Eur Respir J 2012; 40: 416–423
DOI: 10.1183/09031936.00091011

Pioglitazone attenuates endotoxin-induced acute lung injury by reducing neutrophil recruitment

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ABSTRACT: 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 the progression of ALI. As pioglitazone holds potent pleiotropic anti-inflammatory effects, we explored its effects during ALI.

C57Bl/6 mice were exposed to aerosolised lipopolysaccharides (LPSs) (500 μg·mL^{-1}) and their alveolar, interstitial and intravascular neutrophils were assessed 4 h later. Lung permeability changes were evaluated by fluorescein isothiocyanate-dextran clearance and protein content in the bronchoalveolar lavage fluid. *In vitro*, human isolated neutrophils were pretreated with piolitazone (10 μM, for 1 or 3 h) and then activated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine. Neutrophil activation, adhesion, release and formation of reactive oxygen species (ROS) and phagocytosis were measured thereafter.

Pioglitazone treatment before or after induction of ALI significantly diminished alveolar (reduction by 73% and 67%, respectively) and interstitial neutrophil influx (reduction by 55% and 63%, respectively) and reduced lung permeability changes (reduction by 64% and 58%, respectively) indicating a protective role of pioglitazone treatment in ALI. Moreover, pioglitazone significantly reduced degranulation and adhesion of neutrophils without affecting ROS formation and release or bacterial phagocytosis.

Pioglitazone reduces recruitment and activation of neutrophils thereby preventing LPS-induced ALI. Our results imply a potential role for pioglitazone treatment in the management of ALI.

KEYWORDS: Acute lung injury, neutrophils, pioglitazone

Acute lung injury (ALI) is a life-threatening disease with an age-adjusted incidence of 86.2 per 100,000 persons a year [1]. Despite all innovations in intensive care medicine, the mortality of ALI remains up to 40%. ALI is characterised by an increased permeability of the alveolar-capillary barrier resulting in lung oedema with protein-rich fluid consequently leading to impairment of arterial oxygenation. Sepsis is a major cause for the development of ALI, wherein Gram-negative bacteria are predominant. Lipopolysaccharides (LPS) inhalation mimics human Gram-negative ALI, inducing neutrophil recruitment, pulmonary oedema and finally impairment of gas exchange [2]. Recruitment of neutrophils is a key event in the 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 the 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 changes in permeability [6, 7], whereas proteases of neutrophilic origin, such as neutrophil elastase, have been suggested to be important in the degradation of surfactant proteins, epithelial cell apoptosis, and coagulation [8, 9]. Moreover, neutrophils produce vast quantities of reactive oxygen species (ROS) and reactive nitrogen species. Besides their important antimicrobial effector function, neutrophil-derived oxidants promote deleterious pro-inflammatory effects, and thus are a major cause of neutrophil-dependent tissue injury in ALI [3].

Peroxisome proliferator-activated receptors (PPAR) are known as transcription factors that belong to the

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Received:
May 29 2011
Accepted after revision:
Nov 24 2011
First published online:
Jan 20 2012

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

This article has supplementary material accessible from www.erj.ersjournals.com

nuclear hormone receptor superfamily. PPARs are ligand-activated transcription factors, containing three isoforms (α, β, and γ) being encoded by unique genes. Besides their importance in the regulation of both lipid and carbohydrate metabolism, PPARs, especially PPAR-α and PPAR-γ, have received much attention for their potent anti-inflammatory effects [10]. Previous studies have suggested that PPAR-γ ligands reduce the expression of inflammatory cytokine genes and the production of inflammatory cytokines [11, 12]. Consequently, PPAR-α and PPAR-γ 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-γ agonists in models of allergic airway inflammation and bleomycin-induced ALI [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 ALI and may be a suitable potential target for therapy. Thus, we address the effect of pioglitazone treatment in a model of neutrophil-dependent ALI.

