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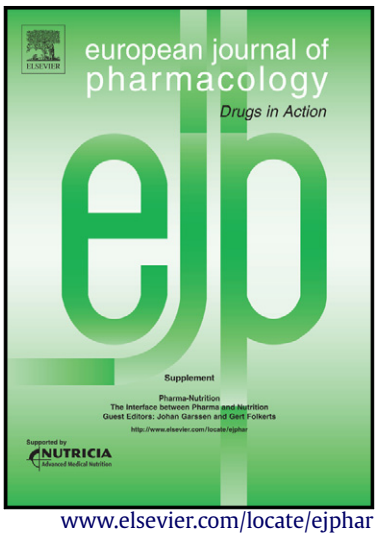

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PII: S0014-2999(13)00642-0
DOI: http://dx.doi.org/10.1016/j.ejphar.2013.08.033
Reference: EJP68793

To appear in: *European Journal of Pharmacology*

Received date: 20 November 2012
Revised date: 14 August 2013
Accepted date: 30 August 2013

Cite this article as: Karla P.S. Barbosa, Laise A.M. Santos, Edlene L. Ribeiro, Ingrid T. Fragoso, Sura W.S. Rocha, Ana K.S. Nunes, Maria E.R. França, Bruna S. Silva, Amanda K.S.e Silva, Mariana A.M. Donato, Fabiana O.S. Gomes, Teresinha G. Silva, Ivan R. Pitta, Marina R. Pitta, Maria C.A. Lima, Flávia D.T. Uchôa, Suely L. Galdino, Christina A. Peixoto, Reduction of carrageenan-induced acute pulmonary inflammation in mice by novel thiazolidinedione derivative LPSF/RA-4, *European Journal of Pharmacology,* http://dx.doi.org/10.1016/j.ejphar.2013.08.033

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Reduction of carrageenan-induced acute pulmonary inflammation in mice by novel thiazolidinedione derivative LPSF/RA-4

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[a] Laboratório de Ultraestrutura, Instituto Aggeu Magalhães, FIOCRUZ, Recife, Brazil.

[b] Laboratório de Microscopia e Microanálise, Centro de Tecnologias Estratégicas do Nordeste, Recife, Brazil.

[c] Laboratório de Planejamento e Síntese de Fármacos, UFPE, Recife, Brazil.

*Corresponding author: Karla Patricia de Sousa Barbosa, Departamento de Entomologia, Centro de Pesquisas Aggeu Magalhães, Av. Moraes Rego s/n, Campus da UFPE - Cidade Universitária, 50670-420, Recife, PE, Brazil. Fax: 55-81-21012500, Phone: 55-81-21012551. E-mail address: karlapsb@gmail.com

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ABSTRACT

A number of studies have demonstrated the biological activities of peroxisome proliferator-activated receptors. However, few studies have addressed the effects of the agonists of these receptors on lung diseases. The aim of the present study was to evaluate the anti-inflammatory action of a novel synthetic thiazolidine derivative (*5Z*)-

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3-benzyl-5-(*1H*-indol-3-ylmethylene)-thiazolidine-2,4-dione (LPSF/RA-4) on acute lung inflammation (pleurisy) induced by carrageenan. Forty mice were randomly allocated to the following groups: I) saline control group (sham); II) carrageenan (CAR) group; III) CAR + LPSF/RA-4 group treated with LPSF/RA-4 (60 <mu>mol /kg); and IV) INDO group treated with indometacin (5 mg/kg). Total cell counts and the measure of nitric oxide (NO) were performed in pleural exudates. Lung fragments were processed for light microscopy, transmission electron microscopy, immunohistochemistry and Western blotting. The influx of leukocytes and NO levels were significantly reduced following treatment with LPSF/RA-4 and INDO. Histopathological and ultrastructural analyses of the CAR group revealed evident tissue alterations, such as oedema, infiltrates of inflammatory cells and emphysema. These alterations were significantly reduced in the groups treated with LPSF/RA-4 or INDO. Immunohistochemistry revealed an increase in inflammatory markers (COX-2, iNOS, TNF-α and IL-1β) in the lung tissue of the CAR group, whereas the groups treated with LPSF/RA-4 and INDO exhibited significant reductions in such immunomarkers. Western blot analysis revealed an increased expression of COX-2 and IL-1 in the CAR group, which was reduced by treatment with LPSF/RA-4. The present findings demonstrate the potent anti-inflammatory action of the novel derivative thiazolidinedione LPSF/RA-4 in acute lung injury induced by carrageenan.

Keywords: Peroxisome proliferator-activated receptor; pleurisy; acute lung injury; thiazolidinedione

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1. INTRODUCTION

Lung diseases, such as asthma and chronic obstructive pulmonary disease, constitute serious public health problems worldwide. While potent anti-inflammatory drugs, such as the glucocorticoids, are available for treatment, these drugs have undesired side effects and exhibit limited efficacy. Thus, there is a need for novel forms of treatment for these diseases (Belvisi et al., 2006).

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the family of nuclear receptors related to retinoid, glucocorticoid and thyroid hormone receptors (Evans, 1988). PPARs have been identified in different lung tissue cells, including cells associated with inflammation in the lung. Three isotypes have been identified [alpha (α), beta/delta (β/δ) and gamma (γ)]. These isotypes are encoded by three separate genes, with different tissue distributions and functions (Desvergne and Wahli, 1999). The suggestion that activation of PPARs may exhibit potent anti-inflammatory and immunomodulatory activity (Serhan, 1996; Serhan and Devchand, 2001) has led to increased interest in studying these receptors in different models of lung inflammation (Belvisi et al., 2006; Crisafulli et al., 2010; Buss et al., 2012).

In recent years, the anti-inflammatory effect of PPAR agonists has been evaluated. Selective binders with high-affinity for each of the PPARs and dual specificity ligands are also available. There is evidence that pioglitazone acts through the activation of both PPAR-α and PPAR-γ, while rosiglitazone acts only through PPAR-γ (Buss et al., 2012).

Acute lung injury (pleurisy) induced by carrageenan is a useful model for the assessment of the contribution of mediators involved in cell alterations during the

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inflammatory process (Cuzzocrea et al., 2008). The initial phase of carrageenan-induced acute inflammation (0 to 1 h) has been attributed to the release of histamine, 5-hydroxytryptamine and bradykinin, followed by a late phase (1 to 6 h) sustained mainly by the release prostaglandins and attributed to the induction of cyclooxygenase-2 (COX-2) in the tissue (Salvemini et al., 1996; Nantel et al., 1999; Crisafulli et al., 2010). The recruitment of polymorphonuclear (PMN) cells from the circulation to the inflamed tissue plays a key role in the breakdown and remodeling of injured tissue (Salvemini et al., 1995). Moreover, macrophages participate in the progression of experimental pleurisy by producing pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin-1 beta (IL-1β) (Crisafulli et al., 2010).

Nitric oxide (NO) is a simple free radical that acts as a mediator of immune system function and cell signal transduction (Jorens et al., 1993). Relatively large amounts of NO can be synthesised by inducible NO synthase (iNOS). Different cells are capable of synthesising and releasing NO in the lung, including neutrophils, macrophages, endothelial cells, epithelial cells and vascular smooth muscle cells (Asano et al., 1994; Moncada, 1994).

The aim of the present study was to investigate the anti-inflammatory action of a novel thiazolidinedione denominated LPSF/RA-4 in a model of carrageenan-induced pleurisy through the analysis PMN cell infiltration, NO synthesis (nitrite concentration), lung injury (histology and ultrastructure) and the expression of the inflammation mediators / enzymes TNF-α, IL-1β, iNOS and COX-2.

