PPAR-α Contributes to the Anti-Inflammatory Activity of 17β-Estradiol

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ABSTRACT

Because studies have shown that 17β-estradiol (E2) produces anti-inflammatory effects after various adverse circulatory conditions, we have recently demonstrated that E2 significantly reduced the acute lung injury. Moreover, previous results suggest that peroxisome proliferator-activated receptor-α (PPAR-α), an intracellular transcription factor activated by fatty acids, plays a role in the control of inflammation. With the aim to characterize the role of PPAR-α in estrogen-mediated anti-inflammatory activity, we tested the efficacy of E2 in an experimental model of lung inflammation, carrageenan-induced pleurisy, comparing ovariectomized wild-type (WT) and PPAR-α lacking (PPAR-αKO) mice. Results indicate that E2-mediated anti-inflammatory activity is weakened in PPAR-αKO mice, compared with WT control groups. In particular, E2 was less effective in PPAR-αKO, compared with WT mice, in inhibition of cell migration as well as lung injury, NF-κB activation, TNF-α production, and inducible nitric-oxide synthase (iNOS) activation. Moreover, macrophages from PPAR-αKO were less susceptible to E2-induced iNOS inhibition in vitro compared with macrophages from WT mice. Moreover, the results indicate that PPAR-α was required for estrogen receptor up-regulation, following E2 treatment. These results show for the first time that PPAR-α contributes to the anti-inflammatory activity of E2.

The inflammatory process is invariably characterized by the production of a number of factors including prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor, and cytokines and by the release of chemicals from tissues and migrating cells. Carrageenan (CAR)-induced local inflammation is commonly used to evaluate the anti-inflammatory effects of nonsteroidal drugs. Therefore, carrageenan-induced pleurisy is a useful model to assess the contribution of cells and mediators involved in tissue alterations during the inflammatory process. In particular, the initial phase of acute inflammation (0–1 h), which is not inhibited by nonsteroidal 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 prostaglandin release and attributed to the induction of inducible cyclooxygenase (COX)-2 in the tissue (Nantel et al., 1999). The onset of the carrageenan-induced acute inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radical, as well as to the release of other neutrophil-derived mediators (Salvemini et al., 1996). It is well established that gender or physiological states like pregnancy or menopause have a major influence on the manifestation of several pathologies with a high inflammatory component. These observations led us to postulate that female sex hormones, particularly estrogens, might play a relevant role in the control of immune response and inflammatory conditions. These results support our hypothesis that female sex hormones, particularly estrogens, might play a relevant role in the control of immune response and inflammatory conditions.

ABBREVIATIONS: CAR, carrageenan; COX, cyclooxygenase; RA, rheumatoid arthritis; ER, estrogen receptor; E2, 17β-estradiol; INOS, inducible nitric-oxide synthase; PPAR-α, peroxisome proliferator-activated receptor-α; NF-κB, transcription factor nuclear factor; PPAR-αKO, mice lacking PPAR-α; WT, wild type; O VX, ovariectomy; PBS, phosphate-buffered saline; ICAM, intercellular adhesion molecule; GITR, glucocorticoid-induced tumor necrosis factor receptor; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; TdT, terminal deoxynucleotidyltransferase; TNF-α, tumor necrosis factor-α; PMSF, phenylmethylsulphonyl fluoride; TBS, Tris-buffered saline; HPRT, hypoxanthine phosphoribosyl-transferase; Ct(0), cycle threshold; LPS, lipopolysaccharide; IFN-γ, interferon-γ; PVDF, polyvinylidene difluoride; iB, inhibitor of nuclear factor-αB.

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tion. Several animal models support this view; however, the mechanisms underlying these effects remain poorly understood. A confusing element in the comprehension of the reciprocal influence of estrogens and the immune system in pathological and physiological states are linked to the observation that the hormone may have opposite activities. For instance, it is well known that the estrogen-dependent maturation of follicles is an inflammatory-like reaction (Modugno et al., 2005). Likewise, the incidence of autoimmune disorders like systemic lupus erythematosus is much higher in women than in men, and menopause decreases symptoms (Ackerman, 2006). Finally, elevated levels of estrogens are associated with several markers of systemic inflammation. On the other hand, several other lines of evidence support the concept that estrogens have anti-inflammatory activity. For example, the incidence of rheumatoid arthritis (RA) is lower in fertile women than in men (Kay and Wingrave, 1983). The same is true for cardiovascular insults, possibly because women have lower incidence of chronic inflammation-related atherothrombotic lesions (Cushman et al., 1999). The effects of estrogens are mediated through well-characterized intracellular receptors (estrogen receptor; ER) α and β (Kiuper and Gustafsson, 1997). In general, 17β-estradiol (E2) has been associated with a proinflammatory activity because in the reproductive tract, which represents E2’s most studied target organ, the hormone stimulates epithelial cell proliferation and increases vascular permeability, edema, and influx of macrophages and eosinophils in the uterine stroma. However, various observations have clearly demonstrated that E2 significantly inhibits the cytokine-dependent induction of inducible nitric-oxide synthase (iNOS) in vitro (Zancam et al., 1999) as well as reduces the development of acute lung injury induced by carrageenan in rats (Cuzzocrea et al., 2001). Peroxisome proliferator-activated receptor-α (PPAR-α) is an intracellular transcription factor, activated by fatty acids, that plays a role in inflammation. Moreover, it has been previously shown that PPAR-α cross-talks with estrogen signaling (Campbell et al., 2003). In addition, it has been reported that PPAR-α activation can result in inhibition of NF-κB activation and the consequent expression of inflammatory genes (De Bosscher et al., 2006). Our data in experimental models of acute inflammation show that mice lacking PPAR-α (PPAR-αKO) develop an increased inflammation compared with wild-type (WT) mice (Cuzzocrea et al., 2006a). Moreover, the absence of PPAR-α results in a reduced anti-inflammatory response to dexamethasone treatment (Cuzzocrea et al., 2008). In addition, treatment with appropriate doses of PPAR-α agonists can inhibit inflammatory diseases development (Crisafulli and Cuzzocrea, 2009). With the aim to evaluate the role of PPAR-α in estrogen-mediated anti-inflammatory activity, we tested the efficacy of E2 in an experimental model of lung inflammation, carrageenan-induced pleurisy, comparing ovarioctomized PPAR-αKO and WT mice. For the first time, results indicate that E2-mediated anti-inflammatory activity is weakened in PPAR-αKO mice, compared with WT control groups.

