

# Peroxisome Proliferator-Activated Receptor- $\alpha$ Contributes to the Anti-Inflammatory Activity of Glucocorticoids<sup>S</sup>

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## ABSTRACT

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

increased in WT mice when combined with PPAR- $\alpha$  agonist treatment. In particular, DEX was less effective in PPAR- $\alpha$ KO, compared with WT mice, as evaluated by inhibition of NF- $\kappa$ B, of TNF- $\alpha$  production, of cell migration, of cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase activation. Interestingly enough, macrophages from PPAR- $\alpha$ KO were less susceptible to DEX-induced COX-2 inhibition in vitro compared with WT mice. However, PPAR- $\alpha$  transfection in PPAR- $\alpha$ KO macrophages, with consequent receptor expression, resulted in reconstitution of susceptibility to DEX-induced COX-2 inhibition to levels comparable with that obtained in WT macrophages. It is noteworthy that the DEX effect on macrophages in vitro was significantly increased in WT cells when combined with PPAR- $\alpha$  agonist treatment. These results indicate that PPAR- $\alpha$  can contribute to the anti-inflammatory activity of GCs.

The inflammatory process is invariably characterized by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor, and by the release of chemicals from tissues and migrating cells (Tomlinson et al.,

1994). Carrageenan-induced local inflammation is commonly used to evaluate anti-inflammatory effects of nonsteroidal anti-inflammatory drugs. 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 (0–1 h), which is not inhibited by nonsteroidal anti-inflammatory drugs such as indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine, and bradykinin followed by a late phase (1–6 h), mainly sustained by

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**ABBREVIATIONS:** COX-2, cyclooxygenase-2; GC, glucocorticoid; PPAR- $\alpha$ , peroxisome proliferator-activated receptor- $\alpha$ ; DEX, dexamethasone; inducible nitric-oxide synthase; GR, glucocorticoid receptor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ICAM-1, intercellular adhesion molecule-1; PBS, phosphate-buffered saline; CAR, carrageenan; GILZ, glucocorticoid-induced leucine zipper; NF- $\kappa$ B, nuclear factor- $\kappa$ B; WT, wild type; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ ; siRNA, small interfering RNA; PPAR- $\alpha$ KO, mice lacking peroxidase proliferator-activated receptor- $\alpha$ ; PPAR- $\alpha$ WT, wild-type control mice; GW6471, *N*-((2*S*)-2-(((1*Z*)-1-methyl-3-oxo-3-(4-(trifluoromethyl)phenyl)prop-1-enyl)amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl)propanamide.

prostaglandin release and attributed to the induction of inducible cyclooxygenase (COX-2) in the tissue (Nantel et al., 1999). It seems 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, and to the release of other neutrophil-derived mediators (Salvemini et al., 1996).

Glucocorticoids (GCs) are effective anti-inflammatory agents widely used in the treatment of inflammatory and autoimmune diseases (Hoffman, 1993; Kwak and Longo, 1996; Frankfurt and Rosen, 2004; 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 nongenomic and genomic signals (Diamond et al., 1990; Lösel and Wehling, 2003; Bianchini et al., 2006). In particular, mechanisms including enzyme activation, such as the glucocorticoid receptor (GR)-associated src kinase and phospholipase and  $Ca^{2+}$  mobilization are a result of GC interaction with the GR (Cifone et al., 1999; Falkenstein et al., 2000; Marchetti et al., 2003; Lépine et al., 2004). After 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 NF- $\kappa$ B, an important player in inflammatory process. Multiple mechanisms are involved in GC-mediated anti-inflammatory activity in addition to direct GR/NF- $\kappa$ B interaction, such as GC-induced up-regulation of I $\kappa$ B and glucocorticoid-induced leucine zipper (GILZ), two proteins able to bind and inhibit NF- $\kappa$ B activation (Ray and Prefontaine, 1994; Auphan et al., 1995; Scheinman et al., 1995; Cannarile et al., 2006; Di Marco et al., 2007). After NF- $\kappa$ B inhibition, a number of inflammatory parameters are blocked, including cytokine production, cell migration, COX-2 and inducible nitric-oxide synthase (iNOS) activation, and tissue damage (Barnes and Karin, 1997; Yamamoto and Gaynor, 2001; Gilroy et al., 2004; Cuzzocrea et al., 2007).

Peroxisome proliferator activated receptor- $\alpha$  (PPAR- $\alpha$ ) is an intracellular transcription factor activated by fatty acids that plays a role in inflammation (Colville-Nash et al., 1998; Neve et al., 2001; Yang et al., 2002; Moraes et al., 2006). Previous studies indicate that PPAR- $\alpha$  expression is induced by GCs and can mediate some of the GC effects, such as modulation of insulin sensitivity and resistance, and can contribute to GC-induced hyperglycemia and blood pressure increase (Lemberger et al., 1994; Bernal-Mizrachi et al., 2003; Cassuto et al., 2005; Bernal-Mizrachi et al., 2007). In addition, it has been reported that PPAR- $\alpha$  activation can result in inhibition of NF- $\kappa$ B activation and inflammatory gene expression (Staels et al., 1998; Delerive et al., 1999, 2000; Okamoto et al., 2005). Our recent results in disease models of colitis and pleurisy show that mice lacking PPAR- $\alpha$  (PPAR- $\alpha$ KO) develop an increased inflammation compared with wild-type (WT) mice. Moreover, treatment with appropriate doses of PPAR- $\alpha$  agonists can inhibit inflammatory disease development (Cuzzocrea et al., 2004, 2006a).

With the aim of characterizing the role of PPAR- $\alpha$  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- $\alpha$ KO and WT mice. We also tested the possible synergism of combined treatment with DEX and clofibrate, a PPAR- $\alpha$  agonist. Results indicate that DEX-mediated anti-inflammatory activity is weakened in PPAR- $\alpha$ KO mice compared with WT controls and that it is increased in WT mice when combined with PPAR- $\alpha$  agonist treatment.

## Materials and Methods

### In Vivo Experiments

**Animals.** Mice (6–7 weeks old, 20–27 g) with a targeted disruption of the PPAR- $\alpha$  gene (PPAR- $\alpha$ KO) and littermate wild-type controls (PPAR- $\alpha$ WT) were purchased from Harlan Nossan (Milan, Italy). Mice homozygous for the PparatnJGonz-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 were 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 the United States (Animal Welfare Assurance number A5594-01, Department of Health and Human Services, Washington, DC).

**Carrageenan-Induced Pleurisy.** Mice were anesthetized 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%  $\lambda$ -carrageenan (0.1 ml) was injected into the pleural cavity as described previously (Cuzzocrea et al., 2006a). The inflammatory cells (approximately 70% of macrophages) in the pleural exudate were suspended in phosphate-buffered saline (PBS) and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

**Experimental Groups.** Mice (either PPAR- $\alpha$ WT or PPAR- $\alpha$ KO) were randomly allocated into the following groups: 1) CAR WT group: PPAR- $\alpha$ WT mice were subjected to carrageenan-induced pleurisy ( $n = 10$ ) and were treated with saline solution (DEX vehicle); 2) CAR PPAR- $\alpha$ KO group: PPAR- $\alpha$ KO mice were subjected to carrageenan-induced pleurisy ( $n = 10$ ) and were treated with saline solution (DEX vehicle); 3) CAR WT + DEX group: same as for CAR WT group with DEX administration (1 mg/kg i.p. bolus) 1 h before carrageenan ( $n = 10$ ); 4) CAR PPAR- $\alpha$ KO + DEX group: same as for CAR PPAR- $\alpha$ KO group with DEX administration (1 mg/kg i.p. bolus) 1 h before carrageenan ( $n = 10$ ); 5) sham WT group: 100  $\mu$ l of saline solution instead of carrageenan was administered to the PPAR- $\alpha$ WT mice ( $n = 10$ ), and they were treated with saline solution (DEX vehicle); 6) sham PPAR- $\alpha$ KO group: 100  $\mu$ l of saline solution instead of carrageenan was administered to the PPAR- $\alpha$ KO mice ( $n = 10$ ), and they were treated with saline solution (DEX vehicle); 7) sham + WT DEX group: same as for sham WT group with DEX administration (1 mg/kg i.p. bolus) 1 h before saline ( $n = 10$ ); and 8) sham + PPAR- $\alpha$ KO DEX group: same as for sham PPAR- $\alpha$ KO group with DEX administration (1 mg/kg i.p. bolus) 1 h before saline ( $n = 10$ ).

In a separate set of experiment mice, PPAR- $\alpha$ WT mice were randomly allocated into the following groups: 1) CAR group: WT mice were subjected to carrageenan-induced pleurisy ( $n = 10$ ) and were treated with DMSO 10% solution (clofibrate vehicle); 2) CAR + clofibrate group same as the PPAR- $\alpha$ WT CAR group with clofibrate administration (100 mg/kg i.p. bolus) 1 h before carrageenan ( $n = 10$ ); 3) CAR + DEX group: same as the CAR group with DEX administration (0.01 mg/kg i.p. bolus) 1 h before carrageenan ( $n = 10$ ); 4) 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) 1 h before carrageenan ( $n = 10$ ); 5) sham group: 100  $\mu$ l of saline solution instead of carrageenan was administered to the mice ( $n = 10$ ), and they were treated with saline solution (DEX vehicle); 6) sham + DEX group: same as sham group with DEX administration (0.01 mg/kg i.p. bolus) 1 h before saline ( $n = 10$ ); 7) sham clofibrate group: same as for sham with clofibrate administration (100 mg/kg i.p. bolus) 1 h before saline ( $n = 10$ ); and 8) sham + clofibrate + DEX group: same as the PPAR- $\alpha$ WT sham group with clofibrate (100 mg/kg i.p. bolus) and DEX (0.01 mg/kg i.p. bolus) administration 1 h before saline ( $n = 10$ ).

