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PPAR-α contributes to the anti-inflammatory activity of glucocorticoids

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Running title: PPAR-α contributes to the anti-inflammatory activity of GCs

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ABSTRACT

Glucocorticoids (GCs) are effective anti-inflammatory agents widely used in therapeutic approach to treatment of acute and chronic inflammatory diseases. Previous results suggest that Peroxisome proliferator activated receptor alpha-α (PPAR-α), an intracellular transcription factor activated by fatty acids, plays a role in control of inflammation. With the aim to characterize the role of PPAR-α in GC-mediated anti-inflammatory activity, we tested the efficacy of dexamethasone (DEX), a synthetic GC specific for GR, in an experimental model of lung inflammation, carrageenan-induced pleurisy, comparing mice lacking PPAR-α (PPAR-αKO) with wild type (WT) mice. We also tested the possible synergism of combined treatment with DEX and clofibrate, a PPAR-α agonist. Results indicate that DEX-mediated anti-inflammatory activity is weakened in PPAR-αKO mice, as compared to WT controls, and that is increased in WT mice when combined with PPAR-α agonist treatment.

In particular, DEX was less effective in PPAR-αKO, compared to WT mice, as evaluated by inhibition of NF-kB, of TNF-α production, of cell migration, of cycloxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) activation. Interestingly enough, macrophages from PPAR-αKO were less susceptible to DEX-induced COX-2 inhibition in vitro as compared to WT mice. However, PPAR-α transfection in PPAR-αKO macrophages, with consequent receptor expression, resulted in reconstitution of susceptibility to DEX-induced COX-2 inhibition to levels comparable to that obtained in WT macrophages. Notably, the DEX effect on macrophages in vitro was significantly increased in WT cells when combined with PPAR-α agonist treatment.

These results indicate that PPAR-α can contribute to the anti-inflammatory activity of GCs.

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INTRODUCTION

The inflammatory process is invariably characterized by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and by the release of chemicals from tissues and migrating cells (Tomlinson et al., 2004). Carrageenan-induced local inflammation is commonly used to evaluate anti-inflammatory effects of non-steroidal drugs (NSAIDs). Therefore, carrageenan-induced local inflammation (pleurisy) is a useful model to assess the contribution of mediators involved in cellular alterations during the inflammatory process.

In particular, the initial phase of acute inflammation (0-1h), which is not inhibited by NSAIDs such us indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine and bradykinin, followed by a late phase (1-6h) mainly sustained by prostaglandin release and attributed to the induction of inducible cyclo-oxygenase (COX-2) in the tissue (Nantel et al., 1999). It appears that the onset of the carrageenan-induced acute inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical, as well as to the release of other neutrophil-derived mediators (Salvemini et al., 1999).

Glucocorticoids (GCs) are effective anti-inflammatory agents widely used in treatment of inflammatory and autoimmune diseases (Frankfurt and Rosen, 2004; Hoffman, 1993; Kwak and Longo, 1996; Rhen and Cidlowski, 2005). Moreover, release of endogenous steroids, produced under the control of hypothalamus-hypothesis axis, represents a physiological mechanism involved in the homeostatic control of inflammatory process development (Buckingham et al., 1996; Schleimer, 2000; Webster et al., 2002). GCs act through many molecular mechanisms including non-genomic and genomic signals (Diamond et al., 1990; Losel and Wehling, 2003; Bianchini et al., 2006). In particular, mechanisms including enzymes activation, such as the case of glucocorticoid receptor (GR)-associated src kinase and phospholypase, and Ca^{++} mobilization are consequent to the GCs interaction with the GR

(Cifone et al., 1999; Falkenstein et al., 2000; Lepine et al., 2004; Marchetti et al., 2003). Following GC/GR interaction, GR is activated, translocates to the nucleus and modulates gene transcription either by stimulation or inhibition. Moreover, activated GR can interact, by protein-to-protein interaction, with other transcription factors such as the case of NF-κB, an important player in inflammatory process. Multiple mechanisms are involved in GC-mediated anti-inflammatory activity in addition to direct GR/NF-κB interaction, as for example GC-induced up-regulation of IκB and Glucocorticoid-Induced Leucine Zipper (GILZ), two proteins able to bind and inhibit NF-κB activation (Auphan et al., 1995; Cannarile et al., 2006; Di Marco et al., 2007; Ray and Prefontaine, 1994; Scheinman et al., 1995). Consequent to NF-κB inhibition, a number of inflammatory parameters are blocked including cytokines production, cell migration, cycloxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) activation and tissue damage (Barnes and Karin, 1997; Cuzzocrea et al., 2007; Gilroy et al., 2004; Yamamoto and Gaynor, 2001).

Peroxisome proliferator activated receptor-α (PPAR-α) is an intracellular transcription factor, activated by fatty acids, which play a role in inflammation (Colville-Nash et al., 1998; Moraes et al., 2006; Neve et al., 2001; Yang et al., 2002). Previous studies indicate PPAR-α expression is induced by GCs and can mediate some of the GCs effects, such as modulation of insulin sensitivity and resistance, and can contribute to GC-induced hyper-glycaemia and blood pressure increase (Bernal-Mizrachi et al., 2003; Bernal-Mizrachi et al., 2007; Cassuto et al., 2005; Lemberger et al., 1994). In addition, it has been reported that PPAR-α activation can result in inhibition of NF-kB activation and inflammatory genes expression (Delerive et al., 1999; Delerive et al., 2000; Okamoto et al., 2005; Staels et al., 1998). Our recent results, in diseases models of colitis and pleurisy, show that mice lacking PPAR-α (PPAR-αKO) develop an increased inflammation as compared to wild type (WT) mice. Moreover,

treatment with appropriate doses of PPAR-α agonists can inhibit inflammatory diseases development (Cuzzocrea et al., 2004; Cuzzocrea et al., 2006a).

With the aim to characterize the role of PPAR-α in GC-mediated anti-inflammatory activity, we tested the efficacy of dexamethasone (DEX), a synthetic GC specific for GR, in an experimental model of lung inflammation, carrageenan-induced pleurisy, comparing PPAR-αKO and WT mice. We also tested the possible synergism of combined treatment with DEX and clofibrate, a PPAR-α agonist. Results indicate that DEX-mediated anti-inflammatory activity is weakened in PPAR-αKO mice, as compared to WT controls, and that is increased in WT mice when combined with PPAR-α agonist treatment.

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MATERIAL AND METHODS

In vivo experiments

Animals

Mice (6-7 weeks old, 20-27 g) with a targeted disruption of the PPAR-α gene (PPAR-αKO) and littermate wild-type controls (PPAR-αWT) were purchased from Jackson Laboratories (Harlan Nossan, Italy). Mice homozygous for the PparatniJGonz targeted mutation mice are viable, fertile and appear normal in appearance and behavior (Cuzzocrea et al., 2006a). The study was approved by the University of Messina Animal Care Review Board. The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986), and USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA).

Carrageenan-induced pleurisy

Mice were anaesthetized with isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.1 ml) or saline containing 2% λ-carrageenan (0.1 ml) were injected into the pleural cavity as previously described (Cuzzocrea et al., 2006a). The inflammatory cells (approximately 70% of macrophages) in the pleural exudate were suspended in phosphate buffer saline (PBS) and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

Experimental groups

Mice (either PPAR-αWT or PPAR-αKO) were randomly allocated into the following groups: (i) CAR WT group. PPAR-αWT mice were subjected to carrageenan-induced pleurisy (N=10) and were treated with saline solution (DEX vehicle), (ii) CAR PPAR-αKO group. PPAR-αKO mice were subjected to carrageenan-induced pleurisy (*N*=10) and were treated

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with saline solution (DEX vehicle); (iii) *CAR WT + DEX group.* Same as for CAR WT group with DEX administration (1 mg/kg i.p. bolus) 1 h prior to carrageenan (N=10); (iv) CAR PPAR-αKO + DEX group: Same as for CAR PPAR-αKO group with DEX administration (1 mg/kg i.p. bolus) 1 h prior to carrageenan (N=10); (v) Sham WT group: 100 µl of saline solution instead of carrageenan were administered to the PPAR-αWT mice (N=10) and were treated with saline solution (DEX vehicle); (vi) Sham PPAR-αKO group. 100 µl of saline solution instead of carrageenan were administered to the PPAR-αKO mice (*N*=10) and were treated with saline solution (DEX vehicle); (vii) Sham + WT DEX group. Same as for Sham WT group with DEX administration (1 mg/kg i.p. bolus) 1 h prior to saline (N=10). (viii) Sham + PPAR-αKO DEX group. Same as for Sham PPAR-αKO group with DEX administration (1 mg/kg i.p. bolus) 1 h prior to saline (N=10).

In a separate set of experiment mice PPAR-αWT were randomly allocated into the following groups: (i) CAR group. WT were subjected to carrageenan-induced pleurisy (N=10) and were treated with DMSO 10% solution (clofibrate vehicle), (ii) CAR + clofibrate group same as the PPAR-αWT CAR group with clofibrate administration (100 mg/kg i.p. bolus) 1 h prior to carrageenan (N=10); (iii) CAR + DEX group same as the CAR group with DEX administration (0.01 mg/kg i.p. bolus) 1h prior to carrageenan (N=10); (iv) CAR + clofibrate + DEX group same as the CAR group with clofibrate (100 mg/kg i.p. bolus) and DEX administration (0.01 mg/kg i.p. bolus) 1h min prior to carrageenan (N=10); (v) Sham group. 100 µl of saline solution instead of carrageenan were administered to the mice (N=10) and were treated with saline solution (DEX vehicle); (vi) Sham + DEX group. Same as Sham group with DEX administration (0.01 mg/kg i.p. bolus) 1 h prior to saline (N=10); (vii) Sham clofibrate group. Same as for Sham with clofibrate administration (100 mg/kg i.p. bolus) 1 h prior to saline (N=10); (viii) Sham + clofibrate + DEX group. Same as the PPAR-αWT Sham group with clofibrate (100 mg/kg i.p. bolus) and DEX (0.01 mg/kg i.p. bolus) administration

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1 h prior to saline (N=10).

Histological examination

Lung tissues samples were taken 4 h after injection of carrageenan. Lung tissues samples were fixed for 1 week in 10 % (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were then de-paraffinized with xylene, stained with hematoxylin and eosin. All sections were studied using Axiovision Ziess (Milan, Italy) microscope.

Localization of nitrotyrosine, ICAM-1, TNF-α GILZ and iNOS by immunohistochemistry

The tissues were fixed in 10% PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After de-paraffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). Sections were incubated overnight with 1) anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS), with anti-ICAM-1 polyclonal antibody (CD54) (1:500 in PBS, v/v) (DBA, Milan, Italy), with anti-TNF-α polyclonal antibody (1:100 in PBS, v/v), with anti-GILZ rabbit polyclonal antibody (1:100 v/v) or with anti-iNOS (1:500 in PBS, v/v) (Santa Cruz, DBA, Milan, Italy). Specific labeling was detected (Bruscoli et al., 2006) with a biotin-conjugated goat anti-rabbit, donkey anti-goat or goat anti-mouse IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). To verify the binding specificity for ICAM-1, TNF-αGILZ or anti-iNOS some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found in the sections indicating that the immunoreactions

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were positive in all the experiments carried out. In order to confirm that the immunoreactions for the nitrotyrosine were specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10mM) to verify the binding specificity.

Determination of myeloperossidase (MPO) activity

Myeloperossidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Mullane et al., 1985). 4 h after intrapleural injection of carrageenan, lung tissues were obtained and weighed. Each piece of tissue was homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C and was expressed in units per gram weight of wet tissue.