Materials and Methods

In Vivo Experiments. Animals. Mice (6–7 weeks old, 20–27 g) with a targeted disruption of the PPAR-α gene (PPAR-αKO) and littermate WT mice were purchased from Jackson Laboratories (Harlan Nossan, Italy). Mice homozygous for the PparatniJGonz-targeted mutation mice are viable, fertile, and seem to be normal in appearance and behavior (Cuzzocrea et al., 2008). The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with regulations in Italy (Ministerial Decree 116192), Europe (Official Journal of the European Community L 358/1 12/18/1986), and the United States (Animal Welfare Assurance No. A5594-01, Department of Health and Human Services). The University of Messina Animal Care Review Board approved the study.

Ovariectomy. All surgical procedures were performed under halothane (2%) anesthesia followed by nitrous oxide/O2 anesthesia for approximately 18 min. Ovariectomy (OVX) was performed as described previously (Poli et al., 1994). Control groups (sham) underwent anesthesia and surgery without removal of the ovaries.

Carrageenan-Induced Pleurisy. Mice were anesthetized with isoflurane, and a skin incision was made at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.1 ml) or saline containing 2% α-carrageenan (0.1 ml) was injected into the pleural cavity as described previously. 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 chamber after vital trypan blue staining.

Experimental Groups. OVX mice (either WT or PPAR-αKO) were randomly allocated into the following groups: 1) CAR WT group, WT mice were exposed to carrageenan-induced pleurisy (n = 10) and were treated with saline solution (E2 vehicle); 2) CAR PPAR-αKO group, PPAR-αKO mice were exposed to carrageenan-induced pleurisy (n = 10) and were treated with saline solution (E2 vehicle); 3) CAR WT + E2 group, same as for CAR WT group with E2 administration (50 µg/kg i.p.) 1 h before carrageenan (n = 10); 4) CAR PPAR-αKO + E2 group, same as for CAR PPAR-αKO group with E2 administration (50 µg/kg i.p.) 1 h before carrageenan (n = 10); 5) sham WT group, 100 µl of saline solution instead of carrageenan was administered to the WT mice (n = 10) and they were treated with saline solution (E2 vehicle); 6) sham PPAR-αKO group, saline solution (100 µl) instead of carrageenan was administered to the PPAR-αKO mice (n = 10) and they were treated with saline solution (E2 vehicle); 7) sham WT + E2 group, same as for sham WT group with E2 administration (50 µg/kg i.p.) 1 h before saline (n = 10); and 8) sham PPAR-αKO + E2 group, same as for sham PPAR-αKO group with E2 administration (50 µg/kg i.p.) 1 h before saline (n = 10). To confirm that the PPAR-α receptor is involved in E2 anti-inflammatory response, the effects of E2 treatment were also observed in the following groups of WT or PPAR-αKO mice: cycling in the estrogenic phase (N-OVX) and N-OVX mice treated with E2 (50 µg/kg i.p.) 1 h before the carrageenan challenge (N-OVX + E2). Each experimental group was composed of 10 mice.

In a separate set of experiments to evaluate the possible effect of combination therapy with E2 and PPAR-α agonist on carrageenan-induced lung pleurisy, 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 dimethyl sulfoxide 10% solution (clofibrate vehicle); 2) CAR + clofibrate group, same as CAR group but clofibrate was administered 1 h before carrageenan (100 mg/kg i.p. bolus) (n = 10); 3) CAR + E2 group, same as the CAR group but E2 was administered 1 h before carrageenan (10 µg/kg i.p.) (n = 10); 4) CAR + clofibrate + E2 group, same as the CAR group plus clofibrate (100 mg/kg i.p. bolus) and E2 were administered (10 µg/kg i.p.) 1 h before carrageenan (n = 10); 5) sham group, saline solution instead of carrageenan was administered to the mice (n = 10); 6) sham + E2 group, same as sham group but mice received E2 administration (10 µg/kg i.p.) 1 h before saline (n = 10); 7) sham clofibrate group, same as for sham but with clofibrate administration (100 mg/kg i.p. bolus) 1 h before saline (n = 10); and 8) sham + clofibrate + E2 group, same as the WT sham group but mice received clofibrate (100 mg/kg i.p. bolus) and E2 (10 µg/kg i.p.) 1 h before saline (n = 10).
Measurement of Circulating Estrogens by Allen-Doisy Bioassay. The state of proliferation and keratinization of the vaginal epithelium is known to closely reflect the extent of circulating estrogens (Kahnt and Doisy, 1928). Therefore, vaginal smears were performed to assess the phase of the estral cycle in intact rats. Mice that showed only cornified cells were selected for the experiment. Vaginal smears were made 14 days after the ovarectomy to demonstrate the decrease of estrogen.