**Histological Examination.** Lung tissues samples were taken 4 h after injection of carrageenan. Lung tissue 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). Sections were then deparaffinized with xylene and stained with hematoxylin and eosin. All sections were studied using an Axiovision Ziess (Milan, Italy) microscope.

**Localization of Nitrotyrosine, ICAM-1, TNF- $\alpha$ , GILZ, and iNOS by Immunohistochemistry.** The tissues were fixed in 10% PBS-buffered formaldehyde, and 8- $\mu$ m sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H<sub>2</sub>O<sub>2</sub> in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific 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) antinitrotyrosine rabbit polyclonal antibody (1:500 in PBS), with anti-ICAM-1 polyclonal antibody (CD54) [1:500 in PBS (v/v)] (DBA), with anti-TNF- $\alpha$  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). To verify the binding specificity for ICAM-1, TNF- $\alpha$ , GILZ, 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 were positive in all of the experiments carried out. To confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (antinitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.

**Determination of Myeloperoxidase Activity.** Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as described previously (Mullane et al., 1985). Four hours 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<sub>2</sub>O<sub>2</sub>. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1  $\mu$ mol of peroxide per minute at 37°C and was expressed in units per gram weight of wet tissue.

**Measurement of TNF- $\alpha$ .** Lungs were homogenized in PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO), and tissue levels of TNF- $\alpha$  were evaluated. The assay was carried out by a colorimetric commercial kit (Calbiochem-Novabiochem Corporation, San Diego, CA) according to the manufacturer's instructions. All cytokine 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 EGTA, 2 mM EDTA, 5 mM NaN<sub>3</sub>, 10 mM  $\beta$ -mercaptoethanol, 20  $\mu$ M leupeptin, 0.15  $\mu$ M pepstatin A, 0.2 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, and 0.4 nM microcystin. The homogenates were centrifuged (1000g, 10 min), and the supernatant was collected to evaluate contents of iNOS, COX-2, I $\kappa$ B- $\alpha$ , and phospho-NF- $\kappa$ B p65 (Ser536). Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (50  $\mu$ g) were dissolved in Laemmli's sample buffer, boiled, and run on 12% SDS-polyacrylamide gel electrophoresis gel and then transferred to Hybond polyvinylidene difluoride membrane. Membranes were blocked for 60 min in TBS and 5% (w/v) nonfat milk and subsequently probed overnight at 4°C with anti-iNOS (Transduction Laboratories, Lexington, KY) (1:2000), anti-COX-2 (Cayman Chemical, Ann Arbor, MI) (1:2000), anti-I $\kappa$ B- $\alpha$  (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-phospho-NF- $\kappa$ B p65 (Ser536) (1:1000; Cell Signaling Technology, Danvers, MA) antibodies [in TBS, 5% (w/v) nonfat milk and 0.1% Tween 20]. Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1:2,000) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent (Pierce, Rockford, IL). Bands were quantified by densitometric analysis performed with a quantitative imaging system.

**Real-Time RT-PCR.** Total RNA was prepared using TRIzol from Invitrogen (Milan, Italy). RT-PCR was done using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). The initial template molecules in the samples were measured in triplicate and expressed as the mean  $\pm$  S.E.M. For real-time RT-PCR analysis, probes were selected in the connecting region of two exons: specific primers for GILZ; sense, 5'-GGTGGCCCTAGACAACAAGA-3', and antisense, 5'-TCTTCTAAGCAGCTCAGCA-3'; primers for COX2: sense, 5'-CATCCTGACCCACTCAAGG-3', and antisense, 5'-AGAAGGCTTCCCAGCTTTTG-3'; primers for PPAR- $\alpha$ : sense, 5'-TCGAATATGTGGGACAAGG-3', and antisense, 5'-GACAGGCAC-TTGTGAAAACG-3'; primers for GR: sense, 5'-AACTGGAATAGGT-GCCAAGG-3', and antisense, 5'-GAGCACACCAGGCAGAGTTT-3'; and primers for hypoxanthine-guanine phosphoribosyltransferase: sense, 5'-CTGCGTGATTAGCGATGATG-3', and antisense, 5'-ACAGAGGCCACAATGTGAT-3'. PCR was performed with Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Milan, Italy) using DyNAmo HS SYBR GREEN qPCR kit (Finnzymes, Espoo, Finland). 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), and 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  $\Delta$ C(t) method was used. The C(t) value was determined using the Opticon Monitor 2 software (MJ Research, Waltham, MA). Gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase housekeeping mRNA expression.

## In Vitro Experiments

**Peritoneal Macrophages.** Peritoneal macrophages were elicited by intraperitoneal injection of 2 ml of 4% sterile thioglycolate medium (Sigma). After 3 days, mice were sacrificed, and macrophages were harvested as described previously (Nunoshiba et al., 1993). Peritoneal macrophages were grown in DMEM supplemented with 1% glutamine, 2.5% HEPES, 1% solution 10,000 U/ml penicillin and 10,000  $\mu$ g/ml streptomycin, 10% fetal bovine serum (FBS), and 1.2% sodium pyruvate (EuroClone, Milano, Italy). Cells were plated on tissue culture plates for 3 to 4 h, and then nonadherent cells were removed by washing with sterile PBS. Peritoneal macrophages were treated with lipopolysaccharide (LPS) 10  $\mu$ g/ml and interferon (IFN)- $\gamma$  100 U/ml for 24 h in DMEM without FBS in the presence or absence of test compounds. Two hours before the stimulation with LPS and INF- $\gamma$ , we added to the medium DEX 1 and 10  $\mu$ M. The

medium was used for the determination on nitrite/nitrate, and cells were used for Western blot analysis.

In a separate set of experiments, peritoneal macrophages were treated with LPS 10  $\mu\text{g}/\text{ml}$  and IFN- $\gamma$  100 U/ml for 24 h in DMEM without FBS in the presence or absence of test compounds. Two hours before the stimulation with LPS and INF- $\gamma$ , we added DEX (1  $\mu\text{M}$ ) and clofibrate (0.2 mM) to the medium. Four hours later, cells were harvested for real-time analysis.

**Transfection.** Transfection of mouse peritoneal macrophages from PPAR- $\alpha$ KO mice was carried out according to a commercially available protocol using Mouse Macrophage Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD) transfection system. In brief, we transfected  $5 \times 10^5$  peritoneal macrophages with 2  $\mu\text{g}$  of plasmid containing full-length PPAR- $\alpha$  or empty vector pSG5 or, for silencing, 1  $\mu\text{g}$  of siRNA specific for PPAR- $\alpha$  or scrambled oligonucleotides. Each group was cotransfected with 2  $\mu\text{g}$  of phosphorylated enhanced green fluorescent protein vector to check transfection efficiency (all transfections resulted green fluorescent protein-positive in a range of 40 to 50% as evaluated by fluorescence-activated cell sorting analysis). Twenty-four hours after transfection, cells were treated with DEX (1  $\mu\text{M}$ ) followed 2 h later by stimulation with LPS and INF- $\gamma$ . 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  $\mu\text{g}/\text{ml}$  leupeptin, and 10  $\mu\text{g}/\text{ml}$  trypsin inhibitor). Lysates from adherent cells were collected by scraping and were centrifuged at 13,000g for 15 min at 4°C. The supernatants were collected, and protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad), and 50  $\mu\text{g}$  of total protein from each sample was analyzed. Proteins were separated by a 12% SDS-polyacrylamide gel electrophoresis and transferred on nitrocellulose membrane (Hybond ECL Nitrocellulose; GE Healthcare, Milan, Italy). The membrane was blocked with 0.1% TBS-Tween containing 5% nonfat 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- $\kappa\text{B}$  p65 (Ser536) rabbit monoclonal antibody diluted 1:1000 (Cell Signaling Technology); anti-COX-2 diluted 1:2000 (Cayman Chemical); and anti-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) diluted 1:2000; the membranes were washed, and protein bands were detected with SuperSignal West Pico Chemiluminescent (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 described previously (Cuzzocrea et al., 2001). In brief, 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  $\mu\text{M}$ ) at room temperature for 3 h. The total nitrite concentration in the samples was then measured using the Griess reaction by adding 100  $\mu\text{l}$  of Griess reagent 0.1% (w/v) naphthylethylenediamide dihydrochloride in H<sub>2</sub>O and 1% (w/v) sulfanilamide in 5% (v/v) concentrated H<sub>3</sub>PO<sub>4</sub> volume ratio of 1:1 to the 100- $\mu\text{l}$  sample. The optical density at 550 nm was measured using enzyme-linked immunosorbent assay microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations were calculated by comparison with optical density at 550 nm of standard solutions of sodium nitrite prepared in H<sub>2</sub>O.