2. MATERIAL AND METHODS

2.1 Chemistry

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The compound (*5Z*)-3-benzyl-5-(*1H*-indol-3-ylmethylene)-thiazolidine-2,4-dione LPSF/RA-4 was obtained from the Laboratório de Planejamento de Síntese de Fármacos (LPSF) of the Universidade Federal de Pernambuco (Brazil) and was duly identified by nuclear magnetic resonance of hydrogen as well as infrared (IR) and mass spectroscopy (MS). Figure 1 displays the route of LPSF/RA-4 synthesis (Fig. 1). The starting reagent was thiazolidine-2,4-dione, which was reacted with benzyl chloride under basic conditions to obtain the 3-benzyl-thiazolidine-2,4-dione LPSF/RA-1 intermediate (Mourão et al., 2005). Laterally, 2-cyano-3-(*1H*-indol-3-yl)-acrylic acid ethyl ester LPSF/IP-19 (Brandão et al., 2004) was synthesised through Knoevenagel condensation between *1H*-indol-3-carbaldehyde and ethyl cyanoacetate. A Michael-type addition was then performed by reacting the ester LPSF/IP-19 with intermediate 3-benzyl-thiazolidine-2,4-dione to form the final indol-thiazolidine-2,4-dione LPSF/RA-4 compound.

Reactions were monitored with analytical thin layer chromatography in silica gel 60 F254 plates and visualised under UV light (254 nm). Melting points were determined on a Quimis 340 capillary melting point apparatus and were not corrected. Infrared spectra were recorded as KBr discs using a BRUKER (IFS 66) infrared spectrophotometer. Nuclear magnetic resonance ^{1}H NMR and ^{13}C NMR spectra were recorded in a VMMRS 400 MHz VARIAN spectrometer using tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as the solvent. Chemical shifts (δ, ppm) were assigned according to the internal standard signal of TMS in DMSOd_6 (δ = ppm). Coupling constants (J) are reported in Hz. ^{1}H NMR spectra are reported in the following order: chemical shift, multiplicity, number and type of proton and coupling constant(s). Mass spectra with ESI mass were obtained in a Bruker HCT ultra mass spectrometer.

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Molecular mass was estimated from the m/z ratio of the deprotonated molecular ions of the formula $(M-H)^-$ in negative ionisation mode.

The LPSF/RA-4 derivative was isolated as a single isomer. X-ray crystallographic studies and ^{13}C-NMR have demonstrated a preferred Z configuration for 5-benzylidene-thiazolidinones (Tan et al., 1986; De Simone et al., 1995). The presence of the arylidene proton peak in the synthesised (*5Z*)-3-benzyl-5-(*1H*-indol-3-ylmethylene)-thiazolidine-2,4-dione LPSF/RA-4 derivatives in ^{1}H NMR confirmed the completion of the nucleophilic addition reaction. The compound was also confirmed by MS data in negative mode. The IR spectrum of the compound showed peaks characteristic of the carbonile group and arilidene (HC=). Preparation of (*5Z*)-3-benzyl-5-(*1H*-indol-3-ylmethylene)-thiazolidine-2,4-dione LPSF/RA-4: Equimolar amounts of 3-benzyl-thiazolidine-2,4-dione (200 mg) and 2-cyano-3-phenyl-acrylic acid ethyl ester (230 mg) were reacted using absolute ethanol (8 mL) as the solvent and morpholine (1 mL) as the catalyst. The reaction mixture was heated to 50 °C for 8 h and then cooled to room temperature. The solid that precipitated out was filtered under vacuum and washed with water and absolute ethanol. MF: $C_{19}H_{14}N_2O_2S$; MW: 334.39; MP: 237^{o}C; Yield: 46%; Rf 0.56 n-hexane/ethyl acetate 7:3. IR (υ, cm^{-1}; KBr): 3418, 1723, 1661, 1594; ^{1}H NMR (400 MHZ, DMSO d_6): s (1H, NH indol) 12.19, m (2H, indol) 7.29-7.18, d (1H indol) 7.93-7.90 J= 7.2, d (1H indol) 7.52-7.50 J= 7.2, s (1H indol) 7.83, s (1H =CH) 8.22, s (2H NCH_2) 4.84, m (5H benzylic) 7.39-7.28. ^{13}C NMR (δ ppm, DMSO-d_6, DEPT): 44.5 (CH_2), 110.5 (C), 112.6 (CH), 113.5 (C), 118.5 (CH), 121.3 (CH), 123.3 (CH), 126.3 (CH), 126.8 (C), 127.6 (2CH), 127.8 (CH), 128.7 (2CH), 129.3 (C), 135.9 (CH chain), 136.3 (C), 165.5 (C=O), 167.2 (C=O). MS (m/z) relative intensity: $[M-H]^-$ 332.9 (100), MS2[333.0]$^-$, 241.8 (4), 199.8 (7), 171.8(100) (Fig. 1).

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2.2 Animals

Male Swiss mice weighing 18 to 25 g were lodged under standardised conditions (22 ± 2 °C, alternating 12-h periods of light and dark and 50% to 60% relative humidity) and allowed free access to standard mouse chow and water prior to the experiment. This study was approved by Ethics Committee for Animal Experimentation of the Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (Brazil) under process number N° 12/2010.

2.3 Experimental Design

The mice were randomly allocated to the following groups: I) sham group, which received only saline [0.1 ml, intrapleural (i.pl.) route] (N = 10); II) CAR group, subjected to carrageenan-induced pleurisy (N = 10); III) CAR + LPSF/RA-4 group, which received LPSF/RA-4 [60 <mu>mol /kg, intraperitoneal (i.p.) route, solubilised in 3% cremophor and diluted in sterile 0.9% saline] 0.5 h prior to carrageenan-induced pleurisy (N = 10); and IV) CAR + INDO group, which received indometacin (5 mg/kg, i.p. route) 0.5 h prior to carrageenan-induced pleurisy (N = 10). Indometacin (INDO) was used as the reference anti-inflammatory drug.

Dose-response assays were first performed to evaluate the optimum LPSF/RA-4 dosage. Prior to pleurisy induction, the animals were treated with different doses of LPSF/RA-4 (10, 30 or 60 <mu>mol /kg, i.p. route). Inflammatory parameters were analysed 4 h after carrageenan injection, based on previous studies (Guiara et al., 1986; Henriques et al., 1990). Subsequently, all procedures as well as the immunohistochemical and western blot analyses were performed only with the optimum dose.

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2.4 Induction of pleurisy

Mice were anaesthetised with an intramuscular injection of a combination of ketamine hydrochloride 10% (115 mg/kg) and xylazine 2% (10 mg/kg). Following confirmation of anaesthesia, the animals received a single intrapleural (i.pl.) injection of 0.1 mL sterile saline (NaCl 0.9%) or saline sterile containing λ-carrageenan (1%) on the right side of the chest. Four hours after the injection of carrageenan, the animals were killed by CO_2 inhalation. The chest was carefully opened and the pleural cavity was washed with 1.0 mL of sterile phosphate buffered saline (PBS), pH 7.6 (Fröde et al., 2009). The exudate and washing solution were removed by aspiration. Any exudate contaminated with blood was discarded. Fluid samples were collected from the pleural cavity to determine the total leukocyte content in a Neubauer chamber with the dilution of the exudate in Trypan blue stain (1:20). Fluid leakage samples were collected for further determination of total leukocytes and NO levels.

2.5 Measurement of NO

The Griess colorimetric reaction was employed for the measurement of NO. This method allows the detection of the oxidation of NO into nitrite ($NO2^-$) in the pleural fluid. Briefly, 50 µl of pleural fluid were added to a 96-well ELISA plate, followed by the same volume of Griess reagent, which is composed of 1% sulphanilamide diluted in 2.5% H_3PO_4 (solution A) and N-1-naphtyl-ethylenediamine also diluted in 2.5% H_3PO_4 (solution B). Assays were performed in duplicate. To prepare a standard curve, a solution of sodium nitrite at an initial concentration of 100 µM was serially diluted in PBS. After incubation for 10 minutes in the dark, reading in the spectrophotometer was performed at 490 nm. The absorbance of different samples

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was compared with the standard curve and the results were expressed as mean ± standard error of the duplicate, using GraphPad Prism software programme (v. 5.0).

