injury (ALI) is a life-threatening disease with an age-adjusted incidence of 86.2 per 100,000 person-years [1]. Despite all innovations in intensive care medicine, the mortality of ALI remains up to 40%. ALI is characterized by an increased permeability of the alveolar-capillary barrier resulting in lung edema with protein-rich fluid consequently leading to impairment of arterial oxygenation. Sepsis is a major cause for development of acute lung injury, wherein Gram-negative bacteria are predominant. LPS inhalation mimics human Gram-negative ALI, inducing neutrophil recruitment, pulmonary edema and finally impairment of gas exchange [2]. Recruitment of neutrophils is a key event in development of ALI [1, 3] leading to plasma leakage and deterioration of oxygenation. The importance of neutrophils in ALI is supported by studies where lung injury is abolished by depletion of neutrophils [4, 5]. Much of the neutrophil-dependent ALI is mediated by granule proteins released from activated neutrophils. For example, azurocidin and α-defensins were implied to directly alter permeability changes [6], [7], whereas proteases of neutrophilic origin such as neutrophil elastase have been suggested to be important in degradation of surfactant proteins, epithelial cell apoptosis, and coagulation [8, 9]. Moreover, neutrophils produce vast quantities of reactive oxygen (ROS) and nitrogen (RNS) species. Besides their important antimicrobial effector function, neutrophil-derived oxidants promote deleterious pro-inflammatory effects thus being a major cause of neutrophil-dependent tissue injury in ALI [3].

Peroxisome proliferator-activated receptors (PPAR) are known as transcriptions factors belonging to the nuclear hormone receptor superfamily. PPARs are ligand-activated transcription factors, with the three isoforms (α, β, and γ) being encoded by
unique genes. Besides their importance in regulation of lipid and carbohydrate metabolism, PPARs, especially PPAR\(\alpha\) and PPAR\(\gamma\), have received much attention for their potent anti-inflammatory effects [10]. Previous studies have suggested that PPAR\(\gamma\) ligands reduce the expression of inflammatory cytokine genes and the production of inflammatory cytokines [11, 12]. Consequently, PPAR\(\alpha\) and PPAR\(\gamma\) agonists may be helpful in the treatment of acute inflammatory diseases such as ALI [13]. In this context, several studies have proven a beneficial role for PPAR\(\gamma\)-agonists in models of allergic airway inflammation and bleomycin-induced acute lung injury [14]. [15].

Although previous *in vitro* and *in vivo* studies have revealed the anti-inflammatory effects of pioglitazone, there is less known about the effects of pioglitazone on neutrophils in ALI. Recruitment of neutrophils, release of granule proteins and generation of ROS by neutrophils display key events in acute lung injury and may suit as potential target for therapy. Hence, we address the effect of pioglitazone treatment in a model of neutrophil-dependent ALI.
Methods

Animals: Male C57Bl/6 mice, 8 weeks of age, were obtained from Janvier SAS (Le Genest Saint Isle, France). Neutrophils were depleted by intraperitoneal injection of Ly6G-specific monoclonal antibody 1A8 (100µg per mouse 12 hours and 0 hours before LPS inhalation, BioXcell, West Lebanon, N. Hamp.). Mice with intact white blood cell count were treated with pioglitazone (2 µg/g bodyweight) or NaCl 0.9 % by intraperitoneal injection 12 hours and 0 hours before LPS inhalation or 1 hour after LPS inhalation, respectively. All experiments were approved by the local ethical authorities.

Murine model of acute lung injury: Aerosolized LPS from Salmonella enteritidis (Sigma Co., St. Louis, MO) dissolved in 0.9% saline (500 µg/ml) was utilized to induce neutrophil-infiltration in the lung. Six mice were exposed simultaneously to aerosolized LPS in a custom-built box (22 cm in length; 10 cm in diameter) connected to an air nebulizer (MicroAir, Omron Healthcare, Vernon Hills, IL) for 30 minutes. Control mice were exposed to saline aerosol (n=8). Neutrophil counts in bronchoalveolar lavage (BAL) and lung tissue (interstitium and pulmonary vasculature) were assessed 4 hours after inhalation. 30 min before euthanasia, 5 µl FITC-Ly-6G (Gr1) (eBioscience) and 100µl Fluorescein isothiocyanate–Dextran (30 mg/ml FITC-Dextran; 70 kDa, Sigma-Aldrich) were applied by tail vein injection to label intravascular neutrophils. The mice were anesthetized with an intraperitoneal injection of ketamine (125 mg/kg body weight; Sanofi-Cefa GmbH Düsseldorf, Germany) and xylazine (12.5 mg/kg b.w.; Phoenix Scientific). The trachea was dissected and cannulated (PortexFineBore Polythene Tubing, 0.28 mm inner diameter (ID)/ 0.61 mm outer diameter (OD), Smiths Medical International, Keene, NH). 5x 0.5 ml PBS was injected and withdrawn. Thereafter, the ribcage was opened
by a midline incision and the pulmonary vasculature was rinsed with 15 ml ice-cold PBS with 0.5 mM EDTA after cutting the inferior cava vein to facilitate exsanguination. The lungs were removed, minced and digested with liberase (1:20; 25 mg Liberase RI/ml aqua, Roche Mannheim Germany). Digested lungs were passed through a cell strainer (70 µm; MiltenyiBiotec GmbH, BergischGladbach, Germany) and the resulting single cell suspension was centrifuged for 5 min at 300 g. The pellets were resuspended in 1 ml hank’s balanced salt solution with 0.3 mmol / l EDTA and 0.1 % BSA. The bronchoalveolar lavage (BAL) fluid was centrifuged for 5 min at 300g (Suppl. Fig 1).

