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Rosiglitazone, a ligand of the peroxisome proliferator-activated receptor-γ, reduces acute inflammation

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are related to retinoid, steroid and thyroid hormone receptors. The PPAR-γ receptor subtype appears to play a pivotal role in the regulation of cellular proliferation and inflammation. The thiazolidinedione rosiglitazone (Avandia) is a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, that was recently approved by the Food and Drug Administration for treatment of type II diabetes mellitus. In the present study, we have investigated the effects of rosiglitazone in animal models of acute inflammation (carrageenan-induced paw oedema and carrageenan-induced pleurisy). We report here for the first time that rosiglitazone (given at 1, 3 or 10 mg/kg i.p. concomitantly with carrageenan injection in the paw oedema model, or at 3, 10 or 30 mg/kg i.p. 15 min before carrageenan administration in the pleurisy model) exerts potent anti-inflammatory effects (e.g. inhibition of paw oedema, pleural exudate formation, mononuclear cell infiltration and histological injury) in vivo. Furthermore, rosiglitazone reduced: (1) the increase in the staining (immunohistochemistry) for nitrotyrosine and poly (ADP-ribose) polymerase (PARP), (2) the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), intercellular adhesion molecules-1 (ICAM-1) and P-selectin in the lungs of carrageenan-treated rats. In order to elucidate whether the protective effect of rosiglitazone is related to activation of the PPAR-γ receptor, we also investigated the effect of a PPAR-γ antagonist, bisphenol A diglycidyl ether (BADGE), on the protective effects of rosiglitazone. BADGE (30 mg/kg i.p.) administered 30 min prior to treatment with rosiglitazone significantly antagonized the effect of the PPAR-γ agonist and thus abolished the anti-inflammatory effects of rosiglitazone. We propose that rosiglitazone and other potent PPAR-γ agonists may be useful in the therapy of inflammation.

Keywords: PPAR (peroxisome proliferator-activated receptor); Rosiglitazone; Inflammation, acute; iNOS (nitric oxide synthase inducible); Cyclooxygenase-2; ICAM-1 (intercellular adhesion molecule-1)

1. Introduction

The inflammatory process is invariably characterised by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and by a release of chemicals from tissues and migrating cells (Tomlinson et al., 1994). Carrageenan-induced local inflammation is commonly used to evaluate non-steroidal anti-inflammatory drugs (NSAID). Therefore, carrageenan-induced local inflammation (paw oedema or pleurisy) is a useful model to asses the contribution of mediators involved in vascular changes associated with acute inflammation.

In particular, the initial phase of inflammation (oedema, 0–1 h) which is not inhibited by NSAID such us 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 more recently has been attributed to the induction of inducible cyclo-oxygenase (COX-2) in the tissue (Nantel et al., 1999). It appears that the onset of the carrageenan local inflammation has been linked to neutrophil infiltration and

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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).

Furthermore, there is a large amount of evidence that the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contribute to tissue damage (Dawson et al., 1999; Cuzzocrea et al., 1997). Inhibitors of nitric oxide synthase (NOS) activity reduce the development of carrageenan-induced inflammation and support a role for nitric oxide (NO) in the pathophysiology associated with this model of inflammation (Cuzzocrea et al., 1997; Wei et al., 1995). In addition to NO, peroxynitrite is also generated in carrageenan-induced inflammation (Cuzzocrea et al., 1997).

Peroxisome proliferator activated receptors (PPAR) are ligand activated transcription factors belonging to the nuclear hormone receptor superfamily, which includes the classical steroid, thyroid, and retinoid hormone receptors as well as many orphan receptors. So far, three PPAR isotypes have been identified and are commonly designated PPARα, PPARß and PPARγ. The actions of PPAR were originally thought to be limited to the control of lipid metabolism and homeostasis. Recent studies, however, showed that PPAR activation can regulate inflammatory responses and cellular proliferation and differentiation as well as apoptosis (Escher and Wahli, 2000; Corton et al., 2000). Therefore, it is possible that PPAR-γ trans-represses the expression of pro-inflammatory mediators at the transcriptional level by inhibiting NF-κB, STAT-1 and activation protein-1 (AP-1) signalling (Ricote et al., 1998). There is also evidence to support a role of PPARγ in various physiopathological conditions including cancer, atherosclerosis and diabetes. Synthesized ligands, thiazolidinedione derivatives (TZD), such as rosiglitazone, pioglitazone and roglitazone, are used as oral antihyperglycemic agents in the therapy of non-insulin-dependent diabetes mellitus (Vamecq and Latruffe, 1999; Kadowaki, 2000). One such compound, rosiglitazone binds with a high affinity to PPARγ (Lehmann et al., 1995) All TZD tested to date, i.e., rosiglitazone, pioglitazone, and troglitazone, bind and activate the PPARγ isotype with K_d that parallel their antidiabetic activity in vivo (Lehmann et al., 1995; Willson et al., 1996). In addition to TZD, other synthetic compounds have been identified as PPARγ activators. It has been also shown that several NSAID, such as indomethacin, ibuprofen, fenoprofen, and flufenamic acid, bind and activate PPARγ and promote adipocyte differentiation (Lehmann et al., 1997). In addition, recent studies have also shown that PPARγ may participate in control of inflammation, especially, in modulating the production of inflammatory mediators (Jiang et al., 1998; Ricote et al., 1998). Recent studies have shown that bisphenol A diglycidyl ether (BADGE) is a PPAR-γ antagonist in various cell line including 3T3-L1 and 3T3-F442A cells (Wright et al., 2000). Therefore, our research group have recently, demonstrated in vivo that BADGE is able to revert the protective effect of rosiglitazone (Cuzzocrea et al., in press). In the present study, we have investigated the effects of rosiglitazone on paw oedema and lung injury associated with carrageenan-induced pleurisy. In particular, we have investigated the effect of the PPAR-γ agonist rosiglitazone on (i) PMN infiltration [myeloperoxidase (MPO) activity], (ii) lipid peroxidation [malondialdehyde (MDA) levels], (iii) COX-2 expression (by immunohistochemistry), (iv) the nitration of tyrosine residues (an indicator of the formation of peroxynitrite) (by immunohistochemistry), (v) poly (ADP-ribose) polymerase (PARP) activation, (vi) iNOS expression and (vii) lung damage (histology). Finally, in order to elucidate whether the protective effects of rosiglitazone is related to the activation of the PPAR-γ receptor, we have also investigated if BADGE, the PPAR-γ antagonist, is able to attenuate the protective effects of rosiglitazone.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (300–350 g; Charles River; Milan; Italy, used for the paw oedema study) and Sprague–Dawley rats (140–160 g, Harlan, Milan; Italy, used for the pleurisy study) 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 purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Paw oedema

Paw oedema was induced by subplantar injection into the rat right hind paw of 0.1 ml sterile saline containing 1% λ-carrageenan (control group). A group of rats were treated with rosiglitazone maleate (1, 3 and 10 mg/kg, Alexis, Milan, Italy) administered intraperitoneally (i.p.) concomitantly with carrageenan injection. In some experiments animals treated with 10 mg/kg rosiglitazone maleate, as previously described, were also treated with BADGE, (30 mg/kg/i.p. Fluka) given 30 min before carrageenan injection. The test agents were solubilized in 10% dimethylsulfoxide (DMSO). Control group of animals received the same volume of vehicle instead of the tested agents. The volume of the paw was measured by a plethysmometer (Basile, Italy) immediately after the injection as previously described (Di Rosa and Willoughby, 1971). Subsequent readings of the same paw were carried out at one hour intervals up to 4 h and compared to the initial readings. The increase in paw volume was taken as oedema volume.