2.6 Histological examination

Lung fragments were washed twice in PBS, pH 7.6, fixed in Bouin's solution (75% picric acid, 20% formaldehyde and 5% glacial acetic acid) for 8 hours, dehydrated in an increasing ethanol series, cleared in xylene and embedded in purified paraffin (VETEC, São Paulo, SP, Brazil). Sections measuring 5 µm were cut using a microtome (Leica RM 2125RT), deparaffinised with xylene, stained with haematoxylin-eosin and evaluated under magnification (400 x) using an inverted microscope (Observer Z1, Zeiss MicroImaging GmbH) equipped with a camera and the 4.7.4 Image Analysis programme (AxionCam MRm Zeiss).

2.7 Electron transmission microscopy

Lung fragments were fixed overnight in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer. The specimens were then washed twice in the same buffer and post-fixed in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in Embed 812. Polymerisation was performed at 60 °C for three days (Florêncio et al., 2005). Ultrathin sections were collected on 300-mesh nickel grids, counterstained with 5% uranyl acetate and lead citrate and examined using a Morgani FEI 268D transmission electron microscope.

2.8 Immunohistochemical localisation of COX-2, iNOS, TNF-α and IL-1β

Five sections (thickness: 5 µm) from each group were cut and adhered to slides

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treated with 3-aminopropyltriethoxysilane (Sigma, USA). Briefly, sections were de-paraffinised with xylene and rehydrated in graded ethanol (100 to 70%). To minimise endogenous peroxidase activity, the slides were treated with 10% (v/v) H_2O_2 in water for fifteen minutes. The sections were washed with 0.01 M Phosphate Buffered Saline (PBS), pH 7.2, and blocked with 1% Bovine Serum Albumin (BSA), 0.2% Tween 20 in PBS for 1 h at room temperature. The sections were incubated overnight at 4 ºC with anti-COX-2 antibody (ABCAM, CA, USA, 1:400), anti-iNOS, anti-TNF-α antibody (ABCAM, CA, USA, 1:250) and anti-IL-1β antibody (GenWay, San Diego, CA, 1:250). The antigen-antibody reaction was visualised with avidin-biotin peroxidase (Dako Universal LSAB ® + Kit, Peroxidase) using 3.3-diaminobenzidine as the chromogen. The slices were then weakly counterstained with Harris' haematoxylin and mounted in entellan (Merck, catalogue number: 1079610100). Positive staining resulted in a brown reaction product. Five pictures at the same magnification were quantitatively analysed using the Gimp 2.6 software (GNU Image Manipulation Program, UNIX platforms).

2.9 Western blotting

Lungs were quickly dissected and homogenised in an overhead stirrer (Wheaton, nº 903475) in an extraction cocktail (10 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 10 mg of aprotinin and 100 mM Tris(hydroxymethyl)aminomethane, pH 7.4). Homogenates were centrifuged at 3000 xg for 10 min. The supernatant was collected and stored at -70 ºC until use for immunoblotting. Protein levels were determined using the Bradford method with BSA as the standard (Bradford, 1970). Proteins (40 µg/µl) were separated on 10% (COX-2) or 14% (IL-1β) sodium dodecyl sulphate polyacrylamide by gel electrophoresis under

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reduced conditions and were electrophoretically transferred onto nitrocellulose membranes (Bio Rad, CA, USA, Ref. 162-0115). After blocking overnight at 4 ºC with 5% non-fat milk in TBS-T (Tris-buffered saline 0.1% plus 0.05% Tween 20, pH 7.4), the membranes were incubated at room temperature for three h with rabbit polyclonal antibody against COX-2 (1:1,000 dilution; ABCAM, CA, USA) and IL-1 β (1:2.000 dilution, Genway, San Diego, CA) diluted in TBS-T buffer solution containing 3% non-fat milk. After washing (six times, 10 min each) in TBS-T, the membranes were further reacted with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:80,000 (Ref. A6154) and 1:80,000 (Ref. A5420), respectively, Sigma, USA) diluted in TBS-T with 1% non-fat milk for 1 h 30 min at room temperature. An enhanced chemiluminescence reagent (Super Signal, Pierce, Ref. 34080) was used for the visualisation of the labelled protein bands and the blots were developed on X-ray film (Fuji Medical, Kodak, Ref. Z358487-50EA). For quantification, the pixel density of each band was determined using the ImageJ 1.38 programme (available at http://rsbweb.nih.gov/ij/download.html; developed by Wayne Rasband, NIH, Bethesda, MD, USA). The results were confirmed in three sets of experiments for each protein investigated. Immunoblotting for β-actin was performed as a control for the above protein blots. After visualisation, the protein antibodies were stripped from the membranes, which were re-probed with monoclonal anti-β-actin antibody (1:2.000 dilution, Sigma, USA). Protein densitometry was then performed.

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2.10 Statistical analysis

The GraphPad Prism software programme (version 5.0) was used for the statistical analysis. Data were expressed as mean ± standard error. For the *in vivo* studies, n represents the number of animals studied. Differences between the control and

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treated groups were analysed using analysis of variance (ANOVA), followed by either Dunnett's or Tukey's post hoc test. All p-values less than 0.05 were considered significant.

3. RESULTS

3.1 Effect of LPSF/RA-4 on leukocyte counts

In the CAR group, the injection of carrageenan induced an acute inflammatory response characterised by the accumulation of fluid containing a large amount of total leukocytes ($338.8 \pm 24.54 \times 10^6$ cells/cavity). However, a significantly lower number of total leukocytes were found in the LPSF/RA-4 and INDO groups ($70.16 \pm 3.23 \times 10^6$ and $58.78 \pm 6.50 \times 10^6$ cells/cavity, respectively) (Fig. 2A). The present results also revealed a significant reduction in cellular infiltrate in the lung tissues at both LPSF/RA-4 (10 and 30 <mu>mol /kg). However, the animals these groups exhibited congestion, haemorrhage, oedema and emphysema. The choice of the LPSF/RA-4 dose used in this study (60 <mu>mol /kg) was based on histopathological studies carried out in our laboratory.

3.2 Effect of LPSF/RA-4 on NO levels in pleural exudate

NO levels in the exudate of the CAR group were significantly higher than those in the sham group, whereas no significant differences were found between sham group and either the LPSF/RA-4 or INDO group. Treatment with LPSF/RA-4 caused a reduction of 72.46 ± 7.51 in NO in comparison to the CAR group ($p < 0.05$). Treatment with either LPSF/RA-4 or indomethacin prevented the increases in NO levels in CAR group.

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3.3 Histopathology of lung fragments

Histological analysis of the lung fragments from the sham group revealed well-preserved tissue (Fig. 2C). In contrast, the animals with carrageenan-induced pleurisy exhibited patchy areas of inflammation composed of lymphocytes, neutrophils and macrophages. Mild haemorrhage, congestion, discrete alveolar thickening due to increased cellularity, oedema and emphysema were also observed (Fig. 2D). The lungs from mice pre-treated with LPSF/RA-4 revealed a significantly lower number of the inflammatory cells, lesser alveolar thickening, fewer areas of haemorrhage and a lesser degree of emphysema (Fig. 2E). Treatment with INDO also prevented lung injury (Fig. 2F).

3.4 Effect of inflammation on ultrastructure of lung tissue

The ultrastructural analysis of the lung fragments from the sham group revealed a preserved morphological pattern, with the alveolar epithelium composed of pneumocytes (Fig 3A). Carrageenan-induced pleurisy led to ultrastructural alterations, such as type II pneumocytes with evident lamellar bodies, electrodense granules, vacuoles and myelin bodies (characterising cell damage), vasodilatation in capillary vessels, numerous collagen fibres in the interstitial space (increasing its thickness) and discrete interstitial haemorrhage. Inflammatory cells and activated alveolar epithelial cells were also observed (Fig. 3B). The animals treated with LPSF/RA-4 and INDO (Fig. 3C and 3D, respectively) exhibited a preserved alveolar epithelium similar to that of the sham group, thereby demonstrating attenuated tissue damage.