Flow cytometry: Cell pellets were labeled with PerCP-Cy5.5 anti-mouse Ly-6G, PE anti-mouse CD115, APC-Cy7 anti-mouse CD45 and APC anti-Mouse F4/80 (all eBioscience). Neutrophils were identified by their typical appearance in the forward scatter-side scatter and as CD45⁺ CD115⁻ and PerCP-Gr1⁺ cells (Suppl. Fig 2). Within the lung, FITC-Gr1 antibody was used to distinguish between interstitial neutrophils (CD45⁺, CD115⁻, PerCP-Gr1⁺, FITC-Gr1⁻) and intravascular neutrophils (CD45⁺, CD115⁻, PerCP-Gr1⁺, FITC-Gr1⁺). All flow cytometry studies were performed using a BD FACSCanto II (Becton Dickinson, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Lung Permeability: FITC-Dextran (70 kDa, Sigma-Aldrich) was used to assess vascular leakage. 100 µl FITC-Dextran (30mg/ml) were administered by tail vein injection 30 min prior to euthanasia and dye extravasation was used to assess change in vascular permeability. The fluorescence of the 100 µl BAL supernatant (FluoBAL) and of 50 µl serum (FluoSerum) was measured and permeability volume was expressed in microlitre (VPerm = (FluoBAL / 100 µl) / (FluoSerum / 50 µl) * BAL volume)
Protein concentration of the BAL. The protein content of the BAL supernatants was assessed using the Bio-Rad Protein Assay based on the method of Bradford (Bio-Rad Laboratories Germany). Measurement of absorbance at 595 nm was performed with a microplate reader (infinite 200, Tecan Group Switzerland).

Histology and electron microscopy. After completion of the experiment, one part of the right lung was fixed in formalin, embedded in paraffin and stained with Mayer’s haematoxylin and eosin for histological examination. Another part of the lung was prepared for scanning electron microscopy as described [5].

Neutrophil isolation. Human neutrophils from healthy donors (male, 25-35 years old, no medication) were isolated as described previously [16]. Neutrophils were incubated with pioglitazone 10 µM for 1 or 3 hour respectively.

Degranulation. After incubation with pioglitazone, neutrophils were activated by adding 10 mM fMLP (Sigma) and upregulation of CD11b and CD29 was measured after 30 min using BD FACSCanto II.

Flow chamber. We coated petri dishes with fibronectin or ICAM1 (1µg / ml +10 % BSA) for laminar flow chamber. Neutrophils were treated with pioglitazone (10 µM for 1 or 3 hour). After activation with fMLP (Sigma), neutrophils were perfused at 1 dyne/cm² over fibronectin or ICAM1 and firmly adherent neutrophils were quantified after 4 min in multiple fields (at least 6 fields, 100x magnification).
Phagocytosis. Fluorescent E. coli and opsonizing reagent (Molecular Probes) were reconstituted as indicated by the manufacturer. IgG opsonization was achieved according to the manufacturer’s instructions. Complement opsonization was attained by incubation of bacteria with fresh human serum at 37°C for 1 hour. Opsonized particles were washed and seeded onto neutrophils which had been incubated with pioglitazone 10 µM for 1 or 3 hour. Fluorescence was measured with BD FACSCanto II (Becton Dickinson, San Jose, CA) after 30 min.

