

Estrogen Receptor Antagonist Fulvestrant (ICI 182,780) Inhibits the Anti-Inflammatory Effect of Glucocorticoids

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ABSTRACT

The glucocorticoid receptor (GR) and estrogen receptor (ER) play important roles in both physiological and pathological conditions involving cell growth and differentiation, lipolysis, control of glucose metabolism, immunity, and inflammation. In fact, recent studies suggest that 17β -estradiol, like glucocorticoids, may also have anti-inflammatory properties, even if the molecular mechanisms responsible for these activities have not yet been completely clarified. The present study was designed to gain a better understanding of the possible cross-talk between GR and ER in a model of lung inflammation (carrageenan-induced pleurisy). In particular, we have investigated whether fulvestrant (ICI 182,780), a selective ER- α antagonist, is able to attenuate the well known anti-inflammatory effect of dexamethasone (DEX), a synthetic glucocorticoid, in ovariectomized rats. We show that ICI 182,780, a selective ER- α antag-

onist, reverses the anti-inflammatory activity exhibited by DEX. Moreover, the coadministration of ICI 182,780 significantly inhibited the ability of DEX to reduce: 1) the degree of lung injury, 2) the rise in myeloperoxidase activity, 3) the increase of poly-(ADP-ribose) polymerase activity, tumor necrosis factor α , and interleukin- 1β levels, 4) inducible nitric-oxide synthase, 5) lipid peroxidation, 6) nitrotyrosine formation, 7) cyclooxygenase expression, and 8) the I κ B- α degradation caused by carrageenan administration. In addition, quantitative PCR shows that DEX down-regulates GR and up-regulates glucocorticoid-induced leucine zipper levels, whereas ICI 182,780 does not counteract these effects. In conclusion, these results suggest that the in vivo anti-inflammatory property of DEX is also related to the ER- α .

The immune and inflammatory responses are complex physiological processes that are critically important to the homeostasis and ultimate survival of an organism. Their coordinate regulation must be assured to allow appropriate and timely immune reaction without an over-reaction that might cause damage to the host. The inflammatory process is invariably characterized by a production of prostaglandins,

leukotrienes, histamine, bradykinin, platelet-activating factor and by a release of chemicals from tissues and migrating cells (Vane and Botting, 1987). Carrageenan-induced local inflammation is commonly used to evaluate the efficacy of nonsteroidal anti-inflammatory drugs. Therefore, carrageenan-induced local inflammation (pleurisy) is a useful model to assess the contribution of mediators involved in vascular changes associated with acute inflammation. 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

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ABBREVIATIONS: COX, cyclooxygenase; E₂, estradiol; ER, estrogen receptor; ICI 182,780, fulvestrant; IL, interleukin; OVX, ovariectomy/ovariectomized; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; MDA, malondialdehyde; DEX, dexamethasone; MPO, myeloperoxidase; TNF- α , tumor necrosis factor α ; ROS, reactive oxygen species; PAR, poly(ADP-ribose); iNOS, inducible nitric-oxide synthase; PCR, polymerase chain reaction; RT, reverse transcription/transcriptase; GILZ, glucocorticoid-induced leucine zipper; bp, base pair(s); GR, glucocorticoid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase; AP-1, activator protein-1.

by a late phase (1–6 h) mainly sustained by prostaglandin release and more recently has been attributed to the induction of inducible cyclooxygenase-2 (COX-2) in the tissue (Nantel et al., 1999). The onset of the carrageenan 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., 1996).

We have proposed that endogenous and exogenous estrogens exert anti-inflammatory activity (Cuzzocrea et al., 2000b, 2001b). This concept is now well accepted and has the support of several investigations carried out in a variety of models of inflammation that have demonstrated that estradiol (E_2) suppresses inflammation by inhibiting the expression of multiple genes encoding cytokines, enzymes and adhesion molecules (Cuzzocrea et al., 2001b; Vegeto et al., 2003; Ghisletti et al., 2005). Yet the exact mechanism underlying ER anti-inflammatory effects remains to be elucidated. Using the carrageenan-induced pleurisy model, we have also pointed out that the observed anti-inflammatory effect of E_2 is partially dependent to the complexes with its cognate receptors (ER). In fact, the coadministration of E_2 and tamoxifen or ICI 182,780 significantly blocked the effect of E_2 (Cuzzocrea et al., 2000b).

Other steroids, such as glucocorticoids, are commonly used as therapeutic agents in many acute and chronic inflammatory and autoimmune diseases, in patients who have undergone transplants, and in the treatment of leukemias and lymphomas (Cupps et al., 1982; Barnes and Adcock, 1993). Their therapeutic action has largely been attributed to their anti-inflammatory and immunosuppressive efficacy and to their growth arrest and death-inducing activity (Riccardi et al., 2002).

Similarly to estrogens, glucocorticoids bind to GR that then dimerize and regulate the transcription rate of target genes by binding to specific DNA sequences named glucocorticoid response elements. Glucocorticoids increase the transcription of genes coding for anti-inflammatory proteins, including lipocortin-1, IL-10, IL-1 receptor antagonist, and neutral endopeptidase, but this is unlikely to account for all of the widespread anti-inflammatory actions of glucocorticoids. The effect of glucocorticoids is to inhibit the expression of multiple inflammatory genes (cytokines, enzymes, receptors, and adhesion molecules). This cannot be due solely to a direct interaction between GR and glucocorticoid response elements, because these binding sites are absent from the promoter regions of most inflammatory genes, and it is more likely to be due to a direct inhibitory interaction between activated GR and activated transcription factors, such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), which regulate the inflammatory gene expression. Both GR and ER exert important actions in tissues other than their primary target tissues. In tissues that express both receptors, glucocorticoids often oppose the actions of estrogens. For example, in the mammary gland, glucocorticoids exert antiproliferative effects, whereas estrogens promote cell growth and proliferation (Zhou et al., 1989; Sutherland et al., 1998). In contrast, in bone, glucocorticoids induce bone resorption (Rackoff and Rosen, 1998), whereas estrogens inhibit this action (Gallagher et al., 2001).

Therefore, there is potential for ER/GR interactions at the

level of transcription in numerous cell lines and tissue types (Uht et al., 1997). The cross-talk between the GR and ER in conditions associated with acute inflammation, however, has not yet been investigated.

Materials and Methods

Animals. Female Sprague-Dawley rats (300–350 g; Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1; Dec. 18, 1986).

Ovariectomy. We have demonstrated that endogenous estrogen has anti-inflammatory properties (Cuzzocrea et al., 2001b). This conclusion was based on the observation that inflammatory response in ovariectomized rats was significantly more severe than in intact rats (Cuzzocrea et al., 2001b). Moreover, to study the role of estrogen in inflammation, we have created an estrogen insufficiency by ovariectomy as described previously (Cuzzocrea et al., 2001b). In brief, all surgical procedures were performed under halothane (2%) anesthesia followed by nitrous oxygen/ O_2 anesthesia for approximately 18 min. Ovariectomy (OVX) was performed through a single dorsal midline cutaneous incision followed by bilateral muscle incisions.

Experimental Groups. OVX animals were randomly allocated into the following groups:

CAR + vehicle group. Rats were subjected to carrageenan-induced pleurisy and received the vehicle for DEX (saline i.p. bolus) 30 min before carrageenan ($n = 10$).

DEX 0.25 group. Same as the CAR + vehicle group, but these rats were administered DEX (0.25 mg/kg i.p. bolus) 30 min before carrageenan ($n = 10$).

DEX 0.50 group. Same as the CAR + vehicle group, but these rats were administered DEX (0.5 mg/kg i.p. bolus) 30 min before carrageenan ($n = 10$).

ICI 182,780 group. Same as the CAR + vehicle group, but ICI 182,780 was administered (500 μ g/kg i.p. bolus) to these rats 1 and 30 min before carrageenan administration ($n = 10$).

ICI 182,780 + DEX 0.25 groups. Same as the DEX 0.25 group but ICI 182,780 was administered (500 μ g/kg i.p. bolus) to these rats 1 h before DEX (0.25 mg/kg i.p.) ($n = 10$).

ICI 182,780 + DEX 0.50 groups. Same as the DEX 0.50 group but ICI 182,780 was administered (500 μ g/kg i.p. bolus) to these rats 1 h before DEX (0.50 mg/kg i.p.) ($n = 10$).

Tamoxifen group. Same as the CAR + vehicle group, but tamoxifen was administered (50 μ g/kg i.p. bolus) to these rats 1 and 30 min before carrageenan administration ($n = 10$).

Tamoxifen + DEX 0.5 groups. Same as the DEX 0.5 group, but tamoxifen was administered (50 μ g/kg i.p. bolus) to these rats 1 h before DEX (0.5 mg/kg i.p.) ($n = 10$).

Sham + saline group. Sham-operated group; the surgical procedures were identical to those in the CAR group, except that saline was administered instead of carrageenan.

Sham + DEX 0.25 group. Identical to sham + saline group except for the administration of DEX (0.25 mg/kg i.p. bolus) 30 min before identical surgical procedures ($n = 10$).