**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. (Burlingame, CA).

## Data Analysis

All values in the figures and text are expressed as mean  $\pm$  S.E.M. of the mean of  $n$  observations. For the in vivo studies,  $n$  represents the number of animals studied. Results were analyzed by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. A  $p$  value less than 0.05 was considered significant.

## Results

**Role of Functional PPAR- $\alpha$  Gene in the Anti-Inflammatory Property of DEX in Carrageenan-Induced Pleurisy.** We analyzed the possible role of PPAR- $\alpha$  in the anti-inflammatory property of DEX during short-term inflammation in the lung. For that purpose, we compared the effect of DEX pretreatment in PPAR- $\alpha$ KO 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- $\alpha$ KO mice compared with vehicle-treated controls (Fig. 1a). Treatment with DEX resulted in a significant inhibition of MPO activity in the lung of WT but not PPAR- $\alpha$ KO mice.

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

To confirm that the PPAR- $\alpha$  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- $\alpha$  (Xu et al., 2002), attenuates the protective effects of DEX. In particular, we have demonstrated that coadministration of GW6471 (10 mg/kg i.p. 30 min before DEX administration) significantly blocked the effect of DEX on PMN infiltration in the pleural cavity and decreased lung MPO activity (see Supplementary Fig. S1).

It is well known that inflammatory cell infiltration is dependent on 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- $\alpha$ KO mice and the effect of DEX treatment.

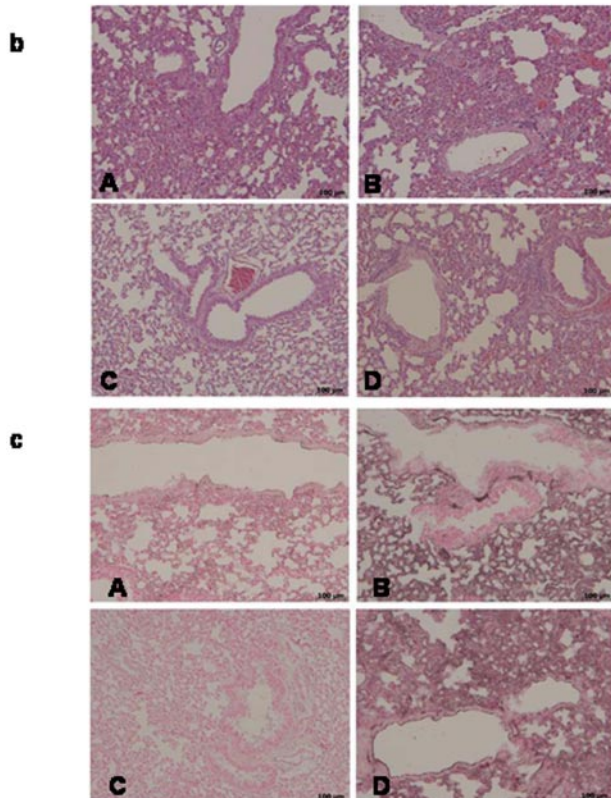
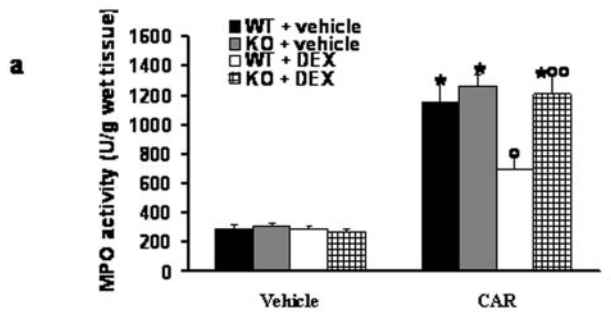
Staining of lung tissue sections obtained from saline-treated controls with anti-ICAM-1 antibody showed a specific staining along bronchial epithelium, demonstrating that ICAM-1 is constitutively expressed (data not shown). Four hours after carrageenan injection, the staining intensity for ICAM-1 was detected along the WT vessels (Fig. 1cA), mainly localized in the vascular endothelium. Moreover, in carrageenan-treated PPAR- $\alpha$ KO 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 PPAR- $\alpha$  gene in PPAR- $\alpha$ KO

mice significantly reduced the DEX-induced inhibition of ICAM-1 expression (Fig. 1cD), further suggesting that PPAR- $\alpha$  is involved in anti-inflammatory activity of DEX.

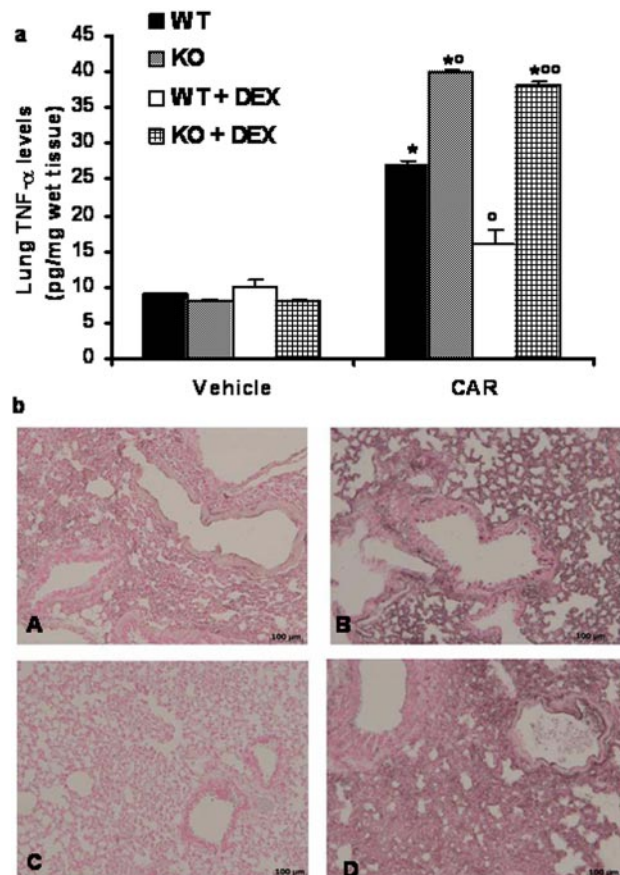
**Role of PPAR- $\alpha$  in DEX-Induced Inhibition of TNF- $\alpha$  Production.** Release of proinflammatory cytokines is an important mechanism responsible for inflammatory processes, including carrageenan-induced pleurisy (Cuzzocrea et al., 2006a,b). At 4 h after carrageenan injection, increased levels of TNF- $\alpha$  protein were observed in lung tissues compared with controls (Fig. 2a; compare columns 1 and 2 with columns 5 and 6). DEX treatment significantly inhibited

TNF- $\alpha$  production in WT (compare columns 7 and 5) but not in PPAR- $\alpha$ KO mice (compare columns 8 and 6). It is noteworthy that lung tissue production of TNF- $\alpha$  was significantly higher in carrageenan-treated PPAR- $\alpha$ KO mice compared with WT animals (compare columns 6 and 5).