Reactive oxygen species. ROS was detected by dihydrodichlorofluoresceindiacetate (DCF, Molecular Probes, Eugene, OR USA) as described previously [17]. Basically, cells were incubated with the profluorescent, lipophilic H2-DCF-DA which can diffuse through the cell membrane. Reaction with intracellular ROS results in the fluorescent molecule DCF (max. emission ~ 530 nm), so that DCF fluorescence can be used as a measure for intracellular ROS levels. Fluorescence intensity was quantified with FACS Canto after 30 min. Similarly, extracellular ROS was measured by singlet oxygen sensor green reagent (Molecular Probes Europe, Leiden Netherlands) as recommended by the manufacturer.

Statistics. All data are expressed as mean ± SD. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). Unpaired Student’s t-test, Mann-Whitney test, or Kruskal-Wallis test with posthoc Dunn tests were used as appropriate. * indicates a p-value < 0.05.
Results

Pioglitazone protects from neutrophil-dependent ALI

After C57Bl/6 mice were exposed to aerosolized lipopolysaccharide (LPS), we observed neutrophil recruitment, plasma leakage, lung (ultra-)structure, and elastase activity in the bronchioalveolar lavage fluid (BALF). Treatment with LPS increased the number of intravascular, interstitial, and alveolar neutrophils as analyzed by flow cytometry (Suppl. Fig 2) of lung homogenates and BALF (Fig 1A). Furthermore, the protein concentration as well as the clearance of fluorescent dextran increased in the BALF by LPS treatment indicating enhanced plasma leakage and edema formation. Moreover, the activity of neutrophil-derived elastase, a protease important in ALI was elevated in LPS-treated animals (Fig 1B). Neutrophil depletion abolishes alveolar fluid efflux and structural changes confirming the previously described importance of neutrophils in ALI (Fig 1A / 1B). To test the potential role of pioglitazone in this model of neutrophil-mediated ALI, mice were treated with pioglitazone prior to LPS exposure. In these experiments we found that pioglitazone reduced the recruitment of neutrophils after LPS inhalation in the intravascular, interstitial, and alveolar compartment of the lung (Fig 1A) and prevented enhanced pulmonary vascular leakage indicated by reduced protein content of the BALF and FITC-Dextran clearance volume (Fig 1B). In addition, treatment with pioglitazone 1 hour after induction of ALI exhibited similar effects (Fig 1A / B). Histological and ultrastructural analyses of lung following LPS-exposure revealed alveolar septal thickening, accumulation of inflammatory cells in the interstitium and the alveoli, and influx of protein-rich fluid into the alveolar space as compared to control mice exposed to aerosolized saline solution. Pioglitazone both before (Fig 2) and after (data not
shown) LPS inhalation abrogated histological alterations of this kind, further supporting its protective role in neutrophil-mediated ALI.

*Pioglitazone reduces neutrophil adhesion to ICAM-1 and fibronectin*

Our *in vivo* data point at the direct reduction of neutrophil recruitment by treatment with pioglitazone. To further confirm this notion, we analyzed the effect of pioglitazone on adhesion of isolated human neutrophils perfused over immobilized ICAM-1 (Fig 3A). Treatment of neutrophils with pioglitazone for 1 hour and 3 hours at 10 µM severely diminished adhesion to ICAM-1. For neutrophils to firmly adhere to ICAM-1, the up-regulation of β₂-integrins from secretory vesicles is a prerequisite. Such mobilization is mediated by secretagogues such as the bacterial wall peptide fMLP. Consequently, we analyzed the effect of pioglitazone on fMLP-induced β₂-integrin up-regulation on neutrophils. After activation of neutrophils with fMLP, expression of β₂-integrin was significantly elevated (Fig 3B). Pioglitazone (10 µM for 1 hour or 3 hours) significantly reduced the fMLP-induced expression of β₂-integrins (Fig 3B), thus offering an explanation for decreased adhesion to ICAM-1 following pioglitazone treatment.

As β₁-integrins, on the other hand, are crucial for extravascular locomotion of neutrophils, we tested the effect of pioglitazone on β₁-integrin up-regulation and neutrophil adhesion to the β₁-integrin-substrate fibronectin. Flow chamber experiments revealed significantly reduced adhesion of neutrophils to fibronectin after pretreatment with pioglitazone (10 µM) for either 1 hour or 3 hours (Fig. 3C). Treatment of neutrophils with fMLP resulted in a trend to increased surface expression of the fibronectin ligand α5β1-integrin, an effect fully reversed by pretreatment with pioglitazone (Fig. 3D).
*Pioglitazone does not impair neutrophil antimicrobial activity*

Besides their contribution to acute lung injury, neutrophils display important antibacterial effector functions in bacterial infections. To analyze if the beneficial anti-inflammatory effect of pioglitazone does not negatively affect these functions we tested the capacity of pioglitazone-treated neutrophils to phagocytose bacteria. Phagocytosis of IgG- (Fig 4A) or complement-opsonized (Fig 4B) FITC-labeled *E. coli* was assessed by flow cytometry. Whereas the bacterial uptake of neutrophils increased after complement-opsonization in comparison to the IgG-opsonization, pioglitazone did not significantly alter bacterial uptake.