Sham + DEX 0.50 group. Identical to sham + saline group except for the administration of DEX (0.50 mg/kg i.p. bolus) to these rats 30 min before identical surgical procedures ($n = 10$).

Sham + ICI 182,780. Identical to sham + saline group except for the administration of ICI 182,780 (500 μ g/kg i.p.) to these rats ($n = 10$).

Sham + ICI 182,780 + DEX 0.25 groups. Same as the sham + DEX 0.25 group but ICI 182,780 was administered (500 μ g/kg i.p. bolus) to these rats 1 h before DEX (0.25 mg/kg i.p.) ($n = 10$).

Sham + ICI 182,780 + DEX 0.50 groups. Same as the sham + DEX 0.50 group but ICI 182,780 was administered (500 μ g/kg i.p. bolus) to these rats 1 h before DEX (0.50 mg/kg i.p.) ($n = 10$).

Sham + Tamoxifen. Identical to sham + saline group except for the administration of Tamoxifen (50 µg/kg i.p.) to these rats ($n = 10$).

Sham + Tamoxifen + DEX. Same as the DEX 0.5 group but tamoxifen was administered (50 µg/kg i.p. bolus) to these rats 1 h before DEX (0.5 mg/kg i.p.) ($n = 10$).

The dose and the time of treatment of ICI 182,780 (500 µg/kg) and DEX was based on studies in our and other laboratories in a model of carrageenan-induced pleurisy (Miyasaka and Mikami, 1982; Cuzzocrea et al., 2000b).

Carrageenan-Induced Pleurisy. Rats 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 (0.2 ml) or saline containing 1% λ -carrageenan (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO₂. The chest was carefully opened, and the pleural cavity rinsed with 2 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 µg/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The inflammatory cells (approximately 70% of macrophages) in the pleural exudate were suspended in PBS and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

Histological Examination. Lung biopsies were taken at 4 h after injection of carrageenan. The biopsies were fixed for 1 week in buffered formaldehyde solution (10% in PBS) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (7 µm) were deparaffinized with xylene, stained with hematoxylin and eosin, and studied using light microscopy (Leitz Dialux 22; Leica, Wetzlar, Germany).

Determination of MPO Activity. MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as described previously (Mullane et al., 1985). At 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 H₂O₂. 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/min at 37°C and was expressed in units per gram weight of wet tissue.

Malondialdehyde Measurement. Thiobarbituric acid-reactant substance measurement, which is considered a good indicator of lipid peroxidation in the lung tissues, was carried out as described previously (Ohkawa et al., 1979). Tissues, collected 4 h after carrageenan administration, were homogenized in 1.15% KCl solution. An aliquot (100 µl) of the homogenate was added to a reaction mixture containing 200 µl of 8.1% SDS, 1500 µl of 20% acetic acid, pH 3.5, 1500 µl of 0.8% thiobarbituric acid, and 700 µl of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000g for 10 min. The optical density at 650 nm was measured using an ELISA microplate reader (SLT-Labinstruments, Salzburg, Austria). Thiobarbituric acid-reactant substances were 1,1,3,3-tetramethoxypropan, 99%, and malondialdehyde (MDA) bis (dimethyl acetyl), 99%, was from Sigma, (Milan, Italy).

Measurement of Cytokines. TNF- α and IL1- β levels were evaluated in the exudate 4 h after the induction of pleurisy by carrageenan injection as described previously (Cuzzocrea et al., 2000a). The assay was carried out using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy) with a lower detection limit of 10 pg/ml.

Nitrite/Nitrate (NO_x) Assay. NO reacts with oxygen to yield nitrate and nitrite. We therefore measured these breakdown prod-

ucts of NO by means of the fluorometric assay for the measurement of nitrite as described previously (Cuzzocrea et al., 2001a). In brief, the nitrate in the sample was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and 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) naphthylethylenediamide dihydrochloride in H₂O and 1% (w/v) sulfanilamide in 5% (v/v) concentrated H₃PO₄; volume, 1:1] to the 100-µl sample. Nitrite concentrations in these samples were determined fluorimetrically, with sodium nitrite used as a standard. The optical density at 550 nm was measured using an ELISA microplate reader (SLT-Lab Instruments). All determinations were performed in duplicate, and results were expressed as NO_x levels in pleural exudates (nanomoles per rat).

Immunohistochemical Localization of iNOS, Nitrotyrosine, and PARP. Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or reactive oxygen species (ROS), was determined by immunohistochemistry as described previously (Cuzzocrea et al., 2001a). At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 7-µm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody [1:500 in PBS (v/v)] or with anti-poly(ADP-ribose) (PAR) goat polyclonal antibody rat [1:500 in PBS (v/v)] or with anti-iNOS monoclonal antibody mouse [1:500 in PBS (v/v)] Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). To confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for PAR or iNOS, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out. Immunocytochemistry photographs ($n = 5$) were assessed by densitometry. The assay was carried out by using Optilab image analysis software (Graftec, Mendon-la-Forêt, France) on a Macintosh computer with a G3 CPU running at 266 MHz.

Real-Time PCR. Total RNA was prepared using TRIzol (Invitrogen, Paisley, Scotland). RT-PCR was done using Moloney murine leukemia virus RT (Invitrogen, San Diego, 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 ER- α yield an amplification product of 124 bp (sense, 5'-GATGGGCTTATTGACCAACC-3'; antisense, 5'-CCAGGCACACTCCAGGAG-3'); for glucocorticoid-induced leucine zipper (GILZ), give rise to a 235-bp fragment (sense, 5'-GGTGGCCCTAGACAACAAGA-3'; antisense, 5'-GTTTCTGGGGCTTCAGGAG-3'); for GR, give a 140-bp fragment (sense, 5'-AAGTGAATAGGTGCCAAGG-3'; antisense, 5'-GAGCTGGATGGAGGAGAGC-3'); and for GAPDH, produce a 287-bp fragment (sense, 5'-GCCTTCCGTGTTCTACCC-3'; antisense, 5'-AGGTCCACCACCTGTTGC-3'). PCR was done in Chromo 4 (Bio-Rad Laboratories, Milan, Italy) using DyNAmo HS SYBR GREEN qPCR kit (Finnzymes Oy, 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 relative amounts of ER, GILZ, GR, and GAPDH mRNA, the comparative $\Delta\Delta C_t$ method was used. The C_t values were determined using the Opticon Monitor 2 software (Bio-Rad). Gene expression was normalized to GAPDH housekeeping mRNA expression.

Western Blot Analysis for iNOS, COX-2, and I κ B- α expression. Lung tissue was homogenized in a buffer containing: 20 mM HEPES, 1.5 mM MgCl₂, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 15 μ g/ml trypsin inhibitor, 3 μ g/ml pepstatin, 2 μ g/ml leupeptin, 40 μ M benzylamine, 1% Nonidet P-40, and 20% glycerol. Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (70 μ g) were dissolved in Laemmli's sample buffer, boiled, and run on a SDS-polyacrylamide minigel (8% polyacrylamide) and then transferred for 40 min at 5 mA/cm² into a 0.45- μ m Hybond polyvinylidene difluoride membrane. Membranes were blocked for 40 min in PBS and 5% (w/v) nonfat milk and subsequently probed overnight at 4°C with mouse monoclonal anti-iNOS (1:10,000; BD Transduction Laboratories, Lexington, KY), anti-COX-2 (1:500; Cayman Chemical, Ann Arbor, MI) antibodies [in PBS; 5% (w/v) nonfat milk and 0.1% Tween 20], or I κ B- α (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) or peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. Immunoreactive bands were visualized using electrochemiluminescence assay detection system according to the manufacturer's instructions. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against α -tubulin protein (1:10,000; Sigma-Aldrich Corp.). Subsequently, the relative expression of the protein bands of iNOS (~130 kDa), COX-2 (~72 kDa), and I κ B- α (~37 kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700; Bio-Rad Laboratories) and a computer program (Molecular Analyst; IBM, White Plains, NY) were quantified by scanning densitometry (GS-700; Bio-Rad Laboratories).

Measurement of Prostaglandin E₂ Levels in the Exudate. The amount of prostaglandin E₂ in the pleural exudates was measured by enzyme immunoassay according to the manufacturer's instruction (Cayman Chemical). Prostaglandin E₂ levels are expressed as picograms per rat.

Materials The ICI 182,780 and tamoxifen were solubilized in 10% ethanol. Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; J. J. Baker, Deventer, Holland).

Statistical Evaluation. All values in the figures and text are expressed as mean \pm S.E.M. from 10 rats for each group. For the in vivo studies, n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissues section collected from all the animals in each group. The results were analyzed by one-way ANOVA followed by a Bonferroni's post hoc test for multiple comparisons. A p value of less than 0.05 was considered significant.