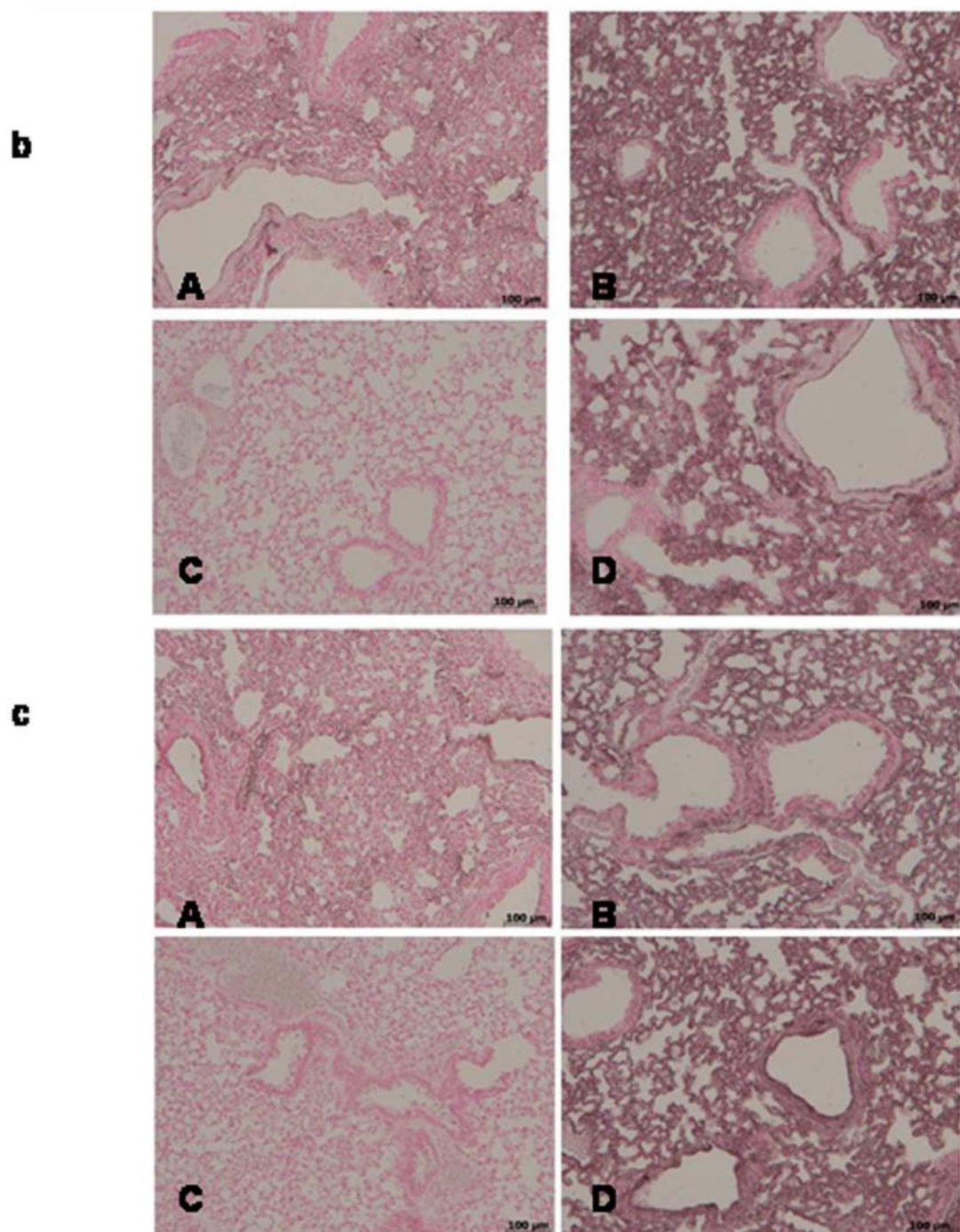
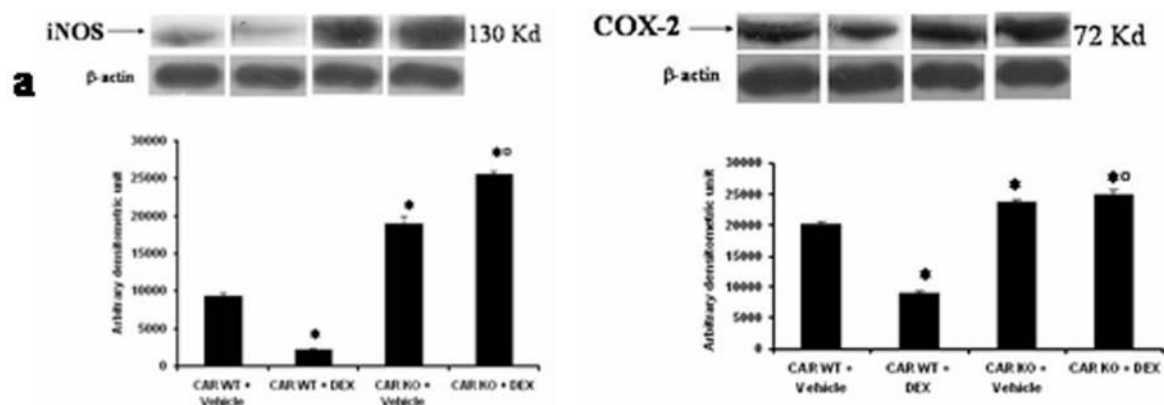
We also evaluated the TNF- $\alpha$  protein expression in the lung tissues by immunohistochemical assay. No positive staining for TNF- $\alpha$  was observed in the lung tissues collected from sham WT mice and from sham PPAR- $\alpha$ KO mice (data not shown). On the contrary, tissue sections obtained from WT animals 4 h after carrageenan administration showed positive staining for TNF- $\alpha$  localized in the infiltrated inflammatory cells, pneumocytes, and vascular wall (Fig. 2bA). In carrageenan-treated PPAR- $\alpha$ KO mice, the staining for TNF- $\alpha$  in the infiltrated inflammatory cells, pneumocytes, and vascular wall was visibly and significantly increased compared with the WT mice (Fig. 2bB). No positive staining for TNF- $\alpha$  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 PPAR- $\alpha$  gene in PPAR- $\alpha$ KO mice significantly blocked the anti-inflammatory effect of DEX (Fig. 2bD). Together, these results indicate that PPAR- $\alpha$



**Fig. 1.** Effect of PPAR- $\alpha$  on the anti-inflammatory property of DEX in carrageenan-induced pleurisy. Four hours after carrageenan administration in WT and PPAR- $\alpha$ KO mice, we measured several parameters. a, lung MPO activity ( $p < 0.01$ ); values shown are mean  $\pm$  S.E.M. of 10 mice for each group, \*,  $p < 0.01$  versus sham; °,  $p < 0.01$  versus carrageenan-WT group; °°,  $p < 0.01$  versus DEX-treated carrageenan-WT group. b, histological examination of lung sections by hematoxylin and eosin coloration: carrageenan-treated WT mice (A), carrageenan-treated PPAR- $\alpha$ KO mice (B), DEX-treated carrageenan-treated WT mice (C), and DEX-treated carrageenan-treated PPAR- $\alpha$ KO mice (D). c, ICAM-1 expression by immunohistochemistry section: carrageenan-treated WT mice (A), carrageenan-treated PPAR- $\alpha$ KO mice (B), DEX-treated carrageenan-treated WT mice (C), and DEX-treated carrageenan-treated PPAR- $\alpha$ KO mice (D). Figure is representative of three different experiments.



**Fig. 2.** Effects of DEX on the release of carrageenan-induced TNF- $\alpha$  in WT and PPAR- $\alpha$ KO mice. a, levels of TNF- $\alpha$  were detected by colorimetric, commercial enzyme-linked immunosorbent assay kit in lung tissues. Values shown are mean  $\pm$  S.E.M. of 10 mice for each group; \*,  $p < 0.01$  versus sham; °,  $p < 0.01$  versus carrageenan-WT group; °°,  $p < 0.01$  versus DEX-treated carrageenan-WT group. b, immunohistochemical localization of TNF- $\alpha$  in the lung section: carrageenan-treated WT mice (A), carrageenan-treated PPAR- $\alpha$ KO mice (B), DEX-treated carrageenan-treated WT mice (C), and DEX-treated carrageenan-treated PPAR- $\alpha$ KO mice (D). Figure is representative of three different experiments.



plays a role in DEX-mediated inhibition of carrageenan-induced TNF- $\alpha$ .

**Role of PPAR- $\alpha$  in DEX-Mediated Inhibition of Carrageenan-Induced NO and COX-2 Production.** iNOS and COX-2 are important mediators of inflammatory processes (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- $\alpha$ KO mice. Results in Fig. 3 indicate that a significant increase of iNOS and COX-2 expression was induced in the lung of WT and PPAR- $\alpha$ KO mice by carrageenan treatment (Fig. 3a). However, although treatment with DEX significantly attenuated iNOS (left) and COX-2 (right) expression of WT mice, it did not counter this increased expression in PPAR- $\alpha$ KO mice. It is noteworthy that iNOS and COX-2 induction by carrageenan administration was more marked in PPAR- $\alpha$ KO than in WT mice.

We also performed immunohistochemical analysis of lung sections obtained from carrageenan-treated WT and PPAR- $\alpha$ KO mice. As shown in Fig. 3, b and c, whereas DEX was effective in inhibiting both iNOS and nitrotyrosine in WT mice, it was less active in PPAR- $\alpha$ KO mice (Fig. 3b, C and D, and c, C and D). It is noteworthy that in carrageenan-treated PPAR- $\alpha$ KO mice, staining for iNOS (Fig. 3bB) and nitrotyrosine (Fig. 3cB) in infiltrated inflammatory cells, pneumocytes, and vascular wall was clearly increased compare with the WT mice (Fig. 3, bA and cA).

**DEX Regulated PPAR- $\alpha$  and GR Expression in Lung Tissues of WT and PPAR- $\alpha$ KO Mice.** It has been shown previously that GCs up-regulate PPAR- $\alpha$  expression (Lemberger et al., 1994). Results here indicated that DEX equally increases PPAR- $\alpha$  in lung homogenates of untreated or carrageenan-treated WT mice (Fig. 4a, columns 2 versus 4). Moreover, DEX also regulates GR expression; in fact WT, or PPAR- $\alpha$ KO mice expressed less GR mRNA upon DEX treatment (Fig. 4b). To note, GR mRNA levels were similar both in untreated or carrageenan-treated mice (Fig. 4b, columns 3 and 4 versus 7 and 8), suggesting that GR expression is not responsible for a decrease of DEX-mediated anti-inflammatory activity.

**Role of PPAR- $\alpha$  in DEX-Mediated Inhibition of Carrageenan-Induced NF- $\kappa$ B Activation.** Most inflammatory mediators, including iNOS, COX-2, interleukin-1 $\beta$ , and TNF- $\alpha$  are controlled by NF- $\kappa$ B, a transcription factor important in inflammatory process that is kept inactive by I $\kappa$ B and GILZ (Beg et al., 1993; Baldwin, 1996; Di Marco et al., 2007). Moreover, activation of NF- $\kappa$ B 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- $\kappa$ B activation in WT and PPAR- $\alpha$ KO mice.