Further to adhesion and migration, neutrophils contribute to ALI by release of ROS. However, ROS displays also important antimicrobial functions of neutrophils. Hence, we investigated the effect of pioglitazone on ROS formation and release of isolated human neutrophils induced by fMLP. After isolation of neutrophils from healthy donors, neutrophils were incubated with pioglitazone (10 µM) for 1 or 3 hours, respectively. fMLP clearly induced formation and release of ROS over time. However, pioglitazone pretreatment failed to affect ROS formation (Fig 4C) and release (Fig 4D), thus implying that pioglitazone does not impair neutrophil antimicrobial activity and that the protective effect of pioglitazone does not stem from effects on ROS release.
Discussion

Despite all innovation in the intensive care, ALI induced by Gram-negative bacteria remains a major challenge. In our study we demonstrate a beneficial effect of pioglitazone in ALI treatment as indicated by reduced edema formation and neutrophil infiltration, both of which are key events during development of acute lung injury.

PPARα- and PPARγ- agonists are developed for treatment of dyslipidaemia and type 2 diabetes. However, recent studies have revealed additional beneficial effects in atherosclerosis and in inflammatory diseases which are partly explained by stabilization of endothelial function [18, 19]. The protective effect of PPARα-agonists of the fibrate class in LPS-induced lung injury has previously been established [20]. With the documented importance of PPARγ in control of neutrophil migration [21], we here investigated the effect of glitazones which might directly reduce the activation and recruitment of the neutrophils, a process that importantly contributes to tissue damage in acute lung injury [3]. Consequently, we analyzed the effects of pioglitazone on neutrophil activity. The importance of neutrophil infiltration in LPS-induced ALI is substantiated in models, where neutrophil adhesion or migration is impaired. In this context it was shown that lack of CXCR2 or blockade of β2-integrins protects from ALI [22, 23]. In our study, pioglitazone prevents intravascular neutrophil adhesion and lung infiltration. As this was addressed in an in vitro assay in absence of other cell types but in presence of substrates typically involved in neutrophil adhesion and migration we conclude that the in vivo effects may to a large part relate to direct interference with surface expression of β1-integrins and β2-integrins. Our results are in line with this previous study, which revealed reduced monocyte adhesion on endothelial cell indicating protective role in acute inflammation of pioglitazone [24]. Interestingly, in our study we found similar effect of pioglitazone
treatment after LPS inhalation in comparison to the treatment before LPS inhalation. This is intriguing as treatment in this way is likely relies on rapidly occurring anti-inflammatory activities. A possible explanation might be the reduced expression of endothelial cell adhesion molecules [25, 26]. Especially decreased expression of P-selectin following treatment with glitazones may offer an explanation for reduced neutrophil recruitment [26]. Furthermore, reduced oxidative stress [27] and decreases in the release of lipid mediators [28] in response to glitazones may offer alternative explanations for reduced neutrophil lung infiltration when treatment is initiated after LPS inhalation.

Rapid upregulation of $\beta_2$-integrins on neutrophils is typically a result of mobilization of preformed granules. $\beta_2$-integrins are localized in secretory vesicles, a compartment discharged when neutrophil-endothelial interaction is established. Secretory vesicles are also rich in azurocidin [29] a protein previously associated with neutrophil-mediated permeability changes [5, 30, 31]. Hence, reduced surface-expression of $\beta_2$-integrins following fMLP stimulation not just explains reduced adhesive capacity, but may also point at impaired release of granule proteins relevant to ALI. In line, we found lower elastase activity in BAL fluids from mice treated with pioglitazone. Elastase aggravates ALI by increasing endothelial and epithelial permeability [32, 33], proteolytic cleavage of surfactant proteins [34] and induction of apoptosis [35]. The in vivo importance of neutrophil elastase in ALI is further corroborated in studies using elastase deficient mice [36] or employing specific inhibitors [37]. Although the release of ROS displays an important antimicrobial mechanism, overproduction of ROS can cause tissue damage in sepsis and ALI [38]. In animal models of ALI, neutrophil-derived ROS caused lung injury as shown by histological examination and permeability measurements [39, 40]. In addition, it was evidenced that ROS can disrupt intercellular tight junctions of the endothelium by phosphorylation of focal
adhesion kinase [41]. Hence, deficiency or blockade of NADPH oxidase prevents from ALI [42-44]. However, in our study pioglitazone fails to affect ROS release. Hence, the protective effect of pioglitazone appears to primarily arise from decreases in neutrophil degranulation, adhesion, and recruitment.