Results

Effects of DEX on carrageenan-Induced Pleurisy. In a preliminary study, to analyze the possible influence of estrogen receptor on DEX anti-inflammatory property during acute inflammation, we examined the effect of ICI 182,780 and tamoxifen, two ER antagonists, on carrageenan-induced pleurisy, evaluated at 4 h after carrageenan administration. The injection of carrageenan into the pleural cavity of rats caused an inflammatory reaction characterized by exudate formation (Fig. 1a) Compared with the number of cells collected from the pleural space of sham-treated rats, injection of carrageenan induced a significant increase of the number of inflammatory cells (Fig. 1b). The important presence of inflammatory cells in the pleural cavity seemed to be correlated with the influx of neutrophils into the lung tissue. Therefore, we investigated the effect of ER antagonists and DEX treatment on the neutrophils infiltration in the lung tissues by measurement of the MPO activity. MPO activity was significantly elevated at 4 h after carrageenan administration in lung tissue from sham-treated rats (Fig. 2). The presence of pleural exudate (Fig. 1a), the number of inflammatory cells (Fig. 1b) in the pleural cavity, and the neutrophil infiltration in lung tissue (Fig. 2) 4 h after carrageenan administration was significantly attenuated in a dose-dependent manner by the i.p. injection of DEX. Coadministration of ICI 182,780 (0.5 mg/kg) or tamoxifen (0.5 mg/kg) significantly blocked the anti-inflammatory effect of DEX (Figs. 1 and 2). Treatment with ICI 182,780 (0.5 mg/kg) or tamoxifen (0.5 mg/kg) alone did not affect the acute inflammatory response (Figs. 1 and 2). Based on these findings, which are in agreement with our preliminary study (Cuzzocrea et al., 2000), in which we have clearly demonstrated that ICI 182,780 (0.5

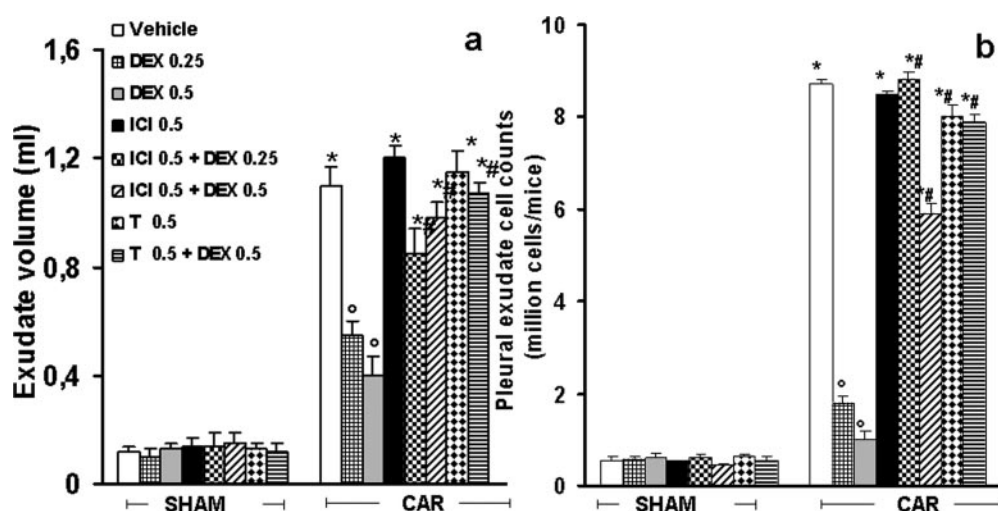


Fig. 1. Effect of DEX on carrageenan-induced inflammation. Volume exudate (a) and accumulation of polymorphonuclear cells (b) in pleural cavity at 4 h after carrageenan injection. DEX (0.25 and 0.50 mg/kg i.p.) significantly reduced pleural exudation and leukocyte infiltration in a dose-dependent fashion. Coadministration of the selective ER antagonist ICI 182,780 (0.5 mg/kg) or tamoxifen (0.5 mg/kg) and DEX significantly blocked the effect of the steroid. Treatment with ICI 182,780 (0.5 mg/kg) or tamoxifen (0.5 mg/kg) alone did not affect the acute inflammatory response (a and b). Data are means \pm s.e.m. from 10 rats for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

mg/kg) or tamoxifen (0.5 mg/kg) in this experimental setting act in a manner similar to that of ER antagonists, all further experiments were performed using as ER antagonist only ICI 182,780 in OVX rats subjected to carrageenan-induced pleurisy.

Effects of DEX on Carrageenan-Induced NO Production and iNOS Expression. NO levels were also significantly increased in the pleural exudate obtained from carrageenan-injected rats (Fig. 3a). Pretreatment of rats with DEX significantly reduced (in a dose-dependent manner) the NO levels in the pleural exudates (Fig. 3a). Coadministration of ICI 182,780 significantly blocked the effect of DEX (Fig. 3a). ICI 182,780 (0.5 mg/kg) treatment alone did not affect the carrageenan-induced production of NO levels in the pleural exudates (Fig. 3a). Immunohistochemical analysis of lung sections from carrageenan-treated rats revealed a positive staining for iNOS (Fig. 3c; see densitometry analysis in Fig. 4). In contrast, no staining for iNOS was found in the lungs from carrageenan-injected rats that had been pretreated with DEX (0.5 mg/kg) (Fig. 3d; see densitometry analysis in Fig. 4). Coadministration of ICI 182,780 blocked the inhibitory effect of the DEX (0.5 mg/kg) on iNOS expression (Fig. 3e; see densitometry analysis in Fig. 4). Note that staining was absent in lung tissue obtained from the sham group (Fig. 3b; see densitometry analysis in Fig. 4). In addition, Western blot analysis of lung homogenates showed a significant increase of iNOS expression 4 h after carrageenan injection compared with sham-treated rats (Fig. 5, a and b). DEX (0.5 mg/kg) treatment was also able to attenuate iNOS expression (Fig. 5, a and b). Coadministration of ICI 182,780 significantly blocked the effect of DEX on the iNOS protein expression (Figs. 5, a and b). ICI 182,780 treatment alone did not affect the carrageenan-induced iNOS expression (data not shown).

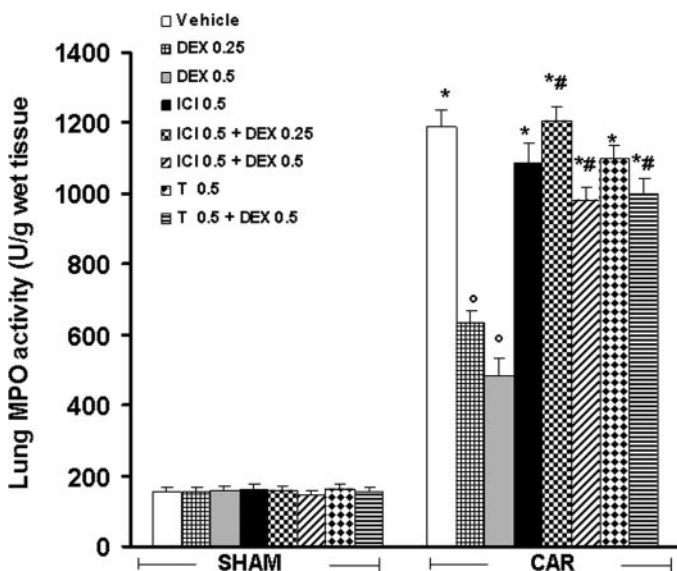


Fig. 2. Effect of DEX on MPO activity in the lung. MPO activity was significantly increased in the lungs of the carrageenan-treated rats in comparison to sham rats. DEX (0.25 and 0.50 mg/kg i.p.) significantly reduced the carrageenan-induced increase in MPO activity in a dose-dependent manner. Coadministration of ICI 182,780 (0.5 mg/kg) or tamoxifen (0.5 mg/kg), and DEX significantly blocked the effect of the steroid. ICI 182,780 (0.5 mg/kg) or tamoxifen (0.5 mg/kg) treatment alone did not affect the carrageenan-induced MPO activity. Data are means \pm s.e.m. from 10 rats for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

Effects of DEX on Carrageenan-Induced Nitrotyrosine Formation, Lipid Peroxidation and PARP Activation. Immunohistochemical analysis of lung sections from rats injected with carrageenan also revealed a positive staining for nitrotyrosine (Fig. 6b; see densitometry analysis in Fig. 4). In contrast, no positive staining for nitrotyrosine was found in the lungs from carrageenan-injected rats, which had been pretreated with DEX (Fig. 6c; see densitometry analysis in Fig. 4). Coadministration of ICI 182,780 significantly inhibited the ability of DEX to reduce nitrotyrosine formation (Fig. 6d, see densitometry analysis in Fig. 4). No positive staining was observed in lungs obtained from the sham group (Fig. 6a, see densitometry analysis in Fig. 4). ICI 182,780 treatment alone did not affect the carrageenan-induced nitrotyrosine formation (data not shown). In addition, 4 h after carrageenan-induced pleurisy, lung was also investigated for MDA levels, indicative of lipid peroxidation. As shown in Fig. 6e, MDA levels were significantly increased in the lung from carrageenan-treated rats. Lipid peroxidation in lung tissue was significantly attenuated in a dose-dependent manner by the i.p. injection of DEX (Fig. 6e). Coadministration of ICI 182,780 significantly blocked the effect of DEX on lipid peroxidation (Fig. 6e). ICI 182,780 treatment alone did not affect the carrageenan-induced lipid peroxidation (Fig. 6e). In addition, immunohistochemical analysis of lung sections obtained from carrageenan-treated rats also revealed a positive staining for PAR, an index of PARP activation (Fig. 6g; see densitometry analysis in Fig. 4). In contrast, no staining for PAR was found in the lungs from carrageenan-injected rats that received DEX (Fig. 6h; see densitometry analysis in Fig. 4). Coadministration of ICI 182,780 blocked the effect of DEX on PAR formation (Fig. 6i; see densitometry analysis in Fig. 4). There was no staining for PAR in lungs obtained from the sham group (Fig. 6f; see densitometry analysis in Fig. 4). ICI 182,780 treatment alone did not affect the carrageenan-induced PARP activation (data not shown).