METHODS

Animals

8-week-old male C57Bl/6 mice were obtained from Janvier SAS (Le Genest Saint Isle, France). Neutrophils were depleted by intraperitoneal injection of Ly6G-specific monoclonal antibody 1A8 (100 μg per mouse 12 h and 0 h before LPS inhalation; BioXcell, West Lebanon, NH, USA). Mice with intact white blood cell counts were treated with either pioglitazone (2 μg per g of bodyweight) or NaCl 0.9 % by intraperitoneal injection 12 h and 0 h before LPS inhalation ort 1 h after LPS inhalation, respectively. All experiments were approved by the local ethical authorities.

Murine model of ALI

Aerosolised LPS from *Salmonella enteritidis* (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 0.9% saline (500 $\mu g \cdot mL^{-1}$) was utilised to induce neutrophil infiltration in the lung. Six mice were exposed simultaneously to aerosolised LPS in a custom-built box (22 cm in length 10 cm in diameter) connected to an air nebuliser (MicroAir; Omron Healthcare, Vernon Hills, IL, USA) for 30 min. Eight control mice were exposed to saline aerosol. Neutrophil counts in the bronchoalveolar lavage fluid (BALF) and lung tissue (interstitium and pulmonary vasculature) were assessed 4 h after inhalation. 30 min before euthanasia, 5 μL of anti-mouse-Ly-6G (Gr-1) fluorescein isothiocyanate (FITC) (Gr1; eBioscience, San Diego, CA, USA) and 100 μl FITC-dextran (30 $mg \cdot mL^{-1}$ FITC-dextran, 70 kDa; Sigma-Aldrich Co.) were applied *via* a tail-vein injection to label intravascular neutrophils. The mice were anesthetised with an intraperitoneal injection of ketamine (125 mg per kg body weight; Sanofi-Cefa GmbH, Düsseldorf, Germany) and xylazine (12.5 mg per kg body weight; Phoenix Scientific, St Joseph, MO, USA). The trachea was dissected and cannulated (PortexFineBore polythene tubing, 0.28 mm inner diameter/0.61 mm outer diameter; Smiths Medical International, Keene, NH, USA). 5×0.5 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 $\cdot mL^{-1}$ aqua; Roche, Mannheim, Germany). Digested lungs were passed through a cell strainer (70 μm; Miltenyi Biotec GmbH, Bergisch Gladbach, 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 $mmol \cdot L^{-1}$ EDTA and 0.1% bovine serum albumin (BSA). BALF was centrifuged for 5 min at 300 g (fig. S1).

Flow cytometry

Cell pellets were labelled with PerCP-Cy5.5 anti-mouse Ly-6G, PE anti-mouse CD115, APC-Cy7 anti-mouse CD45 and APC anti-mouse F4/80 (eBioscience). Neutrophils were identified by their typical appearance in the forward scatter side scatter and as CD45+, CD115- and PerCP-Gr1+ cells (fig. S2). Within the lung, FITC-Gr1 antibody was used to distinguish between interstitial neutrophils (CD45+, CD115-, PerCP-Gr+, FITC-Gr1-) and intravascular neutrophils (CD45+, CD115-, PerCP-Gr1+, FITC-Gr1+). All flow cytometry studies were performed using a BD FACS Canto II (Becton Dickinson, San Jose, CA, USA) and data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

Lung permeability

FITC-dextran was used to assess vascular leakage. 100 μL FITC-dextran (30 $mg \cdot mL^{-1}$) were administered by tail-vein injection 30 min prior to euthanasia and dye extravasation was used to assess the change in vascular permeability. The fluorescence of the 100 μL BALF supernatant ($Fluo_{BALF}$) and of 50 μL serum ($Fluo_{Serum}$) was measured and permeability volume was expressed in μL:

$$V_{Perm} = (Fluo_{BALF} \cdot 100\ \mu L^{-1}) / (Fluo_{Serum} \cdot 50\ \mu L^{-1}) \times \text{BALF volume}$$

Protein concentration of the BALF

The protein content of the BALF supernatants was assessed using the Bio-Rad Protein Assay based on the method of Bradford (Bio-Rad Laboratories GmbH, Munich, Germany). Measurements of absorbance at 595 nm were performed with a microplate reader (Infinite® 200 PRO; Tecan Group Ltd, Männedorf, 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 previously [5].

