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The Cyclopentenone Prostaglandin 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 Attenuates the Development of Acute and Chronic 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 seems to play a pivotal role in the regulation of cellular proliferation and inflammation. Recent evidence also suggests that the cyclopentenone prostaglandin (PG) 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15*d*-PGJ_2), which is a metabolite of prostaglandin D_2, functions as an endogenous ligand for PPAR-γ. We postulated that 15*d*-PGJ_2 would attenuate inflammation. In the present study, we have investigated the effects of 15*d*-PGJ_2 of acute and chronic inflammation (carrageenan-induced pleurisy and collagen-induced arthritis, respectively) in animal models. We report for the first time, to our knowledge, that 15*d*-PGJ_2 (given at 10, 30, or 100 μg/kg i.p. in the pleurisy model or at 30 μg/kg i.p every 48 h in the arthritis model) exerts potent anti-inflammatory effects (e.g., inhibition of pleural exudate formation, mononuclear cell infiltration, delayed development of clinical indicators, and histological injury) in vivo. Furthermore, 15*d*-PGJ_2 reduced the increase in the staining (immunohistochemistry) for nitrotyrosine and poly (ADP-ribose) polymerase and the expression of inducible nitric-oxide synthase and cyclooxygenase-2 in the lungs of carrageenan-treated mice and in the joints from collagen-treated mice. Thus, 15*d*-PGJ_2 reduces the development of acute and chronic inflammation. Therefore, the cyclopentenone prostaglandin 15*d*-PGJ_2 may be useful in the therapy of acute and chronic inflammation.

The cyclopentenone prostaglandin PGJ_2 is formed by dehydration within the cyclopentenone ring of the endogenous prostaglandin PGD_2. PGJ_2 is metabolized further to yield Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15*d*-PGJ_2). Several members of the cyclopentenone family of prostaglandins possess antineoplastic, antiviral activity and anti-inflammatory properties (Straus and Glass, 2001).

Most actions of the cyclopentenone prostaglandins seem to be secondary to their interaction with other cellular target proteins rather than mediated by binding to G-protein-coupled prostanoid receptors. For instance, 15*d*-PGJ_2 is a high-affinity ligand for PPAR-γ. PPAR-γ is a nuclear hormone receptor that regulates gene expression by heterodimerizing with the retinoid X receptor. Binding of the activated heterodimer to promoter region of specific target genes results in either the activation or the suppression of the target gene. Various PPAR-γ ligands have been reported to possess anti-inflammatory properties in vitro (Jiang et al., 1998) and in vivo (see below). It is possible that PPAR-γ *trans*-represses the expression of pro-inflammatory mediators at the transcriptional level by inhibiting NF-κB, signal transducers and activators of transcription-1, and activation protein-1 signaling (Ricote et al., 1998).

Other activities of the cyclopentenone prostaglandins are mediated by the reactive α,β-unsaturated carbonyl group located in the cyclopentenone ring. For instance, 15*d*-PGJ_2 attenuates the activation of the transcription factor NF-κB by preventing the phosphorylation of its inhibitor protein by

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ABBREVIATIONS: 15*d*-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; NF-κB, nuclear factor-κB; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; PARP, poly(ADP-ribose) polymerase; CIA, collagen-induced arthritis; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; CII, bovine type 2 collagen; CFA, complete Freund's adjuvant; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; MDA, malondialdehyde; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL, interleukin.

inhibitory kinase kinase (Rossi et al., 1997). It is now widely accepted that 15*d*-PGJ_2 attenuates the NF-κB–mediated transcriptional activation of many pro-inflammatory genes by PPAR-γ–dependent and -independent mechanisms (Straus and Glass, 2001). For instance, 15*d*-PGJ_2 attenuates the formation of the cytokines TNF-α and IL-12 (Drew and Chavis, 2001), the expression of the adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (Pasceri et al., 2000) and the expression of the inducible, pro-inflammatory proteins cyclooxygenase-2 (COX-2), cytosolic phospholipase A_2 (Tsubouchi et al., 2001), and inducible nitric-oxide (NO) synthase (iNOS) (Ricote et al., 1998; Colville-Nash et al., 1998). However, there is also evidence that 15*d*-PGJ_2 may enhance the formation of the pro-inflammatory chemokine IL-8 in human macrophages/monocytes stimulated with endotoxin in a PPAR-γ-dependent fashion (Zhang et al., 2001).

A recent report by Kawahito et al. (2000) documents that 15*d*-PGJ_2 and the PPAR-γ ligand troglitazone reduce the degree of inflammation (i.e., suppression of pannus formation and mononuclear cell infiltration) associated with adjuvant-induced arthritis in female Lewis rats. This study was designed to gain a better understanding of the effects of 15*d*-PGJ_2 in rodent models of acute and chronic inflammation. To achieve this goal, we have investigated the effects of this cyclopentenone prostaglandin in rodent models of acute (carrageenan-induced pleurisy) and chronic [collagen-induced arthritis (CIA)] inflammation. In particular, we have investigated the effects of 15*d*-PGJ_2 on the lung injury associated with carrageenan-induced pleurisy and on the joint injury associated with collagen-induced arthritis. To gain a better insight into the mechanism(s) of action of the observed anti-inflammatory effects of 15*d*-PGJ_2, we have also investigated the effects of 15*d*-PGJ_2 on expression of iNOS and COX-2, the nitration of cellular proteins by peroxynitrite, and the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP).

Materials and Methods

Animals. Nine-week-old male BALB/c and DBA/1J mice (weight, 20–25 g; Charles River, Milan, Italy) were used for these studies. The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) and with European Economic Community regulations (O.J. of E.C. L358/1 12/18/1986).

Experimental Groups. For the pleurisy study, 60 BALB/c mice were allocated into one of the following groups: 1) administration of carrageenan only (CAR group, $n = 10$); 2) 15*d*-PGJ_2 given as an i.p. bolus 15 min before carrageenan (10, 30, or 100 μg/kg) (CAR + 15*d*-PGJ_2 group, $n = 30$); 3) administration of vehicle for 15*d*-PGJ_2 [10% dimethyl sulfoxide (DMSO)] administered alone (VEH group, $n = 10$); and 4) a sham-operated group in which identical surgical procedures to the CAR group was performed, except that the 10% DMSO was administered instead of carrageenan (SHAM group, $n = 10$).

For the arthritis study, 40 DBA/1J mice were allocated into one of the following groups: 1) collagen-administration only (Arthritic group, $n = 10$); 2) 15*d*-PGJ_2 given as an i.p. bolus every 48 h starting from day 24 (30 μg/kg) (Arthritis + 15*d*-PGJ_2 group, $n = 10$); 3) administration of vehicle for 15*d*-PGJ_2 (10% DMSO) administered alone (VEH group, $n = 10$); and 4) a sham-operated group in which 0.01 M acetic acid was administered instead of collagen (SHAM group, $n = 10$).

Carrageenan-Induced Pleurisy. Carrageenan-induced pleurisy was induced as described previously (Cuzzocrea et al., 2000a). Mice were anesthetized with isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.2 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 was rinsed with 2 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 μg/ml). The exudate and washing solution were removed by aspiration, and the total volume was measured. Any exudate that was contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudate were suspended in phosphate-buffer saline (0.01 M PBS, pH 7.4) and counted with an optical microscope in a Burker's chamber after vital Trypan Blue staining.