Effects of DEX on the Release of Pro-Inflammatory Cytokine Induced by Carrageenan. At 4 h after the injection of carrageenan, increased levels of TNF- α and IL-1 β were observed in pleural exudate compared with that from control rats (Fig. 7). DEX treatment attenuated, in a dose-dependent manner, the release of TNF- α and IL-1 β (Fig. 7). Coadministration of ICI 182,780 blocked the effect of the DEX (Fig. 7). ICI 182,780 treatment alone did not affect the carrageenan-induced TNF- α and IL-1 β production in pleural exudates (Fig. 7).

Effects of DEX on PGE₂ Production and COX-2 Expression. PGE₂ is one of the primary prostaglandins formed from the metabolism of arachidonic acid by the cyclooxygenases. The levels of prostaglandin E₂ in the pleural exudate from carrageenan-injected rats were appreciably increased, and DEX treatment significantly attenuated these levels (Fig. 8a). Western blot analysis of lung homogenates from carrageenan-injected rats also revealed an increased expression of COX-2 compared with sham-treated rats that was significantly attenuated by DEX treatment (Fig. 8, b and c). Coadministration of ICI 182,780 significantly blocked the effect of the steroid on the COX-2 activity (Fig. 8a) and expression (Fig. 8, b and c). ICI 182,780 treatment alone did not affect the carrageenan-induced COX-2 activity (Fig. 8a) and expression (data not shown).

Effects of DEX on Carrageenan-Induced I κ B- α Degradation in Lung Tissue. NF- κ B plays a critical role in the expression of many genes involved in immune and inflammatory responses. The activation of NF- κ B is the consequence of phosphorylation of two specific serines near the N terminus of I κ B- α . The phosphorylation of I κ B- α leads to its ubiquitination, which targets the protein for degradation by the 26S proteasome, resulting in its degradation and consequent NF- κ B activation and nuclear translocation.

Because the expressions of pro-inflammatory mediators, such as iNOS, COX-2, and TNF- α , are known to be modulated by NF- κ B (Christman et al., 1998), and DEX reduces NF- κ B activation, we examined the I κ B- α degradation by Western blot experiments. Results in Fig. 9, a and b, show that carrageenan injection induced a decrease of I κ B- α levels compared with sham group, whereas DEX treatment significantly prevented this degradation. Coadministration of ICI 182,780 significantly blocked the effect of DEX

on the I κ B- α expression (Fig. 9, a and b). ICI 182,780 treatment alone did not affect the carrageenan-induced the I κ B- α degradation.

These results confirm previous observations suggesting that DEX can reduced NF- κ B activation and are consistent with the role of NF- κ B in regulation of inflammatory mediators such as COX-2, iNOS, and TNF- α (Barnes and Adcock, 1993; Riccardi et al., 2002).

Effects of DEX on Carrageenan-Induced Lung Injury. Histological examination of lung sections from carrageenan-injected rats revealed significant tissue damage (Fig. 10b). Thus, histological examination from lung sections of carrageenan-injected rats, compared with sections from saline-treated animals, showed edema, tissue injury, and infiltration of the tissue with inflammatory cells (Fig. 10, a and b). DEX (0.5 mg/kg) reduced the degree of lung injury and polymorphonuclear cell accumulation (Fig. 10c). Coadministration of ICI 182,780 (0.5 mg/kg) significantly blocked the

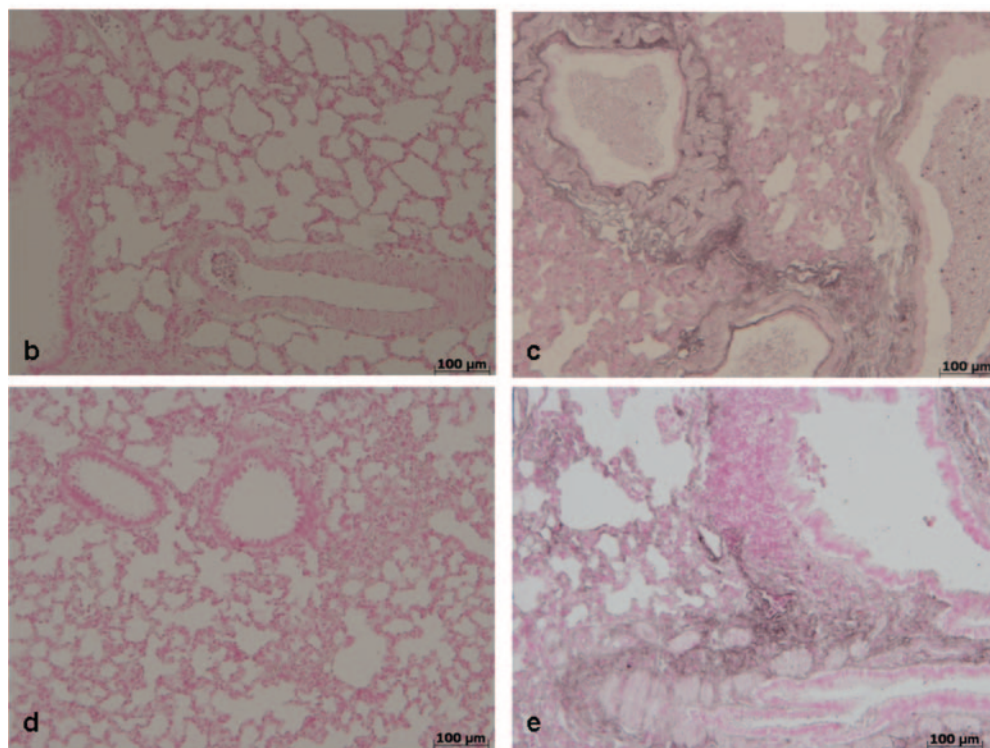
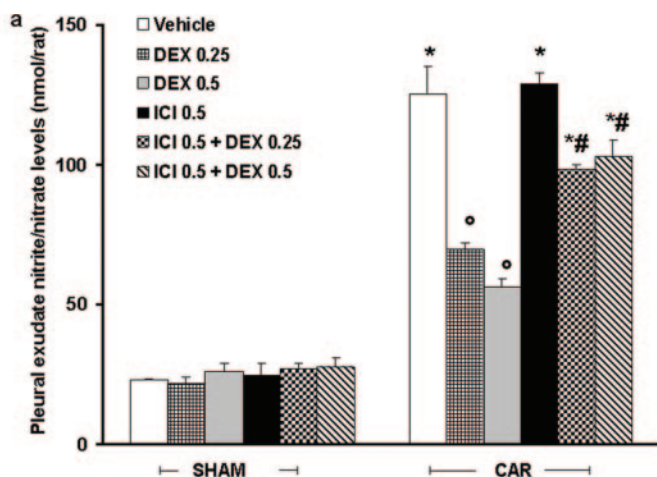


Fig. 3. Effect of DEX on NO production and iNOS expression. Nitrite and nitrate (a) levels in pleural exudate at 4 h after carrageenan administration were significantly increased versus sham group. DEX (0.25 and 0.50 mg/kg i.p.) significantly reduced the carrageenan-induced elevation of nitrite and nitrate exudates levels in a dose-dependent manner (a). Coadministration of ICI 182,780 and DEX significantly blocked the effect of the steroid (a). Treatment with ICI 182,780 (0.5 mg/kg) alone did not affect the carrageenan-induced nitrite and nitrate production in pleural exudate (a). In addition, there was no staining for iNOS (b) in lungs obtained from the sham group. Four hours after carrageenan injection, positive staining for iNOS (c) was localized mainly in macrophages. There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pretreated with DEX (0.5 mg/kg i.p.) (d). Coadministration of ICI 182,780, and DEX (0.5 mg/kg) significantly blocked the effect of the steroids (e). Figure is representative of at least three experiments performed on different experimental days. Data are means \pm S.E.M. from 10 rats for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

anti-inflammatory effect of DEX (Fig. 10d). ICI 182,780 treatment alone did not affect the carrageenan-induced lung injury (data not shown).