Measurement of TNF-α

Lungs were homogenized in PBS containing 2 mmol/L of phenylmethylsulphonyl fluoride (PMSF) (Sigma Chemical Co.) and tissue levels of TNF-α were evaluated. The assay was carried out by a colorimetric commercial kit (Calbiochem-Novabiochem Corporation, USA) according to the manufacturer instructions. All cytokines determinations were performed in duplicate serial dilutions.

Protein extraction and western blot analysis

Tissue samples from lungs were homogenized with a Polytron homogenizer in a buffer containing 0.32 M sucrose, 10 mM tris-HCl, pH 7,4, 1 mM ethylene glycol tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM pepstatin A, 0.2 mM PMSF, 50 mM NaF, 1 mM

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sodium orthovanadate, 0.4 nM microcystin. The homogenates were centrifuged (1000 g, 10 min) and the supernatant was collected to evaluate contents of iNOS, COX-2, IκB-α and phospho-NF-κB p65 (ser536). Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin (BSA) as standard. Equal amounts of protein (50 μg) were dissolved in Laemmli's sample buffer, boiled and run on 12% SDS-PAGE gel and then transferred to hybond polyvinylidene difluoride membrane. Membranes were blocked for 60 min in TBS and 5% (w/v) non fat milk and subsequently probed overnight at 4°C with anti-iNOS (Transduction Laboratories) (1:2000), anti-COX-2 (Cayman) (1:2000), anti-IκB-α (Santa Cruz; 1:1000) or anti-phospho-NF-κB p65 (ser536) (Cell Signaling Technology; 1:1000) antibodies (in TBS, 5% w/v not-fat milk and 0.1% Tween-20). Blots were then incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG (1:2.000) (Jackson ImmunoResearch Laboratories, USA) for 1 hour at room temperature. Immunoreactive bands were detected SuperSignal West Pico Chemioluminescent (PIERCE). Bands were quantified by densitometric analysis performed with a quantitative imaging system.

Real Time RT-PCR

Total RNA was prepared using Trizol from Gibco-BRL (Life Technologies, Paisley, Scotland). RT-PCR was done using Moloney murine leukemia virus (M-MLV) RT (Invitrogen, San Diego, CA). The initial template molecules in the samples were measured in triplicate and expressed as the mean ± SEM. For real-time RT-PCR analysis, probes were selected in the connecting region of two exons: specific primers for GILZ, (sense 5'-GGTGGCCCTAGACAACAAGA-3', antisense 5'- TCTTCTAAGCAGCTCAGCA-3'). Primers for COX2: sense 5'-CATCCTGACCCACTTCAAGG-3', antisense 5'-AGAAGGCTTCCCAGCTTTTG-3'. Primers for PPAR-α: sense 5'-TCGAATATGTGGGGACAAGG-3', antisense 5'-GACAGGCACTTGTGAAAACG-3'. Primers for GR: sense 5'-AACTGGAATAGGTGCCAAGG-3', antisense 5'-

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GAGCACACCAGGCAGAGTTT-3'. Primers for HPRT: sense 5'-CTGCGTGATTAGCGATGATG-3', antisense 5'-ACAGAGGGCCACAATGTGAT-3'. PCR were performed with Chromo4 Real-Time PCR Detection System (Bio-Rad, Milan, Italy) using DyNAmo HS SYBR GREEN qPCR kit (Finnzymes; Celbio). The following experimental run protocol was used: denaturation program (95°C for 15 min), amplification and quantification program, repeated 40 times (95°C for 20 s, 58°C for 20 s, 72°C for 20 s), melting curve program (70-95°C with a heating rate of 0.5 °C per second). For calculation of the amounts of specific mRNA, the Comparative ΔC(t) method was used. The C(t) value was determined using the Opticon Monitor 2 software (MJ Research Bio Rad). Gene expression was normalized to HPRT housekeeping mRNA expression.

In vitro experiments

Peritoneal macrophages

Peritoneal macrophages were elicited by i.p. injection of 2 ml of 4 % sterile thioglycolate medium (TG) (Sigma). After 3 days mice were sacrificed, and macrophages were harvested as described (Nunoshiba et al., 1993). Peritoneal macrophages was grown in DMEM supplemented with 1% glutamine, 2.5% Hepes, 1% solution 10000 units/mL Penicillin-10000 µg/ml Streptomycin, 10 % foetal bovine serum (FBS) and 1.2 % Na pyruvate (EuroClone, Europe). Cells were plated on tissue culture plates for 3-4 h, and then non-adherent cells were removed by washing with sterile PBS. Peritoneal macrophages were treated with lipopolysaccharide (LPS) 10 µg/ml and interferon (IFN)-γ 100 U/ml for 24 h in DMEM without FBS in presence or absence of test compounds. 2 hours before the stimulation with LPS and INF-γ we added to the medium DEX 1 µM and 10 µM. The medium was used for the determination on nitrite/nitrate, and cells were used for western blot analysis.

In a separate set of experiment, peritoneal macrophages were treated with LPS 10 µg/ml and IFN-γ 100 U/ml for 24 h in DMEM without FBS in presence or absence of test compounds. 2

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h before the stimulation with LPS and INF-γ, we added DEX (1 μM) and clofibrate (0.2 mM) to the medium. 4 h later cells were harvested for Real Time analysis.

Transfection

Transfection of mouse peritoneal macrophages from PPAR-αKO mice was carried out according to a commercially available protocol using Mouse Macrophage Nucleofector Kit (Amaxa Biosystems) transfection system. Briefly, we transfected 5 x 10^5 peritoneal macrophages with 2 μg of plasmid containing full-length PPAR-α or empty vector pSG5 or, for silencing, 1 μg of siRNA specific for PPAR-α or scrambled oligo. Each group was co-transfected with 2 μg of pEGFP vector to check transfection efficiency (all transfections resulted GFP positive in a range of 40-50% as evaluated by FACS analysis). 24 h after transfection, cells were treated with DEX (1 μM) followed 2 h later by stimulation with LPS and INF-γ. Cells were harvested 2 h after stimulation and prepared for Real Time analysis.

Western blot analysis

Cells were washed in cold PBS and lysed for 10 min at 4°C with 1 ml of lysis buffer (20 mM Tris pH 7.5, 1 % Nonidet P-40, 1 mM orthovanadate, 1 mM PMSF, 10 mM NaF, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml trypsin Inhibitor). Lysates from adherent cells were collected by scraping and centrifuged at 13000 g for 15 min at 4°C. The supernatants were collected and protein concentration in cell lysates was determined by Bio-Rad Protein Assay (BioRad, Richmond CA) and 50 μg of total protein from each sample was analyzed. Proteins were separated by a 12 % SDS-polyacrylamide gel electrophoresis and transferred on nitro-cellulose membrane (Hybond ECL Nitrocellulose, Amersham, Rainham, UK). The membrane was blocked with 0.1 % TBS-Tween containing 5 % non-fat milk for 1 h at room temperature. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4°C; anti-Phospho-NF-κB p65 (ser536) Rabbit mAb diluted 1:1000 (Cell Signaling technology. US); anti-COX-2 diluted 1:2000 (Cayman chemical, USA); anti-

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iNOS TYPE II diluted 1:2000 (Transduction Laboratories). After the incubation, the membranes were washed three times with 0.1 % TBS Tween and were incubated for 1 h with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, USA) diluted 1:2000; the membranes were washed and protein bands were detected with SuperSignal West Pico Chemioluminescent (PIERCE). Densitometric analysis was performed with a quantitative imaging system.

Measurement of nitrite-nitrate concentration

Total nitrite in medium, an indicator of NO synthesis, was measured as previously described (Cuzzocrea et al., 2001). Briefly, the nitrate in the sample was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) (160 μM) at room temperature for 3 h. The total nitrite concentration in the samples was then measured using the Griess reaction, by adding 100 μl of Griess reagent 0.1% (w/v) naphthylethylendiamide dihydrochloride in H_2O and 1% (w/v) sulphanilamide in 5% (v/v) concentrated H_3PO_4 vol. 1:1 to the 100 μl sample. The optical density at 550 nm (OD_{550}) was measured using ELISA microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in H_2O.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company (Milan, Italy). Secondary and nonspecific IgG antibodies for immunohistochemical analysis were from Vector Laboratories Inc.

Data analysis

All values in the figures and text are expressed as mean ± SEM of the mean of n observations. For the in vivo studies, n represents the number of animals studied. Results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple

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comparisons. A p-value less than 0.05 was considered significant.

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RESULTS

Role of functional PPAR-α gene in the anti-inflammatory property of DEX in carrageenan-induced pleurisy

We analyzed the possible role of PPAR-α in the anti-inflammatory property of DEX during acute inflammation in the lung. For that purpose, we compared the effect of DEX pre-treatment in PPAR-αKO and WT mice subjected to carrageenan-induced pleurisy. At 4 h after intrapleural carrageenan administration, there was a significant increase ($p<0.01$) of PMNs, as evaluated by MPO activity, in the lung of WT and PPAR-αKO mice as compared to vehicle-treated controls (Fig 1a). Treatment with DEX resulted in a significant inhibition of MPO activity in the lung of WT but not of PPAR-αKO mice.

No histological alterations were observed in the lung tissues collected from sham WT mice and from sham PPAR-αKOmice (data not shown). On the contrary, histological examination of lung sections of carrageenan-treated WT and PPAR-αKO mice, showed tissue injury as well as inflammatory cells infiltration (Fig. 1bA and bB respectively). Treatment with DEX resulted in a significant reduction of tissue injury in WT (Fig. 1bC) but not of PPAR-αKO mice (Fig. 1bD). These results indicate that the DEX effect in PPAR-αKO mice was less marked than in WT thus suggesting that PPAR-α is important for the DEX-mediated anti-inflammatory effect.

In order to confirm that the PPAR-α receptor is involved in DEX-mediated anti-inflammatory effects, we also investigated whether GW6471, which functional studies have indicated to be an antagonist of PPAR-α (Xu et al., 2002), attenuates the protective effects of DEX. In particular, we have demonstrated that Co-administration of GW6471 (10 mg/kg i.p. 30minutes before DEX administration) significantly blocked the effect of DEX on PMNs infiltration in the pleural cavity and decreased lung MPO activity (see Fig. Supplementary 1).

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It is well known that inflammatory cell infiltration is dependent from adhesion molecule expression including ICAM-1 (Hallahan and Virudachalam, 1997; Park et al., 2006). We evaluated the ICAM-1 expression in the lung tissue of carrageenan-treated WT and PPAR-αKO mice as well the effect of DEX treatment.

Staining of lung tissue sections obtained from saline-treated controls with anti-ICAM-1 antibody showed a specific staining along bronchial epithelium demonstrating that ICAM-1 is constitutively expressed (not shown). 4 h after carrageenan injection, the staining intensity for ICAM-1 was detected along the WT vessels (Fig 1cA) mainly localized in the vascular endothelium. Moreover, in carrageenan-treated PPAR-αKO mice, the staining for ICAM-1 in the vascular wall was visibly and significantly increased in comparison with that of WT mice (Fig. 1cB). Marked inhibition for ICAM-1 staining was observed in the lung tissues of WT mice after treatment with DEX (Fig. 1cC). On the contrary, the absence of a functional PPARα gene in PPAR-αKO mice significantly reduced the DEX-induced inhibition of ICAM-1 expression (Fig 1cD) further suggesting that PPAR-α is involved in anti-inflammatory activity of DEX.