A basal level of I $\kappa$ B- $\alpha$  was detected in the lung tissues from sham WT mice and from sham PPAR- $\alpha$ KO mice. After carrageenan treatment, I $\kappa$ B- $\alpha$  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- $\alpha$ KO mice (Fig. 5a, compare lines 5 and 6). Furthermore, carrageenan administration caused a significant increase in p65 phosphorylation at Ser536 in the lung tissues from WT and PPAR- $\alpha$ KO mice, and DEX inhibited p65 phosphorylation of WT but not of PPAR- $\alpha$ KO mice. It is noteworthy that carrageenan-induced increase of p65 phosphorylation in the lung tissues of PPAR- $\alpha$ KO was slightly more marked than that of WT mice (Fig. 5b, compare columns 4 and 3).

GILZ has been proposed as a DEX-induced protein able to bind and inhibit p65NF- $\kappa$ B activity (Di Marco et al., 2007). We evaluated, by real-time PCR, the effect of DEX treatment in WT and PPAR- $\alpha$ KO cells on GILZ expression. Results in Fig. 6a indicate that DEX induced an increase of GILZ mRNA expression in WT but not in PPAR- $\alpha$ KO mice. Moreover, this difference was even enhanced in carrageenan-treated WT mice, thus indicating that PPAR- $\alpha$  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- $\alpha$ KO mice (Fig. 6b, A and B). On the contrary, tissue sections obtained from WT and PPAR- $\alpha$ KO animals 4 h after carrageenan administration showed positive staining for GILZ localized in the infiltrating inflammatory cells (Fig. 6b, C and 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; see also arrows in 6bE1) and in the vascular endothelial cells (see arrows, 6bE2). On the contrary, the absence of a functional PPAR- $\alpha$  gene in PPAR- $\alpha$ KO mice resulted in a clear reduction of this DEX-induced GILZ expression (Fig. 6bF). Together, these results indicate that PPAR- $\alpha$  plays a role in DEX-induced I $\kappa$ B and GILZ up-regulation and in NF- $\kappa$ B inhibition.

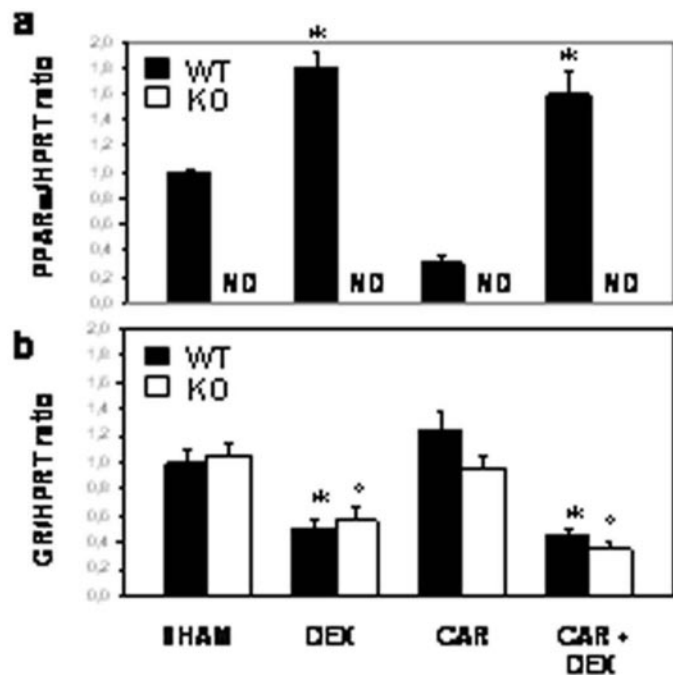
**Combination Therapy with Clofibrate and DEX Reduces Pleural Infiltration of Macrophages, Lung Infiltration of PMNs, and Lung Injury.** Previous results suggest that PPAR- $\alpha$  can favor DEX anti-inflammatory activity. We performed experiments to evaluate the possible effect of combination therapy with clofibrate, a PPAR- $\alpha$  agonist, and DEX on carrageenan-induced lung injury in WT mice. In a preliminary set of experiments, we performed dose-response experiments with clofibrate and DEX. Results indicated that a combination of clofibrate and DEX significantly reduced, in a dose-dependent manner, the PMN infiltration in the pleural cavity and lung MPO activity (see Supplementary Fig. S2). 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, PMN infiltration in the pleural cavity, and lung MPO activity (see Supplementary Fig. S2). Moreover, results of histology in Fig. 7a also confirm that although clofibrate (at a 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

**Fig. 3.** a, representative Western blot analysis of iNOS (left) and COX-2 (right) expression from lung tissue of carrageenan-treated WT and PPAR- $\alpha$ KO mice with or without DEX administration. Shown immunoblots are representative of one of three different experiments. The bottom of each Western blot is represented a densitometry analysis expressed as mean  $\pm$  S.E.M. of three different experiments. \*,  $p < 0.01$  versus Sham; °,  $p < 0.01$  versus carrageenan-WT group. b, effects of DEX on carrageenan-induced iNOS expression (b) or nitrotyrosine formation (c) evaluated by immunohistochemistry: carrageenan-treated WT mice (A), carrageenan-treated PPAR- $\alpha$ KO mice (B), DEX-treated carrageenan-treated WT mice (C); and DEX-treated carrageenan-treated PPAR- $\alpha$ KO mice (D). Figure is representative of three different experiments.

tissue injury, combination of the two agents (clofibrate + DEX) resulted in a detectable inhibition (Fig. 7aD).

The above histological alteration in the lung seemed to correlate with the influx of leukocytes into the lung tissue and 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. PMN 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. PMN infiltration in the pleural cavity and lung MPO activity was significantly reduced in carrageenan-treated mice that received intraperitoneal 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 intraperitoneal injection of clofibrate or DEX alone (Fig. 7b). These results suggest that contemporary agonist stimulation of PPAR- $\alpha$  and GR results in a potentiated anti-inflammatory activity.

**Effect of DEX on Macrophage Inflammatory Markers.** Together, the above results indicate that PPAR- $\alpha$  can favor 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- $\alpha$  gene deletion on DEX anti-inflammatory property, we carried out in vitro experiments using elicited peritoneal macrophages from both WT and PPAR- $\alpha$ KO mice. In particular, macrophages were treated with LPS (10  $\mu$ g/ml) and IFN- $\gamma$  (100 U/ml) for 24 h. As shown in Fig. 8, unstimulated (vehicle) peritoneal macrophages from WT or PPAR- $\alpha$ KO mice



**Fig. 4.** Effect of DEX on GR and PPAR- $\alpha$  expression. a, PPAR- $\alpha$  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 under *Materials and Methods*. PCR analysis was assessed in triplicate. Results are expressed as mean  $\pm$  S.E.M. \*,  $p < 0.01$  versus SHAM WT group; \*\*,  $p < 0.01$  versus SHAM PPAR- $\alpha$ KO group.

produced small amounts of measurable nitrite, the stable end product of NO. Activation of the cells with LPS and IFN- $\gamma$  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- $\alpha$ KO mice incubated with LPS and IFN- $\gamma$  compared with the corresponding WT cells. When DEX (10  $\mu$ M) was added to WT macrophages, 2 h before LPS and IFN- $\gamma$  stimulation, a significant decrease of nitrite/nitrate production in cell medium was observed (Fig. 8a, compare columns 7 and 5). On the contrary, the absence of a functional PPAR- $\alpha$  gene in macrophages from PPAR- $\alpha$ KO resulted in a lack of DEX effect (Fig. 8a, compare columns 8 and 6).

We also performed experiments to determine the DEX effect on LPS and IFN- $\gamma$ -induced COX-2 protein expression. To that purpose, COX-2 protein expression in whole lysates from peritoneal WT or PPAR- $\alpha$ KO macrophages stimulated with LPS and IFN- $\gamma$  and pretreated with DEX, as described above, was evaluated. As shown in Fig. 8b, LPS and IFN- $\gamma$  caused a significant increase of COX-2 protein levels at 24 h compared with unstimulated cells. When WT cells were pretreated with DEX (1 and 10  $\mu$ M), 2 h before LPS and IFN- $\gamma$  stimulation, COX-2 protein expression was inhibited in a concentration-dependent manner with respect to LPS and IFN- $\gamma$ -stimulated cells (Fig. 8b). The absence of a functional PPAR- $\alpha$  gene in macrophages from PPAR- $\alpha$ KO 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- $\kappa$ B activation (Sakurai et al., 2003; Buss et al., 2004). As shown in Fig. 8c, DEX treatment completely inhibited NF- $\kappa$ B activation in macrophages of WT mice whereas only partially in macrophages of PPAR- $\alpha$ KO mice, further suggesting that PPAR- $\alpha$  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 Fig. 7. To that purpose we evaluated COX-2 mRNA levels macrophages from WT and PPAR- $\alpha$ KO mice, activated by LPS and IFN- $\gamma$ , treated with DEX (0.01  $\mu$ M) or DEX (0.01  $\mu$ M) plus clofibrate (0.2 mM). Results in Fig. 8d indicate that clofibrate increased DEX-inhibiting activity of COX-2 expression. It is noteworthy that clofibrate alone (0.2 mM) did not change COX-2 expression levels. These results were further confirmed in dose-response experiments showing that a combination of DEX and clofibrate results in a significant COX-2 inhibition (see Supplementary Fig. S3).