Neutrophil isolation

Human neutrophils from healthy donors (males aged 25–35 yrs and taking no medication) were isolated as described previously [16]. Neutrophils were incubated with pioglitazone 10 μM for 1 or 3 h.

Degranulation

After incubation with pioglitazone, neutrophils were activated by adding 10 mM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine

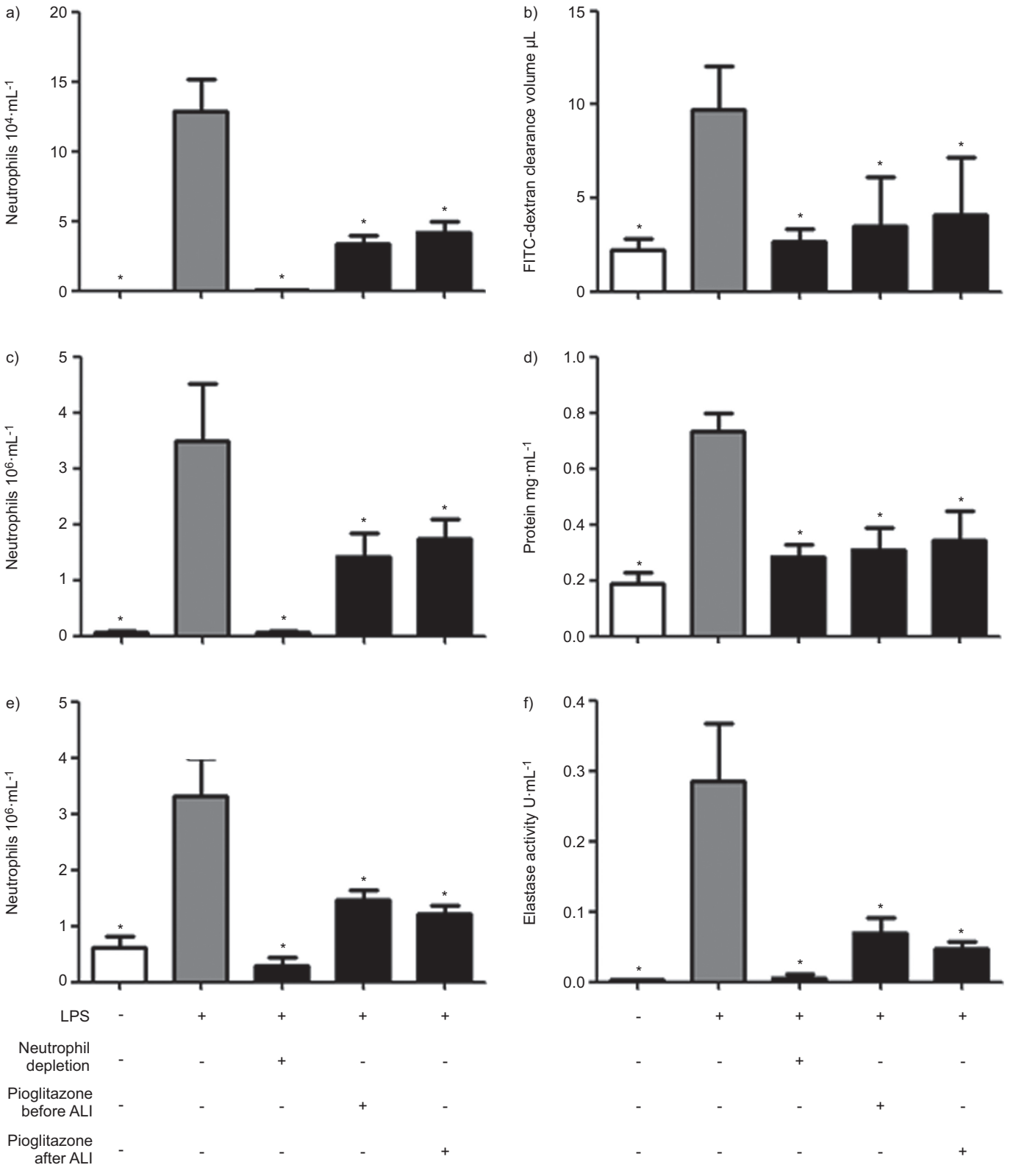

FIGURE 1. Pioglitazone reduces lipopolysaccharide (LPS)-induced acute lung injury (ALI) by interference with neutrophil recruitment. Mice were challenged with LPS *via* inhalation and sacrificed 4 h later. In addition, neutrophils were depleted by antibody injection or mice were treated with piogliatzone (2 µg per g body weight) 12 h and 1 h before, or 1 h after LPS exposure as indicated. Quantification of a) alveolar c) interstitial and e) intravascular neutrophils in mice treated as indicated. Lungs