2.3. Carrageenan-induced pleurisy

Carrageenan-induced pleurisy was induced as previously described (Cuzzocrea et al., 1998). Rats were anesthe-

tised 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% (w/v) 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 CO_2. 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 measured. Any exudate, which 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 leukocytes in the exudate were suspended in phosphate-buffer saline (PBS, 0.01 M, pH7.4) and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

2.4. Experimental groups for carrageenin-induced pleurisy

Rats were randomly allocated into the following groups: (i) CAR + vehicle group. Rats were subjected to carrageenan-induced pleurisy and received the vehicle for rosiglitazone (10% dimethylsulfoxide (DMSO) (v/v) i.p. bolus 30 min prior to carrageen ($N = 10$), (ii) Rosiglitazone group. Same as the CAR + vehicle group but were administered rosiglitazone (3, 10 and 30 mg/kg i.p. bolus) 30 min prior to carrageenan ($N = 10$), (iii) BADGE + Rosiglitazone groups. Same as the Rosiglitazone group but BADGE was administered (30 mg/kg i.p. bolus) 30 min prior to rosiglitazone (30 mg/kg i.p.) ($N = 10$), (iv) Sham + saline group. sham-operated group in which identical surgical procedures to the CAR group was performed, except that the saline was administered instead of carrageenan Sham + BADGE group. Identical to Sham + saline group except for the administration of BADGE (30 mg/kg i.p. bolus) 30 min prior to identical surgical procedures ($N = 10$).

2.5. Histological examination

Lung biopsies were taken 4 h after injection of carrageenan. Lung biopsies were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were then deparaffinized with xylene, stained with hematoxylin and eosin. All sections were studied using light microscopy (Dialux 22 Leitz).

2.6. Measurement of cytokines

TNF-α and IL-1β levels were evaluated in the exudate 4 h after the induction of pleurisy by carrageenan injection as previously described (Cuzzocrea et al., 2002). The assay was carried out using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem, Milan, Italy) with a lower detection limit of 10 pg/ml.

2.7. Measurement of nitrite–nitrate concentration

Total nitrite in exudates, an indicator of nitric oxide (NO) synthesis, was measured as previously described (Cuzzocrea et al., 1998). Briefly, the nitrate in the sample was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and â-nicotinamide adenine dinucleotide 3′ -phosphate (NADPH)(160 μM) at room temperature for 3 h. The total nitrite concentration in the samples was then measured using the Griess reaction, by adding 100 μl of Griess reagent (0.1% (w/v) naphthylethylendiamide dihydrochloride in H_2O and 1% (w/v) sulphanilamide in 5% (v/v) concentrated H_3PO_4; vol. 1:1) to the 100 μl sample. The optical density at 550 nm (OD_{550}) was measured using ELISA microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in H_2O.

2.8. Determination of nitric oxide synthase activity

The calcium-independent conversion of L-arginine to L-citrulline in the homogenates of either pleural macrophages or lungs (obtained 4 h after carrageenan treatment in the presence or absence of rosiglitazone) served as an indicator of inducible nitric oxide synthase (iNOS) activity (Cuzzocrea et al., 1998). Cells or tissues were homogenised on ice using a tissue homogenizer in a homogenation buffer composed of 50 mM Tris–HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM PMSF (pH 7.4). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates as previously described (Cuzzocrea et al., 1998). Briefly, homogenates (30 μl) were incubated in the presence of [^{3}H]L-arginine (10 μM, 5 kBq per tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (2 mM) for 20 min at 22 °C. Reactions were stopped by dilution with 0.5 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50 W (Na^+ form) columns and the eluted [^{3}H]L-citrulline activity was measured by a Beckman scintillation counter.

2.9. Measurement of prostaglandin E_2 in the pleural exudate

The amount of PGE_2 present in the pleural fluid of rats collected 4 h after carrageenan administration. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem, La Jolla, CA, USA).

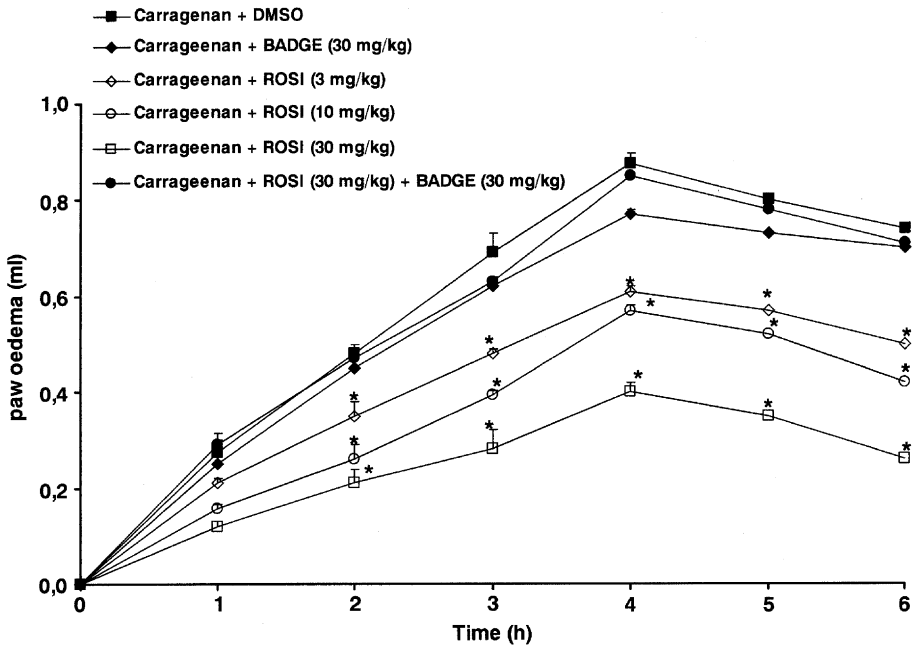

Fig. 1. Effect of rosiglitazone (ROSI) (3, 10 and 30 mg/kg i.p.) on paw edema development elicited by carrageenan in the rat. ROSI (3, 10 and 30 mg/kg i.p.) reduced the carrageenan-induced paw edema development in a dose dependent manner. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist. Data are means ± S.E.M. from 10 rats for each group. *$P<0.01$ versus carrageenan group at the indicated time points.

2.10. Assessment of cyclo-oxygenase activity

Lung tissue, obtained 4 h after the induction of pleurisy by carrageenan injection, was homogenised at 4 °C in a buffer containing the following protease inhibitors: HEPES (20 mM, pH 7.2), sucrose (320 mM), Dithiotreitol (1 mM), styrosporin (10 μg/ml), aprotinin (2 μg/ml), and leupeptin (10 μg/ml). Homogenates were incubated at 37 °C for 30 min in the presence of excess arachidonic acid (30 μM). The samples were boiled and centrifuged at 10,000 × *g* for 5 min. The concentration of 6-keto-$PGF_{1\alpha}$ present in the supernatant was then mea-