Histological Examination. Lung tissues samples were taken 4 h after injection of carrageenan. Lung tissues samples were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Sections, stained with hematoxlyn and eosin, were then deparaffinized with xylene. All sections were prepared for use by using AxioVision Zeiss (Milan, Italy) microscope.

Localization of Nitrotyrosine. ICAM-1, P-Selectin, Bax, Bcl-2, CD30, CD30 Ligand, and iNOS were expressed in units per gram weight of wet tissue.

Measurement of Myeloperoxidase Activity. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte extravasation, was detected with a biotin-conjugated goat anti-rabbit, donkey anti-mouse IgG, or anti-rabbit IgG peroxidase conjugate. The sections were incubated overnight with 1) anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v), anti-ICAM-1 polyclonal antibody (CD54) (1:500 in PBS, v/v), anti-P-selectin polyclonal antibody (1:100 in PBS, v/v), anti-GITR rabbit polyclonal antibody (1:100 v/v) or anti-iNOS (1:500 in PBS, v/v), anti-Bax polyclonal antibody (1:100 in PBS, v/v), anti-Bcl-2 polyclonal antibody (1:100 in PBS, v/v), anti-CD30 polyclonal antibody (1:100 in PBS, v/v), or anti-CD30 ligand polyclonal antibody (1:100 in PBS v/v, Santa Cruz, DBA, Milan, Italy). Specific labeling was detected with a biotin-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Milan, Italy). Sections were incubated overnight with 1) anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v), anti-ICAM-1 polyclonal antibody (CD54) (1:500 in PBS, v/v), anti-P-selectin polyclonal antibody (1:100 in PBS, v/v), anti-GITR rabbit polyclonal antibody (1:100 v/v) or anti-iNOS (1:500 in PBS, v/v), anti-Bax polyclonal antibody (1:100 in PBS, v/v), anti-Bcl-2 polyclonal antibody (1:100 in PBS, v/v), anti-CD30 polyclonal antibody (1:100 in PBS, v/v), or anti-CD30 ligand polyclonal antibody (1:100 in PBS v/v, Santa Cruz, DBA, Milan, Italy). Specific labeling was detected with a biotin-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories). To verify the binding specificity for ICAM-1, P-selectin, Bax, Bcl-2, GITR, CD30, CD30 ligand, and 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 the experiments. To confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (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 (Mulan et al., 1985). MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C and was expressed in units per gram weight of wet tissue.

TUNEL Assay. TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer’s instructions (Apotag HRP kit; DBA, Milano, Italy). In brief, sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H2O2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed in PBS. The sections were then deparaffinized with xylene. All sections were prepared for use by using AxioVision Zeiss (Milan, Italy) microscope.

Protein Extraction and Western Blot Analysis. Lung tissues samples were taken 4 h after injection of carrageenan. Lung tissues samples were fixed in 10% PBS-buffered formaldehyde, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Sections, stained with hematoxlyn and eosin, were then deparaffinized with xylene. All sections were prepared for use by using AxioVision Zeiss (Milan, Italy) microscope.

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Western Blot Analysis. Peritoneal macrophages were washed twice with ice-cold PBS, harvested, and resuspended in extraction buffer A containing 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 2 mM EDTA, 5 mM Na$_2$HPO$_4$, 10 mM β-mercaptoethanol, 50 mM NaF, 0.2 mM PMSF, 0.15 µM pepstatin A, 20 µM leupeptin, and 1 mM sodium orthovanadate and centrifuged at 1,000g for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 µM leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000g at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. Protein concentrations were estimated by the Bio-Rad protein assay by using bovine serum albumin as standard.

Fifty micrograms of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Hybond-P PVDF Membrane; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The filter was then blocked with 1/100 PVDF Membrane; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The filter was then blocked with 1/100

Materials. Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company (Milan, Italy). Secondary and nonspecific IgG antibodies for immunohistochemical analysis were obtained from Vector Laboratories. Specific monoclonal antibodies against iNOS (1:2000; BD Biosciences Transduction Laboratories, Lexington, KY) or IκB-α (1:1000; Santa Cruz Biotechnology, Inc.), phospho-NF-κB p65 (serine 536) (Cell Signaling, 1:1000), or NF-κB p65 (1:1000; Santa Cruz Biotechnology, Inc.). Thereafter, filters were incubated with the secondary antibody (anti-mouse IgG or anti-rabbit IgG peroxidase conjugate 1:5000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature. Subsequently, blots were developed by using enhanced chemiluminescence detection reagents (Pierce) and exposed to Kodak X-Omat film. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against the β-actin or in lamin B1 protein. The protein bands on X-ray film were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories).