Induction of Collagen-Induced Arthritis. Bovine type 2 collagen (CII) was dissolved in 0.01 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C and was frozen at −70°C until required. Complete Freund's adjuvant (CFA) was prepared by the addition of *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. Before injection, CII was emulsified with an equal volume of CFA. CIA was induced as described previously (Szabó et al., 1998; Cuzzocrea et al., 2000b). On day 1, mice were injected intradermally at the base of the tail with 100 μl of the emulsion (containing 100 μg of CII). On day 21, a second injection of CII in CFA was administered.

Clinical Assessment of Collagen-Induced Arthritis. Mice were evaluated daily for arthritis by using a macroscopic scoring system: 0, no signs of arthritis; 1, swelling and/or redness of the paw or one digit; 2, two joints involved; 3, more than two joints involved; and 4, severe arthritis of the entire paw and digits (Cuzzocrea et al., 2000b). The arthritic index for each mouse was calculated by adding the four scores of individual paws. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile, Comerio, Italy).

Assessment of Arthritis Damage. At day 35, animals were sacrificed while under anesthesia, and paws and knees were removed and fixed in 10% (w/v) PBS-buffered formaldehyde for histological examination performed by an investigator blinded to the treatment regimen. The following morphological criteria were used for scoring: 0, no damage; 1, edema; 2, presence of inflammatory cells; and 3, bone resorption (Cuzzocrea et al., 2000b).

Histological Examination. Lung biopsies were taken 4 h after injection of carrageenan, and paws and knees were taken 35 days after induction of CIA. 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). The joints were trimmed, placed in decalcifying solution for 24 h, embedded in paraffin, and sectioned at 5 μm. Sections were then deparaffinized with xylene and stained with Mallory-Azon stain (lung sections) or with hematoxylin and eosin (joint sections). All sections were studied using light microscopy (Dialux 22; Leitz, Midland, Ontario, Canada).

Radiography. Mice were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and arthritic mouse hind paws was performed using an X-ray machine (X12; Philips, Eindhoven, The Netherlands) with a 40-kW exposition for 0.01 s. Radiographic scoring was performed by an investigator blinded for the treatment regime, and the following radiograph criteria were considered and scored accordingly: 0, no bone damage; 1, tissue swelling and edema; 2, joint erosion; and 3, bone erosion and osteophyte formation.

Measurement of Cytokines. TNF-α and IL-1β levels were evaluated in the exudate 4 h after the induction of pleurisy by carrageenan injection and in the plasma from CIA mice as described previously (Cuzzocrea et al., 2000b). The assay was carried out using a colorimetric commercial enzyme-linked immunosorbent assay kit (Calbiochem-Novabiochem, Milan, Italy) with a lower detection limit of 10 pg/ml.

Measurement of Plasma Nitrite Concentration. Total nitrite in mouse plasma, an indicator of NO synthesis, was measured as described previously (Cuzzocrea et al., 2001). In brief, the nitrate in the sample was first reduced to nitrite by incubation with 670 mU/ml nitrate reductase and 160 μM β-NADPH 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 water and 1% (w/v) sulfanilamide in 5% (v/v) concentrated H_3PO_4; volume 1:1] to a 100-μl sample. The optical density at 550 nm (OD_{550}) was measured using enzyme-linked immunosorbent assay microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in water.

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 15*d*-PGJ_2) served as an indicator of iNOS activity (Cuzzocrea et al., 1998c). Cells or tissues were homogenized on ice using a tissue homogenizer in a homogenization buffer composed of 50 mM Tris-HCl, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. Conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline was measured in the homogenates as described previously (Cuzzocrea et al., 1998c). In brief, homogenates (30 μl) were incubated in the presence of 10 μM L-[^{3}H]arginine (5 kBq per tube), 1 mM NADPH, 30 nM calmodulin, 5 μM tetrahydrobiopterin, and 2 mM EGTA for 20 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer, pH 5.5, containing 2 mM EGTA and 2 mM EDTA. Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted L-[^{3}H]citrulline activity was measured by a scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Measurement of Prostaglandin E_2 in the Pleural Exudate. The amount of PGE_2 present in the pleural fluid of mice was measured using radioimmunoassay without prior extraction or purification as described previously (Sautebin et al., 1995).

Assessment of Cyclooxygenase Activity. Lung tissue obtained 4 h after the induction of pleurisy by carrageenan injection was homogenized at 4°C in a buffer containing the following protease inhibitors: 20 mM HEPES, pH 7.2, 320 mM sucrose, 1 mM dithiothreitol, 10 μg/ml styrosporin, 2 μg/ml aprotinin, and 10 μg/ml leupeptin. Homogenates were incubated at 37°C for 30 min in the presence of excess 30 μM arachidonic acid. 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 measured by radioimmunoassay as described previously (Tomlinson et al., 1994). Protein concentration in each homogenate was measured using the Bradford assay with bovine serum albumin used as standard (Bradford, 1976).