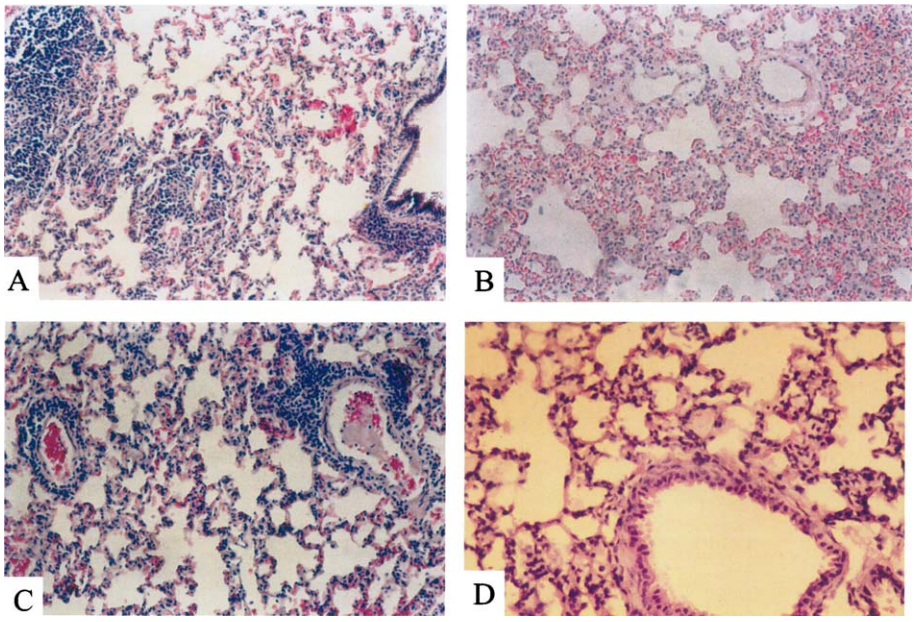

Fig. 2. Effect of rosiglitazone (ROSI) on lung injury. Lung section from a carrageenan-treated rats (A) demonstrating interstitial haemorrhage and PMN accumulation. Lung section from a carrageenan-treated rats after administration of ROSI (30 mg/kg) (B) demonstrating reduced interstitial haemorrhage and cellular infiltration. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist (C). No histological alteration was observed in the lung sections taken from saline-treated animals (D), Original magnification: × 125. Figure is representative of at least three experiments performed on different experimental days.

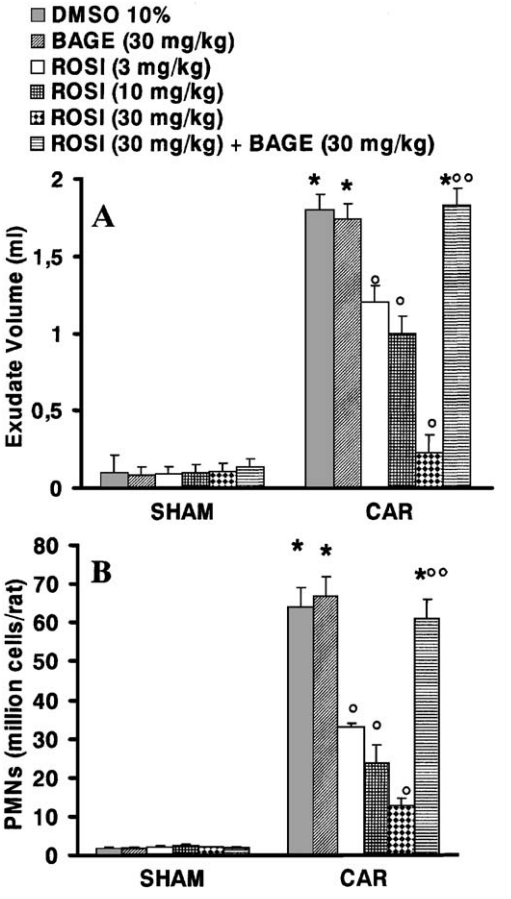

Fig. 3. Effect of rosiglitazone (ROSI) on carrageenan-induced inflammation. Volume exudate (A) and accumulation of polymorphonuclear cells (B) in pleural cavity at 4 h after carrageenan injection. ROSI (3, 10 and 30 mg/kg i.p.) significantly reduced pleural exudation and leukocyte infiltration in a dose dependent fashion. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist. Data are means ± S.E.M. from 10 rats for each group. *$P<0.01$ versus sham. °$P<0.01$ versus carrageenan. °°$P<0.01$ versus ROSI.

sured by radioimmunoassay as previously described (Tomlinson et al., 1994). Protein concentration in each homogenate was measured using the Bradford assay with bovine serum albumin (BSA) used as standard (Bradford, 1976).

2.11. Immunohistochemical localisation of COX-1 and COX-2

Lung biopsies were fixed in 10% (w/v) PBS-buffered formalin and 8 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 permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific binding was minimised 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 avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with a 1:500 dilution of either the primary anti-COX-1 or anti-COX-2 monoclonal antibody (DBA) or with control solutions, which included buffer alone or non-specific, purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase (DBA).

2.12. Immunohistochemical localisation of ICAM-1, P-selectin, 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 previously described (Cuzzocrea et al., 2001). At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization,

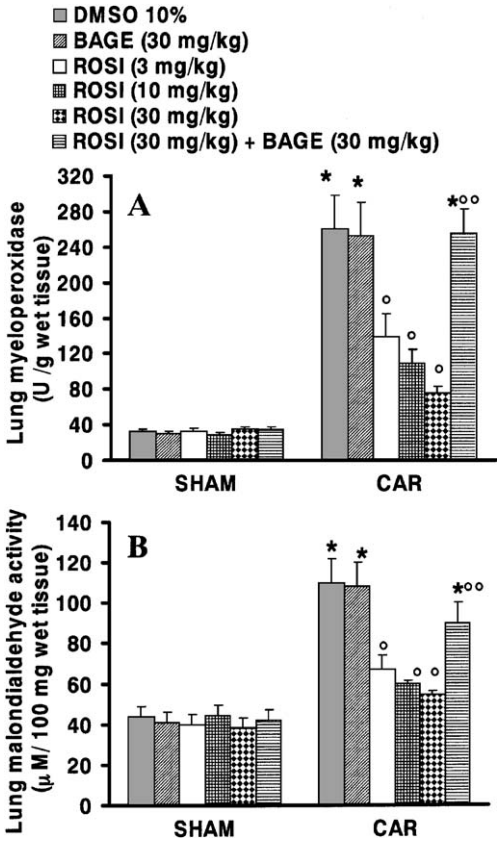

Fig. 4. Effect of rosiglitazone (ROSI) on myeloperoxidase activity and malondialdehyde levels in the lung. Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) levels (B) in the lungs of carrageenan-treated rats killed after 4 h. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated rats in comparison to SHAM rats. ROSI (3, 10 and 30 mg/kg i.p.) reduced the carrageenan-induced increase in MPO activity and MDA levels in a dose dependent manner. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist. Data are means ± S.E.M. from 10 rats for each group. *$P<0.01$ versus sham. °$P<0.01$ versus carrageenan. °°$P<0.01$ versus ROSI.

endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimised 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), respectively. Sections were incubated overnight with (1) rabbit anti-human polyclonal antibody directed towards P-selectin (CD62P) which reacts with rat and with mouse anti-rat antibody directed at ICAM-1 (CD54) (1:500 in PBS, w/v) (DBA) or (2) with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-poly (ADP-ribose) goat policlonal antibody rat (1:500 in PBS, v/v) or with anti-iNOS policlonal antibody rat (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). In order 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 ICAM-1, P-selectin, PAR or iNOS some