Effects of DEX on ER, GR, and GILZ mRNA Levels on Carrageenan-Induced Pleurisy. To evaluate a possible modulation of ER mRNA levels in our experimental model, we performed quantitative PCR in lung tissue as shown in Fig. 11a. Carrageenan treatment, with or without DEX administration, did not change ER mRNA levels, and ICI 182,780 coadministration had no effect compared with the group with DEX alone (Fig. 11a). The same experiment was performed to evaluate changes in GR and GILZ mRNA levels, two effects typical of glucocorticoid action and consequent to glucocorticoid/GR interaction and direct transcription regulation (Barnes and Adcock, 1993; D'Adamio et al., 1997; Sutherland et al., 1998; Riccardi et al., 2002). As expected,

DEX administration in carrageenan-injected rats induced a significant down-regulation of GR levels (Fig. 11c), but coadministration with ICI 182,780 did not influence this effect (Fig. 11c). Moreover, as expected, DEX up-regulated GILZ levels (Fig. 11b) but ICI 182,780 did not counteract this effect (Fig. 11b).

Taken together, these results indicate that DEX administration down-regulates GR and up-regulates GILZ levels, two transcription-dependent events consequent to DEX/GR interaction, and that the ER-specific inhibitor ICI 182,780 does not counteract these effects, thus suggesting that ICI 182,780 effect is not due to interaction and competition with GR.

Discussion

This study provides the first evidence that there is a cross-talk between the GR and ER in conditions associated with experimental acute inflammation. This cross-interaction might be therapeutically relevant when steroidal anti-inflammatory compounds are administered to women treated with ER antagonists.

We demonstrated here that the carrageenan-induced pleurisy is a good model to study glucocorticoid anti-inflammatory activity and we showed that DEX treatment reduced: 1) the development of carrageenan-induced pleurisy, 2) the infiltration of the lung with polymorphonuclear leukocytes, 3) the degree of pro-inflammatory cytokine production in the lung, 4) the expression of iNOS, 5) the degree of lung lipid peroxidation in the lung, 6) the degree of tissue lung injury caused by carrageenan injection, and 7) NF- κ B activation preventing I κ B- α degradation. In particular, the investigation of exudate formation, polymorphonuclear leukocyte infiltration, and MPO accumulation demonstrated that ER antagonists ICI 182,780, such as the SERM tamoxifen, was effective in preventing the effects of DEX, thus suggesting that an interaction between GR and ER contributes to DEX activity. The mechanism involved in the blockade of DEX anti-inflammatory activity by ER antagonists remains to be fully elucidated.

Glucocorticoids are potent anti-inflammatory and immunosuppressive drugs. Their therapeutic effects are largely due to glucocorticoid/GR interaction and to their ability to inhibit many functions of inflammatory cells. In fact, glucocorticoids are known to inhibit production of many CK, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, granulocyte macrophage-colony-stimulating factor, TNF- α , and γ -interferon (Kunicka et al., 1993; Almawi et al., 1996; Fushimi et al., 1998), and to induce TGF- β production (Batuman et al., 1995). Moreover, glucocorticoids inhibit COX-1, COX-2, and prostanoid formation (Jun et al., 1999). Several studies indicate that TNF- α , IL-1 β , and iNOS help to propagate the extension of local or systemic inflammatory process (Iuvone et al., 1997; Hoyos et al., 2005). We confirm in the present study that acute inflammation in the rat results in the production of the pro-inflammatory cytokines TNF- α and IL-1 β , as well as iNOS protein expression. We found that DEX pretreatment attenuated the production of TNF- α and IL-1 β as well as the expression of iNOS. We have also demonstrated that the coadministration of the ER antagonist ICI 182,780 significantly blocked the effect of DEX.

There is good evidence in this and in other experimental models of inflammation that an enhanced formation of pro-

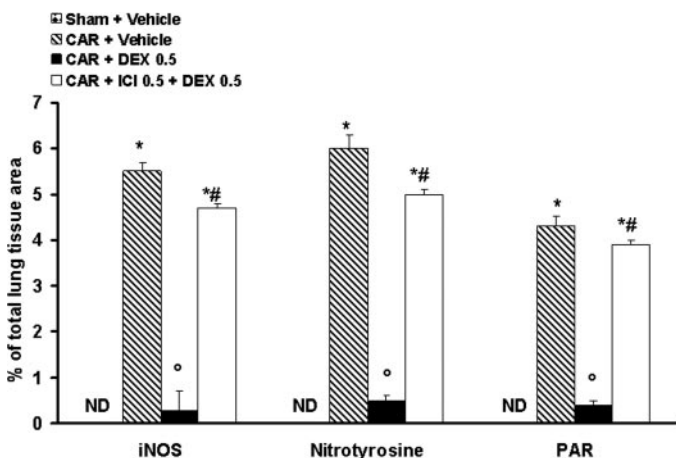


Fig. 4. Typical densitometry evaluation. Densitometry analysis of immunohistochemistry photographs ($n = 5$) for iNOS, nitrotyrosine, and PARP from lung tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh computer. N.D., not detected. Data are expressed as percentage of total tissue area. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR.

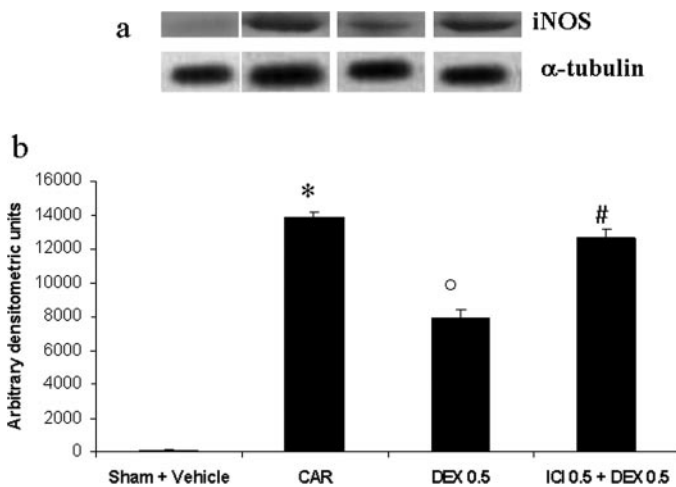


Fig. 5. Western Blot analysis of iNOS in the lung. A representative Western Blot analysis of iNOS from lung tissue of carrageenan-treated rats (a) and its densitometry analysis (b) showed that DEX-treated rats expressed lower levels of iNOS protein compared with CAR group. Coadministration of ICI 182,780, and DEX significantly blocked the effect of the steroid. Immunoblotting in a is representative of one of five to six analyzed. The results in b are expressed as mean \pm S.E.M. from five to six lung tissues. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