were lavaged and b) fluorescein isothiocynate (FITC)-dextran clearance, d) protein concentration and f) elastase activity were assessed in bronchoalveolar lavage fluid of mice treated as indicated. Control n=8, LPS n=10, LPS+neutrophil depletion n=9, and pioglitazone n=8. *: $p<0.05$ compared with LPS-treated animals.

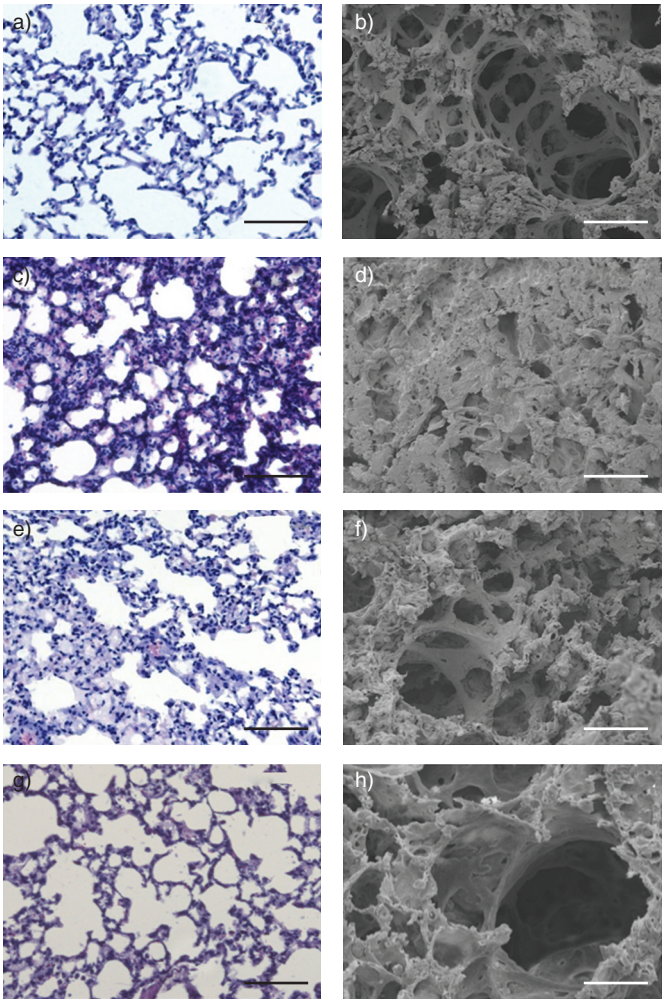

FIGURE 2. Pioglitazone prevents lipopolysaccharide (LPS)-induced structural changes in the lung tissue. a, c, e and g) Representative histological and b, d, f and h) scanning electron microscopic images of lungs from mice treated as follows: a and b) control; c and d) LPS; e and f) pioglitazone before LPS; g and h) pioglitazone after LPS. n=5 for each group. a, c, e and g) Scale bars=250 μm. b, d, f and h) Scale bars=50 μm.

(fMLP; Sigma-Aldrich Co.) and upregulation of CD11b and CD29 was measured after 30 min using BD FACS Canto II.