Role of PPAR-α in DEX-induced inhibition of TNF-α production

Release of pro-inflammatory cytokines is an important mechanism responsible for inflammatory process including carrageenan-induced pleurisy (Cuzzocrea et al., 2006a; Cuzzocrea et al., 2006b). At 4 h after carrageenan injection, increased levels of TNF-α protein were observed in lung tissues when compared to controls (Fig. 2a, compare columns 1 and 2 with columns 5 and 6). DEX treatment significantly inhibited TNF-α production in WT (compare column 7 to column 5) but not in PPAR-αKO mice (compare column 8 to column 6). Notably, lung tissues production of TNF-α was significantly higher in carrageenan-treated PPAR-αKO mice as compared to WT animals (compare column 6 to column 5).

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We also evaluated the TNF-α protein expression in the lung tissues by immunohistochemical assay. No positive staining for TNF-α was observed in the lung tissues collected from sham WT mice and from sham PPAR-αKO mice (data not shown). On the contrary, tissue sections obtained from WT animals, 4 h after carrageenan administration, showed positive staining for TNF-α localized in the infiltrated inflammatory cells, pneumocytes as well as in vascular wall (Fig. 2bA). In carrageenan-treated PPAR-αKO mice, the staining for TNF-α in the infiltrated inflammatory cells, pneumocytes as well as in vascular wall was visibly and significantly increased in comparison with the WT mice (Fig. 2bB). No positive staining for TNF-α was observed in the lung tissues collected from WT mice that have been treated with DEX (Fig. 2bC). On the contrary, the absence of a functional PPARα gene in PPAR-αKO mice significantly blocked the anti-inflammatory effect of DEX (Fig. 2bD).

Together these results indicate that PPAR-α play a role in DEX-mediated inhibition of carrageenan-induced TNF-α

Role of PPAR-α in DEX-mediated inhibition of carrageenan-induced NO and COX-2 production

iNOS and COX-2 are important mediators of inflammatory process (Salvemini et al., 1994; Vane et al., 1994). We performed experiments to evaluate the possible effect of DEX on carrageenan-induced iNOS and COX-2 in WT and PPAR-αKO mice. Results in figure 3 indicate that a significant increase of iNOS and COX-2 expression was induced in the lung of WT and PPAR-αKO mice by carrageenan treatment (Fig. 3a). However, while treatment with DEX significantly attenuated iNOS (left) and COX-2 (right) expression of WT mice, it did not counter this increased expression in PPAR-αKO mice. Notably, iNOS and COX-2 induction by carrageenan administration was more marked in PPAR-αKO than in WT mice.

We also performed immunohistochemical analysis of lung sections obtained from carrageenan-treated WT and PPAR-αKO mice. As shown in figures 3b and 3c, while DEX

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was effective in inhibiting both iNOS and nitrotyrosine in WT, was less active in PPAR-αKO mice (Fig. 3bC-D and Fig. 3cC-D). Notably, in carrageenan-treated PPAR-αKO mice, staining for iNOS (Fig. 3bB) and nitrotyrosine (Fig. 3cB) in infiltrated inflammatory cells, pneumocytes and in cells of the vascular wall, was clearly increased in comparison with the WT mice (Fig. 3bA and Fig. 3cA).

DEX regulates PPAR-α and GR expression in lung tissues of WT and PPAR-αKO mice.

It has been previously shown that GCs up-regulate PPAR-α expression (27). Results here indicated that DEX equally increases PPAR-α in lung homogenates of not treated- or carrageenan-treated WT mice (Fig. 4a, column 2 vs. 4). Moreover, DEX also regulates GR expression; in fact WT or PPAR-αKO mice expressed less GR mRNA upon DEX treatment (Fig 4b). To note, GR mRNA levels were similar both in not treated- or carrageenan-treated mice (Fig. 4b, columns 3-4 versus 7-8), suggesting that GR expression is not responsible for decrease of DEX-mediated anti-inflammatory activity.

Role of PPAR-α in DEX-mediated inhibition of carrageenan-induced NF-kB activation

Most inflammatory mediators, including iNOS, COX-2, IL-1β and TNF-α are controlled by NF-kB, a transcription factor important in inflammatory process, which is kept inactive by IkB and GILZ (Baldwin, 1996; Beg et al., 1993; Di Marco et al., 2007). Moreover, activation of NF-kB transactivation potential is increased by phosphorylation of the p65 subunit (Viatour et al., 2005). We performed experiments to evaluate the possible effect of DEX on carrageenan-induced NF-kB activation in WT and PPAR-αKO mice.

A basal level of IkB-α was detected in the lung tissues from sham WT mice and from sham PPAR-αKOmice. After carrageenan treatment, IkB-α levels were substantially reduced (Fig. 5a). This reduction was countered by DEX treatment ($P<0.01$). However the DEX effect was significantly more evident in WT than in PPAR-αKO mice (Fig. 5a, compare line 5 to line 6). Furthermore, carrageenan administration caused a significant increase in p65 phosphorylation

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at Ser536 in the lung tissues from WT and PPAR-αKOmice and DEX inhibited p65 phosphorylation of WT but not of PPAR-αKO mice. Of note, carrageenan-induced increase of p65 phosphorylation in the lung tissues of PPAR-αKO was slightly more marked than that of WT mice (Fig. 5b, compare column 4 to column 3).

GILZ has been proposed as a DEX-induced protein able to bind and inhibit p65NF-kB activity (Di Marco et al., 2007). We evaluated, by real time PCR, the effect of DEX treatment in WT and PPAR-αKO cells on GILZ expression. Results in Figure 6a indicate that DEX induced an increase of GILZ mRNA expression in WT but not in PPAR-αKO mice. Moreover, this difference was even enhanced in carrageenan-treated WT mice thus indicating that PPAR-α contributes to DEX-induced GILZ expression.

We then evaluated the GILZ protein expression in lung tissues by immunohistochemical assay. No positive staining for GILZ was observed in the lung tissues collected from sham WT mice and PPAR-αKO mice (Fig. 6b A, B). On the contrary, tissue sections obtained from WT and PPAR-αKO animals 4 h after carrageenan administration, showed positive staining for GILZ localized in the infiltrating inflammatory cells (Fig. 6b C, D). In carrageenan-treated WT mice, which have been treated with DEX, the staining for GILZ was visibly and significantly increased mainly localizing in infiltrating macrophages, PMNs and lymphocytes (Fig. 6bE bottom panel, see also arrows in the figure 6bE1) as well in the vascular endothelial cells (see arrows 6bE2). On the contrary, the absence of a functional PPAR-α gene in PPAR-αKO mice resulted in a clear reduction of this DEX-induced GILZ expression (Fig. 6bF). Together these results indicate that PPAR-α play a role in DEX-induced IkB and GILZ up-regulation and in NF-kB inhibition.

Combination therapy with clofibrate and DEX reduces pleural infiltration of macrophages, lung infiltration of PMNs and lung injury.

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Previous results suggest PPAR-α can favour DEX anti-inflammatory activity. We performed experiments to evaluate the possible effect of combination therapy with clofibrate, a PPAR-α agonist, and DEX on carrageen-induced lung injury in WT mice. In a preliminary set of experiments, we performed dose response experiments with clofibrate and DEX. Results indicated that combination of clofibrate and DEX significantly reduced, in a dose dependent manner, the PMNs infiltration in the pleural cavity and lung MPO activity (see Fig. Supplementary 2). Please note that clofibrate (at 100 mg/kg i.p) or DEX (at 0.01 mg/Kg i.p.) alone did not inhibit lung tissue injury, PMNs infiltration in the pleural cavity as well lung MPO activity (see Fig. Supplementary 2). Moreover, results of histology in figure 7a also confirm that while clofibrate (at suboptimal dose of 100 mg/kg i.p., Fig. 7aB) or DEX (at the suboptimal dose of 0.01 mg/Kg i.p., Fig. 7aC) alone did not inhibit lung tissue injury, combination of the two agents (clofibrate + DEX) resulted in a detectable inhibition (Fig. 7aD).

The above histological alteration in the lung appeared to correlate with the influx of leukocytes into the lung tissue as well as in the pleural cavity (Fig. 7b). Therefore, we investigated the effect of the combination therapy on neutrophils infiltration in the pleural cavity and in the lung tissues by measurement of MPO activity. PMNs infiltration in the pleural cavity (Fig 7b, left) and MPO activity (Fig. 7b, right) were significantly elevated 4 h after carrageenan administration in WT mice. PMNs infiltration in the pleural cavity and lung MPO activity was significantly reduced in carrageenan-treated mice that received i.p. injection of the combination therapy with clofibrate and DEX (Fig. 7b). No reduction of PMN infiltration in the pleural cavity and lung MPO activity was found in carrageenan-treated mice that received i.p. injection of clofibrate or DEX alone (Fig. 7b).

These results suggest that contemporary agonist stimulation of PPAR-α and GR results in a potentiated anti-inflammatory activity.

Effect of DEX on macrophage inflammatory markers.

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Together above results indicate PPAR-α can favour the anti-inflammatory activity of DEX. Macrophages are important players in inflammatory processes in vivo (Moraes et al., 2006). To further analyze the effect of PPAR-α gene deletion on DEX anti-inflammatory property we carried out in vitro experiments using elicited peritoneal macrophages from both WT and PPAR-αKO mice. In particular, macrophages were treated with LPS (10 μg/ml) and IFN-γ (100 U/ml) for 24 h. As shown in figure 8, un-stimulated (vehicle) peritoneal macrophages from WT or PPAR-αKO mice, produced small amounts of measurable nitrite, the stable end product of NO. Activation of the cells with LPS and IFN-γ for 24 h significantly increased the nitrite/nitrate ($P<0.001$) production in peritoneal macrophages from WT (Fig. 8a). The nitrite/nitrate production was significantly increased in peritoneal macrophages from PPAR-αKO mice incubated with LPS and IFN-γ in comparison with the corresponding WT cells. When DEX (10 μM) was added to WT macrophages, 2 h before LPS and IFN-γ stimulation, a significant decrease of nitrite/nitrate production in cell medium was observed (Fig. 8a, compare column 7 to column 5). On the contrary, the absence of a functional PPARα gene in macrophages from PPAR-αKO resulted in lack of DEX effect (Fig. 8a, compare column 8 to column 6).

We also performed experiments to determine the DEX effect on LPS and IFN-γ-induced COX-2 protein expression. To that purpose, COX-2 protein expression in whole lysates from peritoneal WT or PPAR-αKO macrophages stimulated with LPS and IFN-γ and pre-treated with DEX, as described above, was evaluated. As shown in Fig. 8b, LPS and IFN-γ caused a significant increase of COX-2 protein levels at 24 h, compared to un-stimulated cells. When WT cells were pre-treated with DEX (1, 10 μM), 2 h before LPS and IFN-γ stimulation, COX-2 protein expression was inhibited in a concentration-dependent manner respect to LPS and IFN-γ-stimulated cells (Fig. 8b). The absence of a functional PPAR-α gene in

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macrophages from PPAR-αKO mice significantly weakened this DEX effect on COX-2 expression (Fig 8b).

We also evaluated in the same experiments the Ser536 phosphorylation, as a measure of NF-kB activation (Buss et al., 2004; Sakurai et al., 2003). As shown in Figure 8c, DEX treatment completely inhibited NF-kB activation in macrophages of WT mice, while only partially in macrophages of PPAR-αKO mice, further suggesting PPAR-α can contribute to the anti-inflammatory activity of DEX.