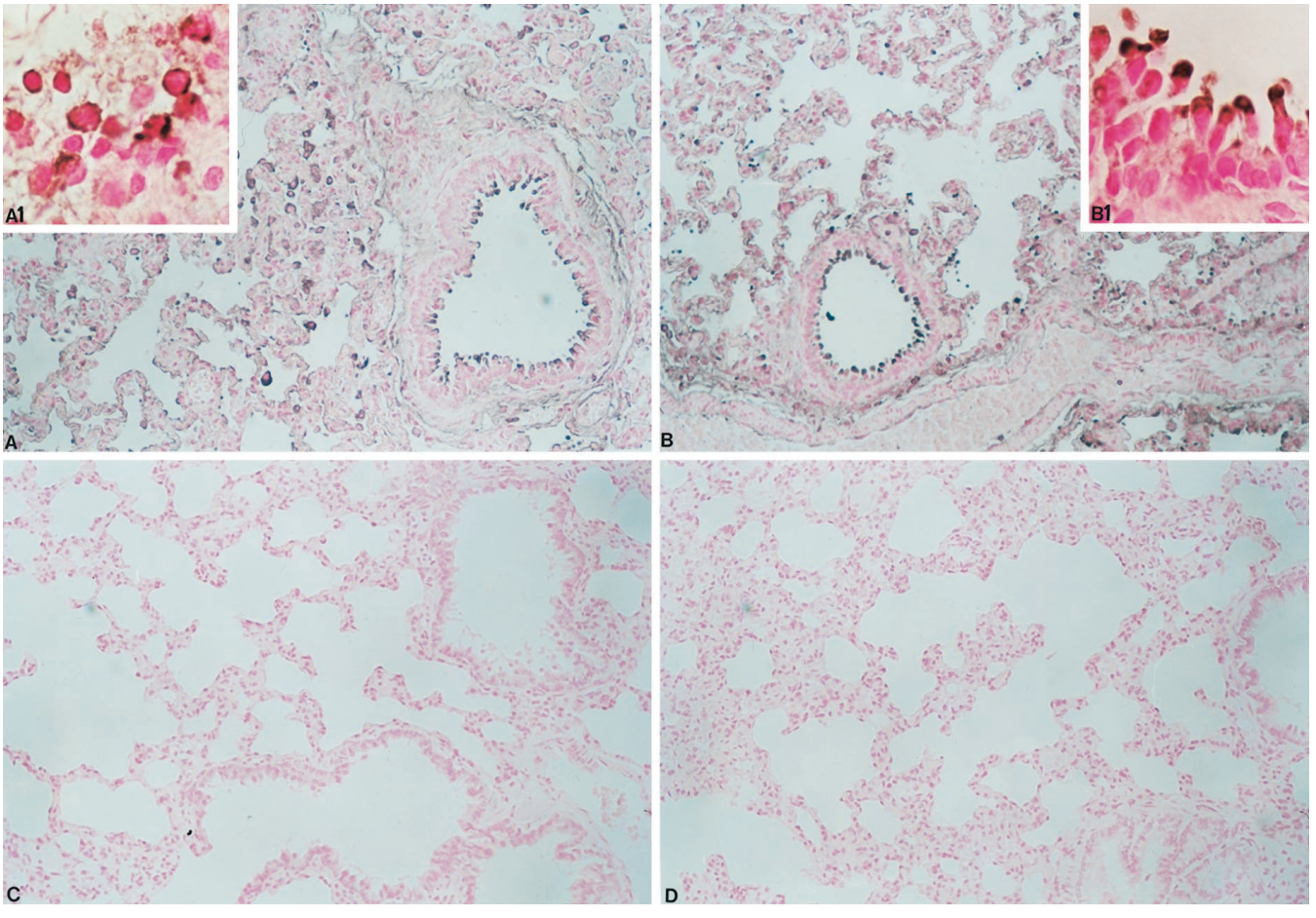

Fig. 1. Immunohistochemical localization of iNOS and COX-2 in the lung. Four hours after carrageenan injection, positive staining for iNOS (A and A1) and COX-2 (B and B1) was localized mainly in macrophages. There was a marked reduction in the immunostaining in the lungs of carrageenan-treated mice pretreated with 100 μg/kg 15*d*-PGJ_2 (C and D). Original magnifications in A through D, 125×; A1 and B1, 375×. This figure is representative of at least three experiments performed on different experimental days.

TABLE 1

Effect of 15-deoxy-12,14-PGJ_2 (15*d*-PGJ_2) on carrageenan-induced inflammation, NO formation, and PG production in the pleural exudate

Data are means ± S.E. means of 10 rats for each group.

	Volume Exudate	PMNs Infiltration	Nitrite/Nitrate	Lung iNOS Activity	PGE_2	Lung 6-KETO-$PGF_{1\alpha}$
	ml	*million cells/mouse*	*nmol/mouse*	*fmol/mg/min*	*pg/mouse*	*pg/mg*
Sham + Vehicle	0.11 ± 0.03	2.2 ± 0.4	4.2 ± 1.1	4.0 ± 0.6	N.D.	N.D.
Sham + 15*d*-PGJ_2 (10 μg/kg)	0.12 ± 0.04	2.1 ± 0.5	3.9 ± 2.3	4.3 ± 0.5	N.D.	N.D.
Sham + 15*d*-PGJ_2 (30 μg/kg)	0.11 ± 0.05	2.2 ± 0.7	4.4 ± 1.0	4.1 ± 0.5	N.D.	N.D.
Sham + 15*d*-PGJ_2 (100 μg/kg)	0.10 ± 0.03	2.1 ± 0.55	4.0 ± 1.6	3.9 ± 0.9	N.D.	N.D.
CAR + vehicle	1.15 ± 0.10*	80 ± 4.1**	70 ± 2.2*	160 ± 3.2*	180 ± 4.5*	200 ± 5.4*
CAR + 15*d*-PGJ_2 (10 μg/kg)	0.52 ± 0.11**	60 ± 2.2**	40 ± 2.5**	100 ± 2.4**	90 ± 4.2**	106 ± 2.2**
CAR + 15*d*-PGJ_2 (30 μg/kg)	0.30 ± 0.09**	35 ± 2.1**	22 ± 2.4**	60 ± 4.5**	64 ± 2.4**	80 ± 3.4**
CAR + 15*d*-PGJ_2 (100 g/kg)	0.20 ± 0.10**	20 ± 3.2**	17 ± 3.4**	35 ± 4.1**	38 ± 5.1**	50 ± 2.1**

* $p < 0.01$ versus sham.
** $p <$ versus carrageenan.

Immunohistochemical Localization 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. Nonspecific binding was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with 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 and nonspecific, purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase (DBA).

Immunohistochemical Localization of Nitrotyrosine. Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or ROS, was determined by immunohistochemistry as described previously (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, 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. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20