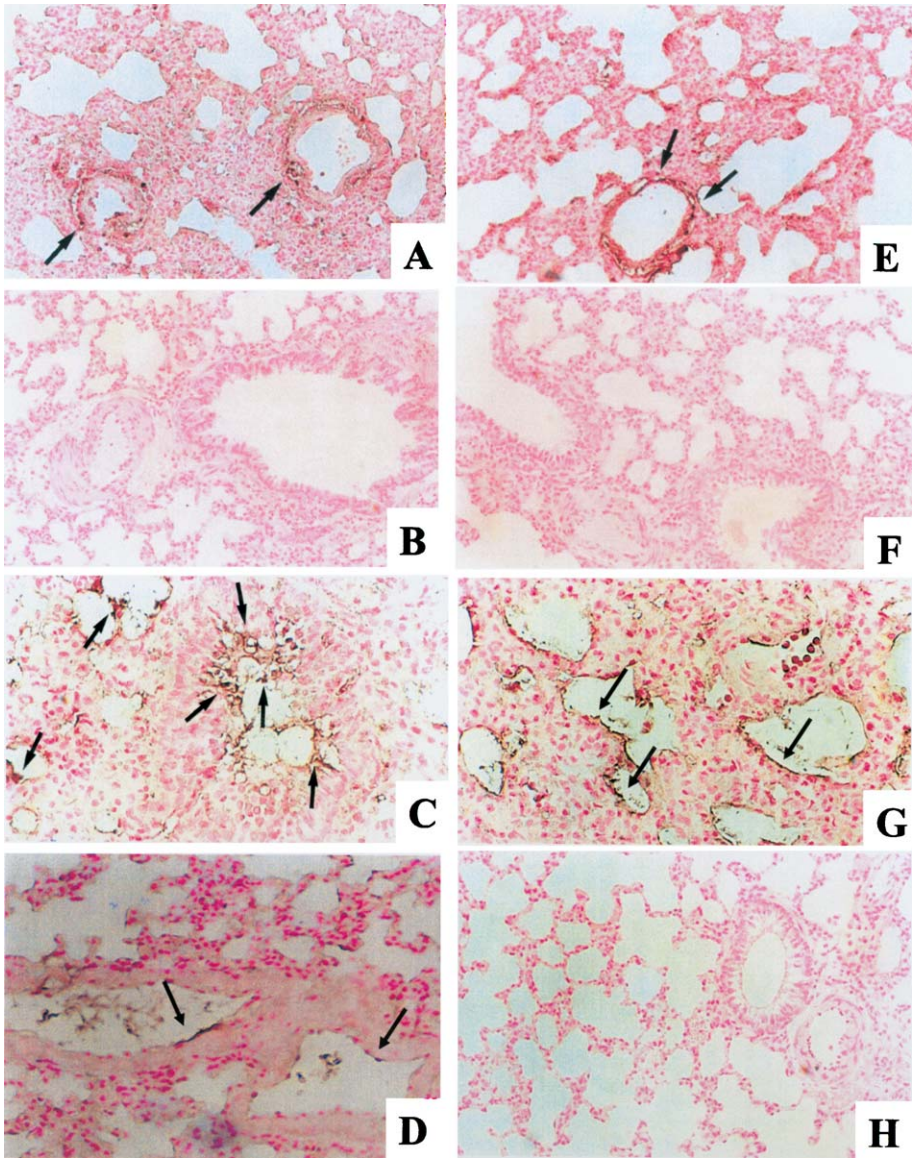

Fig. 5. Immunohistochemical localization of ICAM-1 and P-selectin in the lung. Section obtained from carrageenan-treated rats showed intense positive staining for ICAM-1 (A) and for P-selectin (C) on bronchial epithelium. The degree of bronchial epithelium staining for ICAM-1 (B) and for P-selectin (D) was markedly reduced in tissue section obtained from rosiglitazone-treated rats (30 mg/kg, i.p.). Co-administration of BADGE and rosiglitazone blocked the effect of the PPAR-γ agonist on ICAM-1 (C) and P-selectin expression (F). Staining of lung tissue sections obtained from saline-treated rats with anti-ICAM-1 antibody showed a specific staining along bronchial epithelium demonstrating that ICAM-1 is constitutively expressed (D). No positive staining for P-selectin was found in lung tissue section from saline-treated rats (H). Original magnification: × 150. Figure is representative of at least three experiments performed on different experimental days.

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.

2.13. Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Mullane et al., 1985). At the specified time following injection of carrageenan, lung tissues were obtained and weighed, each piece homogenised in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* 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 hydrogen peroxide. 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 milliunits per g of wet tissue.

2.14. Malondialdehyde measurement

Malondialdehyde (MDA) levels in the lung tissue were determined as an indicator of lipid peroxidation as previously described (Ohkawa et al., 1979). Lung tissue collected at the specified time, was homogenised in 1.15% (w/v) KCl solution. A 100 μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% (w/v) SDS, 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) thiobarbituric acid and 700 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm.

2.15. Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Poole, Dorset, UK). Rosiglitazone was obtained from Alexis. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

2.16. Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (S.E.M.) of *N* observations. For the in vivo studies *N* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Data sets were examined by one- or two-way analysis of variance, and individual group means were then compared with Student's unpaired *t*-test. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of rosiglitazone on the course of the carrageenan-induced paw oedema

Carrageenan injection caused a time-dependent increase of paw volume with a peak occurring at 4 h (Fig. 1). Rosiglitazone (3, 10 or 30 mg/kg/i.p.) significantly inhibited the oedema caused by carrageenan in a dose-related fashion. The inhibitory effect of rosiglitazone (30 mg/kg/i.p.) was completely reversed by the administration of 30 mg/kg/i.p. BADGE (Fig. 1). Treatment of rats with BADGE alone (30 mg/kg) did not modify oedema formation (data not shown).

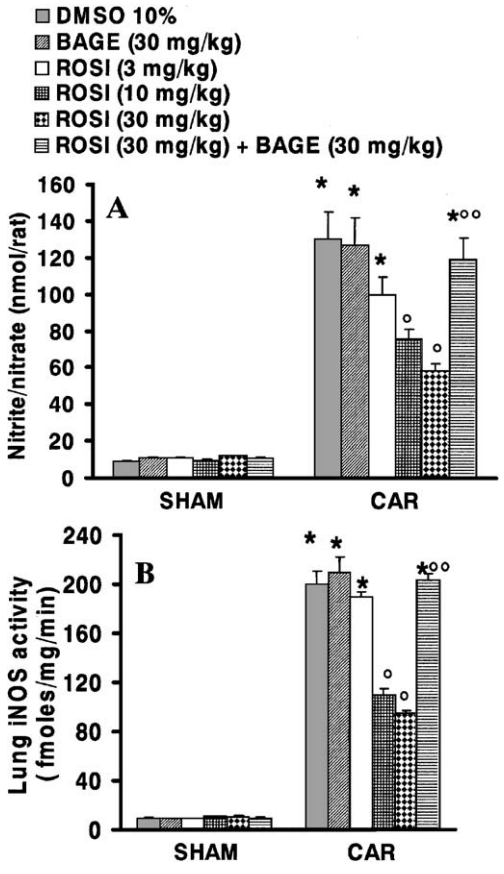

Fig. 6. Nitrite and nitrate concentrations in pleural exudate (A) and pulmonary iNOS activity (B) at 4 h after carrageenan administration. Nitrite and nitrate levels and iNOS activity in carrageenan treated rats were significantly increased versus sham group. ROSI (3, 10 and 30 mg/kg i.p.) significantly reduced the carrageenan-induced elevation of nitrite and nitrate levels and the iNOS activity in a dose dependent manner. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist. Data are means ± S.E.M. from 10 rats for each group. *$P<0.01$ versus sham. °$P<0.01$ versus carrageenan. °°$P<0.01$ versus ROSI.