We also performed experiments with macrophages in vitro to further evaluate the combination therapy effect as shown in figure 7. To that purpose we evaluated COX-2 mRNA levels macrophages from WT and PPAR-αKO mice, activated by LPS and IFN-γ, treated with DEX (0.01 μM) or DEX (0.01 μM) plus clofibrate (0.2 mM). Results in figure 8d indicate that clofibrate increased DEX inhibiting activity of COX-2 expression. To note, clofibrate alone (0.2 mM) did not change COX-2 expression levels. These results were further confirmed in dose response experiments showing that combination of DEX and clofibrate result in a significant COX-2 inhibition (see Fig. Supplementary 3).

PPAR-α reconstitution recovers PPAR-αKO macrophage susceptibility to DEX treatment.

Finally, as further control, macrophages from PPAR-αKO mice were used in transfection experiments aimed to reconstitute PPAR-α expression. As shown by results in figure 9a, PPAR-α expression in cells of PPAR-αKO mice partially but significantly reconstituted the susceptibility to DEX inhibiting activity of COX-2 expression. Moreover, in the same samples, GR levels were comparable (Fig 9c, columns 2-4 vs. 5-7). GR expression levels were also tested in WT macrophages treated or not with siRNA specific for PPAR-α and no detectable differences were found (Fig. 9d, columns 2-4 vs. 5-7).

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All together, these in vitro experiments clearly indicate that PPAR-α contribute to the anti-inflammatory activity of DEX, independent from possible changes in GR expression and are in agreement with results indicating a synergism (Fig. 7 and 8d) of DEX and PPAR-α agonist combination treatment.

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DISCUSSION

In the present paper we show that the absence of PPAR-α, in PPAR-αKO mice, results in a reduced anti-inflammatory response to DEX treatment. Moreover, we also show that the anti-inflammatory activity of DEX is increased, in WT mice, when administered in combination with a PPAR-α agonist.

These results are in agreement with our previous observations indicating that PPAR-αKO mice are more susceptible to induction of inflammation, possibly due to a less efficient physiological anti-inflammatory control exerted by endogenous GCs (Delerive et al., 1999; Genovese et al., 2005; Lovett-Racke et al., 2004; Okamoto et al., 2005). We here confirm these observations for some of inflammatory parameters such as cell tissue infiltrate, TNF-α production, iNOS and COX-2 activity.

GCs, including DEX, are potent anti-inflammatory agents and for that reason are used in therapy of a number of human diseases. Their efficacy resides in part in the capability to counter NF-kB activation, an important transcription factor in inflammation, production of cytokines relevant to the inflammatory process such as for example TNF-α, induction of iNOS and COX-2 as well increase of their enzymatic activity (Auphan et al., 1995; Scheinman et al., 1995; Yamagata et al., 1993). In addition, GCs inhibit adhesion molecules, as for example ICAM-1, and the consequent PMNs and macrophages migration into the inflamed tissue.

PPAR-α itself is also able to directly mediate some anti-inflammatory effects and it has been shown that its agonist-induced activation inhibits a number of inflammatory mechanisms including TNF-α production, iNOS, COX-2 and adhesion molecules expression as well cell infiltration in the tissues (Genovese et al., 2005; Lovett-Racke et al., 2004; Neve et al., 2001). Based on these observations we performed studies in the attempt to determine whether the presence and/or the stimulation of PPAR-α could enhance the GCs anti-inflammatory

efficacy. For that purpose we used an experimental model of acute lung inflammation, performed by treating WT and PPAR-αKO mice with carrageenan, and tested the anti-inflammatory efficacy of DEX, alone or in combination with clofibrate, a PPAR-α agonist.

It is known that NF-kB activation is central in inflammation and that GCs can counter its activity by different mechanisms including the increase of IkB expression (Auphan et al., 1995). Moreover, we have shown that GCs rapidly induce expression of GILZ, another protein able to bind and inhibit NF-kB (Di Marco et al., 2007; Ayroldi et al., 2007). Results here described indicate that DEX-induced IkB and GILZ over-expression is well detected in lung tissues of carrageenan-treated WT but not of carrageenan-treated PPAR-αKO mice. Notably, DEX treatment inhibited p65NF-kB phosphorylation at Ser536, an event associated with NF-kB activation, in WT but not in PPAR-αKO mice. Moreover, similar results were obtained in vitro with isolated peritoneal macrophages stimulated by LPS and INFγ, a treatment able to deliver a strong activation signal (Rossi et al., 2005; Warfel and Zucker-Franklin, 1986). In fact, macrophages treatment with DEX countered the NF-kB activation in WT but not in PPAR-α cells.

There is evidence that production of pro-inflammatory cytokines, such as for example TNF-α, is important to induce local and systemic inflammation and that production of this cytokine can be inhibited by treatment with GCs (Barnes, 2006; Vlahos and Stewart, 1999). When WT and PPAR-αKO mice were treated with DEX a significant inhibition of TNF-α level was measured in WT but not in PPAR-αKO mice.

Up-regulation and activation of COX-2 and iNOS, with consequent nitrite/nitrate production, are typical characteristics of inflammatory process (Luss et al., 1994; Weinberg et al., 1995). GCs can counter these effects and we here show that while DEX treatment significantly inhibited those inflammatory parameters in WT, did not in PPAR-αKO mice. Of note, lack of DEX-induced COX-2 inhibition was also evident in experiments with isolated peritoneal

macrophages from PPAR-αKO mice. Most important, when macrophages from PPAR-αKO mice were transfected with PPAR-α, to reconstitute its expression, they regained the susceptibility to DEX-induced inhibition of COX-2 expression. Notably, these results could also suggest a role of PPAR-α on functions including in vivo interaction with vessel walls that takes place during inflammation (Barlic et al., 2006).

The results here described clearly indicate that the anti-inflammatory efficacy of DEX treatment is favoured by the presence of PPAR-α. Besides, DEX regulates GR and PPAR-α expression, but their levels are similar in healthy or carrageenan-induced pleurisy mice, either WT or PPAR-αKO, thus indicating that impaired DEX anti-inflammatory activity in PPAR-αKO mice is not due to modulation of GR or PPAR-α expression. Moreover, previous studies showed that PPAR-α agonists exert some anti-inflammatory activity (Colville-Nash et al., 1998; Genovese et al., 2005; Lovett-Racke et al., 2004). For that reason we performed experiments to test whether the combination of DEX with clofibrate, a PPAR-α agonist agent, could result in a more efficient anti-inflammatory effect. Results here described indicate that in vivo co-treatment with suboptimal doses of DEX and clofibrate was synergic in inhibition of a number of inflammatory events in WT mice, including PMNs infiltration into the lung tissue. In accordance, in vitro treatment of WT isolated peritoneal macrophages with clofibrate significantly increased the DEX-induced inhibition of COX-2 expression.

The efficacy of GCs treatment in inflammatory and autoimmune diseases is an important therapeutic subject and while some patients obtain clinical improvements from treatment, others are not responsive or even resistant to therapy. As an example, it is known that only a certain percentage of patients affected by inflammatory bowel diseases (IBDs) are cured by GCs treatment while others are not (Farrell and Kelleher, 2003; Orii et al., 2002; Sands, 2007). The reasons of response or not response to therapy are not fully understood and molecular mechanisms, such as for example change of the ratio between different GR

isoforms, pre-existing levels of NF-kB and heat-shock proteins have been drawn out in the attempt to explain and predict sensitivity and resistance (Chikanza, 2002; Meduri et al., 2005; Wikstrom, 2003). Results here discussed suggest a new mechanism contributing to determine the full GCs efficacy and suggest future studies aimed to analyze the possible relevance of PPAR-α in other human inflammatory disease models, such as sepsis and experimental colitis.

In conclusion, our results clearly indicate that PPAR-α can contribute by enhancing the anti-inflammatory activity of DEX in carrageenan-induced pleurisy model. These observations could suggest new therapeutic approaches of combination therapy with GCs and PPAR-α agonists in inflammatory diseases.

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Footnotes

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FIGURES LEGENDS

Figure 1: Effect of PPAR-α on the anti-inflammatory property of DEX in carrageenan-induced pleurisy. 4 h after carrageenan administration in WT and PPAR-αKO mice we measured: a. lung MPO activity ($p < 0.01$); Values shown are mean ± SEM of 10 mice for each group **P*<0.01 *vs.* Sham; °*P*<0.01 *vs*. carrageenan-WT group; °°*P*<0.01 *vs.* DEX-treated carrageenan-WT group. b. Histological examination of lung sections by hematoxylin and eosin coloration: A: carrageenan-treated WT mice; B: carrageenan-treated PPAR-αKO mice; C: DEX-treated carrageenan-treated WT mice; D: DEX-treated carrageenan-treated PPAR-αKO mice. c. ICAM-1 expression by immunohistochemistry section: A: carrageenan-treated WT mice; B: carrageenan-treated PPAR-αKO mice; C: DEX-treated carrageenan-treated WT mice; D: DEX-treated carrageenan-treated PPAR-αKO mice. Figure is representative of 3 different experiments.

Figure 2: Effects of DEX on the release of carrageenan-induced TNF-α in WT and PPAR-αKO mice. a. levels of TNF-α were detected by colorimetric, commercial ELISA kit in lung tissues. Values shown are mean ± SEM of 10 mice for each group**P*<0.01 *vs.* Sham; °*P*<0.01 *vs.* carrageenan-WT group; °°*P*<0.01 *vs*. DEX-treated carrageenan-WT group. b. Immunohistochemical localization of TNF-α in the lung section: A: carrageenan-treated WT mice; B: carrageenan-treated PPAR-αKO mice; C: DEX-treated carrageenan-treated WT mice; D: DEX-treated carrageenan-treated PPAR-αKO mice. Figure is representative of 3 different experiments.

Figure 3: a. Representative western blot analysis of iNOS (left) and COX-2 (right) expression from lung tissue of carrageenan-treated WT and PPAR-αKO mice with or without

DEX administration. Shown immunoblots are representative of one of 3 different experiments. In the bottom panels of each western blot is represented a densitometry analysis expressed as mean ± SEM of 3 different experiments. **P*<0.01 *vs.* Sham; °*P*<0.01 *vs.* carrageenan-WT group. b. Effects of DEX on carrageenan-induced iNOS expression (b) or nitrotyrosine formation (c) evaluated by immunohistochemistry: A: carrageenan-treated WT mice; B: carrageenan-treated PPAR-αKO mice; C: DEX-treated carrageenan-treated WT mice; D: DEX-treated carrageenan-treated PPAR-αKO mice. Figure is representative of 3 different experiments.

Figure 4: Effect of DEX on GR and PPAR-α expression. a. PPAR-α mRNA expression evaluated by Real Time PCR. ND: not detectable. b. GR mRNA expression evaluated by Real Time PCR. SHAM : saline solution-treated mice; DEX: DEX-treated mice, CAR: carrageenan-treated mice; CAR + DEX: carrageenan and DEX-treated mice as described in Material and Methods. PCR analysis was assessed in triplicate. Results are expressed as mean ± SEM. **p* <0.01 vs. SHAM WT group; ***p* <0.01 vs. SHAM PPAR-αKO group.

Figure 5: Effect of DEX on carrageenan-induced NF-kB activation in lung tissue from WT and PPAR-αKOmice. Representative western blot analysis of IkB-α expression (a) and NF-kB/p65 phosphorylation on Ser536 (b) in the lung tissues. Immunoblots showed are representative of one of 3 different experiments. In the bottom panels of each western blot is represented a densitometry analysis expressed as mean ± SEM of 3 different experiments. **P*<0.01 *vs*. Sham; °*P*<0.01 *vs*. carrageenan-WT group; °°*P*<0.01 *vs.* DEX-treated carrageenan-WT group.