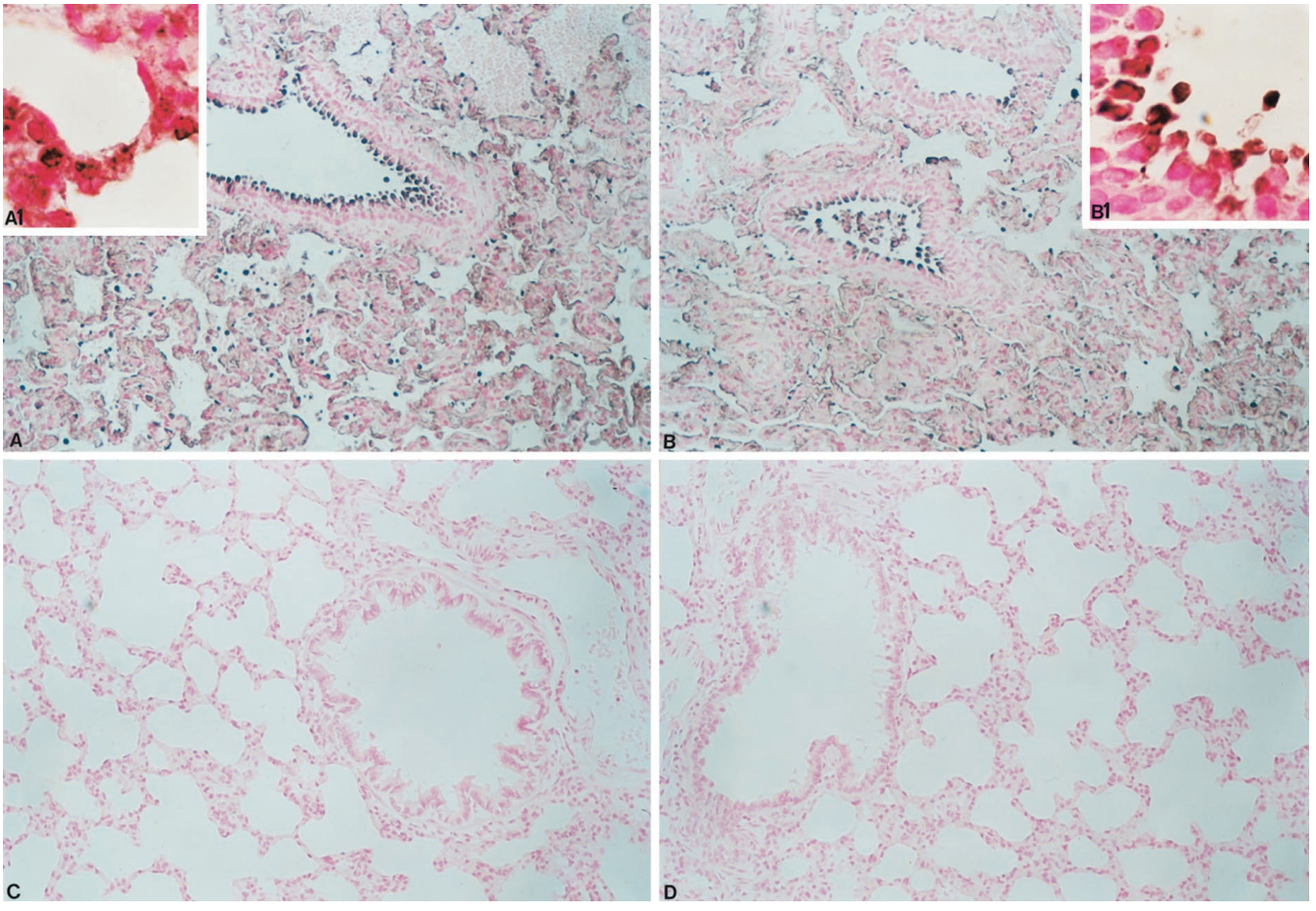

Fig. 2. Effect of 15*d*-PGJ_2 on nitrotyrosine formation and PARP activation. Four hours after carrageenan injection, positive staining for nitrotyrosine (A and A1) and for PARP was observed (B and B1). There was a marked reduction in the immunostaining in the lungs of carrageenan-treated mice pretreated with 15*d*-PGJ_2 (100 μg/kg) (C and D). Original magnifications in A through D, 125×; in A1 and B1, 375×. This figure is representative of at least three experiments performed on different experimental days.

min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA). The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine monoclonal antibody (DBA) or with control solutions including buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA).

Immunohistochemical Localization of Poly(ADP-Ribose). At the specified time after the carrageenan injection, lung tissues 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. Nonspecific adsorption was minimized by incubating the section in 2% (w/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). The sections were then incubated overnight with 1:500 dilution of primary anti-poly(ADP-ribose) (PAR) monoclonal antibody (Alexis Biochemicals, Milan, Italy) or with control solutions, which included buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase (DBA).

Myeloperoxidase Activity. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as described previously (Mullane et al., 1985). At the specified time after injection of carrageenan, lung tissues were obtained and weighed, and each piece was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7.0, 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 1.6 mM tetramethylbenzidine 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 per minute at 37°C and was expressed in milliunits per gram of wet tissue.

Malondialdehyde Measurement. Malondialdehyde (MDA) levels in the lung tissue were determined as an indicator of lipid peroxidation as described previously (Ohkawa et al., 1979). Lung tissue collected at the specified time was homogenized 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 of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3,000*g* for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm.

Materials. Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Poole, Dorset, UK). 15*d*-PGJ_2 was obtained from Cayman (Milan, Italy). All other chemicals were of the highest commercial grade available.

Statistical Evaluation. 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. 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. For the arthritis studies, Mann-Whitney *U* test (two-tailed, independent) was used to compare medians of the arthritic indices (Cuzzocrea et al., 2000b). A *P* value of less than 0.05 was considered significant.

Results

Effects of 15*d*-PGJ_2 on Carrageenan-Induced Pleurisy. All mice treated with carrageenan developed an acute pleurisy characterized by the production of turbid exudate (Table 1). Compared with the number of cells collected from the pleural space of the sham group of mice, injection of carrageenan induced a significant increase in the number of PMNs (Table 1). Pretreatment of mice with 15*d*-PGJ_2 attenuated the volume of the pleural exudate and the number of PMNs within the exudate in a dose-related fashion (Table 1).

NO levels were also significantly increased in the exudate obtained from mice administered carrageenan (Table 1). A significant increase in iNOS activity 4 h after administration of carrageenan was detected in lungs obtained from mice subjected to carrageenan-induced pleurisy (Table 1). Pretreatment of mice with 15*d*-PGJ_2 significantly reduced (in a dose-dependent fashion) both NO levels and iNOS activity (Table 1).

Immunohistochemical analysis of lung sections obtained from carrageenan-treated mice revealed a positive staining for iNOS (Fig. 1, A and A1). In contrast, no staining for iNOS was found in the lungs of carrageenan-treated mice that had

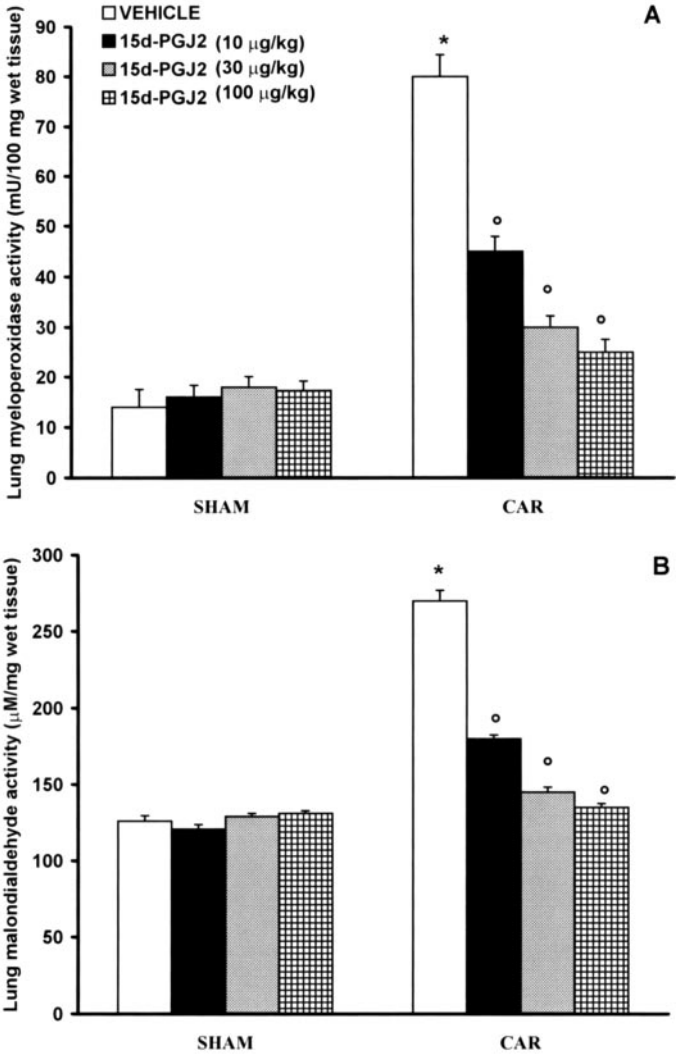

Fig. 3. Effect of 15*d*-PGJ_2 on myeloperoxidase activity and malondialdehyde levels in the lung. MPO activity (A) and MDA levels (B) in the lungs of carrageenan-treated mice killed after 4 h. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated mice in comparison with SHAM mice. 15*d*-PGJ_2 (10–100 μg/kg) reduced the carrageenan-induced increase in MPO activity and MDA levels in a dose-dependent manner. Values shown are mean ± S.E.M. of 10 rats for each group. ∗, $P < 0.01$ versus sham; °, $P < 0.01$ versus carrageenan.