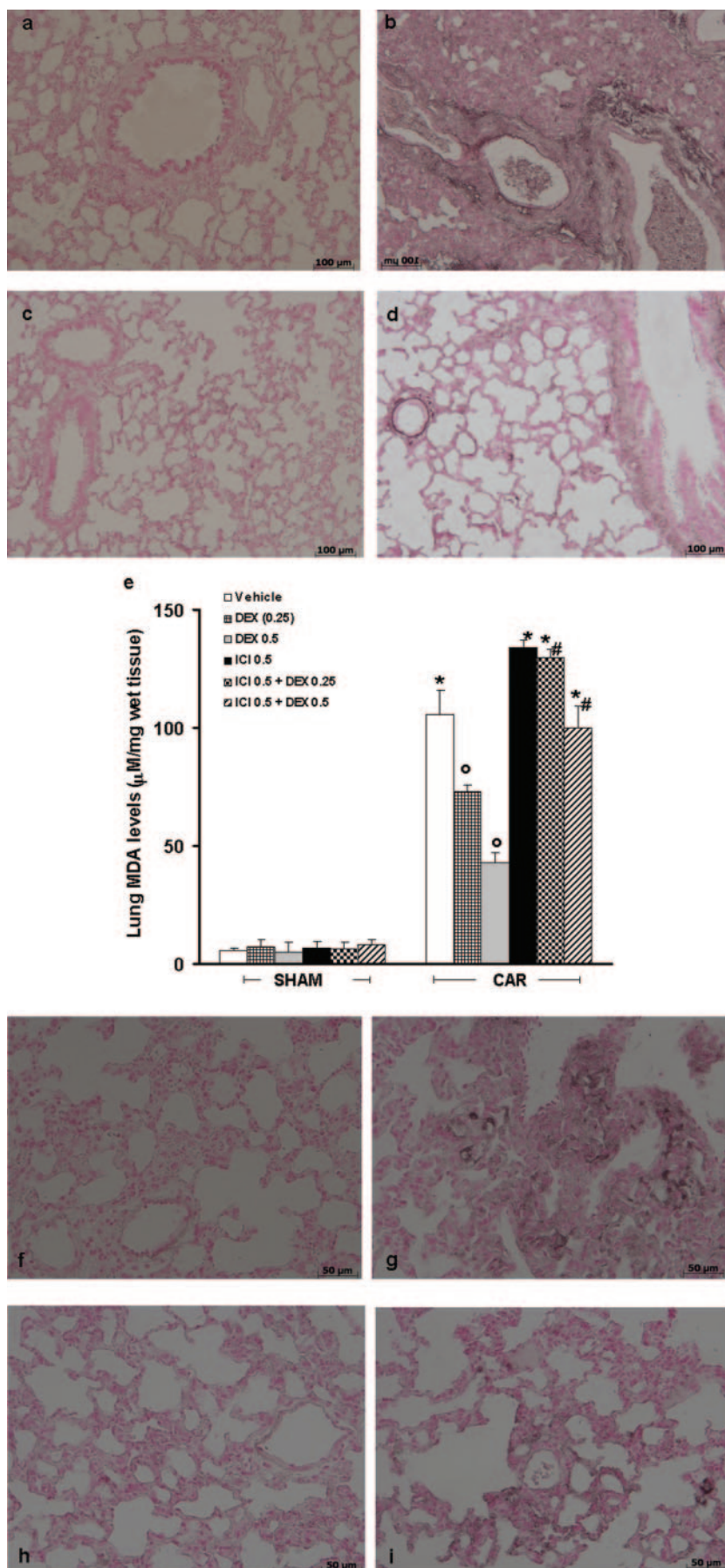


Fig. 6. Effect of DEX on nitrotyrosine formation, lipid peroxidation, and PAR production. There was no staining for nitrotyrosine (a) in lungs obtained from the sham group of rats. Four hours after carrageenan injection, positive staining for nitrotyrosine (b) was observed. There was a marked reduction in the immunostaining for nitrotyrosine in the lungs of carrageenan-treated rats pretreated with DEX (0.5 mg/kg i.p.) (c). Coadministration of ICI 182,780 and DEX (0.5 mg/kg) significantly blocked the effect of the steroids (d). In addition, 4 h after carrageenan administration, a significant increase in MDA levels, an index of lipid peroxidation, was observed in the lungs of the carrageenan-treated rats compared with the sham-treated rats (e). DEX (0.25 and 0.50 mg/kg i.p.) significantly reduced the carrageenan-induced increase in MDA levels in a dose-dependent manner (e). Coadministration of ICI 182,780 and DEX (0.5 mg/kg) significantly blocked the effect of the steroid (e). ICI 182,780 (0.5 mg/kg) treatment alone did not affect the carrageenan-induced lung lipid peroxidation (e). Moreover, there was no staining for PAR (f) in lungs obtained from the sham group of rats. Four hours after carrageenan injection, positive staining for PAR (g) was observed. There was a marked reduction in the immunostaining for PAR in the lungs from the CAR group pretreated with DEX (0.5 mg/kg i.p.) (h). Coadministration of ICI 182,780 and DEX (0.5 mg/kg) significantly blocked the effect of the steroids (i). Figure is representative of at least three experiments performed on different experimental days. Data are means \pm S.E.M. from 10 rats for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

stanoids after the induction of COX-2 contributes to the pathophysiology of local inflammation (Flower and Rothwell, 1994) and also that selective inhibitors of COX-2 exert potent anti-inflammatory effects (Mukherjee, 2002). We also demonstrate here that the increase of the PGE₂ levels, caused by injection of carrageenan into the pleural cavity, is reduced in the exudate from DEX-treated rats. The enhanced formation of PGE₂ is secondary to the expression of COX-2 protein, because 1) there was no increase of the expression of COX-1 protein after carrageenan injection (Posadas et al., 2004) and 2) selective inhibitors of COX-2 activity, including NS-398 (nimesulide) and SC-58125 (celecoxib), abolish the increase of PGE₂ caused by carrageenan injection into the pleural cavity (Nakatsugi et al., 1996). Thus, we confirm that DEX treatment reduced COX-2 protein expression and activity caused by carrageenan injection in the lung. We have also demonstrated that the coadministration of ICI 182,780 significantly blocked the effect of the DEX.

Although the exact molecular mechanisms involved in glucocorticoid-mediated immunomodulation are still not completely understood, it is widely accepted that most anti-inflammatory and immunomodulatory actions are due to inhibition of the activity of transcription factors such as AP-1 and NF- κ B (Uht et al., 1997; Riccardi et al., 2002). In particular, NF- κ B is controlled by I κ B- α , and its degradation results in NF- κ B activation and induction of transcription of many pro-inflammatory genes, including TNF- α , IL-1 β , COX-2, and iNOS, to name but a few (Christman et al., 1998). We report here that carrageenan caused a significant I κ B- α degradation in the lung tissues at 4 h, whereas treatment with DEX significantly prevented I κ B- α degradation. We have also demonstrated that the coadministration of the ER antagonist ICI 182,780 significantly blocked the effect of DEX, thus suggesting that the inhibitory effect of DEX on NF- κ B activation may partially depend on ER activation.

A number of recent studies have demonstrated that the recruitment of cells into an area of inflammation may be mediated also by a novel group of small proteins with relatively specific chemotactic activity for leukocyte subpopulations. Diminished expression of the pro-inflammatory cytokines in the lung tissue from carrageenan-injected rats that received DEX correlated to the reduction of leukocyte infil-

tration as assessed by the specific granulocyte enzyme MPO and to the moderation of the tissue damage as evaluated by histological examination. Neutrophils are recruited into the tissue by local production of cytokines and then contribute to tissue destruction by the production of reactive oxygen metabolites, granule enzymes, and cytokines that further amplify the inflammatory response by acting on macrophages and lymphocytes (Kadl and Leitinger, 2005). Neutrophils were shown to express GR and ER receptor, suggesting a potential role of these receptors in the regulation of neutrophil function (Molero et al., 2002). Other studies have also demonstrated that GR may modulate leukocyte-endothelial cell interactions during inflammation through regulation of endothelial adhesion molecules (Schramm and Thorlacius, 2004). Furthermore, we found that the tissue damage induced by carrageenan was associated with high levels of the tissue thiobarbituric acid-reactant MDA, which is considered a good indicator of lipid peroxidation (Eiserich et al., 1996). An intense immunostaining of nitrotyrosine formation also suggested that a structural alteration of lung had occurred, most probably as a result of the formation of highly reactive nitrogen-derivatives. Recent evidence indicates, in fact, that several chemical reactions, involving nitrite, peroxynitrite, hypochlorous acid, and peroxidases, can induce tyrosine nitration and may contribute to tissue damage (Cuzzocrea et al., 1999). There is a large amount of evidence that the production of ROS (such as hydrogen peroxide, superoxide, and hydroxyl radicals) at the site of inflammation contributes to tissue damage (Cuzzocrea et al., 2001c). Therefore, in this study we clearly demonstrated that DEX treatment prevented lipid peroxidation and nitrotyrosine formation. The reduction of lung lipid peroxidation and nitrotyrosine staining in the DEX-treated animals is more likely to be related to the decreased inflammatory cell infiltration as well as to the inhibitory effect on iNOS expression. We have also demonstrated that the coadministration of ICI 182,780 significantly blocked the effect of DEX. ROS produce strand breaks in DNA that triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARP, which also plays an important role in inflammation (Szabo, 1997). We demonstrate here that DEX treatment reduced the activation of PARP during carrageenan-induced pleurisy in the lung.

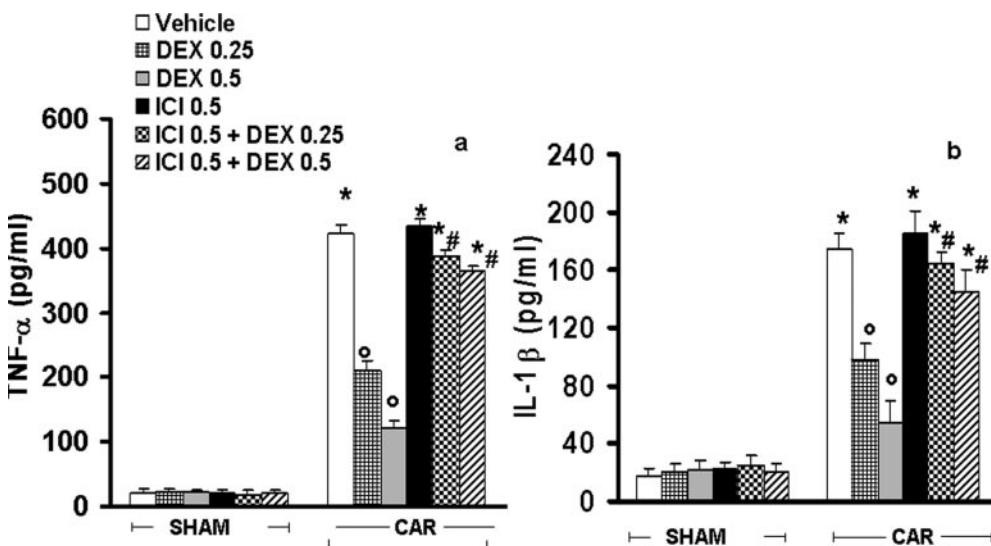


Fig. 7. Exudate levels of TNF- α and IL-1 β . Exudate levels of TNF- α (a) and IL-1 β (b). DEX (0.25 and 0.50 mg/kg i.p.) significantly reduced the carrageenan-induced elevation of cytokine levels in a dose-dependent manner. Coadministration of ICI 182,780 and DEX significantly blocked the effect of the steroid. Treatment with ICI 182,780 (0.5 mg/kg) alone did not effect the carrageenan-induced cytokines production in the pleural exudates. Data are means \pm S.E.M. from 10 rats for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

Thus, we propose that the anti-inflammatory effects of DEX may be due, at least in part, to the prevention of the activation of PARP. Moreover, we have also demonstrated that the coadministration of ICI 182,780 significantly blocked the effect of DEX on nitrotyrosine formation and PARP activation.