Figure 6: Effects of DEX on GILZ expression in lung from WT and PPAR-αKO mice upon carrageenan-induced pleurisy. a. GILZ mRNA expression evaluated by Real Time PCR. SHAM: saline solution-treated mice; DEX: DEX-treated mice, CAR: carrageenan-treated mice; CAR + DEX: carrageenan and DEX-treated mice as described in Material and Methods. PCR analysis was assessed in triplicate. Results are expressed as mean ± SEM. * p <0.01 *vs.* SHAM; ** p <0.01 *vs.* carrageenan-treated WT group. b. Immunohistochemical localization of GILZ in the lung: A: sham WT mice; B: sham PPAR-αKO mice; C: carrageenan-treated WT mice; D: carrageenan-treated PPAR-αKO mice; E-E1-E2: DEX-treated carrageenan-treated WT mice; F: DEX-treated carrageenan-treated PPAR-αKO mice. Figure is representative of 3 different experiments.

Figure 7: Effects of DEX and PPAR-α agonist clofibrate on lung injury. a. Lung section from carrageenan-treated mice demonstrating lung tissue injury (A) characterised by in infiltrating macrophages, PMNs and lymphocytes around alveolar wall (A1) as well as around vessels (A2). Clofibrate (100 mg/kg; B) or DEX (0.01 mg/kg, C) treatment did not attenuate the development of acute lung injury 4 h after carrageenan administration. On the contrary, clofibrate and DEX reduced the degree of lung injury and the inflammatory cells infiltration (D). Figure is representative of 3 different experiments. b. Effects of DEX and PPAR-α agonist clofibrate on the neutrophils infiltration see section b in the pleural cavity (left) and in the lung tissues (right) from WT and PPAR-αKO mice upon carrageenan-induced pleurisy. Values shown are mean ± SEM of 3 different experiments. *P<0.01 *vs.* Sham; °P<0.01 *vs.* carrageenan-WT group.

Figure 8: Effects of DEX in macrophages from WT and PPAR-αKO mice stimulated in vitro by LPS and IFNγ̃a. Nitrite/nitrate production evaluated as described in Material and Methods

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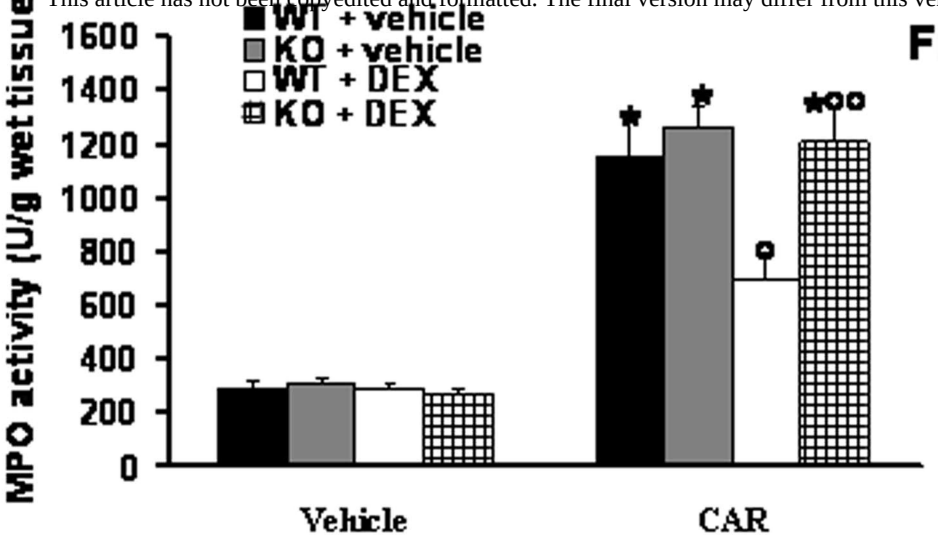
section; b. COX-2 expression assessed by western blot analysis. c. NF-kB activation evaluated by western blot analysis for phosphorylation of Ser536; Immunoblots showed are representative of one of three experiments analysed. In the bottom panels of each western blot is represented a densitometry analysis expressed as mean ± SEM of 3 different experiments; d. Effects of DEX and PPAR-α agonist clofibrate on COX-2 mRNA expression, evaluated by Real Time PCR, in macrophages from WT and PPAR-αKO mice stimulated in vitro by LPS and IFNγ̃ PCR analysis were assessed in triplicate. Results are expressed as mean ± SEM; *p <0.01 *vs.* vehicle-treated macrophages; **p <0.01 *vs.* LPS- and IFNγ-treated WT macrophages; ***p <0.01 *vs.* LPS-, IFNγ- and DEX-treated WT macrophages.

Figure 9: Effects of DEX in macrophages from WT and PPAR-αKO mice stimulated in vitro by LPS and IFNγ for COX-2, GR and PPAR-α expression. COX-2 (a), GR (b) or PPAR-α (c) expression evaluated by Real Time PCR in macrophages from PPAR-αKO mice transfected with empty vector (pSG5) or PPAR-α expression vector. GR (d) or PPAR-α (e) expression evaluated by Real Time PCR in macrophages from PPAR-αKO mice transfected with scramble oligo or siRNA oligo specific for PPAR-α. In the first line arbitrary unit was given to WT macrophages mRNA expression for each experiment. V: vehicle treated-macrophages; LPS: LPS and IFNγ treated-macrophages; LPS + DEX: LPS, IFNγ and DEX treated-macrophages as described in Material and Methods. PCR analyses were assessed in triplicate. Results are expressed as mean ± SEM. *p <0.01 *vs.* vehicle-treated macrophages; ** p <0.01 *vs.* LPS + DEX-treated and pSG5 transfected macrophages.

Fig. 1

a



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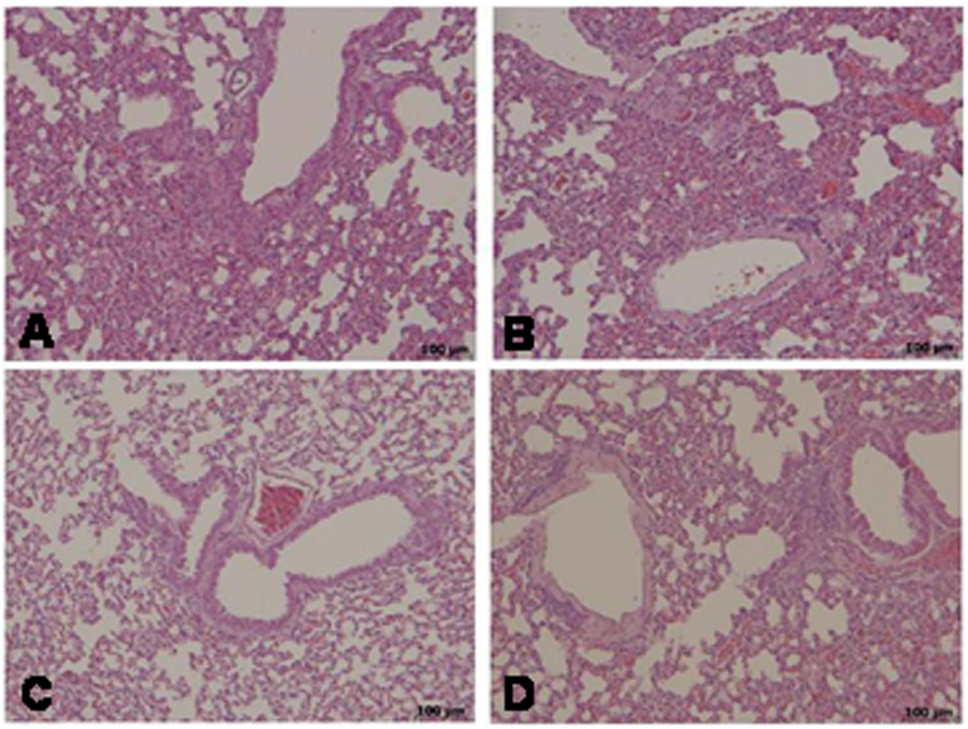

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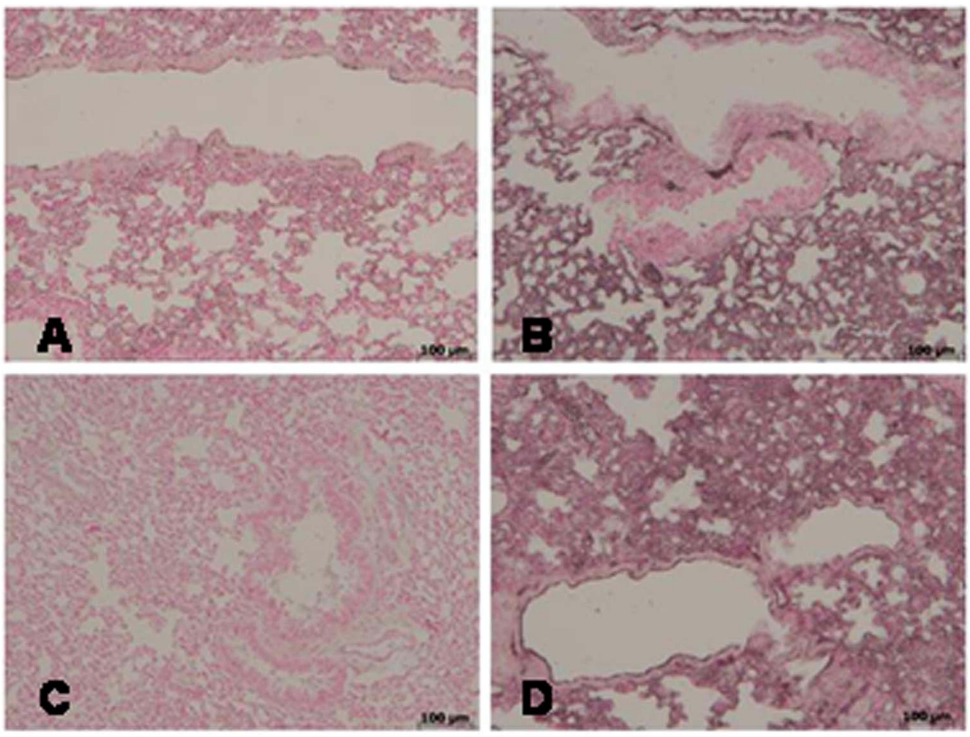