Measurement of Nitrite-Nitrate Concentration. Total nitrite in medium, an indicator of NO synthesis, was measured as described previously (Crisafulli and Cuzzocrea, 2009). The optical density at 550 nm (OD$_{550}$) was measured by using enzyme-linked immunosorbent assay microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations were calculated by comparison with standard solutions of sodium nitrite prepared in H$_2$O.

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Data Analysis. All values in the figures and text are expressed as mean ± S.E.M. 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-α Gene in the Anti-Inflammatory Property of E2 in Carrageenan-Induced Lung Injury and Inflammatory Cells Infiltration. We analyzed the possible role of PPAR-α in the anti-inflammatory property of E2 during acute inflammation in the lung. For that purpose, we compared the effect of E2 pretreatment in ovariectomized PPAR-αKO and WT mice, subjected to carrageenan-induced pleurisy. At 4 h after intrapleural carrageenan administration, there was a significant increase ($P < 0.01$) of the number of PMN cells, collected from the pleural space as well from the lung tissues, as evaluated by cell count and MPO activity, in carrageenan (CAR in the figure)-

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- MPO activity was significantly enhanced in OVX PPAR-αKO and WT mice (Fig. 1, A and B).
- Ovariectomy did not cause significant changes in these parameters in saline-treated PPAR-αKO and WT mice (Fig. 1, A and B).
- E2 treatment also significantly reduced the number of inflammatory cell infiltration in the pleural cavity as well as in the lung MPO activity in N-OVX WT but not in N-OVX PPAR-αKO mice (Fig. 1, A and B).

- Western Blot Analysis. Peritoneal macrophages were washed twice with ice-cold PBS, harvested, and resuspended in extraction buffer A containing 0.32 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 2 mM EDTA, 5 mM Na$_2$HPO$_4$, 10 mM β-mercaptoethanol, 50 mM NaF, 0.2 mM PMSF, 0.15 µM pepstatin A, 20 µM leupeptin, and 1 mM sodium orthovanadate and centrifuged at 1,000g for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 µM leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000g at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. Protein concentrations were estimated by the Bio-Rad protein assay by using bovine serum albumin as standard.

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No histological alterations were observed in the lung tissues collected from sham WT and sham PPAR-α/KO mice (data not shown). On the contrary, histological examination of lung sections of carrageenan-treated ovariectomized WT and PPAR-α/KO mice showed tissue injury as well as inflammatory cell infiltration (Fig. 2A). Treatment with E2 resulted in a significant reduction of tissue injury in ovariectomized WT (Fig. 2Ab) but not in ovariectomized PPAR-α/KO mice (Fig. 2Ad). These results indicate that the E2 effect in ovariectomized PPAR-α/KO mice was less marked than in ovariectomized WT, suggesting that PPAR-α is important for the E2-mediated anti-inflammatory effect.

Inflammatory cell infiltration is dependent on adhesion molecule expression including ICAM-1 and P-selectin. We evaluated the ICAM-1 and P-selectin expression in the lung tissue of ovariectomized carrageenan-treated WT and PPAR-α/KO mice as well as the effect of E2 treatment.

At 4 h after carrageenan injection, the staining intensity for ICAM-1 (Fig. 2B) and P-selectin (Fig. 2C) was detected along the WT vessels mainly localized in the vascular endothelium. Moreover, in ovariectomized carrageenan-treated PPAR-α/KO mice, the staining for ICAM-1 (Fig. 2B) and P-selectin (Fig. 2C) in the vascular wall was visibly and significantly higher compared with WT mice. Marked inhibition for ICAM-1 (Fig. 2Bb) and P-selectin (Fig. 2Cb) staining was observed in the lung tissues from ovariectomized WT mice after treatment with E2. On the contrary, treatment with estrogen (E2) did not inhibit the expression of ICAM and P-selectin in PPAR-α/KO mice, suggesting that PPAR-α is involved in the anti-inflammatory activity of E2. No positive staining for P-selectin and ICAM-1 was detected in lungs of sham WT and sham PPAR-α/KO mice (data not shown).

**Role of PPAR-α in E2-Induced Inhibition of TNF-α Production.** Release of proinflammatory cytokines is an important mechanism that plays a role in inflammatory process, including carrageenan-induced pleurisy (Cuzzocrea et al., 2006b). At 4 h after carrageenan injection, increased levels of TNF-α protein were observed in lung tissues compared with vehicle-treated controls (Fig. 2D). E2 treatment significantly inhibited TNF-α production in ovariectomized WT but not in ovariectomized PPAR-α/KO mice (Fig. 2D). It is noteworthy that lung tissue production of TNF-α was significantly higher in carrageenan-treated PPAR-α/KO mice compared with WT animals (Fig. 2D). Taken together, these
results indicate that PPAR-\(\alpha\) plays a role in E2-mediated inhibition of carrageenan-induced TNF-\(\alpha\).