Flow chamber

We coated Petri dishes with fibronectin or intercellular adhesion molecule (ICAM)-1 (1 μg·mL^{-1} + 10% BSA) for laminar flow chamber. Neutrophils were treated with pioglitazone (10 μM for 1 or 3 h). After activation with fMLP, neutrophils were perfused at 1 dyn·cm^{-2} over fibronectin or ICAM-1 and firmly adherent neutrophils were quantified after 4 min in multiple fields (a minimum of six fields at ×100 magnification).

Phagocytosis

Fluorescent *Escherichia coli* and opsonising reagent (Molecular Probes, Eugene, OR, USA) were reconstituted as indicated by the manufacturer. Immunoglobulin (Ig)G opsonisation was achieved according to the manufacturer's instructions. Complement opsonisation was attained by incubation of bacteria with fresh human serum at 37°C for 1 h. Opsonised particles were washed and seeded onto neutrophils, which had been incubated with pioglitazone 10 μM for 1 or 3 h. Fluorescence was measured with BD FACS Canto II after 30 min.

Reactive oxygen species

ROS was detected by dihydrodichlorofluoresceindiacetate (DCF; Molecular Probes) 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 (maximum emission ~530 nm), so that DCF fluorescence can be used as a measure for intracellular ROS levels. Fluorescence intensity was quantified with FACS Canto II after 30 min. Similarly, extracellular ROS was measured by singlet oxygen sensor green reagent (Molecular Probes Europe, Leiden, the 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., San Diego, CA, USA). Unpaired t-tests, Mann–Whitney test or Kruskal–Wallis test with *post hoc* Dunn tests were used as appropriate.

RESULTS

Pioglitazone protects from neutrophil-dependent ALI

After C57Bl/6 mice were exposed to aerosolised lipopolysaccharide, we observed neutrophil recruitment, plasma leakage, lung (ultra-) structure, and elastase activity in the BALF. Treatment with LPS increased the number of intravascular, interstitial and alveolar neutrophils as analysed by flow cytometry (fig. S2) of lung homogenates and BALF (fig. 1). Furthermore, the protein concentration, as well as the clearance of fluorescent dextran, increased in the BALF with the LPS treatment, thereby indicating enhanced plasma leakage and oedema formation. Moreover, the activity of neutrophil-derived elastase, a protease important in ALI, was elevated in LPS-treated animals (fig. 1). Neutrophil depletion abolishes alveolar fluid efflux and structural changes confirming the previously described importance of neutrophils in ALI (fig. 1). 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. 1) and prevented enhanced pulmonary vascular leakage indicated by reduced protein content of the BALF and FITC-dextran clearance volume (fig. 1). In addition, treatment with pioglitazone 1 h after induction of ALI exhibited similar effects (fig. 1). 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 aerosolised 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.