been pretreated with 15*d*-PGJ_2 (Fig. 1C). Staining was absent in lung tissue obtained from the sham group (data not shown). Immunohistochemical analysis of lung sections obtained from mice treated with carrageenan also revealed a positive staining for nitrotyrosine (Fig. 2, A and A1). In contrast, no positive staining for nitrotyrosine was found in the lungs of carrageenan-treated mice that had been pretreated with 15*d*-PGJ_2 (Fig. 2C). Immunohistochemical analysis of lung sections obtained from mice treated with carrageenan also revealed a positive staining for PAR (Fig. 2, B and B1). In contrast, no staining for PAR was found in the lungs of carrageenan-treated mice pretreated with 15*d*-PGJ_2 (Fig. 2D). There was no staining for either nitrotyrosine or PAR in lungs obtained from the sham group of mice (data not shown).

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 mice were significantly increased (Table 1). The levels of PGE_2 were significantly lower in the exudate obtained from carrageenan-treated mice that had been pretreated with 15*d*-PGJ_2. In lungs from carrageenan-treated mice, the amount of 6-keto-$PGF_{1\alpha}$ was significantly increased compared with sham mice (Table 1). The amount of 6-keto-$PGF_{1\alpha}$ was significantly reduced in the lungs from carrageenan-treated mice pretreated

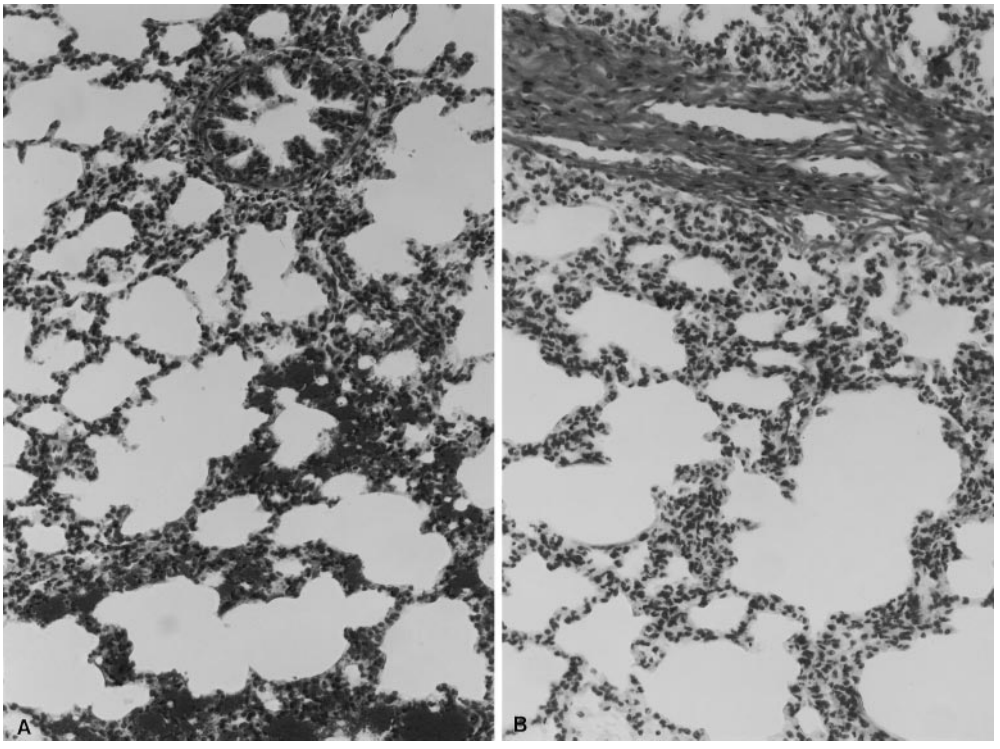

Fig. 4. Effect of 15*d*-PGJ_2 on lung injury. Lung section from a carrageenan-treated mouse (A) demonstrating interstitial hemorrhage and PMN accumulation. Lung section from a carrageenan-treated mouse after administration of 15*d*-PGJ_2 (100 μg/kg) (B) demonstrating reduced interstitial hemorrhage and cellular infiltration. Original magnification, 125×. This figure is representative of at least three experiments performed on different experimental days.

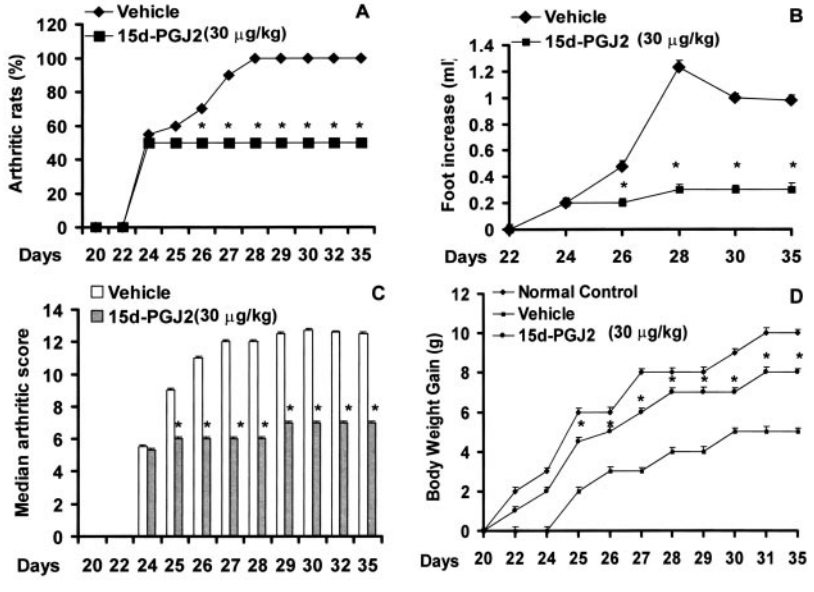

Fig. 5. Effect of 15*d*-PGJ_2 on the onset, on the secondary lesion, and on body weight gain in collagen-induced arthritis. The percentage of arthritic mice (i.e., those showing clinical scores of arthritis >1) are represented (A). There was a significant increase in the arthritic score from day 26 ($P < 0.01$) (C). Paw edema increased in a time-dependent mode between 26 and 35 days post immunization (B). Beginning on day 25, CII-treated mice gained significantly less weight than the normal mice, and this trend continued through to day 35 (D). 15*d*-PGJ_2 had a positive, dose-dependent effect on the percentage of arthritic mice (A), arthritic score (C), weight gain (D), and paw edema (B) of CII-immunized mice. Values are means ± S.E.M. of 10 animals for each group. *, $P < 0.01$ versus control; °, $P < 0.01$ versus CIA.

with 15*d*-PGJ_2 (Table 1). Immunohistochemical analysis of lung sections obtained from carrageenan-treated mice also revealed a positive staining for COX-2, which was localized primarily in alveolar macrophages (Fig. 1, B and B1). In contrast, no positive COX-2 staining was found in the lungs of from carrageenan-treated mice that had been pretreated with 15*d*-PGJ_2 (Fig. 1D). Staining was absent in tissue obtained from the sham group of animals (data not shown).