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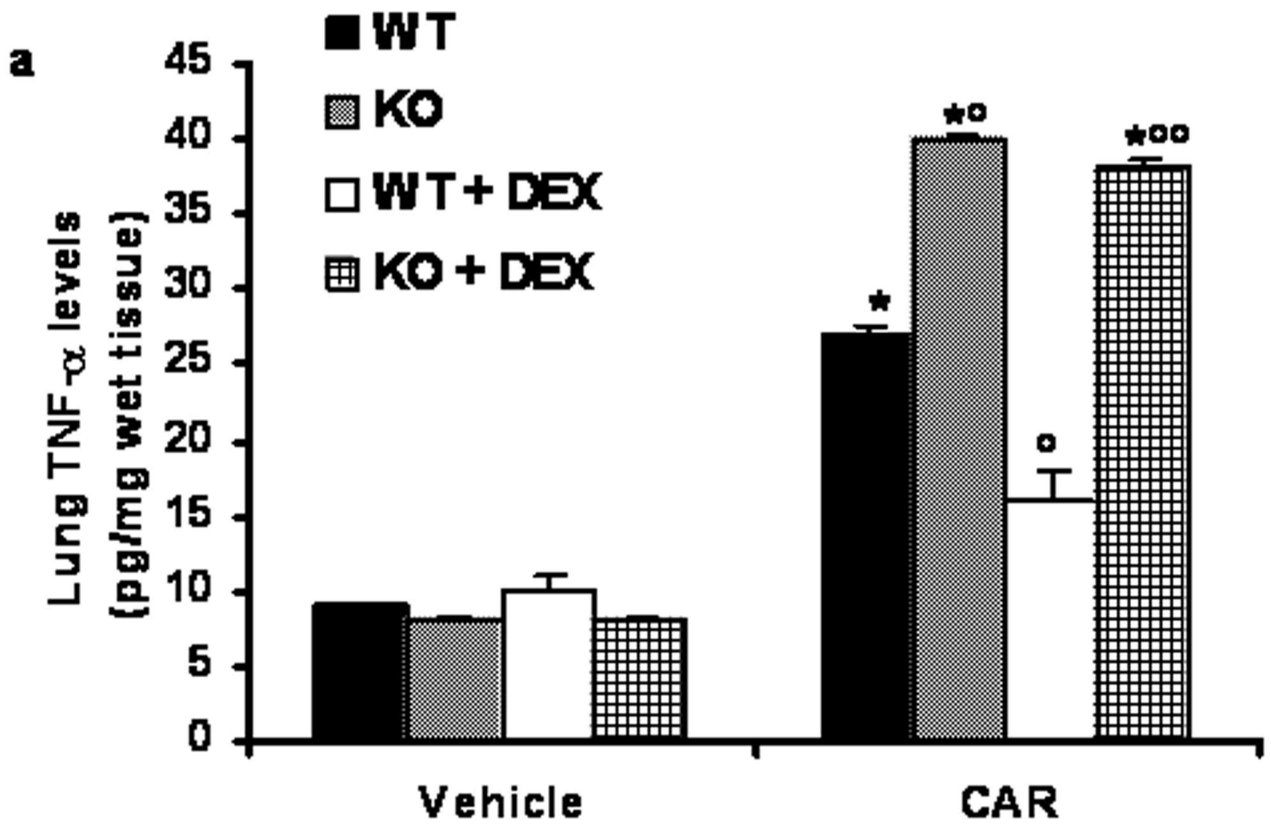
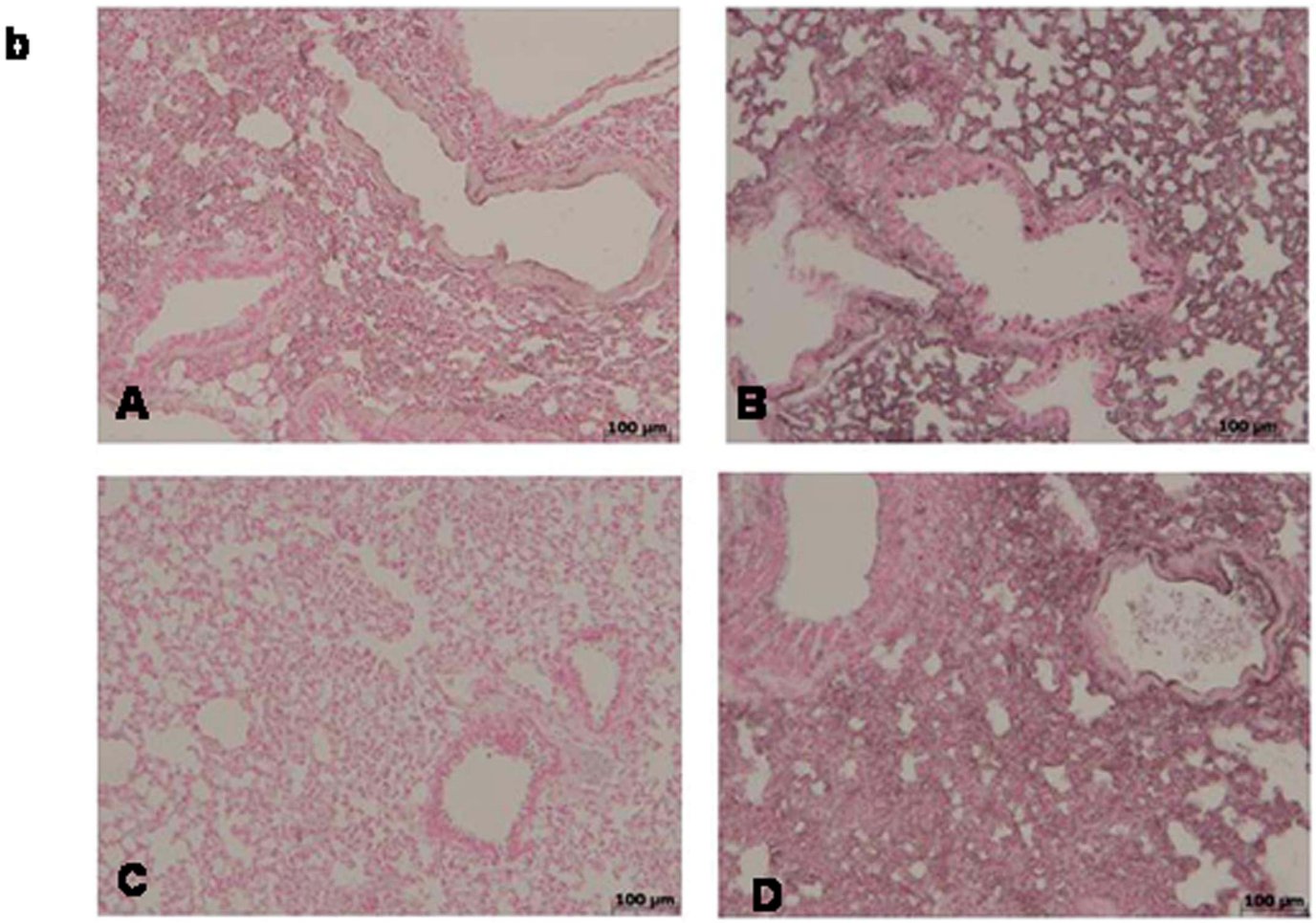
Fig. 2





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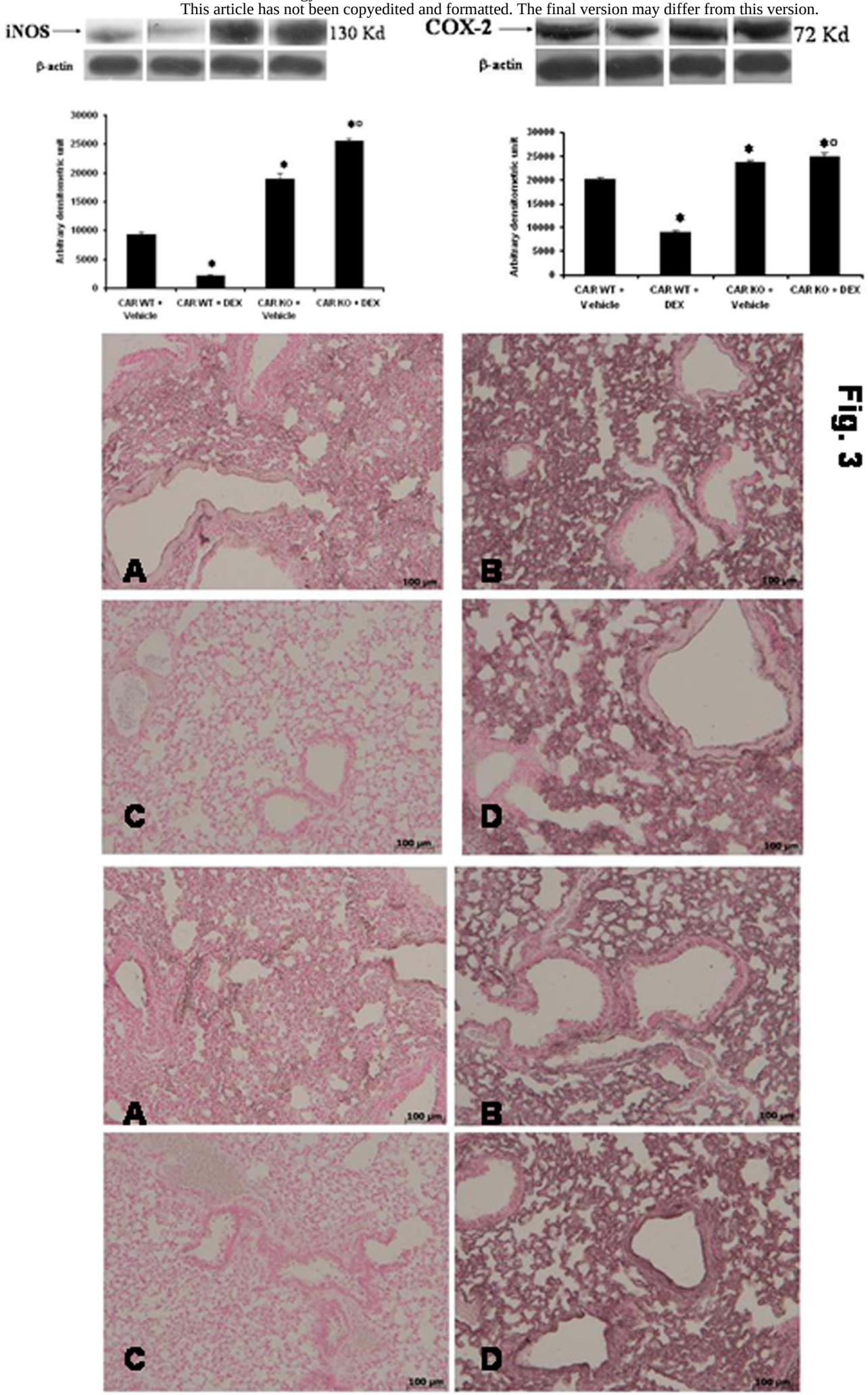

Fig. 3

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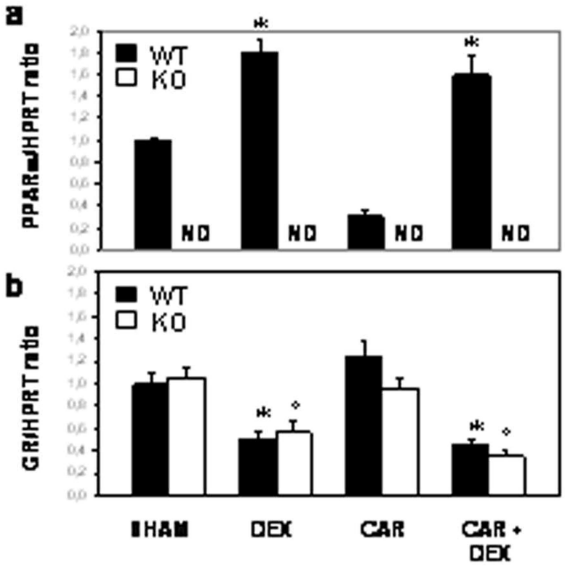