3.5 Effect of LPSF/RA-4 on IL-1β, TNF-α, COX-2 and iNOS levels

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In the present study, no significant staining for IL-1β was found in the lung tissues from the sham group (Fig. 4A). In contrast, lung tissue from the CAR group exhibited a positive reaction for IL-1β in leucocytes, alveolar macrophages, endothelial cells and epithelial cells (Fig. 4B). Treatment with both LPSF/RA-4 and INDO (Fig. 4C and D, respectively) led to a significant reduction in IL-1β staining in comparison to the CAR group (see densitometry analysis, Fig. 4E).

Immunohistochemical analysis for TNF-α revealed no significant labelling in the sham group (Fig 4F). In contrast, lung tissue from the CAR group exhibited a positive staining for TNF-α, especially in pneumocytes, macrophages, infiltrate cells and the vascular wall (Fig. 4G). Treatment with both LPSF/RA-4 and INDO (Fig. 4H and I, respectively) led to a reduction in IL-1β staining in comparison to the CAR group (see densitometry analysis, Fig. 4J).

Lung tissue from the sham group expressed baseline levels of COX-2 (Fig. 5A), whereas lung tissue from the mice treated with carrageenan demonstrated greater COX-2 expression (Fig. 5B). This expression was significantly reduced in the LPSF/RA-4 group (Fig. 5C). The treatment with INDO also inhibited COX-2 expression, but to a lesser extent than that found with LPSF/RA-4 (Fig. 5D, densitometry analysis 5E).

The sham group demonstrated baseline levels of iNOS (Fig. 5F), whereas the CAR group exhibited significant iNOS labelling in alveolar epithelial cells (Fig 5G). Treatment with LPSF/RA-4 and INDO led to a significant reduction in staining for COX-2 (Fig. 5H and I, respectively) in comparison to the CAR group (see densitometry analysis, Fig. 5J).

3.6 Western blotting analysis for COX-2 and IL-1 β

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Baseline levels of COX-2 were detected in the sham group, whereas a significant increased in COX-2 was found in the CAR group. However, treatment with LPSF/RA-4 significantly reduced the expression of COX-2 (Fig. 6A and B), confirming the results obtained in the immunohistochemical analysis performed with lung fragments. In the present study, the entire lung was used in this analysis, which may explain the small increase in the expression of COX-2 found with this technique.

Baseline levels of IL-1β were detected in the sham group, whereas the CAR group exhibited increased levels of IL-1β. As expected, LPSF/RA-4 led to a significant reduction in IL-1β levels in comparison to the CAR group (Fig. 6C and D).

4. DISCUSSION

The present study evaluated the effects of a novel derivative of thiazolidinedione, denominated LPSF/RA-4, on lung acute inflammation induced by carrageenan in mice. The inflammatory process is characterised by the production of histamine, leukotrienes, prostaglandins, platelet-activating factor and bradykinin as well as the release of chemicals from cells (Tomlinson et al., 1994).

Carrageenan-induced pleurisy is a well-characterised experimental model of inflammation commonly used to evaluate cell migration and other inflammatory parameters. Non-steroidal anti-inflammatory drugs are effective in inhibiting both cell migration and exudation (Saleh et al., 1999). In the present study, both LPSF/RA-4 and indometacin were effective in significantly reducing the migration of neutrophils and the influx of mononuclear cells. These findings are consistent with data described in previous studies that also used the pleurisy model for the evaluation of the PPARg agonist rosiglitazone (Buss et al., 2012) and the PPARa agonist pioglitazone (Fröde et al., 2009), demonstrating that glitazones are effective in inhibiting the migration of

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leukocytes following the administration of carrageenan in the pleural cavity of a murine model.

Light microscopy revealed that the carrageenan-induced pleurisy was characterised by cellular infiltrate surrounding blood vessels, extravasated erythrocytes in the perivascular interstitium of arterioles and alveolar haemorrhage. The histological analyses demonstrated that, when administered 0.5 hour prior to CAR, LPSF/RA-4 significantly suppressed morphological changes in the lung, such as oedema, the accumulation of inflammatory cells and alveolar thickness, thereby reducing lung injury. Similarly, the ultrastructural analyses revealed that carrageenan-induced pleurisy led to damage to type II pneumocytes and vasodilatation of capillary vessels. However, treatment with either LPSF/RA-4 or INDO inhibited lung injury, as the treated groups exhibited preserved alveolar epithelium similar to that of the sham group.

In the present study, TNF-α and IL-1β levels were significantly reduced following treatment with LPSF/RA-4 and similar effects were obtained with indometacin, which is the drug of reference for the attenuation of acute pulmonary inflammation. Therefore, this study provides evidence of the beneficial effect of the novel thiazolidinedione-derived LPSF/RA-4 and demonstrates that this effect is associated with an inhibitory effect on the pro-inflammatory cytokines IL-1β and TNF-α, which are potent mediators of the chemotaxis of leukocytes in inflammatory processes (Lampinen et al., 2004). These findings confirm and extend those obtained by Buss et al. (2012), who found that roziglitazone treatment decreased the levels of these cytokines in a murine pleurisy model.

Previous studies have demonstrated that nitric oxide plays a critical role in acute and chronic inflammatory responses (Medeiros et al., 1995). However, treatment with LPSF/RA-4 significantly reduced NO products in the exudates of the lungs affected

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with pleurisy. Similarly, immunohistochemical analyses of the lung tissues also indicated that LPSF/RA-4 reduced levels of iNOS. These effects were similar to those obtained with indometacin. Studies have indicated that inhibitors of NOS activity reduce the development of carrageenan-induced inflammation, which suggests a role for NO in the pathophysiology associated with this model of inflammation (Cuzzocrea et al., 1998; Rubbo et al., 1994).

The NO pathway has similarities with the COX pathway, since both are key regulators of inflammatory responses and have both constitutive and inducible isoforms of their enzymes (Sano et al. 1992; Clancy et al., 2000).

PPARγ agonists reduce iNOS expression and NO production in a dose-dependent manner in macrophages and other inflammatory cells (Crosby et al., 2006) and PPARγ inhibits the induction and production of cytokines, such as IL-1β and TNF-α, by macrophages (Buss et al., 2012).

Cuzzocrea et al. (2004) showed that the PPAR-γ agonist rosiglitazone reduced the expression of COX-2 and iNOS in mouse lungs submitted to carrageenan. The results of present study demonstrate that treatment with LPSF/RA-4 was more effective in inhibiting the expression of COX-2 than treatment with indometacin. However, the difference did not achieve statistical significance, which is likely related to the non-selectivity of indometacin for COX isoforms, as reported in previous studies (Nakano et al., 2007).

The present study shows evidence that the novel thiazolidinedione denominated LPSF/RA-4 has an anti-inflammatory profile, as it inhibits the influx of inflammatory cells and leads to a decrease in lung injury in a pleurisy model induced by carrageenan. This effect is linked to the inhibition of the expression of TNF-α, IL-1β, iNOS and COX-2. The findings suggest that the PPAR ligands may represent novel therapeutic

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agents for lung inflammation. New experiments are currently being designed to evaluate whether this effect is related to the transcriptional regulation of PPAR-γ, PPAR-α or the activation of both receptors.

5. ACKNOWLEDGMENTS

This study was supported by the Brazilian fostering agencies Pernambuco Foundation for Assistance to Science and Technology (FACEPE), Aggeu Magalhães Research Centre of the Oswaldo Cruz Foundation in Recife (CPqAM/FIOCRUZ), Centre for Strategic Technology in the Northeast (CETENE) and the National Institute of Structural Biology and Bioimaging (INBEB).