**Role of PPAR-\(\alpha\) in E2-Mediated Inhibition of Carrageenan-Induced iNOS Expression and Nitrotyrosine Expression.** iNOS is an important mediator of inflammatory process. We performed immunohistochemical analysis of lung sections obtained from ovariectomized carrageenan-treated WT and PPAR-\(\alpha\)-KO mice. As shown in Fig. 3, A and B, whereas E2 was effective in inhibiting both iNOS and nitrotyrosine in WT mice, it was less active in PPAR-\(\alpha\)-KO mice. In fact, in carrageenan-treated PPAR-\(\alpha\)-KO mice, staining for iNOS (Fig. 3Ad) and nitrotyrosine (Fig. 3Bd) in infiltrated inflammatory cells, pneumocytes, and cells of the vascular wall was clearly increased compared to WT mice. No positive staining for iNOS and nitrotyrosine in lungs of sham WT and sham PPAR-\(\alpha\)-KO mice was detected (data not shown).

**Role of PPAR-\(\alpha\) in E2-Mediated Inhibition of Carrageenan-Induced CD30 and CD30 Ligand.** CD30 and CD30 ligand are important mediators of immune response and inflammation, including acute lung inflammation (Polte et al., 2006). We performed experiments to evaluate the possible effect of E2 on carrageenan-induced CD30 and CD30 ligand expression in ovariectomized WT and PPAR-\(\alpha\)-KO mice. As shown in Fig. 3, C and D, whereas E2 was effective in inhibiting both CD30 and CD30 ligand expression in WT mice (Fig. 3, Cb and Db), it was less active in PPAR-\(\alpha\)-KO mice (Fig. 3, Cd and Dd). Moreover, in carrageenan-treated PPAR-\(\alpha\)-KO mice, staining for CD30 (Fig. 3C) and CD30 ligand (Fig. 3D) in infiltrated inflammatory cells, pneumocytes, and cells of the vascular wall was clearly increased compared to WT mice. No positive staining for CD30 and CD30 ligand in lungs of sham WT and sham PPAR-\(\alpha\)-KO mice was detected (data not shown).

**Role of PPAR-\(\alpha\) in E2-Mediated Inhibition of Carrageenan-Induced GITR Expression.** It is well known that GITR is a coactivating molecule that plays a role in immune response induction as well in the inflammatory process (Nocentini et al., 2007). Moreover, we have recently demonstrated that GITR is expressed in the lung tissues from carrageenan-treated mice (Cuzzocrea et al., 2006b). In lung sections from sham WT and sham PPAR-\(\alpha\)-KO mice, no positive staining for GITR was observed (data not shown). In lung sections from carrageenan-treated ovariectomized WT mice, we obtained a positive staining for GITR in infiltrating macrophages, PMNs, and lymphocytes as well as in vascular endothelium (Fig. 4A). E2 treatment significantly reduced the GITR staining in ovariectomized WT but not in PPAR-\(\alpha\)-KO mice (Fig. 4A, b versus d).

**Role of PPAR-\(\alpha\) in E2-Mediated Inhibition of on Apoptosis in Lung Tissues after Carrageenan-Induced Pleurisy.** To investigate whether acute lung inflammation is

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Fig. 3. Effects of E2 on carrageenan-induced iNOS expression (A) or nitrotyrosine formation (B) evaluated by immunohistochemistry: a, carrageenan-treated WT mice; b, E2-treated carrageenan-treated WT mice; c, carrageenan-treated PPAR-\(\alpha\)-KO (KO) mice; d, E2-treated carrageenan-treated KO mice. Immunohistochemical localization of CD30 (C) or CD30 ligand (D) expression evaluated by immunohistochemistry. E2 significantly reduced CD30 and CD30 ligand levels of WT but not KO mice. a, carrageenan-treated WT mice; b, E2-treated carrageenan-treated WT mice; c, carrageenan-treated KO mice; d, E2-treated carrageenan-treated KO mice. The figure is representative of three different experiments.
associated with apoptotic cell death, we measured TUNEL-like staining in lung tissues. At 4 h after carrageenan administration, lung tissues demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 4B). In contrast, E2 treatment significantly reduced apoptotic cells or fragments formation in ovariectomized WT but not in PPAR-α KO mice (Fig. 4B, b versus d). No apoptotic cells were observed in lungs from sham WT and sham PPAR-α KO mice (data not shown). A positive control is also included (normal rodent mammary gland) (Fig. 4Be).

Western Blot Analysis and Immunohistochemistry for Bax and Bcl-2. Bax and Bcl-2 are important molecules in regulation of apoptosis. The presence of Bax in lung homogenates was investigated by Western blot 4 h after carrageenan administration. No basal level of Bax was detected in lung tissues obtained from sham-treated animals (Fig. 5Aa, see densitometry analysis a1) (sham KO is the same as sham WT; data not shown). Bax levels were substantially increased in the lung tissues from carrageenan-treated WT and PPAR-α KO mice (Fig. 5Aa, see densitometry analysis a1). E2 treatment significantly reduced Bax expression in ovariectomized WT but not in PPAR-α KO mice (Fig. 5Aa, see densitometry analysis a1).

To detect Bcl-2 expression, protein extracts from lung tissues were also analyzed by Western blot analysis. A basal level of Bcl-2 expression was detected in lung tissues from sham-treated mice (Fig. 5Ab, see densitometry analysis b1). At 4 h after carrageenan administration, Bcl-2 expression was significantly reduced in the lung tissues from carrageenan-treated WT and PPAR-α KO mice (Fig. 5Ab, see densitometry analysis b1). Treatment of mice with E2 significantly countered the reduction of Bcl-2 expression in ovariectomized WT but not in PPAR-α KO mice (Fig. 5Ab, see densitometry analysis b1).