COX-1 was also detected by immunohistochemical analysis in the lung sections obtained from mice 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 mice treated with 15*d*-PGJ_2 was similar to that observed in lungs obtained either from carrageenan-treated mice or from sham mice (data not shown).

All mice that were treated with carrageenan exhibited a substantial increase in the activities of MPO and MDA in the lungs (Fig. 3, A and B). Pretreatment of mice with 15*d*-PGJ_2 attenuated the increase in MPO and MDA caused by carrageenan in the lung (Fig. 3, A and B). In the sham group, 15*d*-PGJ_2 had no effect on any of the parameters measured (Fig. 3, A and B). Histological examination of lung sections of mice treated with carrageenan showed edema, tissue injury, and infiltration of the tissue with PMNs, lymphocytes, and plasma cells (Fig. 4A). 15*d*-PGJ_2 treatment reduced the lung injury and the infiltration of the tissue with white blood cells (Fig. 4B).

Effects of 15*d*-PGJ_2 in Collagen-Induced Arthritis. CIA developed rapidly in mice immunized with CII and clinical signs (periarticular erythema and edema) of the disease first appeared in mice hind paws between 24 and 26 days postchallenge (Fig. 5A), leading to a 100% incidence of CIA at day 27. In 15*d*-PGJ_2-treated mice, neither the clinical signs nor the histopathological features of CIA were observed in mice forepaws during the 28-day evaluation period. The maximum incidence of CIA in these mice during the complete 35-day study period was 50% (Fig. 5A) ($P < 0.05$).

Hind-paw erythema and swelling increased in frequency and severity in a time-dependent mode with maximum ar-

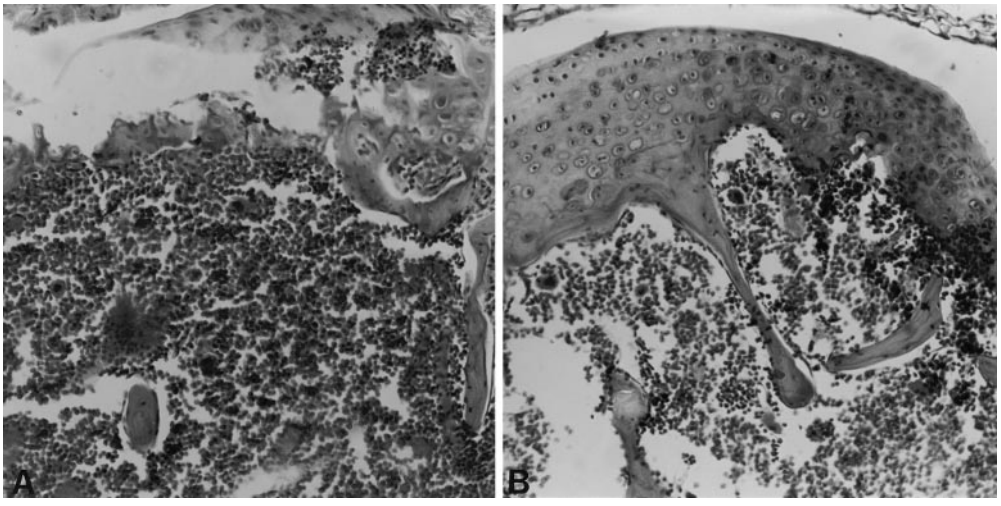

Fig. 6. Effect of 15*d*-PGJ_2 on bone erosion. Representative histology of the inflammatory cells infiltration and bone erosion (A) of an arthritic animal. The degree of inflammatory cell infiltration is reduced (B) in the paws of the 15*d*-PGJ_2-treated arthritic animals. Original magnification, 125×. This figure is representative of at least three experiments performed on different experimental days.

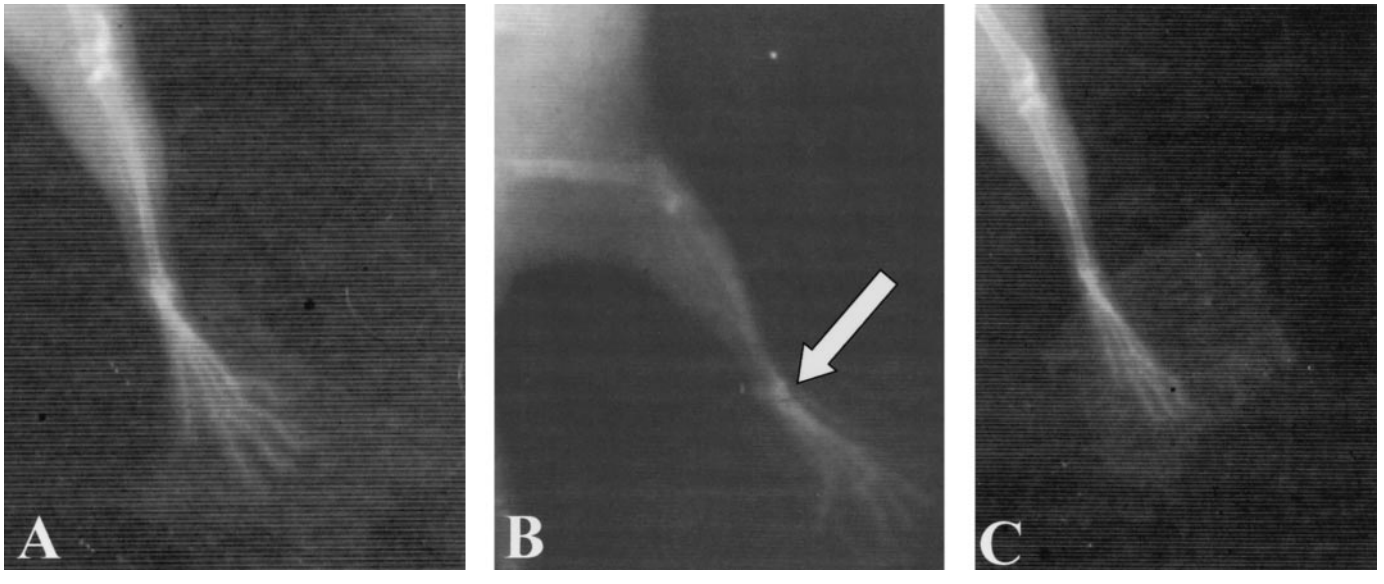

Fig. 7. Radiographic progression of CIA in the tibiotarsal joint of mice with CIA. There was no evidence of pathology in the tibiotarsal joints of SHAM mice (A). The hind paws from CII-immunized (35 days) mice demonstrated bone resorption (arrow) (B). 15*d*-PGJ_2 treatment suppressed joint pathology (arrow) and soft tissue swelling in the mouse hind paw (C). This figure is representative of at least three experiments performed on different experimental days.

thritis indices of approximately 13 observed between 28 and 25 days postimmunization (Fig. 5C). 15*d*-PGJ_2 attenuated the arthritis index between days 25 and 35 post-CII immunization in a dose-dependent fashion (Fig. 5C). There was no macroscopic evidence of either hind-paw erythema or edema in the sham group of mice (Fig. 5C).