These results suggest that some anti-inflammatory effects of DEX are due in part to ER. However, the nature of the GR-ER interaction remains to be defined. The possibilities of

ER cross-interaction with GR may be discussed in the context of what is known on the mechanisms underlying GR anti-inflammatory action:

1. GR interacts with GREs to increase gene transcription. One important GR gene target is annexin-1, a strong inhibitor of phospholipase A2 (Ozawa, 2005). A few studies have shown that the E₂-ER complex increases annexin synthesis, but the ICI 182,780-ER complex should be transcriptionally incompetent and unable to alter annexin-1 cell content interfering with GR action.
2. GR may associate with transcription factors (such as signal transducer and activator of transcription, NF- κ B, and AP-1) and block their access to their responsive elements in the promoter of inflammatory genes or may act on the stability of the I κ B- α that retains NF- κ B in the cytoplasm. The E₂-ER complex is also known to interfere with the activity of the same transcription factors by direct interaction or by inhibiting their translocation within the nuclear compartment. The presence of ICI 182,780 would limit ER communications with other nuclear factors without interfering on GR-nuclear factor interactions due to the abundance of nuclear factors in cells.
3. Finally, DEX-GR is known to negatively regulate the transcription of selected inflammatory genes. The mechanism of such negative regulation is not completely understood; in general, generally nuclear receptors inhibit the transcription of target genes by recruiting corepressors. In this case, ICI 182,780 might limit the availability of corepressors because it has been shown that the ICI 182,780-ER complex is endowed with high affinity for corepressors. Thus, a likely mechanism for the GR-ER interconnection here described may be the subtraction of corepressors due to their binding to the ER-ICI 182,780 complex.

Moreover, various evidence points out the possible interaction for GR-ER (Sengupta and Wasylyk, 2001) at different transcriptional levels in various tissues and cells (Zhou et al., 1989; Sutherland et al., 1998). In this respect, we tested whether both ER and/or GR genes could be regulated at transcriptional level by DEX. Results showed that ER levels did not change upon DEX treatment, whereas GR expression was down-regulated. This fact is not surprising because DEX down-regulates GR expression in other cell types (Mader et al., 1989; D'Adamio et al., 1997). On the other hand, ICI 182,780 did not counteract GR modulation, suggesting that ICI 182,780 effect does not depend from a direct modulation of GR activity. Moreover, we analyzed GILZ expression, a gene rapidly induced by GR activation, tested as a direct GR-target gene, and results showed that ICI 182,780 did not affect GILZ up-regulation induced by DEX. Taken together, these results indicate that ICI 182,780 did not affect GR levels or its direct transcriptional activity (i.e., GILZ up-regulation), thus suggesting that other mechanisms are responsible for ICI 182,780 interaction with DEX. The mechanism by which the ER and GR interact at the level of transcription must involve a process distinct from steroid receptor/hormone response element interactions (Uht et al., 1997). In fact, these interactions are highly specific, as dictated by differences in the DNA-binding regions of the ER and GR and in the sequence specificity of their cognate response elements (Klock et al., 1987). An alternate explana-

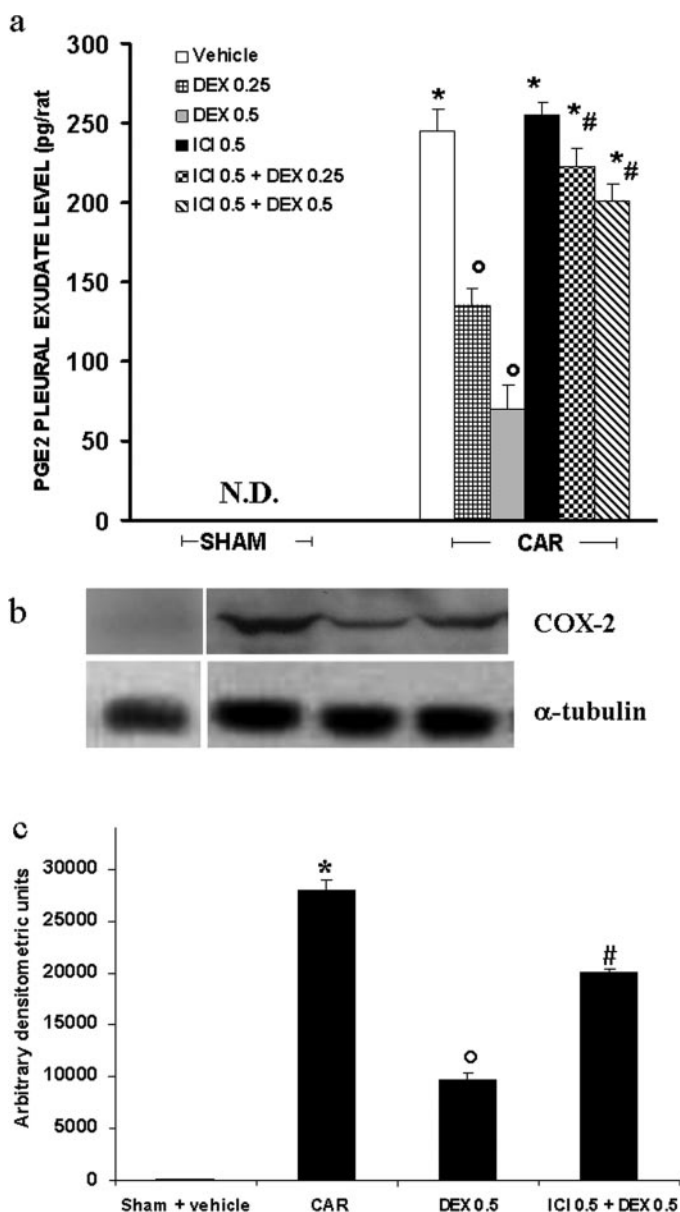


Fig. 8. Effect of DEX on PGE₂ production and COX-2 expression. PGE₂ levels in the pleural exudate from carrageenan-treated rats (a). DEX (0.25 and 0.50 mg/kg i.p.) significantly reduced the carrageenan-induced elevation of PGE₂ exudate levels in a dose-dependent manner (a). In addition, a representative Western blot analysis of COX-2 from lung tissue from carrageenan-treated rats (b) and its densitometry analysis (c) showed that DEX-treated rats expressed lower levels of COX-2 protein compared with CAR-treated rats 4 h after carrageenan administration. Coadministration of ICI 182,780 and DEX significantly blocked the effect of the steroid on PGE₂ production (a). ICI 182,780 treatment alone did not affect the carrageenan-induced the COX-2 activity (a). Immunoblotting in b is representative of one lung tissues of five to six analyzed. The results in c are expressed as mean \pm S.E.M. from five to six lung tissues. Data are means \pm S.E.M. from 10 rats for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

tion could involve interactions between steroid receptors and other transcription factors. ER, GR, and other nuclear hormone receptors have been shown to alter transcription through regulation of the AP-1 response. In fact, estrogens and glucocorticoids have opposing effects at this response element: estrogens stimulate AP-1-activated transcription (Webb et al., 1995), whereas glucocorticoids inhibit it (Dostert and Heinzl, 2004). These mechanisms may be operative in this experimental model and further suggest that the

anti-inflammatory effect of DEX requires the participation of ER.

In conclusion, this study confirms the evidence that DEX causes a substantial reduction of acute inflammation in the rat. In addition, we also demonstrate, for the first time in vivo, that the ER- α antagonist ICI 182,780 significantly attenuates the protective effect of DEX, thus suggesting that the mechanisms underlying the protective effects of DEX are partially ER- α -dependent. Our data provide the first evi-

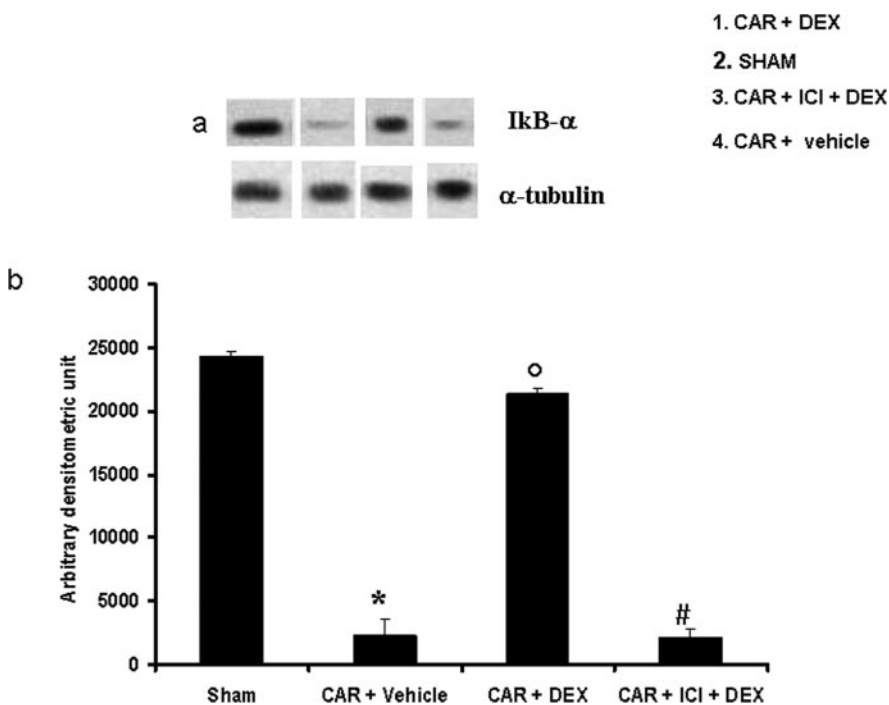


Fig. 9. Effect of DEX on IκB- α degradation. Representative Western blots showing the effects of DEX on IκB- α degradation (a and b) at 4 h after carrageenan injection. Coadministration of ICI 182,780, and DEX significantly blocked the effect of the steroid on IκB- α degradation (a). A representative blot of lysates (a) obtained from five animals per group is shown, and densitometric analysis of all animals is reported. The results in b are expressed as mean \pm S.E.M. from $n = 5$ or 6 lung tissues for each group. *, $P < 0.01$ versus sham; °, $P < 0.01$ versus CAR; #, $P < 0.01$ versus DEX.