Lung samples were also collected 4 h after carrageenan administration to determine the immunohistological staining for Bax and Bcl-2. Lung tissues taken from sham WT (data not shown) and sham PPAR-α KO mice (Fig. 5Ba) did not stain for Bax, whereas lung sections obtained from carrageenan-treated WT and PPAR-α KO mice exhibited positive staining for Bax (Fig. 5B, b and d). E2 treatment significantly reduced the degree of positive staining for Bax in the lung of ovariectomized WT but not in PPAR-α KO mice (Fig. 5B, c versus e). In addition, lung sections from sham WT (data not shown) and sham PPAR-α KO mice (Fig. 5Ca) demonstrated positive staining for Bcl-2, whereas in carrageenan-treated WT and PPAR-α KO mice Bcl-2 staining was significantly reduced (Fig. 5C, b and d). E2 treatment significantly attenuated the loss of positive staining for Bcl-2 in the lung of ovariectomized WT but not in PPAR-α KO mice (Fig. 5C, c versus e).

Effect of E2 on PPAR-α Expression in Lung Tissue. Moreover, to better elucidate the protective effect of E2, we examined the modification of PPAR-α in the lung tissue. Ovariectomy provoked a clear decrease of this specific receptor. Moreover, carrageenan injection in ovariectomized WT mice induced a further reduction of PPAR-α expression. E2 treatment attenuated this reduction, as revealed by densitometry analysis (Fig. 6).

Effect of E2 on Macrophage Inflammatory Markers. Taken together, the above results indicate that PPAR-α can favor the anti-inflammatory activity of E2. Macrophages are important players in inflammatory processes in vivo. To fur-
ther analyze the effect of PPAR-α gene deletion on E2 anti-inflammatory property, we carried out in vitro experiments by using elicited peritoneal macrophages from both ovariec-
tomized WT and PPAR-αKO mice. In particular, macro-
phages were treated with LPS (10 μg/ml) and IFN-γ (100 U/ml) for 24 h. As shown in Fig. 7A, unstimulated (untreated) peritoneal macrophages from WT or PPAR-αKO mice pro-
duced small amounts of measurable nitrite, the stable end product of NO. Stimulation significantly increased the nitrite/nitrate ($P < 0.001$) production by peritoneal macro-
phages from WT and PPAR-αKO mice (Fig. 7A). Moreover, the nitrite/nitrate production was significantly increased in stimulated macrophages from PPAR-αKO mice compared to the corresponding WT cells. When E2 ($10^{-7}$, $10^{-8}$, and $10^{-9}$ M) was added to WT macrophages, 2 h before LPS and IFN-γ stimulation, a significant decrease of nitrite/nitrate production in cell medium was observed in a concentration-dependent manner (Fig. 7A). On the contrary, the absence of a functional PPAR-α gene in macrophages from PPAR-αKO resulted in lack of E2 effect (Fig. 7A).

We also performed experiments to determine the E2 effect on LPS and IFN-γ-induced iNOS protein expression. For that purpose, iNOS protein expression in whole lysates from peri-
toneal macrophages, from WT or PPAR-αKO mice, stimu-
lated with LPS and IFN-γ and pretreated with E2, as de-
scribed above, was evaluated. As shown in Fig. 6B, LPS and IFN-γ caused a significant increase of iNOS protein levels at 24 h, compared to unstimulated (untreated) cells (Fig. 6, compare column 2 to column 1). When WT cells were pre-
treated with E2 ($10^{-7}$ M), 2 h before LPS and IFN-γ stimulation, iNOS protein expression was inhibited in a concen-
tration-dependent manner to LPS and IFN-γ-stimulated cells, respectively (Fig. 7B, compare column 3 to column 2). The absence of a functional PPAR-α gene in macrophages from PPAR-αKO mice significantly weakened this E2 effect on iNOS expression (Fig. 7B).

**Role of PPAR-α on E2-Induced Inhibition of NF-κB Activation.** It is well known that NF-κB activation and the consequent nuclear translocation play a pivotal role in inflam-

Fig. 5. A, representative Western blot analysis of Bax (a) or Bcl-2 (b) expression from lung tissue of carrageenan-treated WT and PPAR-αKO (KO) mice with or without E2 administration. The immunoblots shown are representative of one of three different experiments. In the bottom panels of each Western blot, a representative densitometry analysis is expressed as mean ± S.E.M. of three different experiments. +, $P < 0.01$ versus Sham; ++, $P < 0.01$ versus carrageenan-WT group; +++, $P < 0.01$ versus carrageenan-WT + E2 group. Immunohistochemical localization of Bax expression (B) or Bcl-2 (C) from lung tissue of carrageenan-treated WT and KO mice with or without E2 administration evaluated by immunohistochemistry: a, sham KO mice; b, carrageenan-treated WT mice; c, E2-treated carrageenan-treated WT mice; d, carrageenan-treated KO mice; e, E2-treated carrageenan-treated KO mice. The figure is representative of three different experiments.
agonists have no effect on airway inflammation (Catley et al., 2008). Results here indicated that ERα expression increases in lung homogenates of carrageenan-treated WT mice (Fig. 7E, column 3 versus column 1). It is interesting to note that in carrageenan-treated PPAR-α/KO mice, the increase of ERα levels is significantly reduced compared to carrageenan-treated WT mice (Fig. 7E, column 4 versus column 3). Moreover, E2 treatment affects ERα mRNA levels in WT mice (Fig. 7E, columns 5 versus column 3), but not in PPAR-α/KO mice (Fig. 7E, columns 6 versus 4). Taken together, these results indicate that PPAR-α is important for the modulation of ER expression, thus contributing to the E2-mediated effects.