The rate and the absolute gain in body weight were comparable in normal mice and CII-immunized mice for the first week (Fig. 5D); however, beginning on day 25, the CII-challenged mice gained significantly less weight than the normal mice, and this trend continued through to day 35. 15*d*-PGJ_2 attenuated the weight loss caused by immunization with CII (compared with the respective control group) (Fig. 5D).

The data in Fig. 5B demonstrate a time-dependent increase in hind-paw volume (each value represents the mean of both hind paws) in mice immunized with CII. Maximum paw volume occurred by day 28 in the CII-immunized mice. 15*d*-PGJ_2 significantly suppressed hind-paw swelling from day 24 to 35 postimmunization in a dose-dependent fashion (Fig. 5B). A maximal reduction in response hind-paw swelling of 66% was observed from day 28 to 35. No increase in hind-paw volume over time was observed in the sham mice group (Fig. 5B).

The histological evaluation (at day 35) of the paws in the vehicle-treated arthritic animals revealed signs of severe arthritis, with massive infiltration of the tissue with white blood cells (neutrophils, macrophages, and lymphocytes). In addition, severe or moderate necrosis and sloughing of the synovium were seen, together with the extension of the inflammation into the adjacent musculature with fibrosis and increased mucous production (Fig. 6A; see Fig. 8A for damage score). In the 15*d*-PGJ_2-treated mice, the degree of arthritis was significantly reduced (Fig. 6B; see Fig. 8A for damage score).

A radiographic examination of hind paws from mice at 35 days post-CII immunization revealed bone matrix resorption at the joint margin (Fig. 7A; see Fig. 8B for radiograph score). 15*d*-PGJ_2 markedly reduced the degree of bone resorption (Fig. 7B; see Fig. 8B for radiograph score). There was no evidence of pathology in sham mice (data not shown).

At day 35, all vehicle-treated arthritic animals exhibited a substantial increase in the plasma MDA levels (Fig. 9). Treatment of mice with 15*d*-PGJ_2 significantly attenuated the increase in MDA caused by CIA (Fig. 9). No increases in plasma MDA levels were observed with sham mice (Fig. 9).

Immunohistochemical analysis of joint sections obtained from CII-treated mice revealed a positive staining for nitrotyrosine, which was primarily localized in the synovia (Fig. 10A). In contrast, no positive staining for nitrotyrosine was found in the joint of CII-treated mice, which had been pretreated with 15*d*-PGJ_2 (Fig. 10C). Immunohistochemical analysis of joint sections obtained from CII-treated mice also revealed a positive staining for PAR (Fig. 10B). In contrast, no specific staining for PAR was found in the joint of CIA-treated mice, which had been pretreated with 15*d*-PGJ_2 (Fig. 10D). There was no staining for either nitrotyrosine or PAR in joints obtained from the sham group of mice (data not shown).

Immunohistochemical analysis of joint sections obtained from CII-treated mice revealed a positive staining for iNOS and COX-2 (Fig. 11, A and C). In contrast, no positive iNOS

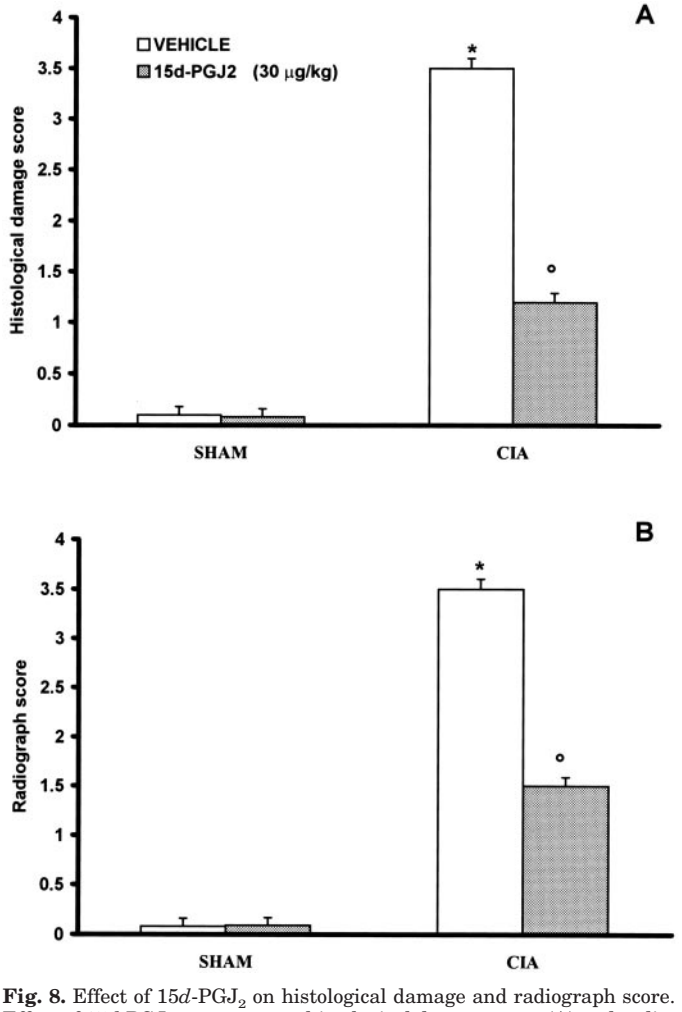

Fig. 8. Effect of 15*d*-PGJ_2 on histological damage and radiograph score. Effect of 15*d*-PGJ_2 treatment on histological damage score (A) and radiograph score (B). Values are means ± S.E.M. of 10 animals for each group. *, $P < 0.01$ versus control; °, $P < 0.01$ versus CIA.

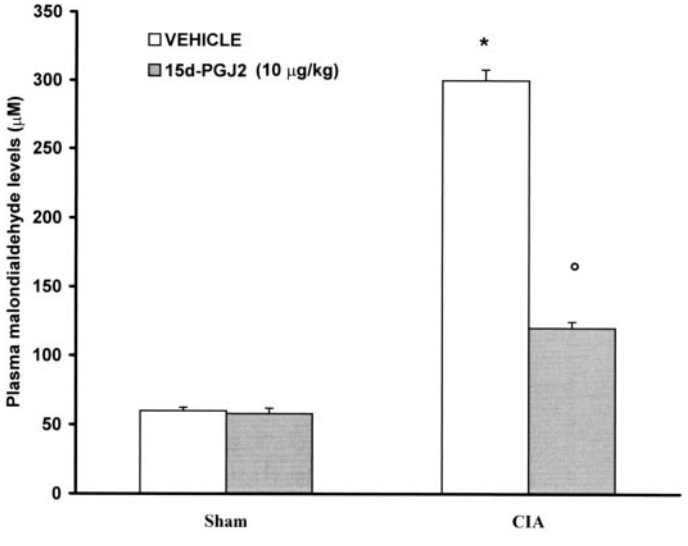

Fig. 9. Effect of 15*d*-PGJ_2 on malondialdehyde levels in the plasma: MDA levels in the plasma of CII-immunized mice killed after 35 days. MDA levels were significantly increased in the plasma of the CII-immunized mice in comparison with SHAM mice (*, $P < 0.01$). 15*d*-PGJ_2 treatment reduced the CIA increase in MDA levels. Values are means ± S.E.M. of 10 mice for each group. *, $P < 0.01$ versus control; °, $P < 0.01$ versus CIA.

and COX-2 staining was found in the joints of CII-treated mice that had been pretreated with 15*d*-PGJ_2 (Fig. 11, B and D).