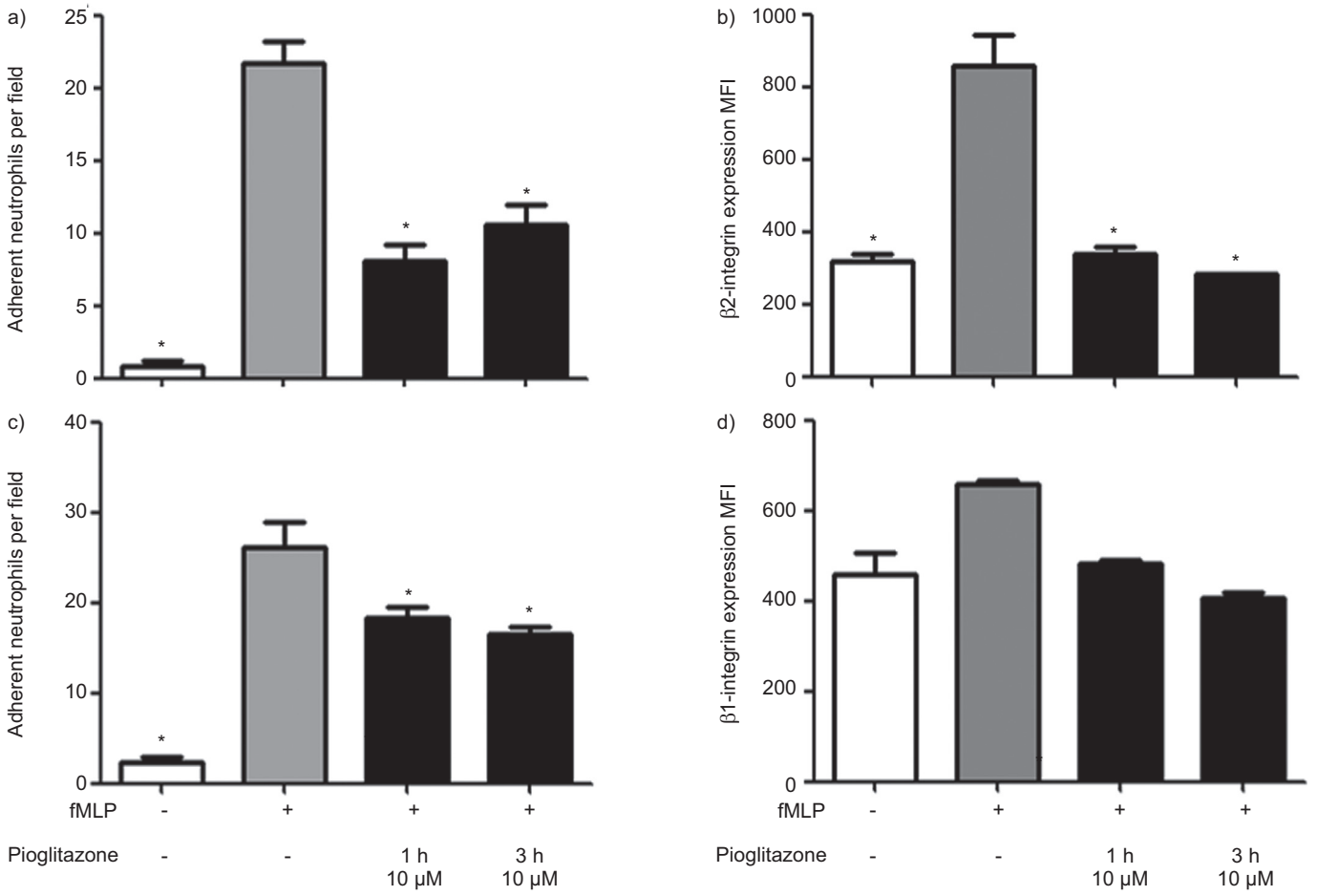

FIGURE 3. Pioglitazone impairs neutrophil adhesion to intercellular adhesion molecule (ICAM)-1 and fibronectin. Isolated human neutrophils were pre-treated with pioglitazone (10 μM, 1 and 3 h) and then activated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). a) Neutrophils were perfused over immobilised recombinant ICAM-1 at 1 dyn·cm^{-2} and the number of adherent cells was enumerated. n=8–10 for each. b) Mean fluorescence intensity (MFI) of surface-expressed β2-integrin as measured by flow cytometry after staining with directly conjugated antibodies. n=3–6 for each. c) Neutrophils were perfused over immobilised fibronectin at 1 dyn·cm^{-2} and the number of adherent cells was enumerated. n=8–10 for each. d) MFI of surface-expressed β1-integrins as measured by flow cytometry after staining with directly conjugated antibodies. n=3–6 for each. *: $p<0.05$ compared to the fMLP group.

Pioglitazone reduces neutrophil adhesion to ICAM-1 and fibronectin

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

As β1-integrins are crucial for extravascular locomotion of neutrophils, we tested the effect of pioglitazone on β1-integrin upregulation and neutrophil adhesion to the β_1-integrin substrate fibronectin. Flow chamber experiments revealed significantly reduced adhesion of neutrophils to fibronectin after pretreatment with pioglitazone (10 μM) for either 1 or 3 h (fig. 3). 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. 3).