6. REFERENCES

Asano, K., Chee, C.B., Gaston, B., Lilly, C.M., Gerard, C., Drazen, J.M., Stamler, J.S., 1994. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. Proc Natl Acad Sci U S A 91, 10089-10093.

Belvisi, M.G., Hele, D.J., Birrel, M.A., 2006. Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. Eur J Pharmacol 533,101–109.

Bradford, M.A., 1970. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brandão, S.S.F., Andrade, A.M.C., Pereira, D.T.M., Barbosa Filho, J.M., Lima, M.C.A., Galdino, S.L., Pitta, I.R., Barbe, J., 2004. A novel way of synthesis of 1,3,5-trisubstituted-2-thioxoimidazolidinones. Heterocyclic Communications 10, 9-14.

Accepted manuscript

ACCEPTED MANUSCRIPT

Buss, Z.S., Medeiros, Y.S., Fröde, T.S., 2012. PPAR-Gamma Agonist Rosiglitazone Attenuates the Inflammation Caused by Carrageenan in the Mouse Model of Pleurisy. Inflammation 35, 280-288.

Clancy, R., Varenika, B., Huang, W., Ballou, L., Attur, M., Amin, A.R., Abramson, S.B., 2000. Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. J Immunol 165, 1582-1587.

Crisafulli, C., Mazzon, E., Paterniti, I., Galuppo, M., Bramanti, P., Cuzzocrea, S., 2010. Effects of Liver x receptor agonist treatment on signal transduction pathways in acute lung inflammation. Respiratory Research 22, 11-19.

Crosby, M.B., Zhang, J., Nowling, T.M., Svenson, J.L., Nicol, C.J., Gonzalez, F.J., Gilkeson, G.S., 2006. Inflammatory modulation of PPAR gamma expression and activity. Clin Immunol.118, 276-283.

Cuzzocrea, S., Zingarelli, B., Caputi, A.P., 1998. Role of peroxynitrite and poly (ADP-ribose) synthetase on cellular energy depletion in carrageenan-induced pleurisy. J Chemother 10, 153-156.

Cuzzocrea, S., Pisano, B., Dugo, L., Ianaro, A., Maffia, P., Patel, N.S., Di Paola, R., Ialenti, A., Genovese, T., Chatterjee, P.K., Di Rosa, M., Caputi, A.P., Thiemermann, C., 2004. Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-gamma, reduces acute inflammation. Eur J Pharmacol 483, 79-93.

Cuzzocrea, S., 2006. Peroxisome proliferator-activated receptors and acute lung injury. Curr Opin Pharmacol 6, 263–270.

Cuzzocrea, S., Bruscoli, S., Mazzon, E., Crisafulli, C., Donato, V., Di Paola, R., Velardi, E., Esposito, E., Nocentini, G., Riccardi, C., 2008. Peroxisome Proliferator-Activated Receptor-a Contributes to the Anti-Inflammatory Activity of Glucocorticoids. Molecular Pharmacology 73, 323–337.

Accepted manuscript

ACCEPTED MANUSCRIPT

De Simone, C.A., Zukerman-Schpector, J., Pereira, M.A., Luu-Duc, C., Pitta, I.R., Galdino, S.L., Amorim, E.L.C., 1995. 3-(4-Bromobenzyl)-5-(4-fluorobenzylidene)-imidazolidine-2,4-dione. Acta Cryst. C51, 2620-2621.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator activated receptors: nuclear control of metabolism. Endocrine Reviews, 20, 649–688.

Evans, R.M.,1988. The steroid and thyroid hormone receptor superfamily. Science 240, 889–895.

Florêncio, M.S., Saraiva, K.L., Peixoto, C.A., 2005. The effects of diethylcarbamazine on the ultrastructure of lung cells in vivo. Tissue Cell 37, 241-246.

Fröde, T.S., Buss, Z.S., Reis, G.O., Medeiros, Y.S. 2009. Evidence of anti-inflamatory effects of pioglitazone in the murine pleurisy model induced by carrageenan. International Immunopharmacology 9, 1394-1400.

Ghiara, P., Bartalini, M., Tagliabue, A., Boraschi, D., 1986. Anti-inflammatory activity of IFN-beta in carrageenan-induced pleurisy in the mouse. Clin Exp Immunol. 66, 606-14.

Henriques, M.G., Weg, V.B., Martins, M.A., Silva, P.M., Fernandes, P.D., Cordeiro, R.S., Vargaftig, B.B., 1990. Differential inhibition by two hetrazepine PAF antagonists of acute inflammation in the mouse. Br J Pharmacol 99, 164-168.

Jorens, P.G., Vermeire, P.A., Herman, A.G., 1993. L-arginine-dependent nitric oxide synthase: a new metabolic pathway in the lung and airways. Eur Respir J 6, 258-266.

Lampinen, M., Carlson, M., Håkansson, L.D., Venge, P., 2004. Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy 59, 793-805.

Moncada, S., 1994. Nitric oxide. J Hypertens Suppl 12, 35-39.

Mourão, R.H., Silva, T.G., Soares, A.L.M., Vieira, E.S., Santos, J.N., Lima, M.C.A., Lima, V.L.M., Galdino, S.L., Barbe, J., Pitta, I.R., 2005. Synthesis and Biological

Accepted manuscript

ACCEPTED MANUSCRIPT

Activity of Novel Acridinylidene and Benzylidene thiazolidinediones. Eur Jour Med Chem 40(11), 1129-1133.

Nakano, M., Denda, N., Matsumoto ,M., Kawamura, M., Kawakubo, Y., Hatanaka, K., Hiramoto, Y., Sato, Y., Noshiro, M., Harada, Y., 2007. Interaction between cyclooxygenase (COX)-1- and COX-2-products modulates COX-2 expression in the late phase of acute inflammation. Eur J Pharmacol 559, 210-218.

Medeiros, M.V., Binhara, I.M., Moreno, Júnior. H., Zatz, R., De Nucci, G., Antunes, E., 1995. Effect of chronic nitric oxide synthesis inhibition on the inflammatory responses induced by carrageenin in rats. Eur J Pharmacol 285,109-114.

Nantel, F., Denis, D., Gordon, R., Northey, A., Cirino, M., Metters, K.M., Chan, C.C., 1999. Distribuition and regulation of cyclooxigenase-2 in carrageenan-induced inflammation. British Journal of Pharmacology 128, 853-859.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M., Freeman, B.A., 1994. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. J Biol Chem 269, 26066-26075.

Saleh, T.S., Calixto, J.B., Medeiros, Y.S., 1999. Effects of anti-inflammatory drugs upon nitrate and myeloperoxidase levels in the mouse pleurisy induced by carrageenan. Peptides 20, 949-956.

Salvemini, D., Seibert, K., Masferrer, J.L., Settle, S.L., Currie, M.G., Needleman, P., 1995. Nitric Oxide Activates the Cyclooxygenase Pathway in Inflammation. Am J Ther 2, 616-619.

Salvemini, D., Wang, Z.Q., Wyatt, P.S., Bourdon, D.M., Marino, M.H., Manning, P.T., Currie, M.G., 1996. Nitric oxide: a key mediator in the early and late phase of

Accepted manuscript

ACCEPTED MANUSCRIPT

carrageenan-induced rat paw inflammation. British Journal of Pharmacology 118, 829–838.

Sano, H., Hla, T., Maier, J.A., Crofford, L.J., Case, J.P., Maciag, T., Wilder, R.L., 1992. In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. J Clin Invest 89, 97-108.

Serhan, C.N., 1996. Inflammation: signalling the fat controller. Nature 384, 23–24.

Serhan, C.N., Devchand, P.R., 2001. Novel anti-inflammatory targets for asthma: a role for PPARgamma? Am J Respir Cell Mol Biol 24, 658 – 661.