3.2. Effects of rosiglitazone on carrageenan-induced pleurisy

Histological examination of lung sections revealed significant tissue damage (Fig. 2A). Thus, when compared to lung sections taken from saline-treated animals (Fig. 2D), histological examination of lung sections of rats treated with carrageenan showed oedema, tissue injury as well as infiltration of the tissue with neutrophils (PMNs) (Fig. 2A). Rosiglitazone reduced the degree of lung injury (Fig. 2B). Furthermore, the injection of carrageenan into the pleural cavity of rats elicited an acute inflammatory response characterised by the accumulation of fluid (oedema) that contained a large amount of PMNs (Fig. 3A and B). Neutrophils also infiltrated the lung tissues (Fig. 4A) and this was associated with lipid peroxidation of lung tissues as evidenced by an increase in the levels of MDA (Fig. 4B). Oedema formation, PMNs infiltration in the pleural cavity, neutrophil infiltration in lung tissue, and lipid peroxidation were significantly attenuated in a dose dependent manner by

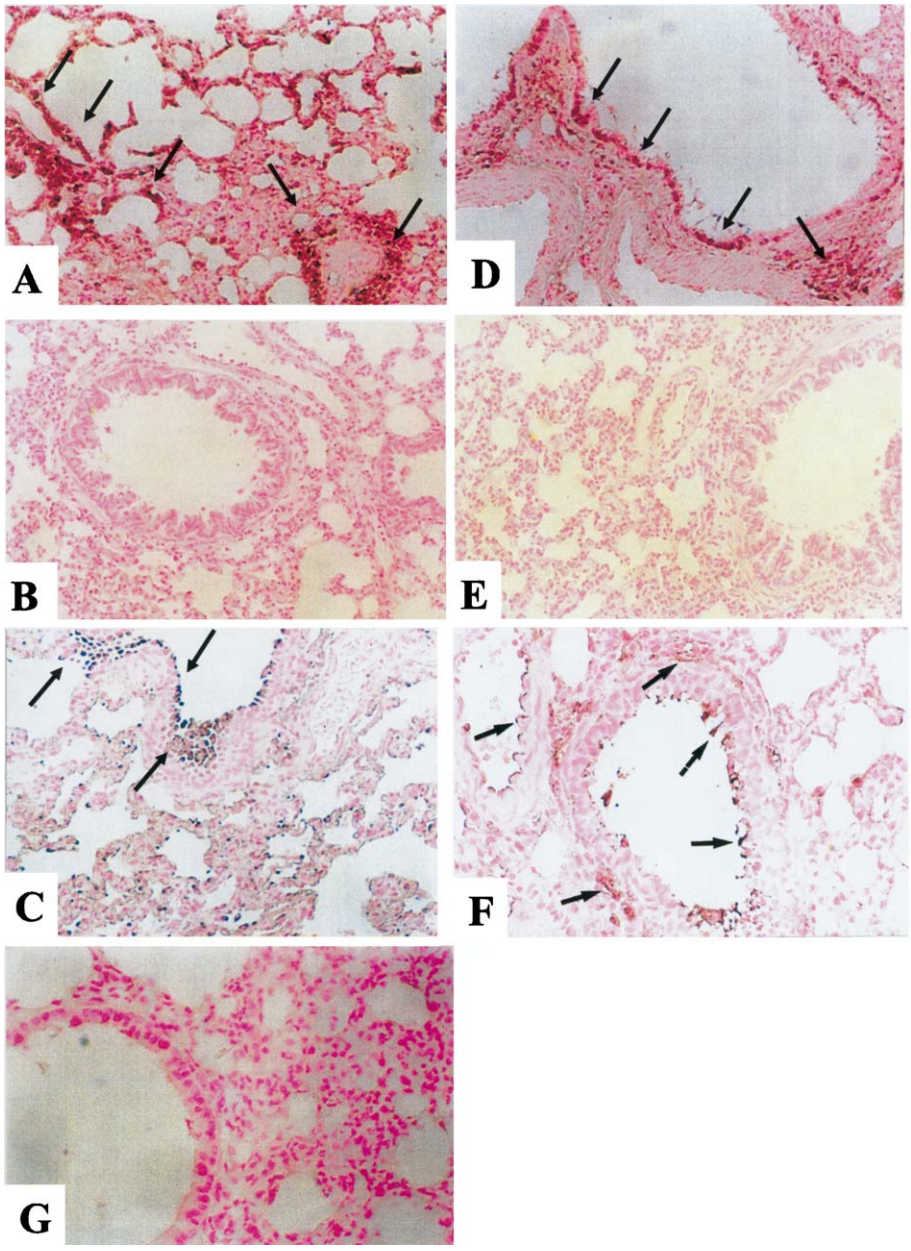

Fig. 7. Immunohistochemical localization of iNOS and COX-2 in the lung. Four hours after carrageenan injection, positive staining for iNOS (A) and COX-2 (D) was localized mainly in macrophages. There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pre-treated with rosiglitazone (30 mg/kg i.p.) (B,E). Co-administration of BADGE and rosiglitazone blocked the effect of the PPAR-γ agonist on iNOS (C) and COX-2 expression (F). There was no staining for iNOS (G) in lungs obtained from the sham group of rats Original magnification: 150 × . This figure is representative of at least three experiments performed on different experimental days.

the i.p. injection of rosiglitazone (Figs. 3 and 4). Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist (Figs. 2C, 3 and 4).

3.3. Effects of rosiglitazone on the expression of adhesion molecules (ICAM-1, P-selectin)

Staining of lung tissue sections obtained from saline-treated rats with anti-ICAM-1 antibody showed a specific staining along bronchial epithelium demonstrating that ICAM-1 is constitutively expressed (see arrows Fig. 5D). No positive staining for P-selectin was found in lung tissue section from saline-treated rats (Fig. 5H). At 4 h after carrageenan injection, the staining intensity for ICAM-1 substantially increased along the bronchial epithelium (see arrows, Fig. 5A). Lung tissue sections obtained from carrageenan-treated rats showed positive staining for P-selectin localized in the bronchial epithelium (Fig. 5E). No positive staining for ICAM-1 or P-selectin was found in the lungs of carrageenan-treated rats that received i.p. injection of rosi-