Pioglitazone does not impair neutrophil antimicrobial activity

Besides their contribution to ALI, neutrophils display important antibacterial effector functions in bacterial infections. To analyse 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-opsonised (fig. 4a) or complement-opsonised (fig. 4b) FITC-labelled *E. coli* was assessed by flow

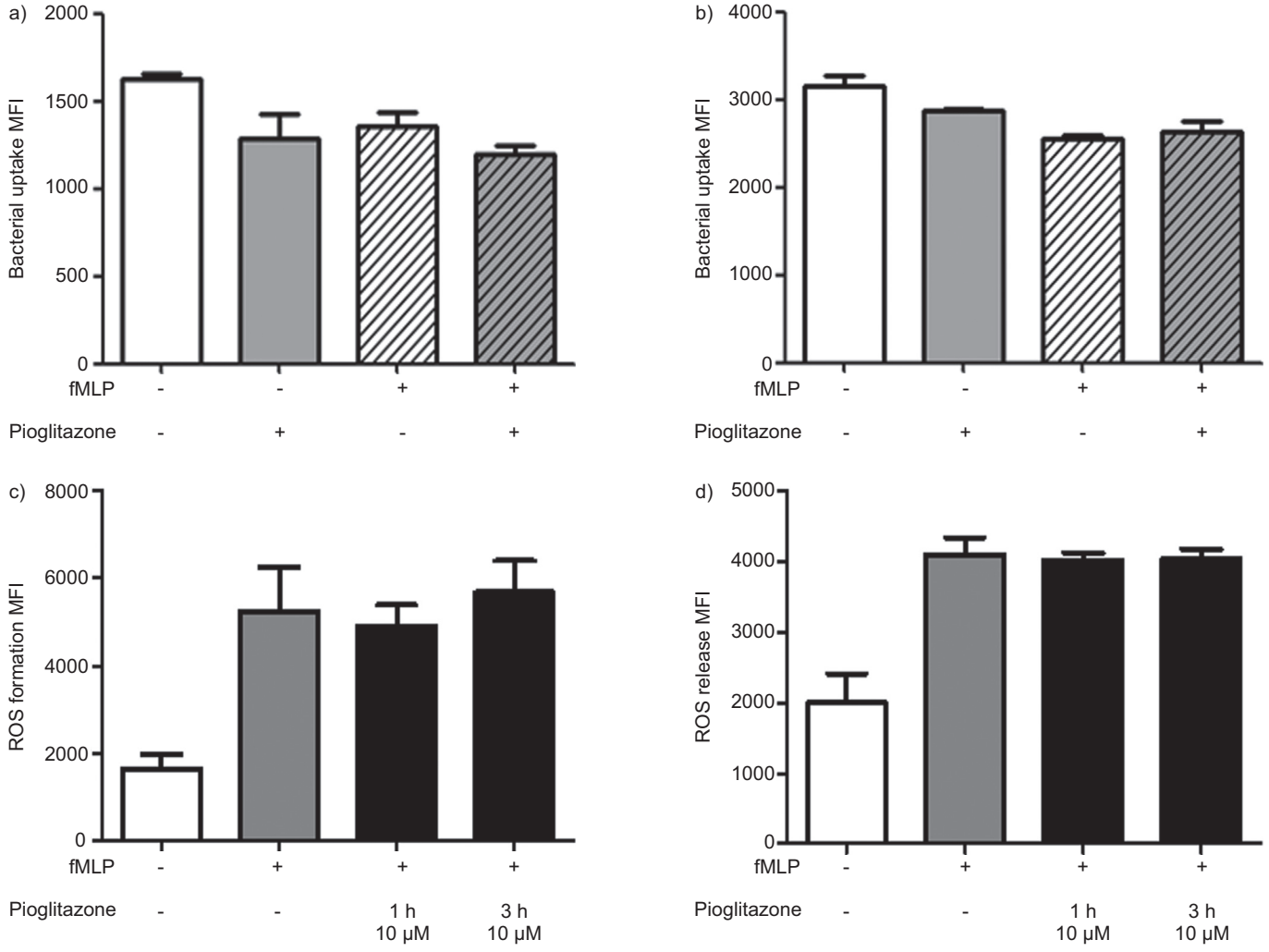

FIGURE 4. Pioglitazone does not affect neutrophil antimicrobial activity. Bacterial uptake of fluorescent a) immunoglobulin G- or b) complement-opsonised *Escherichia coli* by activated or resting neutrophils as assessed by flow cytometry. Neutrophils were activated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and pre-treated with pioglitazone as indicated. n=4. Isolated human neutrophils were pre-treated with pioglitazone (10 μM, 1 and 3 h). c) Neutrophils were labelled with the sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate and reactive oxygen species (ROS) formation was recorded by flow cytometry following fMLP stimulation. Data indicate mean fluorescence intensity (MFI) 30 min after fMLP exposure. n=6 for each. d) Neutrophils were labelled with singlet oxygen green as a marker of extracellular ROS release. Data indicate MFI 30 min after fMLP exposure. n=6 for each. Data are presented as mean±SD.

cytometry. Whereas the bacterial uptake of neutrophils increased after complement opsonisation in comparison to the IgG opsonisation, pioglitazone did not significantly alter bacterial uptake.

Further to adhesion and migration, neutrophils contribute to ALI by release of ROS. However, ROS also displays important antimicrobial functions in 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 h. fMLP clearly induced formation and release of ROS over time. However, pioglitazone pre-treatment 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 innovations in intensive care medicine, 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 oedema formation and neutrophil infiltration, both of which are key events during development of ALI.

PPAR-α and -γ agonists have been developed for treatment of dyslipidaemia and type 2 diabetes. However, recent studies have revealed additional beneficial effects in atherosclerosis and inflammatory diseases, which are partly explained by stabilisation of endothelial function [18, 19]. The protective effect of PPAR-α agonists of the fibrate class in LPS-induced lung injury has previously been established [20]. With the documented importance of PPAR-γ in control of neutrophil migration [21], we 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 ALI [3]. Consequently, we analysed 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 a blockade of β_2-integrins protects from