Tan F., Ang K.P., Fong Y.F., 1986. (Z)- and (E)-5-arylmethylenehydantoins: spectroscopic properties and configuration assignment. J Chem Soc Perkin Trans. II, 12, 1941-1944.

Tomlinson, A., Appleton, I., Moore, A.R., Gilroy, D.W., Willis, D., Mitchell, J.A., Willoughby, D. A., 1994. Cyclo-oxygenase and nitric oxide synthase isoforms in rat carrageenin-induced pleurisy. Br. J. Pharmacol 113, 693-698.

FIGURE LEGENDS:

Figure 1: Synthesis route of (*5Z*)-3-benzyl-5-(*1H*-indol-3-ylmethylene)-thiazolidine-2,4-dione compound LPSF/RA-4

Figure 2: (A) Effect of thiazolidinedione-derived LPSF/RA-4 (10, 30 or 60 μ<mu>mol /kg) on total cell migration in initial phase (4 h) of carrageenan-induced pleurisy in mice. Data are expressed as mean ± S.E.M. of 10 mice in each group. *** $P < 0.0001$ compared with CAR group. (B) Effect of LPSF/RA-4 (60 μ<mu>mol /kg) on NO quantification following carrageenan-induced pleurisy in mice. * $P < 0.013$, LPSF/RA-4 and INDO groups in

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comparison to CAR group. (C-F) Histopathology of lungs from mice following carrageenan-induced injury. (C) Lung sections from sham group. (D) Tissue damage caused by carrageenan-induced pleurisy evidenced in increased cellularity and polymorphonuclear infiltration (white arrow), haemorrhage (asterisk) and thickening of alveolar wall (black arrow). (E) Treatment with LPSF/RA-4 and (F) INDO administered 0.5 hours prior to pleurisy led to reduced lung injury and neutrophil infiltration. Figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± S.E.M. of 10 mice in each group. Scale bar = 20 μm.

Figure 3: Ultrastructural analysis of lung after carrageenan-induced injury and LPSF/RA-4 treatment. (A) Lung sections from mice in sham group (note preserved lung architecture). (B) Lung with carrageenan-induced pleurisy showing inflammatory cells (asterisk) and activated alveolar epithelial cells (arrows) (note cellular debris). (C) Lung treated with LPSF/RA-4 exhibiting well-preserved pneumocytes and alveoli. (D) Ultrathin sections of lungs from INDO group demonstrating reduction in lung injury. Bar = 5 μm.

Figure 4: Effects of LPSF/RA-4 on immunohistochemical localisation of IL-1β and TNF-α. (A) Baseline levels of IL-1b detected in sham group. (B) At 4 h after carrageenan injection, staining intensity for IL-1β substantially increased in alveolar macrophages and epithelial cells. (C) Reduction of staining for IL-1 following administration of LPSF/RA-4 or (D) INDO 0.5 hour prior to carrageenan injection. (E) Densitometry analysis of photographs of immunohistochemistry for IL-1-β from lung tissues. *** $P < 0.002$ in comparison to CAR group. (F) Baseline levels of TNFα in sham group. (G) Positive staining for TNF-α in tissue sections from CAR group mainly located in pneumocytes and inflammatory cells. (H) After LPSF/RA-4 or (I) INDO treatment, reduced degree of positive staining for TNF-α in lung tissues. (J) Densitometry analysis of photographs for TNF-α from lung tissues. *** $P < 0.001$ in comparison to CAR group. Figures are representative of at least 3 experiments performed on

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different experimental days. Data are expressed as mean ± S.E.M. of n = 6 mice in each group. Scale bar = 20 μm.

Figure 5: Effect of LPSF/RA-4 on immunohistochemical localisation of COX-2 and iNOS in lung tissue after carrageenan-induced pleurisy. (A) Baseline levels of COX-2 in sham group. (B) Positive labelling detected in type II pneumocytes in CAR group. (C) Treatment with LPSF/RA-4 significantly reduced COX-2 staining in comparison to CAR group, achieving similar levels to sham group. (D) Indometacin treatment also reduced COX-2 staining. (E) Densitometry analysis of photographs of immunocytochemistry for COX-2 from lung tissues. *** $P < 0.006$ in comparison to CAR group. (F) Low levels of iNOS in sham group. (G) Positive labelling detected in alveolar epithelial cells in CAR group. (H) Treatment with LPSF/RA-4 significantly reduced iNOS staining in comparison to CAR group. (I) Indometacin treatment also reduced iNOS staining. (J) Densitometry analysis of photographs of immunohystochemistry for iNOS in lung tissues. *** $P < 0.0001$ in comparison to CAR group. Figures are representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± S.E.M. of n = 6 mice in each group. Scale bar = 20 μm.

Figure 6: Western blot analysis of COX-2 and IL-1β expression in lung tissues. (A and B) Baseline expression of COX-2 detected in sham group, with significant difference in comparison to CAR group. LPSF/RA-4 treatment reduced expression of COX-2. (A) Representative blot of lysates obtained from pool of 5 animals per group. (B) Data are expressed as mean ± S.E.M. of 3 replicates for each group. (C and D). Baseline expression of IL-1β detected in lung samples of sham group, with significant difference in comparison to CAR group. LPSF/RA-4 treatment reduced expression of IL-1β. (C) Representative blot of lysates obtained from pool of 5 animals per group. (D) Data are expressed as mean ± S.E.M. of 3 replicates for each group. Data analysed by one-way ANOVA, followed by Dunnet's test and Tukey's test. ***$P < 0.0001$ in comparison to CAR group.

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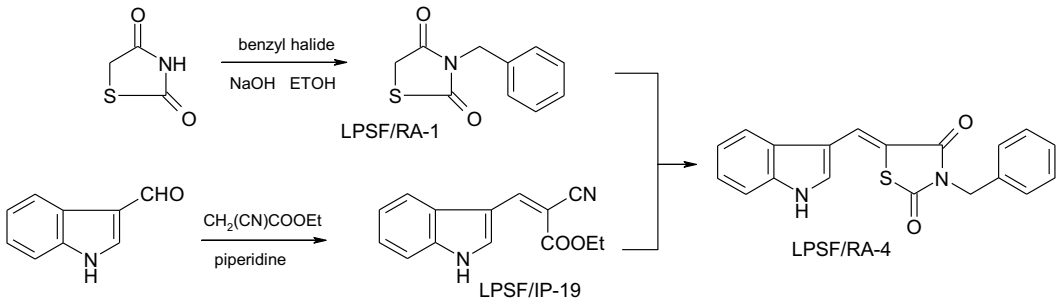