**PPAR- $\alpha$  Reconstitution Recovers PPAR- $\alpha$ KO Macrophage Susceptibility to DEX Treatment.** Finally, as further control, macrophages from PPAR- $\alpha$ KO mice were used in transfection experiments aimed to reconstitute PPAR- $\alpha$  expression. As shown by results in Fig. 9a, PPAR- $\alpha$  expression in cells of PPAR- $\alpha$ KO 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 versus 5–7). GR expression levels were also tested in WT macrophages treated or not with siRNA specific for PPAR- $\alpha$ , and no detectable differences were found (Fig. 9d, columns 2–4 versus 5–7). All together, these in vitro experiments clearly indicate that PPAR- $\alpha$  contributes to the anti-inflammatory activity of



DEX, independent of possible changes in GR expression, and are in agreement with results indicating a synergism (Figs. 7 and 8d) of DEX and PPAR- $\alpha$  agonist combination treatment.

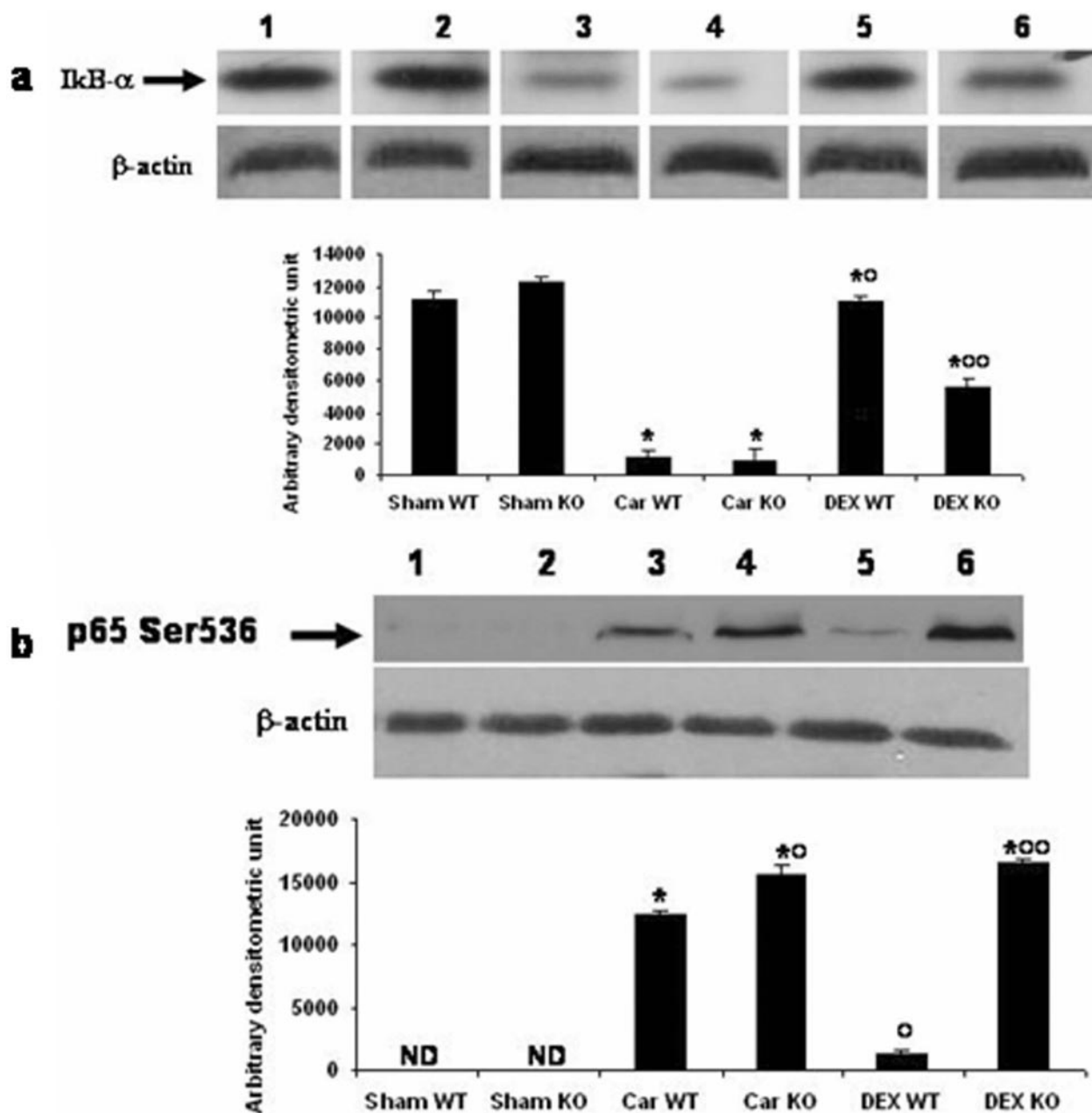
### Discussion

In the present article, we show that the absence of PPAR- $\alpha$  in PPAR- $\alpha$ KO 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- $\alpha$  agonist.

These results are in agreement with our previous observations indicating that PPAR- $\alpha$ KO mice are more susceptible to the induction of inflammation, possibly due to a less efficient

physiological anti-inflammatory control exerted by endogenous GCs (Delerive et al., 1999; Lovett-Racke et al., 2004; Genovese et al., 2005; Okamoto et al., 2005). We here confirm these observations for some of the inflammatory parameters such as cell tissue infiltrate, TNF- $\alpha$  production, iNOS, and COX-2 activity.

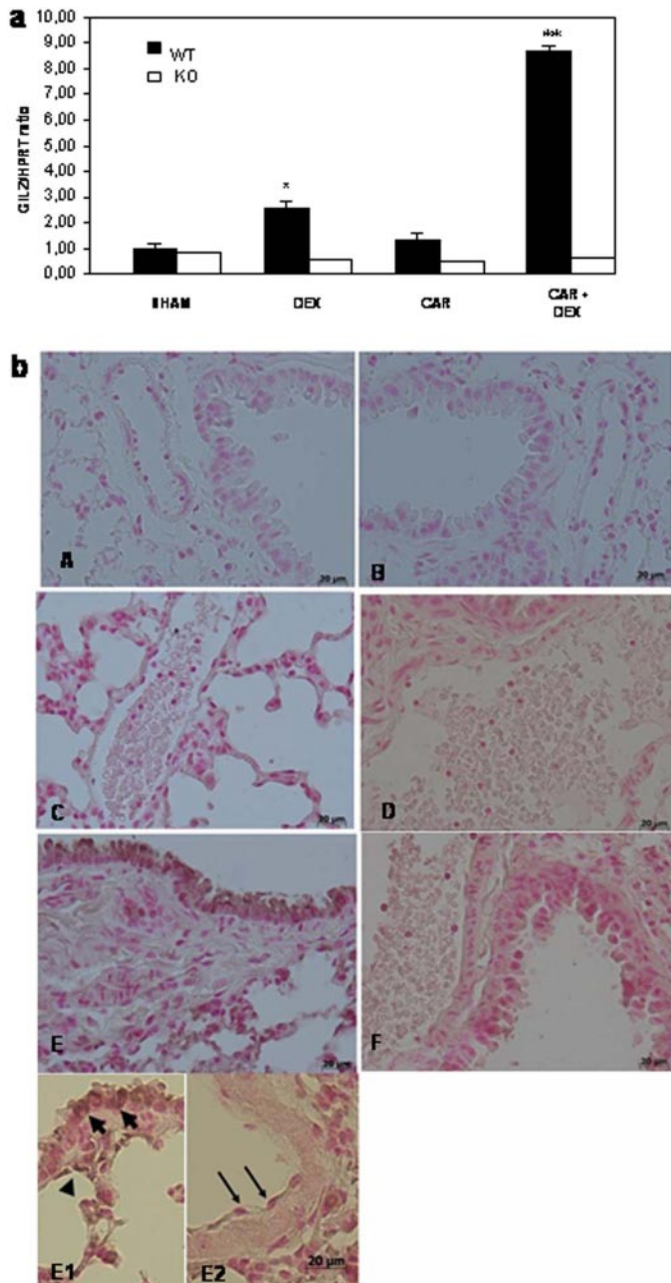
GCs, including DEX, are potent anti-inflammatory agents and for that reason are used in the treatment of a number of human diseases. Their efficacy resides in part in the capability to counter NF- $\kappa$ B activation, an important transcription factor in inflammation, production of cytokines relevant to the inflammatory process such as TNF- $\alpha$ , induction of iNOS and COX-2, and increase of their enzymatic activity (Yamagata et al., 1993; Auphan et al., 1995; Scheinman et al.,



**Fig. 5.** Effect of DEX on carrageenan-induced NF- $\kappa$ B activation in lung tissue from WT and PPAR- $\alpha$ KO mice. Representative Western blot analysis of I $\kappa$ B- $\alpha$  expression (a) and NF- $\kappa$ B/p65 phosphorylation on Ser536 (b) in the lung tissues. Immunoblots shown are representative of one of three different experiments. The bottom of each Western blot is represented a densitometry analysis expressed as mean  $\pm$  S.E.M. of three different experiments. \*,  $p < 0.01$  versus sham; <sup>o</sup>,  $p < 0.01$  versus carrageenan-WT group; <sup>oo</sup>,  $p < 0.01$  versus DEX-treated carrageenan-WT group.

1995). In addition, GCs inhibit adhesion molecules, such as ICAM-1, and the consequent PMN and macrophage migration into the inflamed tissue.