Further to emigration, neutrophils are irreplaceable in bacterial clearance, much of which is mediated by phagocytosis and intracellular bacterial killing [45]. Data from our study indicate that pioglitazone does not negatively affect bacterial uptake and clearance as assessed by ROS formation experiments. Hence, these data suggest that pioglitazone might not impair clearance during bacterial infections and thus further support its clinical applicability. However, further in vivo studies are required to evaluate the effect of pioglitazone on bacterial clearance in a broader setting in life animals.

Conclusion

Pioglitazone attenuates recruitment and activation of neutrophils in a model of ALI and hereby displays beneficial effects. Moreover, pioglitazone treatment after onset of ALI was as effective as treatment before onset of ALI implicating a potential role for glitazones in the management of ALI.
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Figure 1 Pioglitazone reduces LPS-induced acute lung injury by interference with neutrophil recruitment. Mice were challenged with LPS via inhalation and sacrificed 4 hours later. In addition, neutrophils were depleted by antibody injection or mice were treated with pioglitazone (2 µg/g body weight) 12 hours and one hour before or one hour after LPS exposure as indicated. **A:** Quantification of alveolar (top), interstitial (middle), and intravascular neutrophils (bottom) in mice treated as indicated. **B:** Lungs were lavaged and the FITC-dextran clearance (top), protein concentration (middle), and elastase activity (bottom) were assessed in BAL fluids of mice treated as indicated. n for ctrl is 8, n for LPS is 10, n for LPS + neutrophil depletion is 9, and for the pioglitazone treated groups is 8. * indicates significant difference compared to LPS-treated animals.
Figure 2: Pioglitazone prevents LPS-induced structural changes in the lung tissue. Representative histological (left) and scanning electron microscopic (right) images of lungs from mice treated as indicated. n is 5 in each group. Scale bars indicate 50 μm for scanning electron microscopy and 250 μm for histology.
Figure 3: Pioglitazone impairs neutrophil adhesion to ICAM-1 and fibronectin.

Isolated human neutrophils were pre-treated with pioglitazone (10 μM, 1 hour and 3 hours) and then activated with fMLP. **A** Neutrophils were perfused over immobilized recombinant ICAM-1 at 1 dyne/cm² and the number of adherent cells was enumerated. n = 8-10 for each bar; **B** MFI of surface-expressed β₂-integrin as measured by flow cytometry after staining with directly conjugated antibodies. n = 3-6. * indicates significant difference in comparison to the fMLP group. **C** Neutrophils were perfused over immobilized fibronectin at 1 dyne/cm² and the number of adherent cells was enumerated. n = 8-10 for each bar. **D** MFI of surface-expressed of β₁-integrins as measured by flow cytometry after staining with directly conjugated antibodies. n = 3-6. * indicates significant difference in comparison to the fMLP group.
Figure 4: Pioglitazone does not affect neutrophil antimicrobial activity. 

**A / B:** Bacterial uptake of fluorescent IgG- (A) or complement-opsonized (B) *E. coli* by activated or resting neutrophils as assessed by flow cytometry. Neutrophils were activated with fMLP and pretreated with pioglitazone as indicated (n=4).

**C / D:** Reactive oxygen species. Isolated human neutrophils were pre-treated with pioglitazone (10 μM, 1 hour and 3 hours); C Neutrophils were labelled with the sensitive dye H2DCFDA and ROS formation was recorded by flow cytometry following fMLP-stimulation. Data indicate fluorescence intensity 30 min after fMLP exposure. n=6 for each bar. D Neutrophils were labelled with singlet oxygen green as marker of extracellular ROS release. Data indicate fluorescence intensity 30 min after fMLP exposure. n=6 for each bar.
**Endotoxin-induced acute lung injury (ALI)**

Suppl. Figure 1 Experimental outline
Suppl. Figure 2: Gating strategy for compartment-specific identification of neutrophils. A: Neutrophils in the BAL of ctrl mice or LPS treated mice are identified as CD45⁺ CD115⁻ Ly6G⁺. B: Intravascular (IV) neutrophils are discriminated from interstitial (IS) neutrophils by being positive for a FITC conjugated antibody to Ly6G that was injected i.v. 30 minutes before mice were sacrificed.