Fig. 4

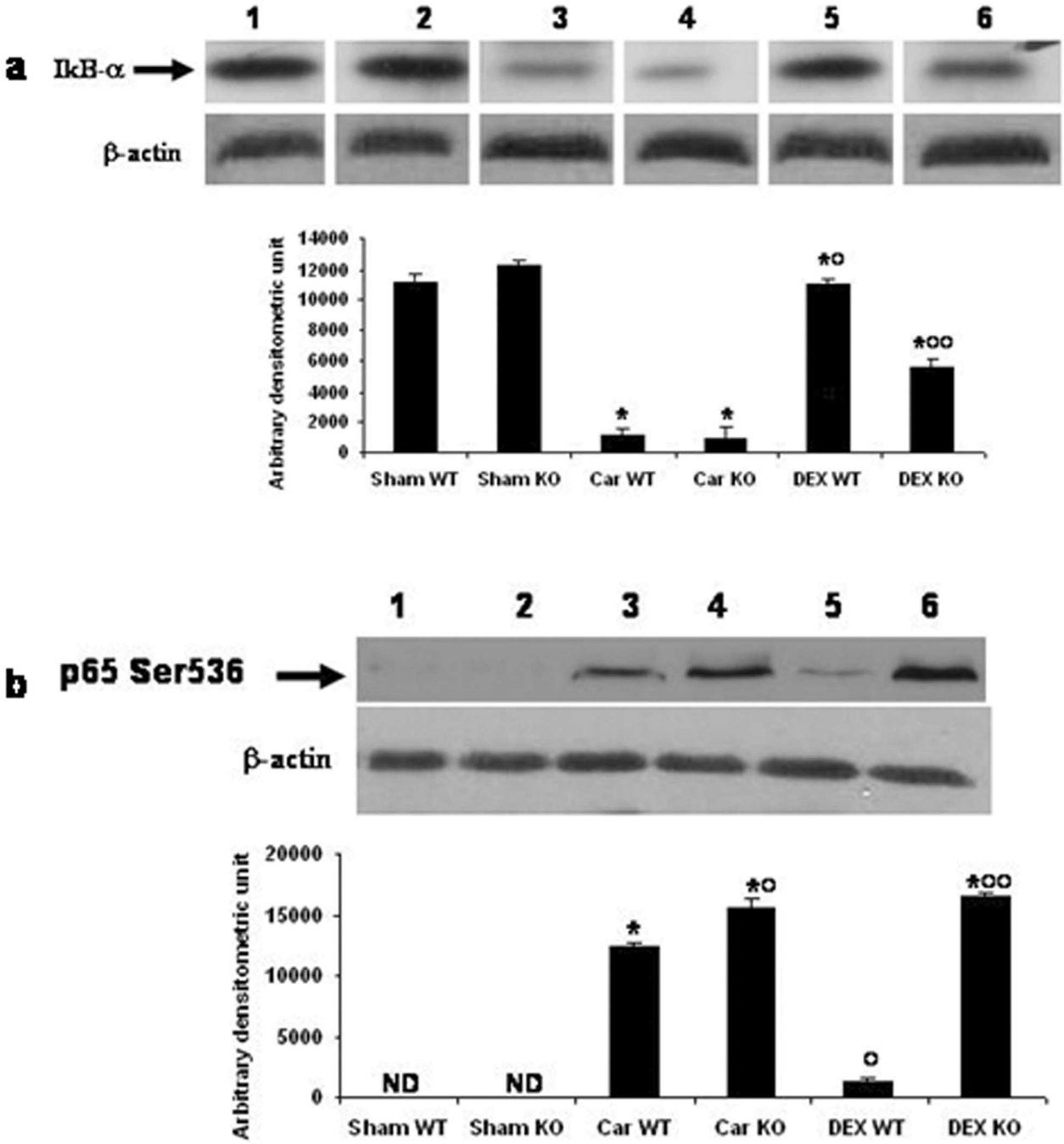
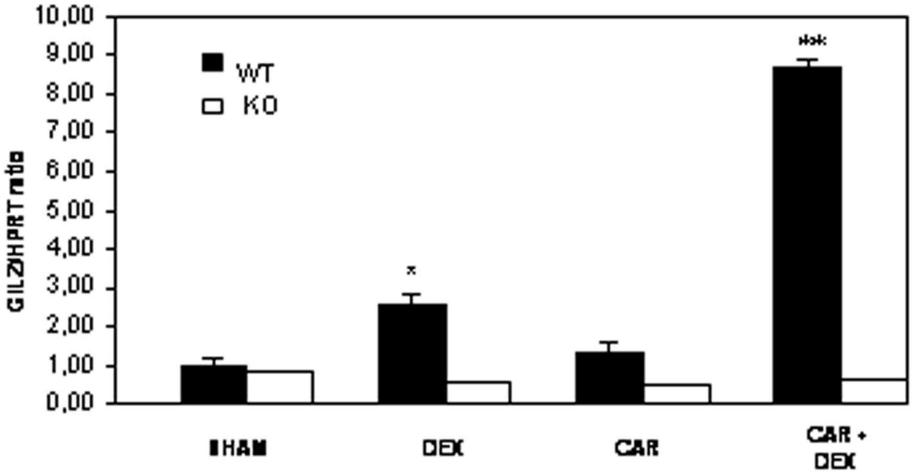


Fig. 5

Fig. 6

a



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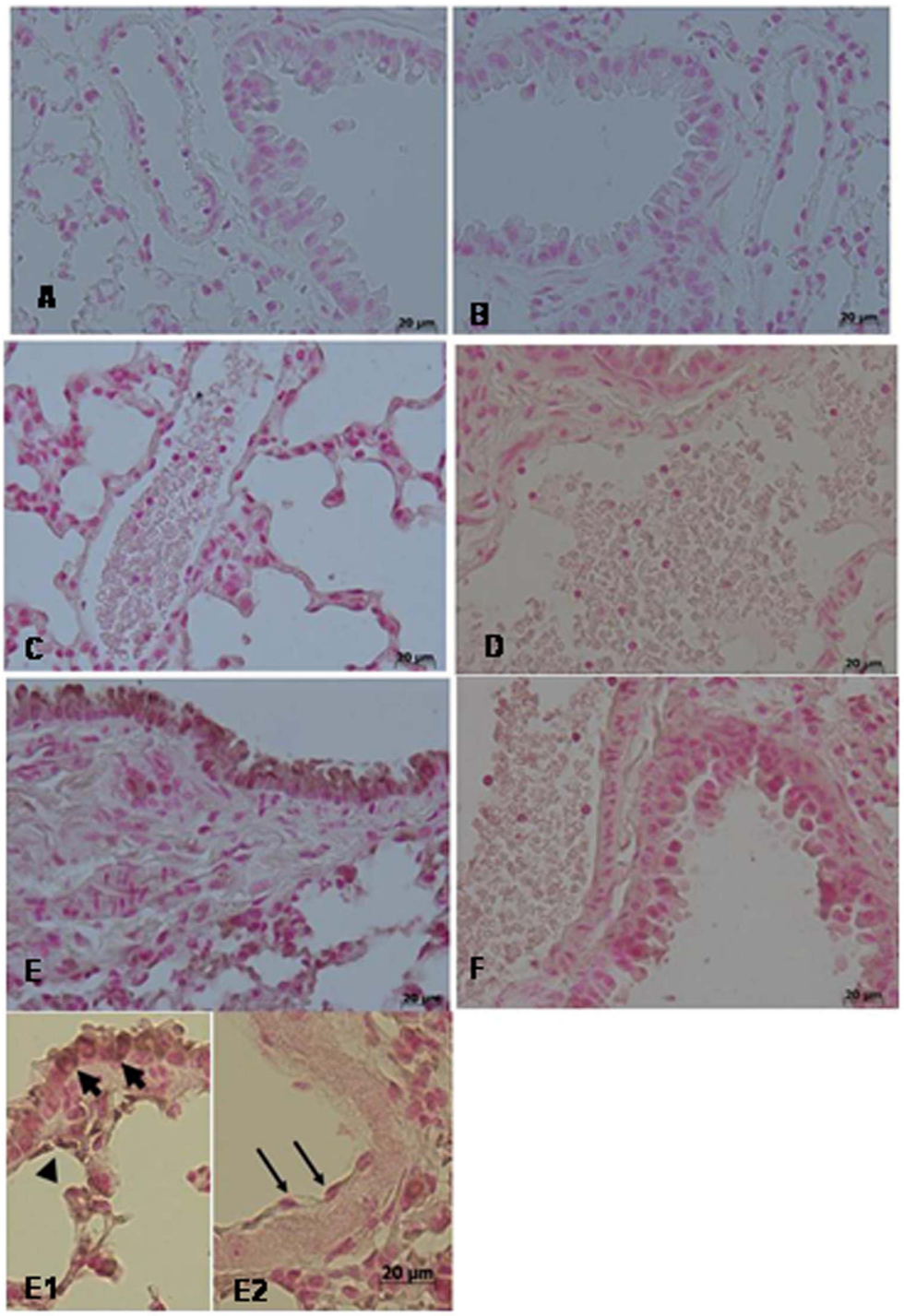