PPAR- $\alpha$  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 mech-



**Fig. 6.** Effects of DEX on GILZ expression in lung from WT and PPAR- $\alpha$ KO 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 under *Materials and Methods*. PCR analysis was assessed in triplicate. Results are expressed as mean  $\pm$  S.E.M.; \*,  $p < 0.01$  versus SHAM; \*\*,  $p < 0.01$  versus carrageenan-treated WT group. **b**, Immunohistochemical localization of GILZ in the lung: sham WT mice (A), sham PPAR- $\alpha$ KO mice (B), carrageenan-treated WT mice (C); carrageenan-treated PPAR- $\alpha$ KO mice (D), DEX-treated carrageenan-treated WT mice (E-E1-E2), and DEX-treated carrageenan-treated PPAR- $\alpha$ KO mice (F). Figure is representative of three different experiments.

anisms, including TNF- $\alpha$  production, iNOS, COX-2, and adhesion molecule expression, as well as cell infiltration in the tissues (Neve et al., 2001; Lovett-Racke et al., 2004; Genovese et al., 2005).

Based on these observations, we performed studies in an attempt to determine whether the presence and/or the stimulation of PPAR- $\alpha$  could enhance the GC's anti-inflammatory efficacy. For that purpose, we used an experimental model of acute lung inflammation performed by treating WT and PPAR- $\alpha$ KO mice with carrageenan and tested the anti-inflammatory efficacy of DEX, alone or in combination with clofibrate, a PPAR- $\alpha$  agonist.

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

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

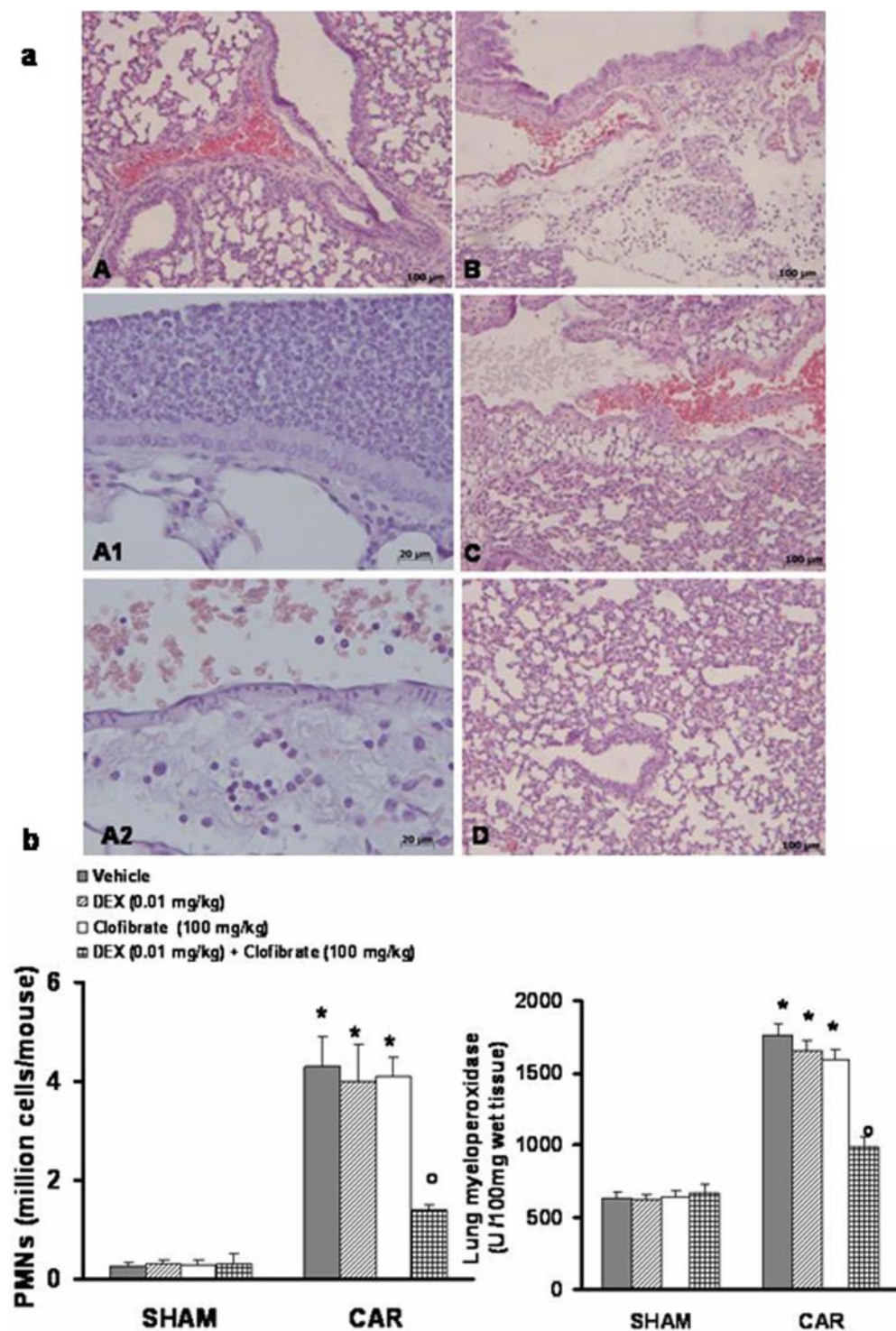
Up-regulation and activation of COX-2 and iNOS, with consequent nitrite/nitrate production, are typical characteristics of the inflammatory process (Luss et al., 1994; Weinberg et al., 1995). GCs can counter these effects, and we here show that whereas DEX treatment significantly inhibited those inflammatory parameters in WT mice, it did not in PPAR- $\alpha$ KO mice. It is noteworthy that a lack of DEX-induced COX-2 inhibition was also evident in experiments with isolated peritoneal macrophages from PPAR- $\alpha$ KO mice. Most important, when macrophages from PPAR- $\alpha$ KO mice were transfected with PPAR- $\alpha$ , to reconstitute its expression, they regained the susceptibility to DEX-induced inhibition of COX-2 expression. It is noteworthy that these results could also suggest a role of PPAR- $\alpha$  on functions, including in vivo interaction with vessel walls that takes place during inflammation (Barlic et al., 2006).

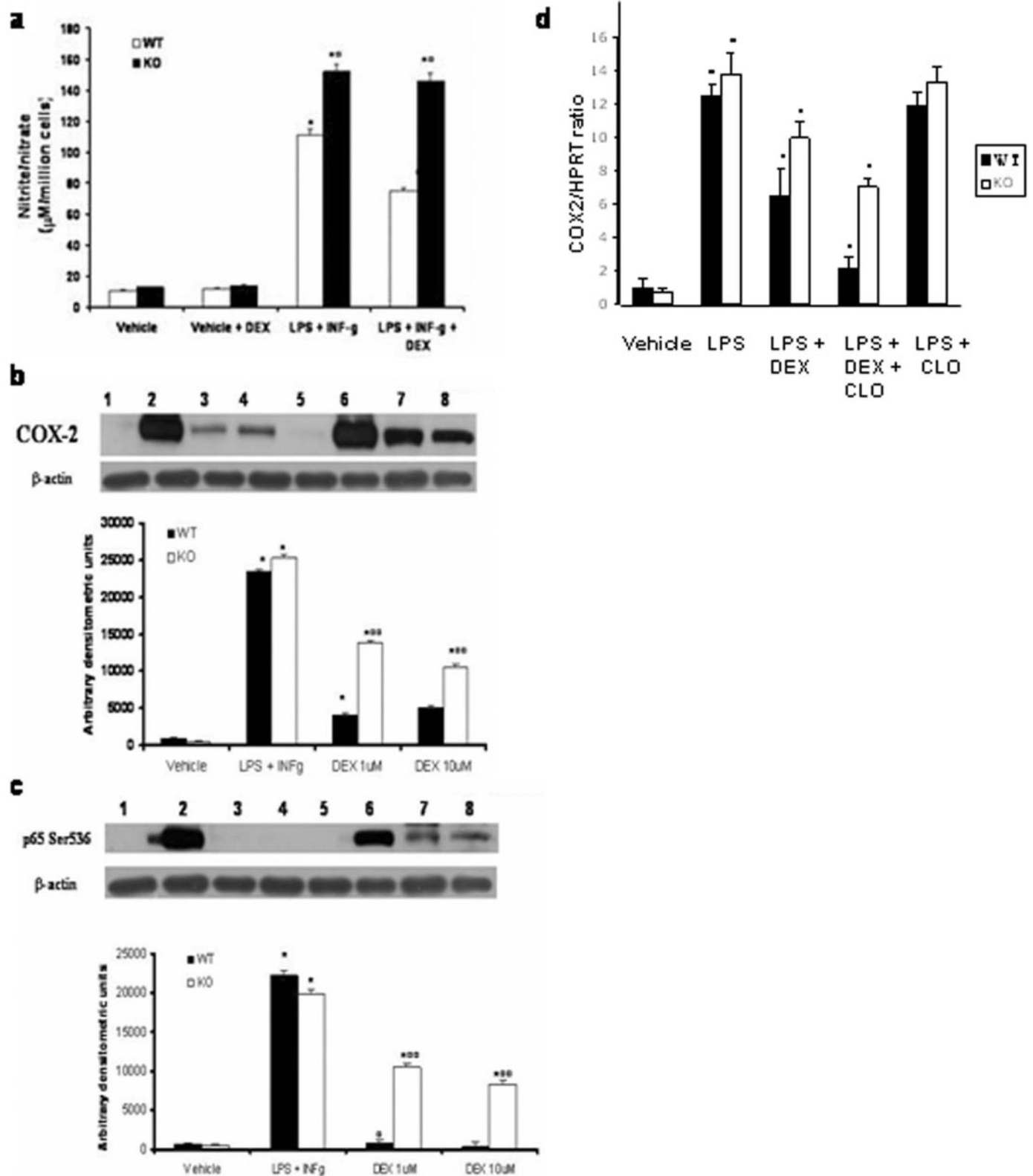
The results described here clearly indicate that the anti-inflammatory efficacy of DEX treatment is favored by the presence of PPAR- $\alpha$ . Besides, DEX regulates GR and PPAR- $\alpha$  expression, but their levels are similar in healthy or carrageenan-induced pleurisy mice, either WT or PPAR- $\alpha$ KO, thus indicating that impaired DEX anti-inflammatory activity in PPAR- $\alpha$ KO mice is not due to modulation of GR or PPAR- $\alpha$  expression. Moreover, previous studies showed that PPAR- $\alpha$  agonists exert some anti-inflammatory activity