Figure 1

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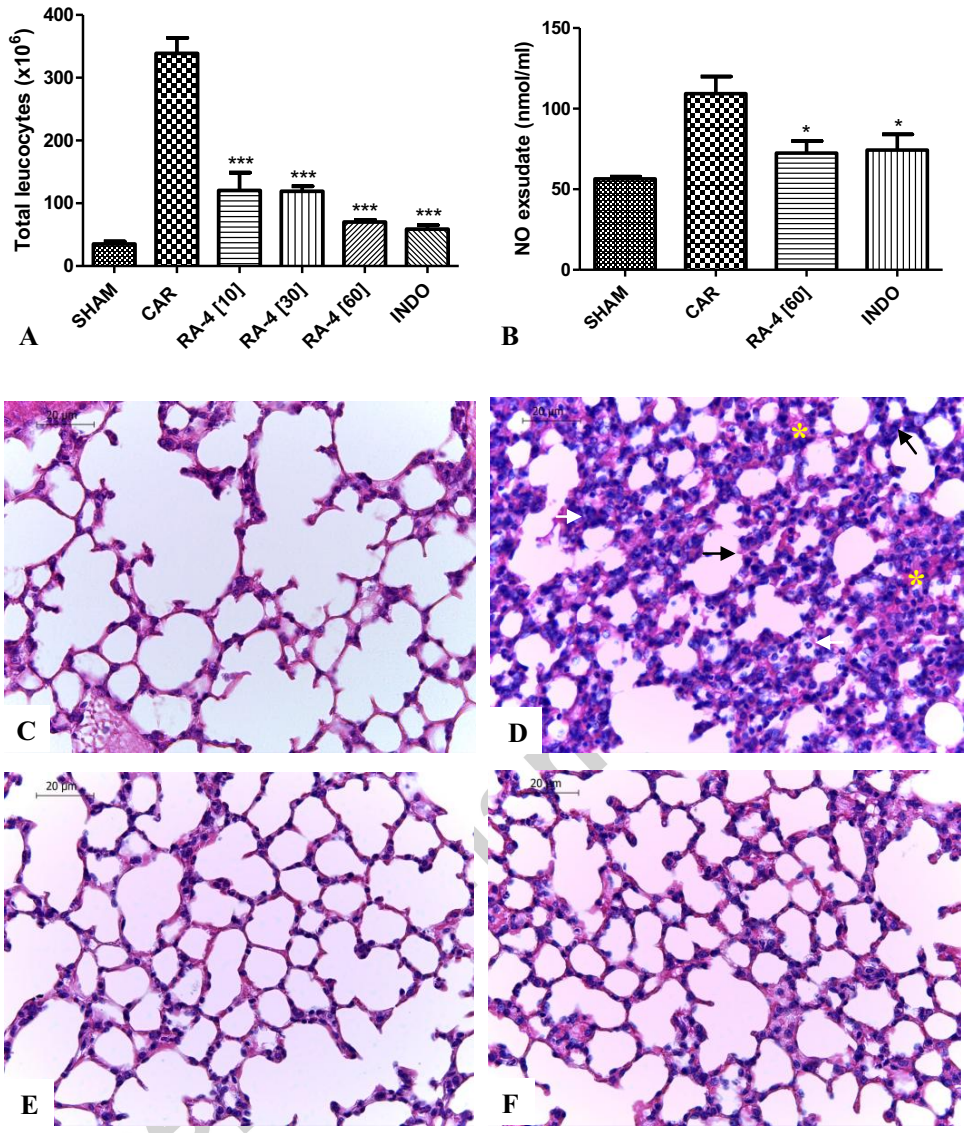

Figure 2

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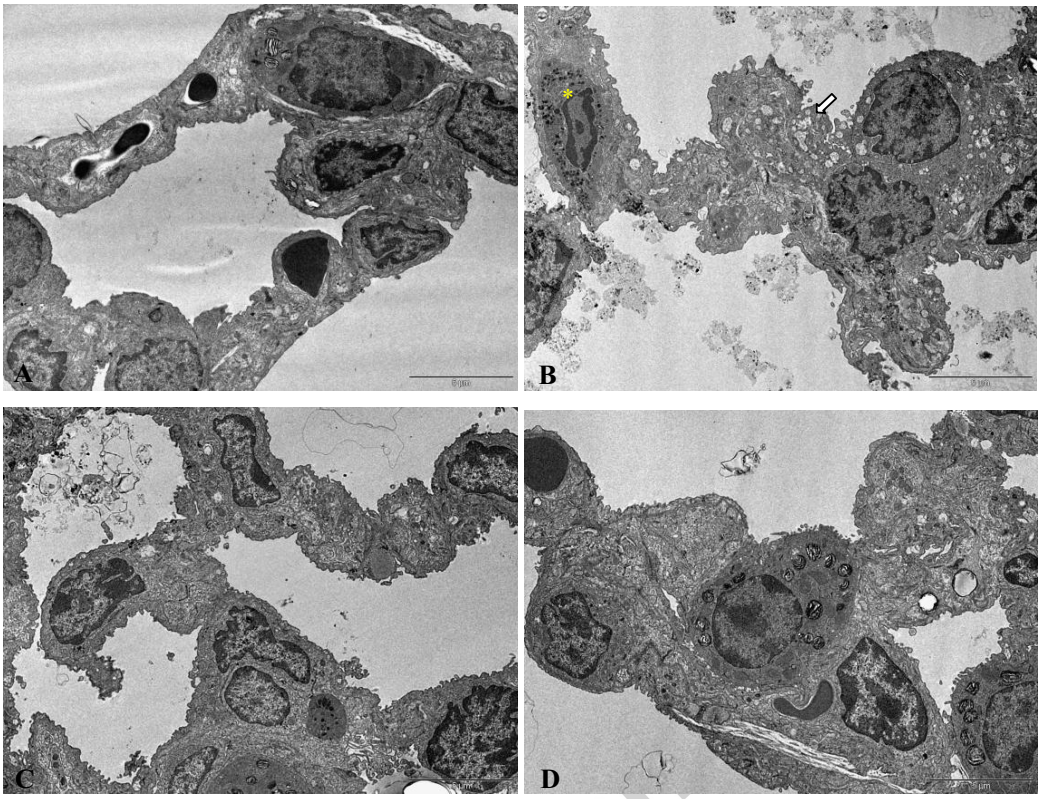

Figure 3

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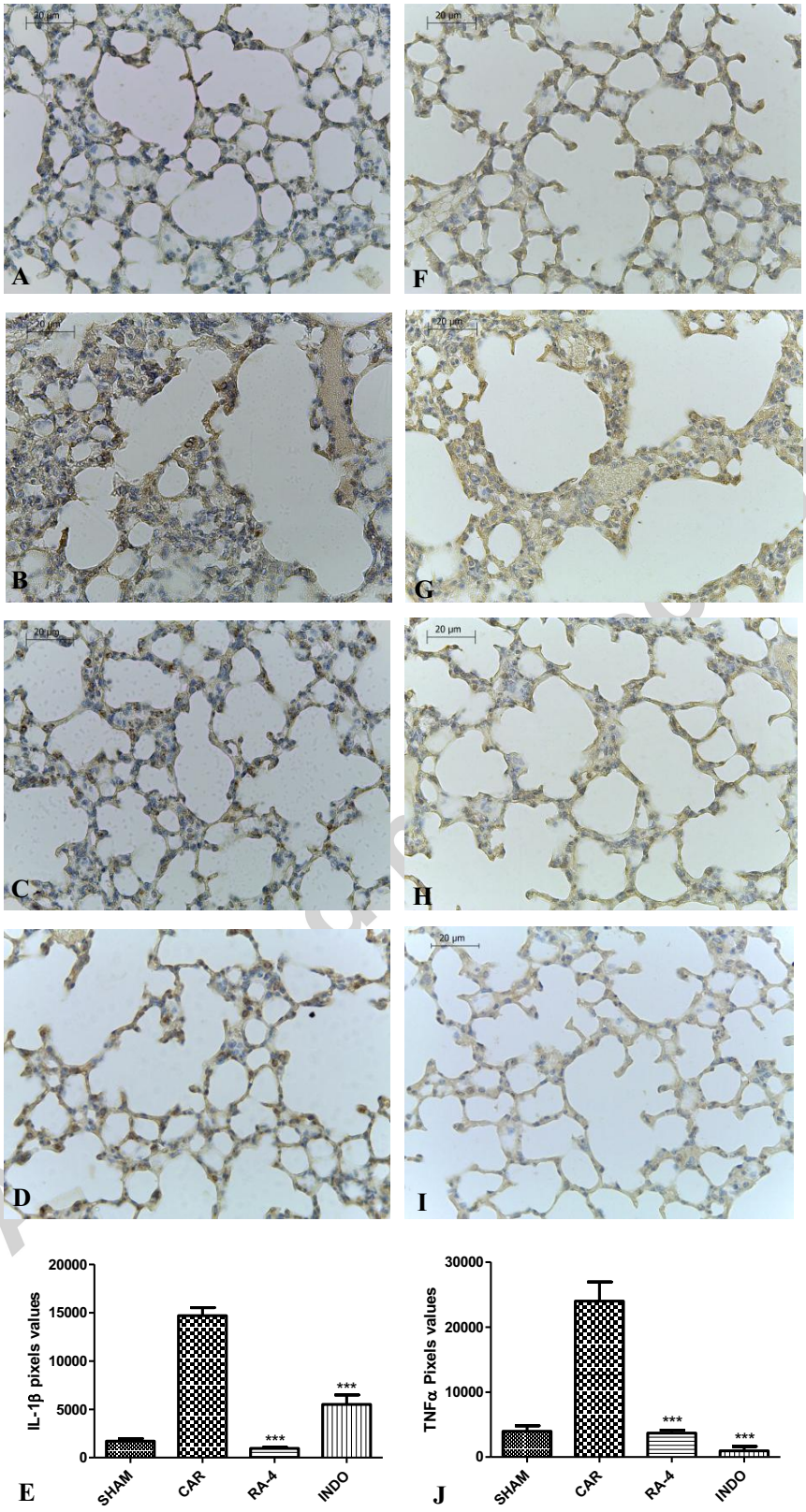