**Combination Therapy with Clofibrate and E2 Reduces Pleural Infiltration of Macrophages and Lung Infiltration of PMNs.** We performed experiments to evaluate the possible effect of combination therapy with clofibrate, a PPAR-α agonist, and E2 on carrageenan-induced lung injury in WT mice. In a preliminary set of experiments, we performed dose-response experiments with clofibrate and E2. Results indicated that a combination of clofibrate (100 mg/kg i.p.) and E2 (10 μg/kg i.p.) significantly reduced, in a dose-dependent manner, the PMN infiltration in the pleural cavity and lung MPO activity. Please note that clofibrate (at 100 mg/kg i.p.) or E2 (at 10 μg/kg i.p.) alone did not inhibit PMN infiltration in the pleural cavity and lung MPO activity. Moreover, although clofibrate (at a suboptimal dose of 100 mg/kg i.p.) or E2 (at the dose of 10 μg/kg i.p.) alone did not inhibit lung tissue injury, combination of the two agents (clofibrate + E2) resulted in a detectable inhibition (Fig. 8, A and B).

**Discussion**

In the present article, we show that the absence of PPAR-α, in PPAR-α/KO mice, results in a reduced anti-inflammatory response to E2 treatment. In particular, we show that PPAR-α plays a role in E2 control of a number of inflammatory parameters such as cell tissue infiltration, lung injury, proinflammatory cytokines production, iNOS induction, and oxidative stress. Diversity and correlation of inflammatory diseases, such as atherosclerosis, systemic lupus erythematosus, or RA, with female sex hormones levels have been reported, thus suggesting that these hormones contribute to the control of inflammation. In particular, estrogens may play a relevant role in the control of immune response, and several experimental models of chronic inflammatory diseases support this hypothesis (Modugno et al., 2005). On the other hand, other lines of evidence suggest that estrogens have anti-inflammatory activity. For example, the incidence of RA is lower in fertile women than in men (Kay et al., 1983), and the same is true for cardiovascular insults, possibly because women have lower incidence of chronic inflammation-related athermanous lesions (Cushman et al., 1999). During pregnancy, multiple sclerosis undergoes remission and is exacerbated in the postpartum period (Confavreux et al., 1998). During menopause, users of hormone replacement therapy show a delayed manifestation of neurodegenerative diseases with inflammatory components like Alzheimer’s (Honjo et al., 1995). They also have a reduced risk of cardiovascular events (Nathan and Chaudhuri, 1997) as well as a slower osteoporosis progression (Horowitz, 1993), possi-
bly due to the estrogens-mediated suppression of inflammatory cytokine production, stimulation of osteoclastogenesis, and bone resorption. Estrogens display anti-inflammatory activity in several animal models including rat adjuvant-induced arthritis (Badger et al., 1999), endotoxin-induced uveitis (Miyamoto et al., 1999), vascular inflammatory reaction (Rhodin et al., 2000), and experimental encephalomyelitis (Offner et al., 2000). The mechanism responsible for the anti-inflammatory activity of estradiol systemic administration may be due to the hormone-dependent blockade of cytokine production, such as IL-1 and TNF-α. Moreover, the anti-inflammatory effect of acute administration of E2 (50 μg/kg) on carrageenan paw edema of male rats was also shown (Esposito et al., 2005). Estrogens might also inhibit macrophage infiltration in the damaged tissues. The centrality of macrophages in disease processes makes macrophage regulation a major target in the prevention, control, and cure of inflammatory processes. Consequently, macrophage-restricted genes may be crucial targets for therapeutic intervention. Macrophages express both of the described intracellular estrogens receptors ERα and ERβ (Vegeto et al., 1999), and their exposure to low concentrations of estradiol results in decreased production of inflammatory agents such as TNF-α (Mendelsohn and Karas, 1999). Moreover, estrogens...
anti-inflammatory efficacy resides in part in the capability to counter activation of NF-κB, an important transcription factor in inflammation, production of cytokines relevant to the inflammatory process such as for example TNF-α, induction of iNOS and COX-2 as well increase of their enzymatic activity (Cuzzocrea et al., 2001). In addition, estrogens inhibit adhesion molecule expression (for example, ICAM-1) and the consequent PMN and macrophage migration into the inflamed tissue (Cuzzocrea et al., 2001). Many studies indicate that PPAR-α is able to directly mediate some anti-inflammatory effects, and it has been shown that its agonist-induced activation inhibits a number of inflammatory mechanisms including TNF-α production, iNOS, COX-2, and adhesion molecule expression as well cell infiltration in the tissues (Cuzzocrea et al., 2004).