(Colville-Nash et al., 1998; Lovett-Racke et al., 2004; Genovese et al., 2005). For that reason, we performed experiments to test whether the combination of DEX with clofibrate, a PPAR- $\alpha$  agonist agent, could result in a more efficient anti-inflammatory effect. Results described here indicate that in vivo cotreatment with suboptimal doses of DEX and clofibrate was synergic in the inhibition of a number of inflammatory events in WT mice, including PMN infiltration into the lung tissue. In accordance with this finding, in vitro treatment of WT isolated peritoneal

macrophages with clofibrate significantly increased the DEX-induced inhibition of COX-2 expression.

The efficacy of GC treatment in inflammatory and autoimmune diseases is an important therapeutic subject, and although some patients obtain clinical improvements from treatment, others are not responsive or are even resistant to therapy. As an example, it is known that only a certain percentage of patients affected by inflammatory bowel diseases are cured by GC treatment, whereas others are not (Orii et al., 2002; Farrell and Kelleher, 2003; Sands, 2007).



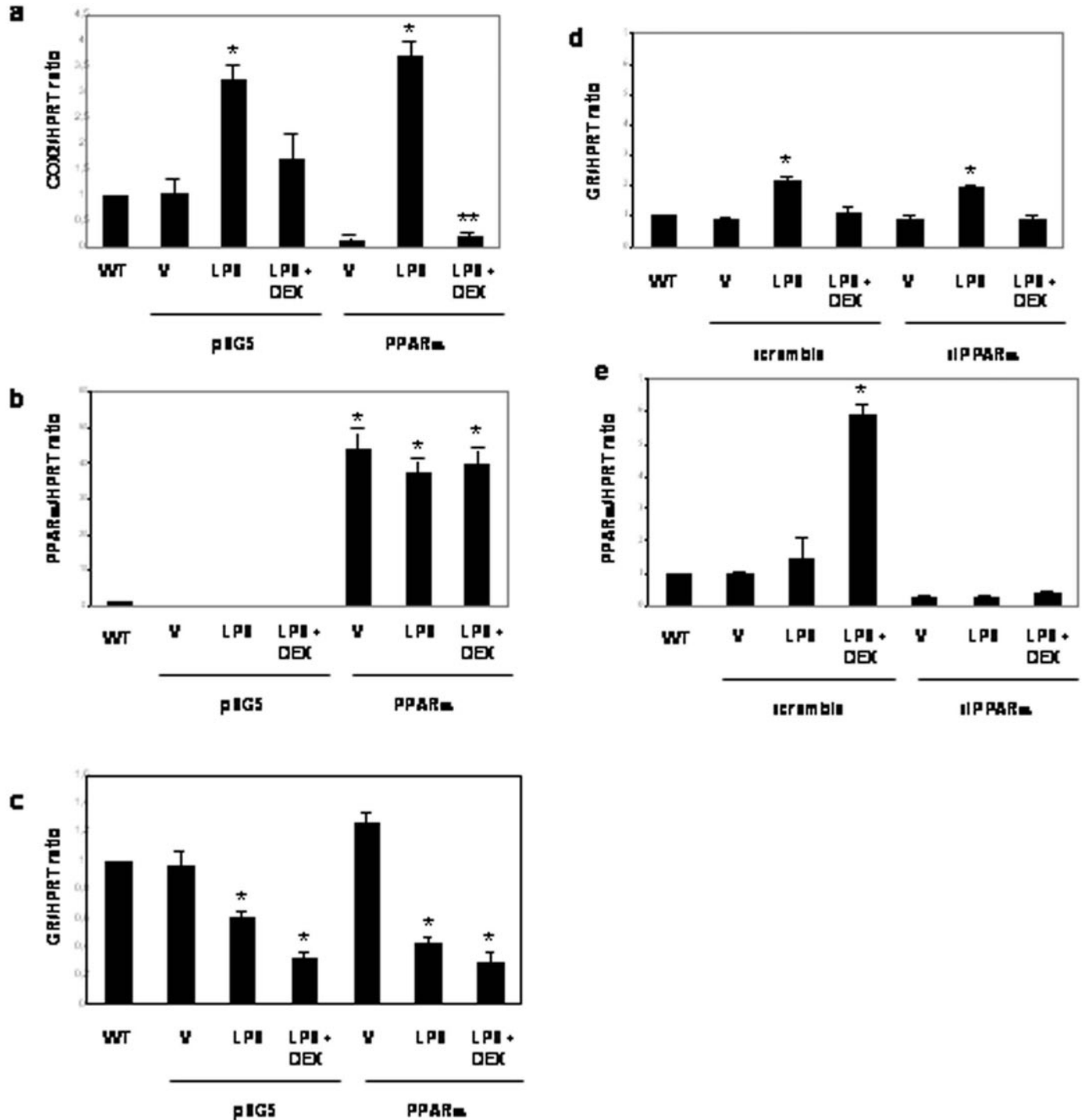


**Fig. 8.** Effects of DEX in macrophages from WT and PPAR- $\alpha$ KO mice stimulated in vitro by LPS and IFN- $\gamma$ . a, nitrite/nitrate production evaluated as described under *Materials and Methods*. b, COX-2 expression assessed by Western blot analysis. c, NF- $\kappa$ B activation evaluated by Western blot analysis for phosphorylation of Ser536. Immunoblots shown are representative of one of three experiments analyzed. The bottom of each Western blot is a densitometry analysis expressed as mean  $\pm$  S.E.M. of three different experiments. d, effects of DEX and PPAR- $\alpha$  agonist clofibrate on COX-2 mRNA expression, evaluated by real-time PCR, in macrophages from WT and PPAR- $\alpha$ KO mice stimulated in vitro by LPS and IFN- $\gamma$ . PCR analyses were assessed in triplicate. Results are expressed as mean  $\pm$  S.E.M.; \*,  $p < 0.01$  versus vehicle-treated macrophages; \*\*,  $p < 0.01$  versus LPS- and IFN- $\gamma$ -treated WT macrophages; \*\*\*,  $p < 0.01$  versus LPS-, IFN- $\gamma$ -, and DEX-treated WT macrophages.

The reasons for response or no response to therapy are not fully understood, and molecular mechanisms, such as change of the ratio between different GR isoforms and pre-existing levels of NF- $\kappa$ B and heat-shock proteins have been drawn out in an attempt to explain and predict sensitivity and resistance (Chikanza, 2002; Wikström, 2003; Meduri et al., 2005).

Results here suggest a new mechanism contributing to determine the full GC efficacy and suggest future studies aimed at analyzing the possible relevance of PPAR- $\alpha$  in other human inflammatory disease models, such as sepsis and experimental colitis.

In conclusion, our results clearly indicate that PPAR- $\alpha$  can



**Fig. 9.** Effects of DEX in macrophages from WT and PPAR- $\alpha$ KO mice stimulated in vitro by LPS and IFN- $\gamma$  for COX-2, GR, and PPAR- $\alpha$  expression. COX-2 (a), GR (b), or PPAR- $\alpha$  (c) expression evaluated by real-time PCR in macrophages from PPAR- $\alpha$ KO mice transfected with empty vector (pSG5) or PPAR- $\alpha$  expression vector. GR (d) or PPAR- $\alpha$  (e) expression evaluated by real-time PCR in macrophages from PPAR- $\alpha$ KO mice transfected with scramble oligonucleotides or siRNA oligonucleotides specific for PPAR- $\alpha$ . In the first line, arbitrary unit was given to WT macrophages mRNA expression for each experiment. V, vehicle treated-macrophages; LPS, LPS- and IFN- $\gamma$ -treated macrophages; LPS + DEX, LPS-, IFN- $\gamma$ -, and DEX-treated macrophages as described under *Materials and Methods*. PCR analyses were assessed in triplicate. Results are expressed as mean  $\pm$  S.E.M. \*,  $p < 0.01$  versus vehicle-treated macrophages; \*\*,  $p < 0.01$  versus LPS + DEX-treated and pSG5 transfected macrophages.

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- $\alpha$  agonists in inflammatory diseases.

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