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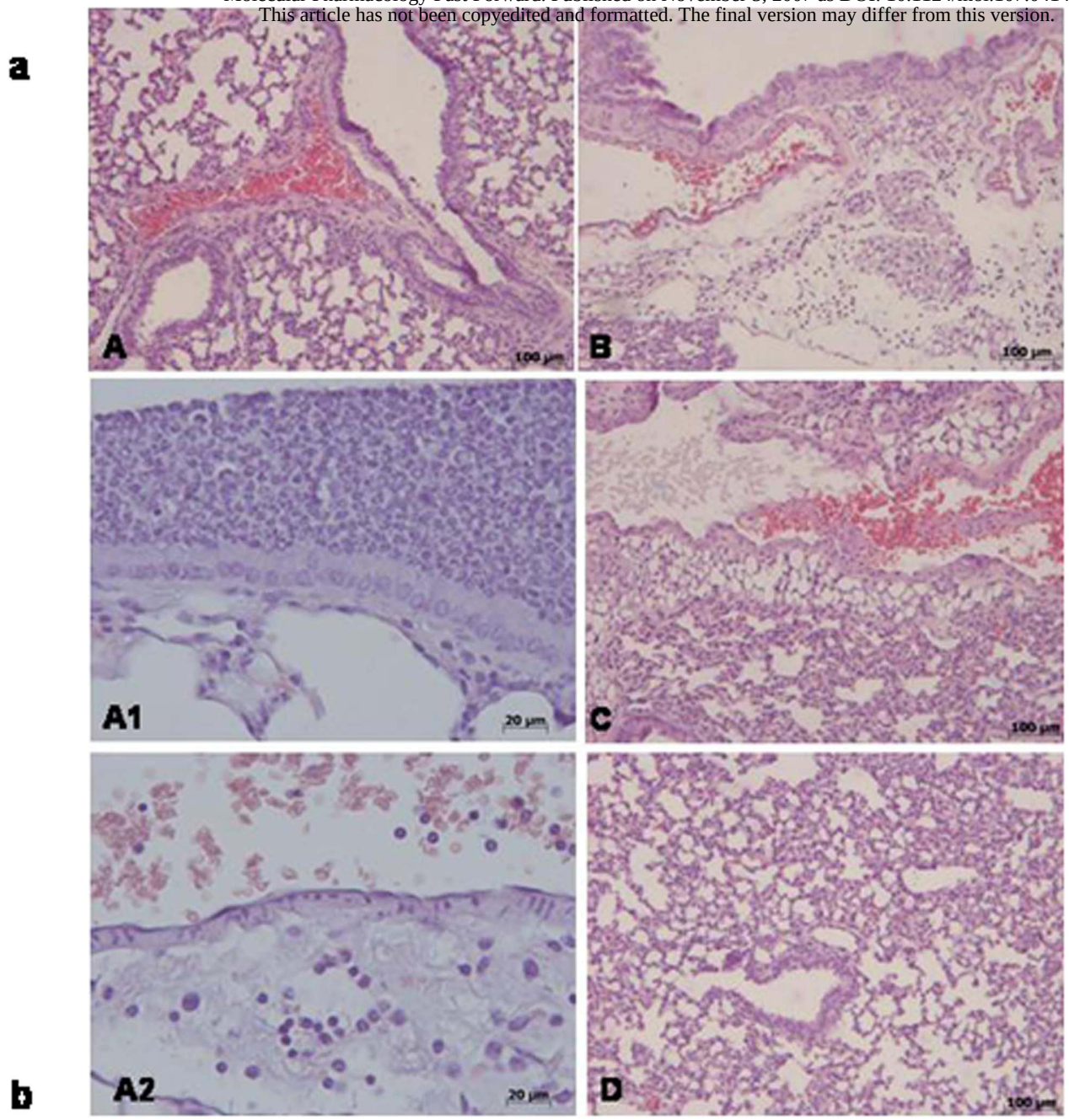
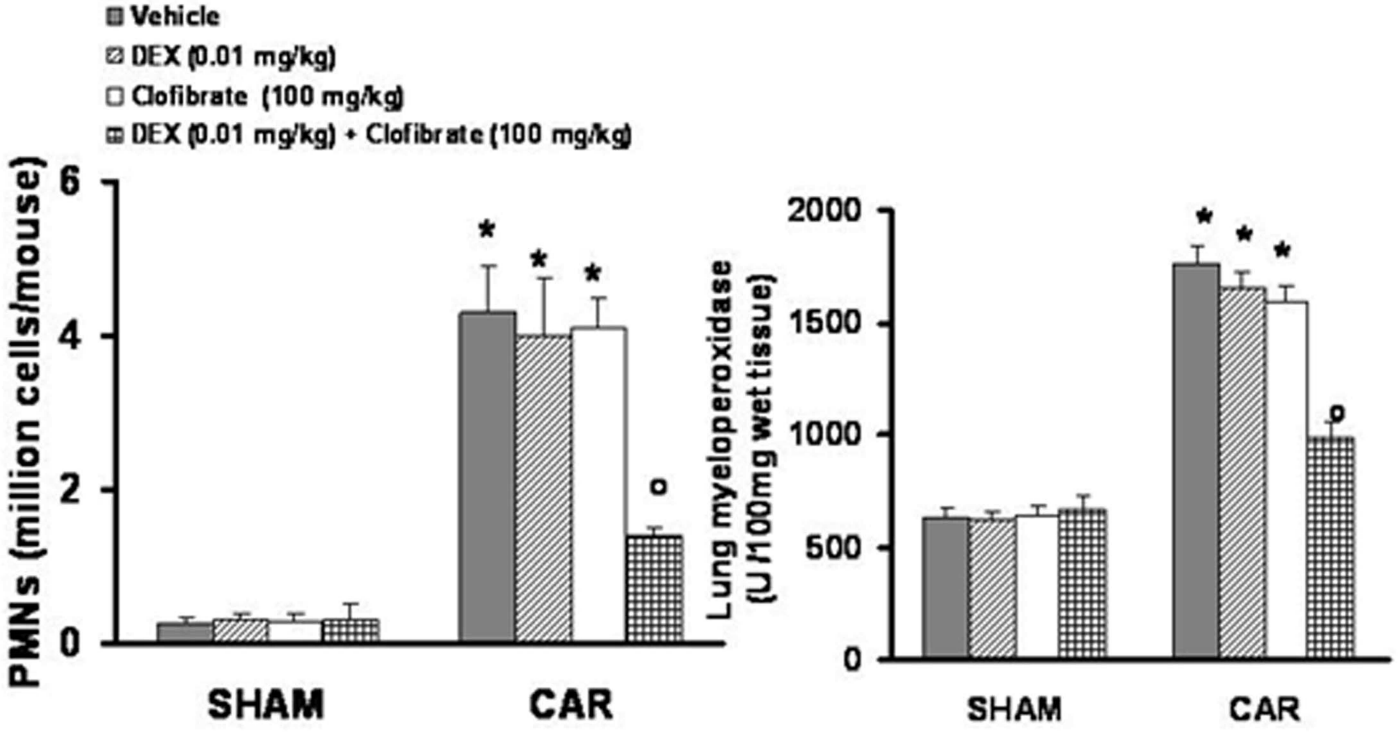
Fig. 7





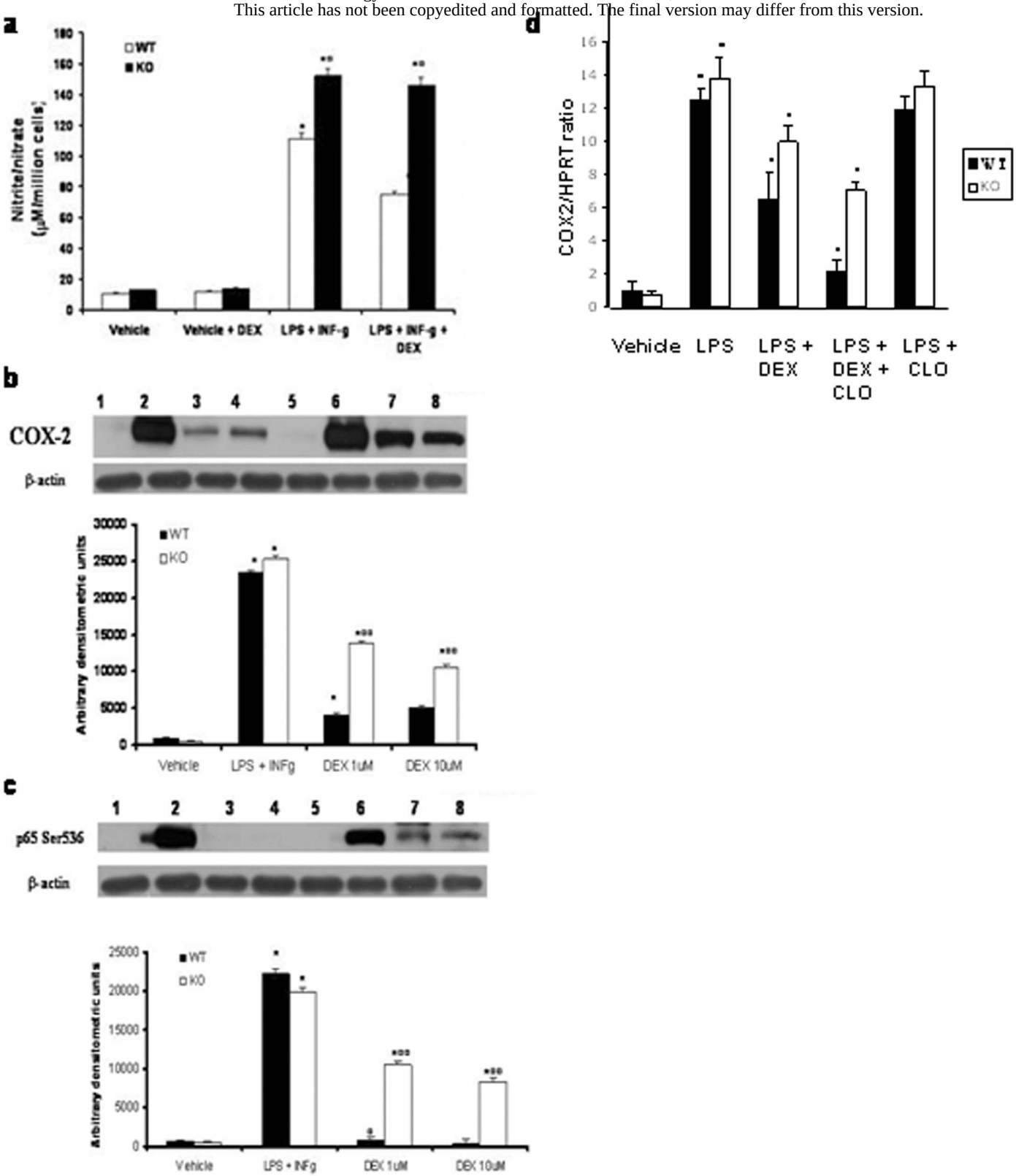


Fig. 8

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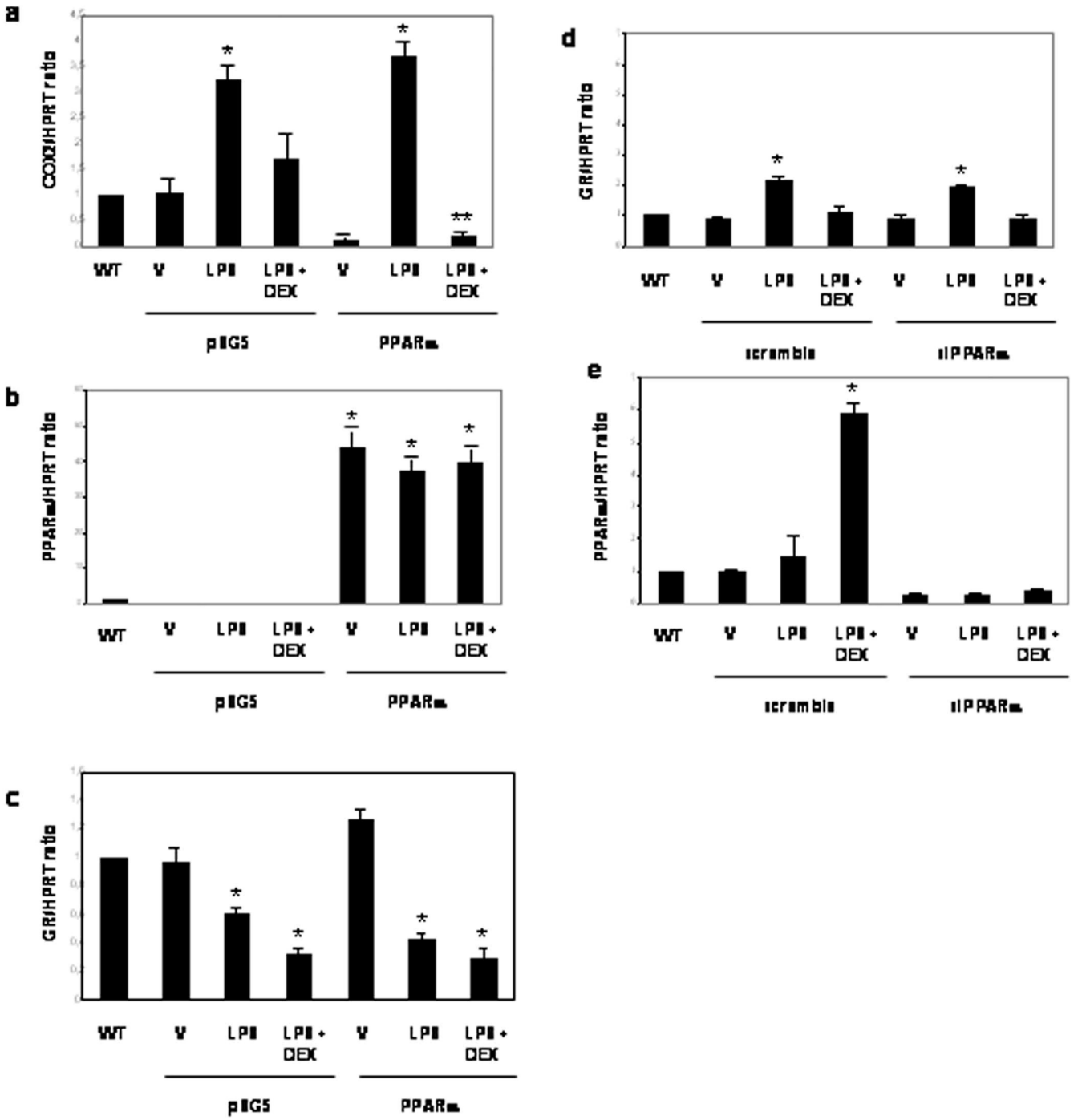

Fig. 9