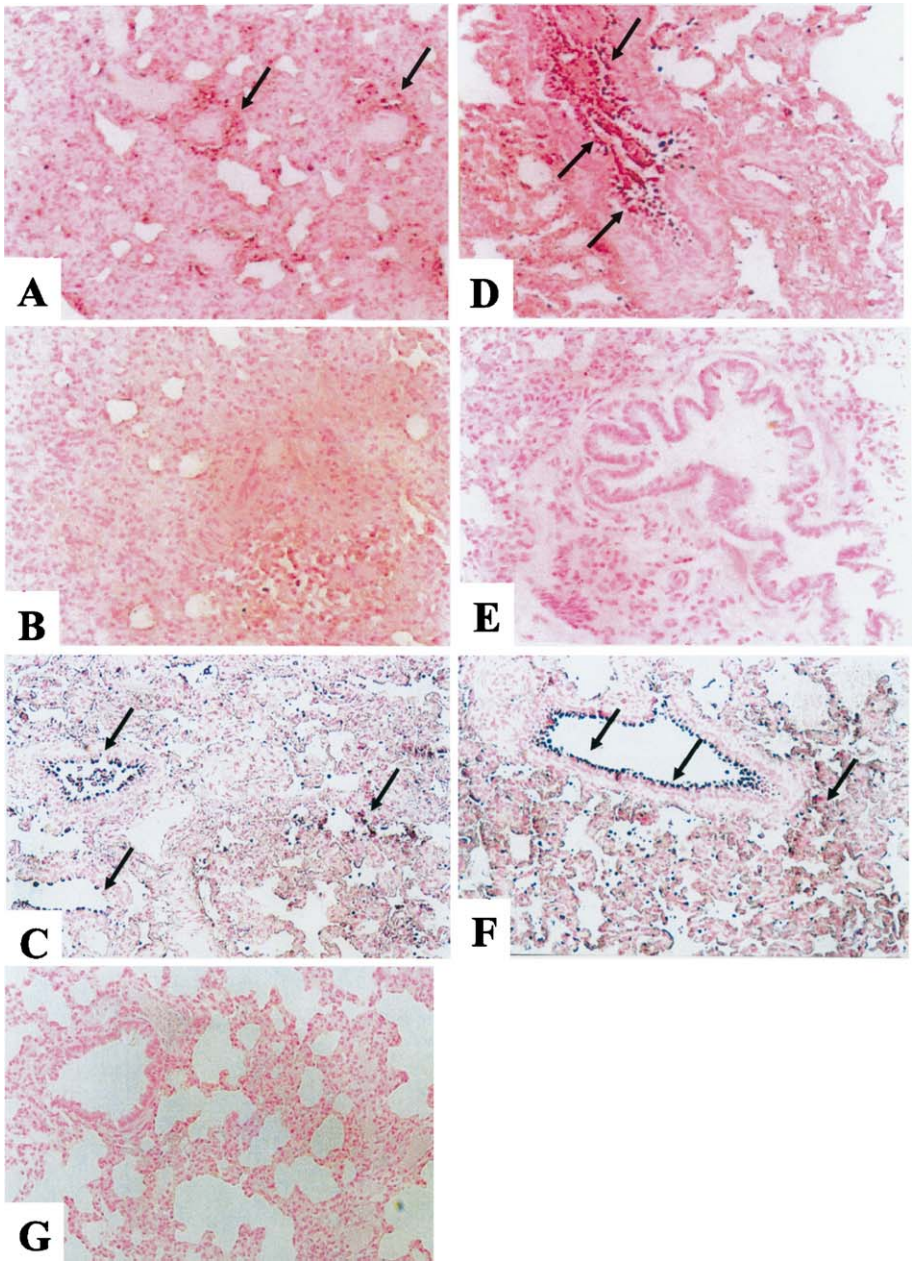

Fig. 8. Effect of rosiglitazone (ROSI) on nitrotyrosine formation and PARP activation. Four hours after carrageenan injection, positive staining for nitrotyrosine (A) and for PAR (D) was observed. There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pre-treated with rosiglitazone (30 mg/kg i.p.) (B,E). Co-administration of BADGE and rosiglitazone blocked the effect of the PPAR-γ agonist on nitrotyrosine (C) and PARP activation (F). There was no staining for nitrotyrosine (G) in lungs obtained from the sham group of rats. Original magnification: 150 ×. This figure is representative of at least three experiments performed on different experimental days.

glitazone (30 mg/kg) (Fig. 5B,F). Co-administration of BADGE and rosiglitazone blocked the effect of the PPAR-γ agonist on ICAM-1 and P-selectin expression (see arrows, Fig. 5C,G). BADGE treatment alone did not affect ICAM-1 and P-selectin (data not shown).

3.4. Effects of rosiglitazone on carrageenan-induced NO production

NO levels were a also significantly increased in the exudate obtained from rats administered carrageenan (Fig. 6A). A significant increase in iNOS activity 4 h after administration of carrageenan was detected in lungs obtained from rats subjected to carrageenan-induced pleurisy (Fig. 6B). Pre-treatment of rats with rosiglitazone significantly reduced (in a dose-dependent fashion) both NO levels and iNOS activity (Fig. 6A,B).

Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist (Fig. 6A,B). Immunohistochemical analysis of lung sections obtained from carrageenan-treated rats revealed a positive staining for iNOS (see arrows, Fig. 7A). In contrast, no staining for iNOS was found in the lungs of carrageenan-treated rats, which had been pre-treated with rosiglitazone (Fig. 7B). Staining was absent in lung tissue obtained from the sham group (Fig. 7G). Co-administration of BADGE and rosiglitazone locked the effect of the PPAR-γ agonist on iNOS expression (see arrows, Fig. 7C). BADGE treatment alone did not effect iNOS expression (data not shown).

3.5. Effects of rosiglitazone on carrageenan-induced nitrotyrosine formation and PARS activation

Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan also revealed a positive staining for nitrotyrosine (see arrows, Fig. 8A). In contrast, no positive staining for nitrotyrosine was found in the lungs of carrageenan-treated rats, which had been pre-treated with rosiglitazone (Fig. 8B). Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan also revealed a positive staining for PAR (Fig. 8D). In contrast, no staining for PAR was found in the lungs of carrageenan-treated rats pre-treated with rosiglitazone (Fig. 8E). There was no staining for either nitrotyrosine (Fig. 8G) or PAR (data not shown) in lungs obtained from the sham group of rats. Co-administration of BADGE and rosiglitazone blocked the effect of the PPAR-γ agonist on nitrotyrosine formation and PARP activation (see arrows, Fig. 8C,F). BADGE treatment alone did not effect nitrotyrosine formation and PARP activation (data not shown).

3.6. Effects of rosiglitazone on carrageenan-induced prostaglandin formation

COX activity in carrageenan-induced pleural exudate and lung homogenates was assessed by measuring the increased formation of PGE_2 in the exudate. The levels of PGE_2 found in the pleural exudate of carrageenan-treated rats was significantly increased (Fig. 9A). The levels of PGE_2 were significantly lower in the exudate obtained from carrageenan-treated rats that had been pre-treated with rosiglitazone (Fig. 9A). In lungs from carrageenan-treated rats, the amount of 6-keto-$PGF_{1\alpha}$ was significantly increased in comparison with sham rats (Fig. 9B). The amount of 6-keto-$PGF_{1\alpha}$ was significantly reduced in the lungs from carrageenan-treated rats pre-treated with rosiglitazone (Fig. 9B). Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist (Fig. 9A,B).

Immunohistochemical analysis of lung sections obtained from carrageenan-treated rats also revealed a positive staining for COX-2, which was primarily localised in alveolar macrophages (see arrows, Fig. 7D). In contrast, no positive COX-2 staining was found in the lungs from carrageenan-treated rats that had been pre-treated with rosiglitazone (Fig. 7E). Staining was absent in tissue obtained from the sham group of animals (data not shown).

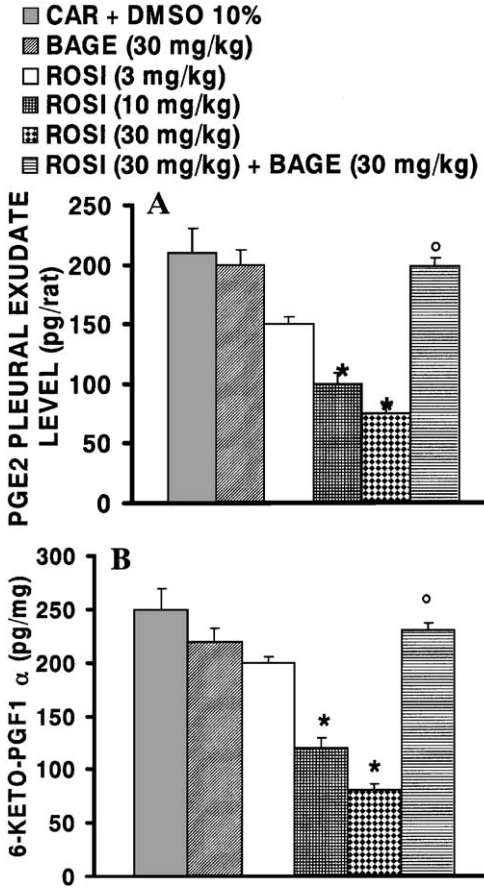

Fig. 9. PGE_2 levels in the pleural exudate (A) and 6-keto-$PGF_{1\alpha}$ in the lungs (B) from carrageenan-treated rats. ROSI (3, 10 and 30 mg/kg i.p.) significantly reduced the carrageenan-induced elevation of PGE_2 and 6-keto-$PGF_{1\alpha}$ in a dose dependent manner. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist. Data are means ± S.E.M. from 10 rats for each group. *$P<0.01$ versus sham. °$P<0.01$ versus carrageenan. °°$P<0.01$ versus ROSI.