ALI [22, 23]. In our study, pioglitazone prevented intravascular neutrophil adhesion and lung infiltration. As this was addressed in an *in vitro* assay in the absence of other cell types but in the presence of substrates typically involved in neutrophil adhesion and migration, we conclude that the *in vivo* effects may, in a large part, relate to direct interference with surface expression of β1-integrins and β2-integrins. Our results are consistent with a previous study that revealed reduced monocyte adhesion on endothelial cells indicating a 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 this mode of treatment probably 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 β2-integrins on neutrophils is typically a result of mobilisation of preformed granules. β2-integrins are localised 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 β2-integrins following fMLP stimulation not only explains reduced adhesive capacity, but may also point to impaired release of granule proteins relevant to ALI. Consistent with this, we found lower elastase activity in BALF 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 is an important antimicrobial mechanism, overproduction of ROS can cause tissue damage in sepsis and ALI [38]. In animal models of ALI, neutrophil-derived ROS cause lung injury, as shown by histological examination and permeability measurements [39, 40]. In addition, it has been shown that ROS can disrupt intercellular tight junctions of the endothelium by phosphorylation of focal adhesion kinase [41]. Hence, deficiency or blockade of reduced nicotinamide adenine dinucleotide phosphate oxidase prevents ALI [38, 42, 43]. However, in our study, pioglitazone failed to affect ROS release. Hence, the protective effect of pioglitazone appears to primarily arise from decreases in neutrophil degranulation, adhesion and recruitment.

After migration, neutrophils are irreplaceable in bacterial clearance, much of which is mediated by phagocytosis and intracellular bacterial killing [44]. 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.

Conclusion

Pioglitazone attenuates recruitment and activation of neutrophils in a model of ALI and, thereby, 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.

SUPPORT STATEMENT

This study was supported by the Deutsche Forschungsgemeinschaft, the German Heart Foundation, the Else-Kröner-Fresenius Foundation and the B. Braun Foundation.

STATEMENT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

The authors wish to acknowledge X. Balaj, S. Roubrocks and S. Winkler (all, Institute for Molecular Cardiovascular Research (IMCAR), RWTH Aachen, Aachen, Germany) for their excellent technical assistance.

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