Figure 4

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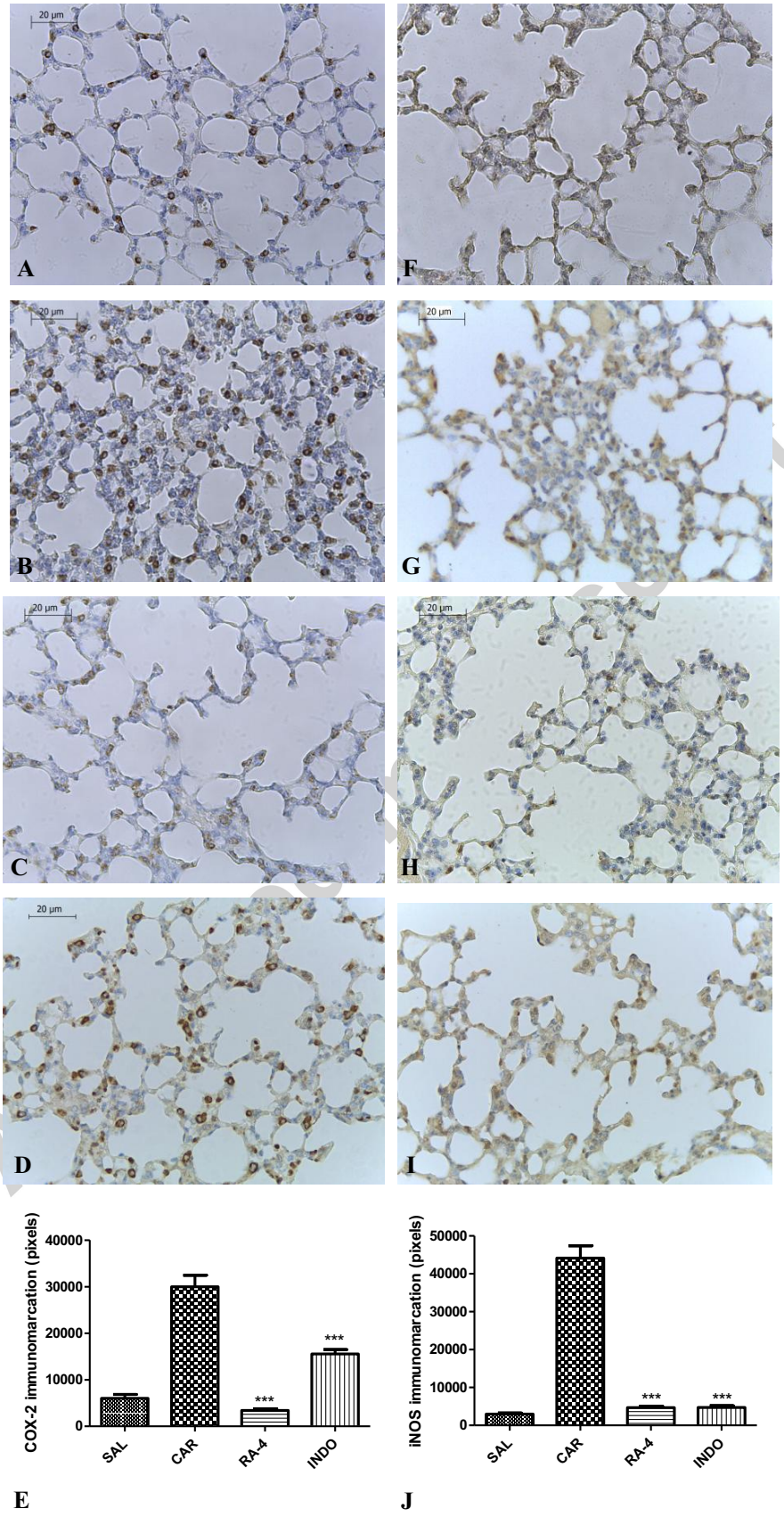

Figure 5

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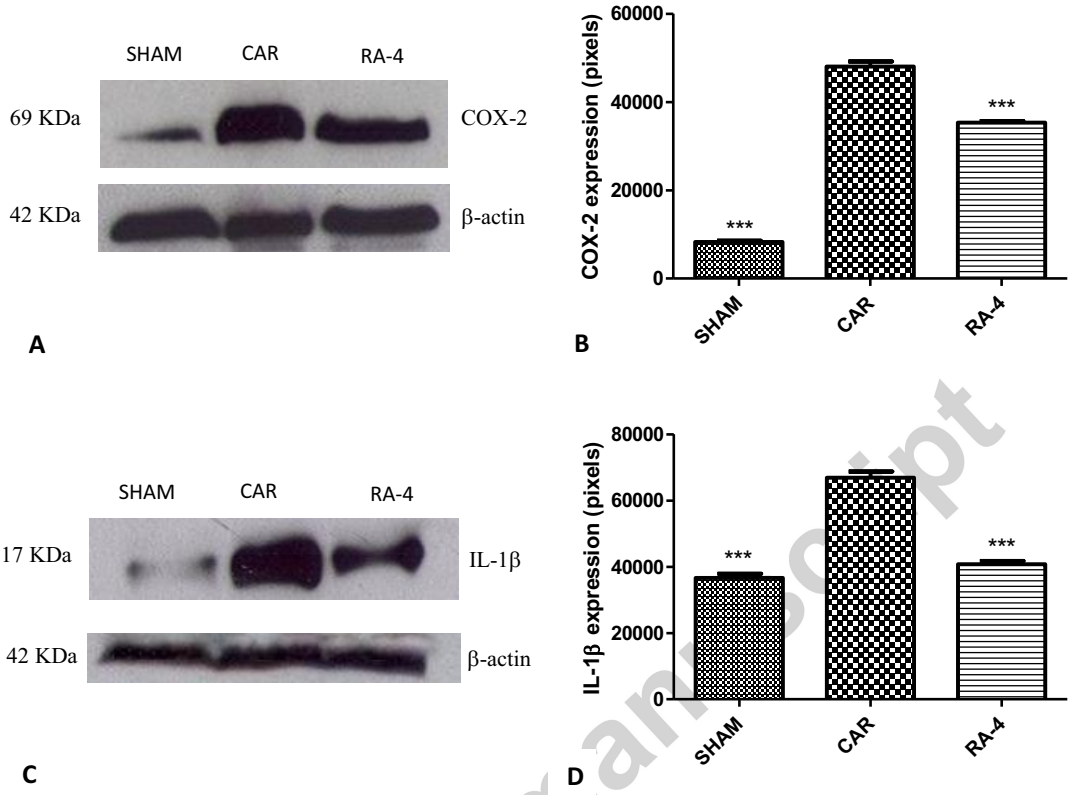

Figure 6

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