Co-administration of BADGE and rosiglitazone blocked the effect of the PPAR-γ agonist on COX-2 expression (see arrows, Fig. 7F). BADGE treatment alone did not effect COX-2 expression (data not shown). COX-1 was also detected by immunohistochemical analysis in the lung sections obtained from rats treated with carrageenan, but the degree of staining was similar to that observed in the lungs of sham animals (data not shown). The degree of staining for COX-1 in lungs of carrageenan-treated rats treated with rosiglitazone was similar to that observed in lungs obtained either from carrageenan-treated rats or from sham rats (data not shown).

3.7. Effects of rosiglitazone on the release of pro-inflammatory cytokine induced by carrageenan

When compared to controls at 4 h after the injection of carrageenan, an increase in the levels of TNF-α and IL-1β, was observed in pleural exudates (Fig. 10A,B). Treatment attenuated the release of TNF-α and IL-1β (Fig. 10A,B). Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist (Fig. 10A,B).

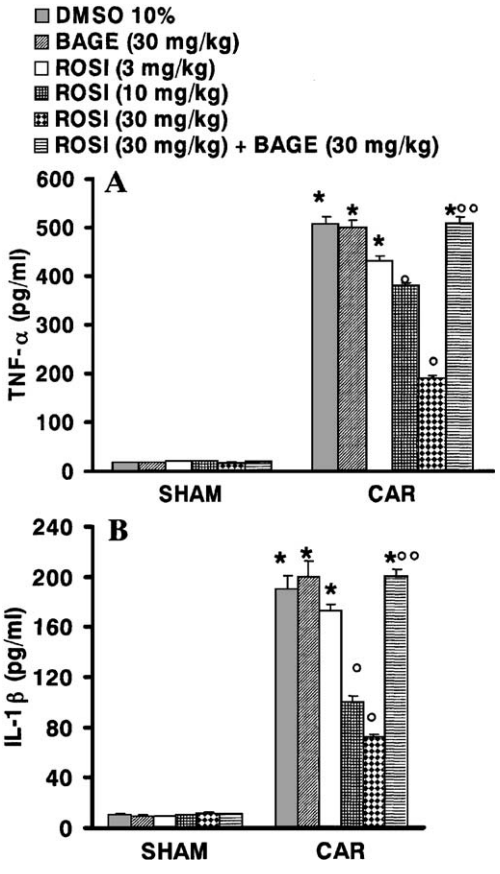

Fig. 10. Exudate levels of TNFα (A) and IL1β (B). ROSI (3, 10 and 30 mg/kg i.p.) significantly reduced the carrageenan-induced elevation of cytokine levels in a dose dependent manner. Co-administration of BADGE and rosiglitazone significantly blocked the effect of the PPAR-γ agonist. Data are means ± S.E.M. from 10 rats for each group. *$P<0.01$ versus sham. °$P<0.01$ versus carrageenan. °°$P<0.01$ versus ROSI.

4. Discussion

Peroxisome proliferator-activated receptors (PPARS) are members of the nuclear hormone receptor superfamily. These receptors which exist in three different isoforms (PPAR-α, PPAR-γ and PPAR-Δ). has been detected in various species whit a tissue-specific differential expression (Youssef and Badr, 2001). PPARs regulate a variety of physiological processes including adipogenesis, glucose metabolism and placental function (Tomlinson et al., 1994; Vane, 1994). Therefore, only recent studies have implicated the various PPAR isoforms in inflammation (Youssef and Badr, 2001; Delerive et al., 2001). In particular, both PPAR-α and PPAR-γ receptor have been reported to regulate the inflammatory response (Chinetti et al., 2000); however the extent of this regulation and indeed its direction, are still controversial. Recently, it has been point out that PPAR-α receptor may exert an anti-inflammatory role by observation that PPAR-α deficient mice exhibited a prolonged inflammatory response when challenged with leukotriene B4 (LTB4) (Devchand et al., 1996). In agreement with these results recently Taylor et al. (2002) have clearly demonstrated the protective effects of several PPAR-α and PPAR-γ subtype-specific agonists, including WY-14643 (delivered in the diet) and perfluorooctanoic acid (PFOA), on the inflammation and hyperalgesia produced by intraplantar carrageenan injection. In contrast, other studies have demonstrated that dietary treatment with PPAR-α agonist increase the release of plasma TNF-α levels after LPS administration an effect that was significantly reduced in PPAR-α deficient mice (Hill et al., 1999). Based on this last evidence a pro-inflammatory role for PPAR-α has been postulated. The contribution of PPAR-γ to the inflammatory response is also unclear (Delerive et al., 2001). PPAR-γ receptor is the molecular target of the thiazolidinedione (TZD) class of antidiabetic drugs, which include rosiglitazone and pioglitazone (Willis et al., 1996).

PPAR-γ is prominently expressed in activated monocytes and tissue macrophages in vivo, including the foam cells of atherosclerotic lesions (Masferrer et al., 1994). Multiple functions have been proposed for PPAR-γ receptor in inflammation but how the PPAR-γ signalling pathway may affect the development of acute inflammation remains unclear. Therefore, in the present study we evaluate the effect of rosiglitazone in carrageenan-induced inflammation which has been well characterized by manly group including ours as a useful model to asses the contribution of mediators involved in acute inflammation and commonly used to evaluate non-steroidal anti-inflammatory drugs.

This study provides the evidence that rosiglitazone attenuates: (i) the development of carrageenan-induced paw oedema, (ii) the development of carrageenan-induced pleurisy, (iii) the infiltration of the lung with PMNs, (iv) the degree of lipid peroxidation in the lung, (v) the expression of ICAM-1 and P-selectin and (vi) the degree of lung injury caused by injection of carrageenan. All of these findings

support the view that rosiglitazone attenuates the degree of acute inflammation in the rat. What, then, is the mechanism by which rosiglitazone reduces acute inflammation?

In order to elucidate whether the anti-inflammatory effect of rosiglitazone observed here is related to activation of the PPAR-γ receptor, we also investigated the effect of a PPAR-γ antagonist, bisphenol A diglycidyl ether (BADGE), on the anti-inflammatory effects of rosiglitazone. We demonstrate that pre-treatment of animals with BADGE attenuates the protective effects of rosiglitazone. Thus, we propose that (i) activation of PPAR-γ reduces the development of acute inflammation, and (ii) that the activation of PPAR-γ contributes to the anti-inflammatory effects of rosiglitazone.

There is evidence that the pro-inflammatory cytokines TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process (Yamamoto et al., 1995; Crofford et al., 1997; Inoue and Kawanishi, 1995). We confirm here that the inflammatory process (carrageenin-induced pleurisy) leads to a substantial increase in the levels of both TNF-α and IL-1β in the exudates which likely contribute in different capacities to the evolution of acute inflammation.