Based on these observations, we performed studies in an attempt to determine whether PPAR-α contributes to the estrogens anti-inflammatory efficacy. For that purpose, we used an experimental model of acute lung inflammation, performed by injecting ovariectomized WT and PPAR-αKO mice with carrageenan, and tested the anti-inflammatory efficacy of E2. Coactivating molecules of the TNF receptor family play a role in immune and inflammatory responses. This is also the case for CD30, which has been shown to play a role in lung inflammation (Polte et al., 2006) and GITR. In this study, we show that E2 inhibits CD30 and CD30L expression in WT but not in PPAR-αKO mice. We have recently demonstrated that GITR plays a role in acute inflammation of the lungs. In fact, mice with a targeted deletion of the GITR gene [GITR(−/−) mice] show a significantly lower carrageenan-induced lung inflammatory response compared to GITR(+/+) mice (Cuzzocrea et al., 2006b). In the present study, we confirmed that in lung sections from carrageenan-treated WT mice, there was a positive staining for GITR in infiltrating macrophages, PMNs, and lymphocytes as well as in vascular endothelium. We also demonstrated that the E2 treatment significantly reduced the GITR expression in ovariectomized WT but not in PPAR-αKO mice (Fig. 6). This effect, considering that GITR, like CD30 and other molecules of the TNF receptor family, acts as a coactivating molecule and induces NF-κB activation, can contribute to E2-mediated anti-inflammatory activity.

It is known that NF-κB activation is central in inflammation and that estrogens modulate its activity by different mechanisms including the increase of IκB expression (Ghisletti et al., 2005). Therefore, we have showed that there is cross-talk between the glucocorticoid receptor and ER in conditions associated with experimental acute inflammation (Cuzzocrea et al., 2001). This cross-interaction might be therapeutically relevant when steroidal anti-inflammatory compounds are administered to women treated with ER antagonists. In this regard, in the present study we have shown that like glucocorticoids, E2 inhibits NF-κB. Our results indicate that E2-induced IκB overexpression is well detected in LPS-stimulated peritoneal macrophages of WT but not PPAR-αKO mice. It is noteworthy that E2 treatment inhibited p65NF-κB nuclear localization, an event associated with NF-κB activation, in WT but not in PPAR-αKO mice. During the inflammatory process, due to NF-κB and other transcription factors activation, there is an increased production of proinflammatory cytokines, such as TNF-α, which is important to induce local and systemic inflammation (Cannarile et al., 2008). When WT and PPAR-αKO mice were treated with E2, a significant inhibition of TNF-α level was measured in WT but not in PPAR-αKO mice. Up-regulation and activation of iNOS, with consequent nitrite/nitrate production, are typical characteristics of inflammatory process. E2 can counter these effects, and we show in this study that E2 treatment significantly inhibited inflammatory parameters in WT but not in PPAR-αKO mice. It is noteworthy that a lack of E2-induced iNOS inhibition was evident in experiments with isolated peritoneal macrophages from PPAR-αKO mice. These results could suggest a role for PPAR-α on functions including in vivo interaction with vessel walls, which takes place during inflammation. Several studies have implicated the formation of reactive oxygen species and reactive nitrogen species in acute inflammation. Nitrotyrosine formation was initially proposed as a specific marker for peroxynitrite (Beckman, 1996). However, there is recent evidence indicating that other reactions can also induce tyrosine nitration (Endoh et al., 1994). Therefore, increased nitrotyrosine staining is considered to be an indication of “increased nitrosative stress.” To confirm the pathological contributions of peroxynitrite to acute lung injury induced by carrageenan administration in mice, we evaluated the formation of nitrotyrosine in the injured tissue. Our results indicate that the immunostaining for nitrotyrosine was reduced in E2-treated WT but not in PPAR-αKO mice.

Apoptosis is characteristic of inflammatory damage, and recent studies have demonstrated the induction of apoptosis in different cell lines in response to reactive oxygen species,
peroxynitrite, and NO (Merrill et al., 1993). By using the TUNEL coloration and Western blot analysis, we have confirmed that E2 plays an important role in the attenuation of apoptosis during acute lung injury. In this regard, we have identified proapoptotic transcriptional changes, including up-regulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2. Antioxidative activities of E2 might in part explain some of the findings reported in the present study. However, the antioxidant activity of E2 is observed at pharmacological concentrations of the hormone and is not blocked by antagonists of the estrogen receptors. Therefore, we propose that the lack of lung injury in E2-treated WT mice reported here is not due to antioxidant action of estrogens, because mice were treated with a dose of the hormone that was not very high. Of significance, the protective effects of E2 were not observed when E2 was administered to PPAR-αKO mice, again indicating that the observed effects of E2 are dependent, at least in part, from PPAR-α receptor. Our results clearly indicate an important contribution of PPAR-α to estrogen-induced anti-inflammatory action; however, the initial molecular mechanisms responsible for this effect remain to be clarified. Results in this study indicate that PPAR-α is important for ER up-regulation. More recently, we have clearly demonstrated that the anti-inflammatory efficacy of dexamethasone treatment is favored by the presence of PPAR-α (Cuzzocrea et al., 2008). The results in this study clearly demonstrate the relevance of the PPAR-α receptor in the regulation of the anti-inflammatory effects of estrogen therapy and suggest future studies aimed to analyze the possible relevance of PPAR-α in other human inflammatory disease models, such as sepsis and experimental colitis.

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References

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