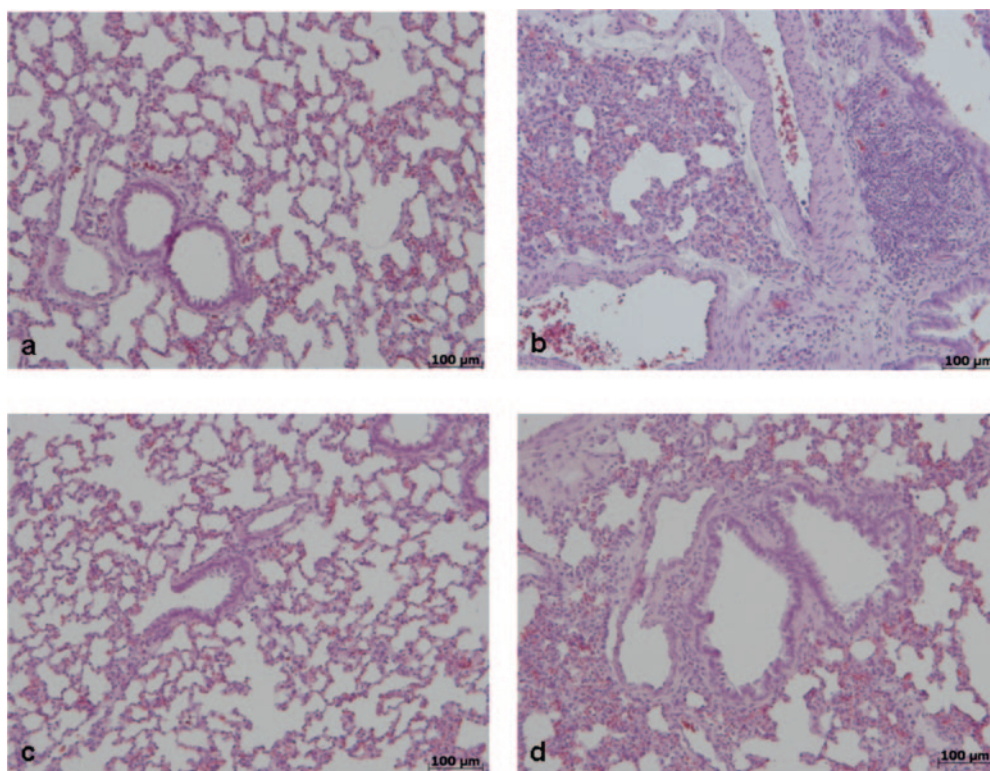


Fig. 10. Effect of DEX on lung injury. a, no histological alteration was observed in the lung sections taken from saline-treated animals. b, lung section from a carrageenan-treated rats demonstrating tissue injury and inflammatory cells accumulation. c, lung section from a carrageenan-treated rats after administration of DEX (0.5 mg/kg) demonstrating reduced tissue injury and cellular infiltration. d, co-administration of ICI 182,780 and DEX (0.5 mg/kg) significantly blocked the effect of the steroids. Figure is representative of at least three experiments performed on different experimental days.

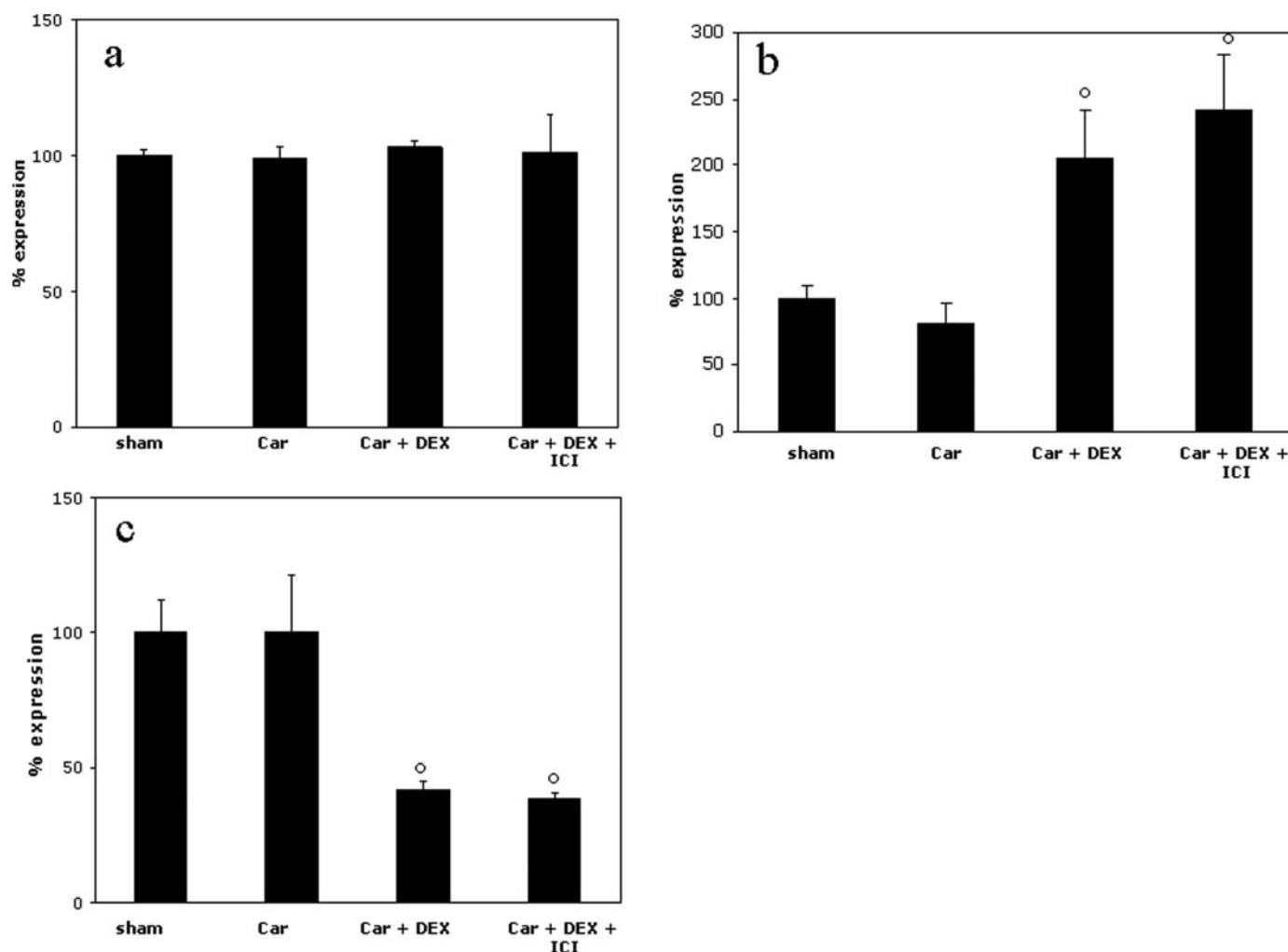


Fig. 11. PCR analysis of ER, GR and GILZ. Effect of DEX on expression levels of ER (a), GILZ (b), and GR (c). Quantitative real-time PCR analysis showed that: a, ER expression levels did not change, compared with CAR-injected rats with or without DEX. Coadministration of ICI 182,780, and DEX did not change the effect of the steroid; b, GILZ was up-regulated in DEX-treated rats after carrageenan administration, and coadministration of ICI 182,780 and DEX did not change the effect of the steroid; c, GR was down-regulated in DEX-treated rats after carrageenan administration, and coadministration of ICI 182,780 and DEX did not change the effect of the steroid. The results are expressed as mean \pm S.E.M. of three independent experiments; *, $P < 0.01$ versus CAR.

dence of a functional cross-interaction between glucocorticoid and estrogen receptors in the inflammatory reactions that might be of interest, particularly in women undergoing therapy with antiestrogenic compounds. Future studies will attempt to better clarify the in vivo mechanism by which the ER and GR interact in modulation of inflammatory process.

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