Recently, several studies have reported that treatment of monocytes and macrophages with high concentrations of PPAR-γ agonists reduced secretion of inflammatory cytokines and inhibited macrophage activation. In particular, treatment of monocytes with TZD reduced release of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. In the present study, we found that pre-treatment of rats with rosiglitazone attenuated the production of TNF-α and IL-1β. We have also demonstrated that the co-administration of BADGE and rosiglitazone blocked these effects of the PPAR-γ agonist. These findings, therefore, confirm (i) that acute inflammation results in the activation and the subsequent expression of pro-inflammatory inflammatory cytokines, and suggest (ii) that rosiglitazone activates the PPAR-γ receptor resulting in the reduction of the release of these of pro-inflammatory cytokines.

A number of recent studies have demonstrated that the recruitment of cells into an area of inflammation may be mediated not only by C5a, leukotrienes, platelet activating factor, or bacterial-derived peptides, but also by a novel group of small proteins with relatively specific chemotactic activity for leukocyte subpopulations. In addition, various studies have gained substantial insight into the importance of specific adhesion molecules and mediators in the following processes which finally result in the recruitment of PMNs at a specific site of inflammation: (1) margination, capture and rolling on the vascular endothelium, (2) activation and firm adhesion, (3) diapedesis through gaps between endothelial cells in post-capillary venules and (4) finally migration along a gradient of chemokines. Activated PMNs, therefore, play a crucial role in the destruction of foreign antigens and the breakdown and remodelling of injured tissue. Leukocyte-endothelial interactions involve a complex interplay among adhesion glycoproteins (i.e. integrins, members of the immunoglobulin superfamily and selectins). One member of the selectin family, P-selectin , is rapidly translocated from the Weibel-Palade bodies to the endothelial cell surface upon activation of endothelial cells with thrombin, histamine, hypoxia-reoxygenation, or oxygen-derived free radicals (Patel et al., 1991). P-selectin promotes rolling of leukocytes on the endothelium. The rolling of leukocytes is the first step in the interactions of leukocytes with the endothelium and facilitates the activation and adherence of PMNs (Patel et al., 1991). The firm adhesion of PMNs to the endothelium, however, is a complex phenomenon, which also involves other endothelium-based adhesion molecules. In fact, endothelial adhesion molecules are considered to play a pivotal role in the localization and development of an inflammatory reaction (Butcher, 1993). Intercellular adhesion molecules (ICAM-1) are adhesion molecules normally expressed at a low basal level, but their expression can be enhanced by various inflammatory mediators such as IL-1 and TNF-α (Wertheimer et al., 1992).

We report in the present study that acute inflammation in the rat results in the expression of ICAM-1 and P-selectin. We found that pre-treatment of rats with rosiglitazone attenuated the expression of P-selection as well as the up-regulation of ICAM-1. We have also demonstrated that the co-administration of BADGE and rosiglitazone blocked these effects of the PPAR-γ agonist. These findings, therefore, suggest (i) that acute inflammation results in the activation and the subsequent expression of pro-inflammatory genes, and (ii) that rosiglitazone activates the PPAR-γ receptor resulting in the reduced expression of pro-inflammatory genes.

The absence of an increased expression of the adhesion molecules in the lung tissue of carrageenan-treated rats which received rosiglitazone (30 mg/kg i.p.) correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme MPO and with the moderation of the tissue damage as evaluated by histological examination.

Neutrophils are recruited into the tissue by local production of cytokines and can then contribute to tissue destruction by the production of reactive oxygen metabolites, granule enzymes, and cytokines that further amplify the inflammatory response by their effects on macrophages and lymphocytes (Chatham et al., 1993) Neutrophils were shown to express PPARγ (Greene et al., 1995) suggesting a potential role of this transcription factor in neutrophil function. Other studies have also demonstrated that PPARγ ligands may modulate leukocyte-endothelial cell interactions during inflammation through regulation of endothelial adhesion molecules.

Furthermore, we found that the tissue damage induced by carrageenan in vehicle-treated mice was associated with high levels of tissue thiobarbituric acid-reactant malondialdehyde, 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 due to 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 (Salvemini et al., 1998) In addition to NO, peroxynitrite is also generated in carrageenan-induced acute inflammation.

Therefore, in this study we clearly demonstrate that rosiglitazone treatment prevent the induction of iNOS and the formation of peroxynitrite.

ROS produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS resulting in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed 'the PARS Suicide Hypothesis' (Szabó et al., 1997) There is recent evidence that the activation of PARS may also play an important role in inflammation (Szabó et al., 1998). We demonstrate here that rosiglitazone treatment reduced the activation of PARS during carrageenan-induced pleurisy in the lung. Thus, we propose that the anti-inflammatory effects of rosiglitazone may be—at least in part—due to the prevention of the activation of PARS. Several cellular mechanisms, including the mode of gene regulation and signal transduction, may account for the anti-inflammatory effect of PPAR-γ ligands. Recently it has been shown that PPAR-γ ligands may act at the transcriptional level, at least in part, through inhibition of AP-1 and NF-κB activity (Fahmi et al., 2001).

Although the exact mechanisms remain unclear, activated PPARγ could downregulate AP-1, NF-κB, and STATs activity by nitration of essential transcription cofactors, such as CBP/p300 and SRC-1 (Staels et al., 1998). PPAR may also antagonize AP-1 and NF-κB activity through protein–protein interaction. Such a mechanism was shown to be utilized by retinoic acid receptor and glucocorticoid receptor with AP-1 or NF-κB (Karin, 1998). NF-κB has been shown to activate, via transcription, the genes encoding pro-inflammatory cytokines (TNF-α, IL-lβ and IL-12), cell adhesion molecules (ICAM-1), inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2). Several studies also support the conclusion that pro-inflammatory cytokines, cell adhesion molecules and NO and prostaglandin E_2 (PGE_2) derived from iNOS and COX-2, respectively, play important roles in the pathogenesis of acute and chronic inflammation.

In particular, there is good evidence in this and in other models of inflammation that an enhanced formation of prostanoids following the induction of COX-2 contributes to the pathophysiology of local inflammation and chronic and also that selective inhibitors of COX-2 exert potent anti-inflammatory effects. We demonstrate here that the increase in the levels of PGE_2 caused by injection of carrageenan into the pleural cavity is reduced in the exudate of rosiglitazone-treated rats. The enhanced formation of PGE_2 is secondary to the expression of COX-2 protein, as (i) there was no increase in the expression of COX-1 protein (detected by immunoistochemistry) after carrageenan injection and (ii) selective inhibitors of COX-2 activity including NS-398 (nimesulide) and SC-58125 (celecoxib) markedly abolished the increase in PGE_2 caused by injection of carrageenan into the pleural space (Harada et al., 1996; Cuzzocrea et al., 2002). Thus, we propose that rosiglitazone reduced the expression of COX-2 protein and activity caused by injection of carrageenan in the lung. This effect of rosiglitazone was abolished by the co-administration of BADGE together with rosiglitazone.

In conclusion, this study provides the evidence that rosiglitazone, a ligand of PPAR-γ causes a substantial reduction of acute inflammation in the rat. In addition, we also demonstrate, for the first time in vivo, that a PPAR-γ antagonist, BADGE, significantly attenuates the protective effect of rosiglitazone. Thus, we demonstrate here that the mechanisms underlying the protective effects of rosiglitazone are dependent on the activation of PPAR-γ. The activation of PPAR-γ by rosiglitazone, in turn, results in a reduction of (i) the expression of iNOS and the nitration of proteins by peroxynitrite, (ii) the formation of the pro-inflammatory cytokines, (iii) the expression of COX-2, (iv) the expression of adhesion molecules and (v) the inflammatory cells infiltration. These findings support the potential use of PPAR-γ ligands as therapeutic agents in the therapy of conditions associated with acute inflammation.

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