Discussion

This study provides the first evidence, to our knowledge, that 15*d*-PGJ_2 attenuates the development of carrageenan-induced pleurisy, the infiltration of the lung with PMNs, the degree of lipid peroxidation in the lung, IκB-α degradation, the degree of lung injury caused by injection of carrageenan, the development of CIA, the infiltration of bone joints by PMNs, and the degree of joint injury in mice treated with type 2 collagen. All of these findings support the view that 15*d*-PGJ_2 attenuates the degree of acute and chronic inflammation in the mouse. What, then, is the mechanism by which 15*d*-PGJ_2 protects the joint against this inflammatory injury?

It has been reported that 15*d*-PGJ_2 inhibits the activation of NF-κB by preventing the phosphorylation of inhibitory kinase kinase and, hence, preventing the degradation of inhibitor κB (Marx et al., 1998). Thus, the anti-inflammatory effects of 15*d*-PGJ2 observed in the present study may be due, at least in part, to the inhibition of the activation of NF-κB by this cyclopentenone prostaglandin. The promoter region of the murine and human COX-2 genes contain binding sites for NF-κB (Feng et al., 1995; Topping and Jones, 1998), and there is evidence that 15*d*-PGJ_2 attenuates the expression of COX-2 in rat synoviocytes (Tsubouchi et al., 2001). An enhanced formation of prostanoids after the induction of COX-2 contributes to the pathophysiology of local and chronic inflammation (Harada et al., 1996; Cuzzocrea et al., 2000b) and selective inhibitors of COX-2 also exert potent anti-inflammatory effects (Futaki et al., 1993; Mitchell et al., 1993; Tomlinson et al., 1994; Harada et al., 1996). Here, we demonstrate that the increase in the levels of PGE_2 caused by injection of carrageenan into the pleural cavity of mice is reduced in the exudate of mice treated with 15*d*-PGJ_2. The enhanced formation of PGE_2 is secondary to the expression of COX-2 protein because there was no increase in the expression of COX-1 protein (as detected by immunohistochemistry) after carrageenan injection and because selective inhibitors of COX-2 activity including NS-398 (nimesulide) and SC-58125 (celecoxib) markedly abolish the increase in PGE_2 caused by injection of carrageenan into the pleural space (Futaki et al., 1993; Mitchell et al., 1993; Harada et al., 1996). Thus, we propose that 15*d*-PGJ_2 reduces the expression of COX-2 protein and activity caused by injection of carrageenan in the lung and in the joints from collagen-treated mice.

There is increasing evidence that an enhanced formation of NO by iNOS also contributes to the inflammatory process (Wei et al., 1995; Salvemini et al., 1996; Cuzzocrea et al., 1998c, 2000a). This study demonstrates that 15*d*-PGJ_2 attenuates the expression of iNOS in the lung from carrag-

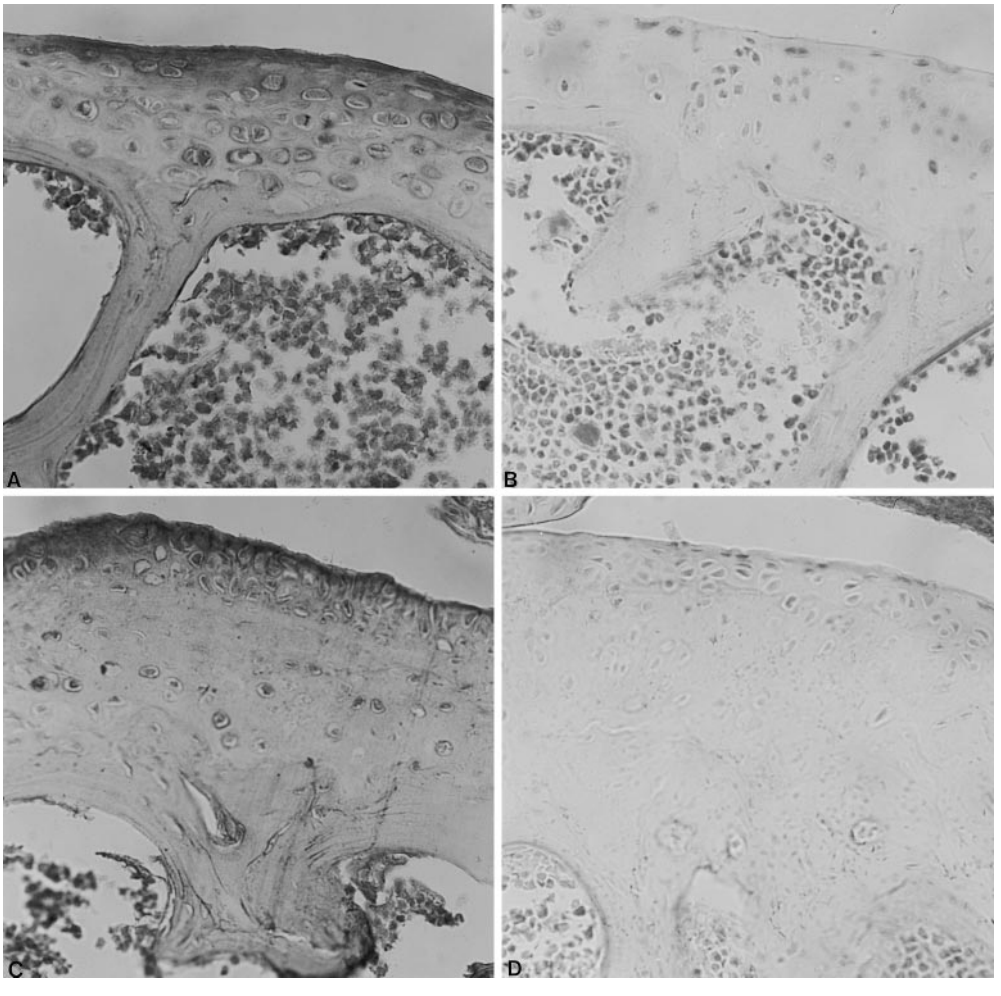

Fig. 10. Effect of 15*d*-PGJ_2 on nitrotyrosine and PARP immunostaining. Nitrotyrosine and PARP immunostaining in the paw of a mouse at 35 days of CIA (A and C). A marked increase in nitrotyrosine and PARP staining is evident in the paws in arthritic mice. There was a marked reduction in the immunostaining in the paw of 15*d*-PGJ_2-treated mice (B and D). Original magnification, 125×. This figure is representative of at least three experiments performed on different experimental days.

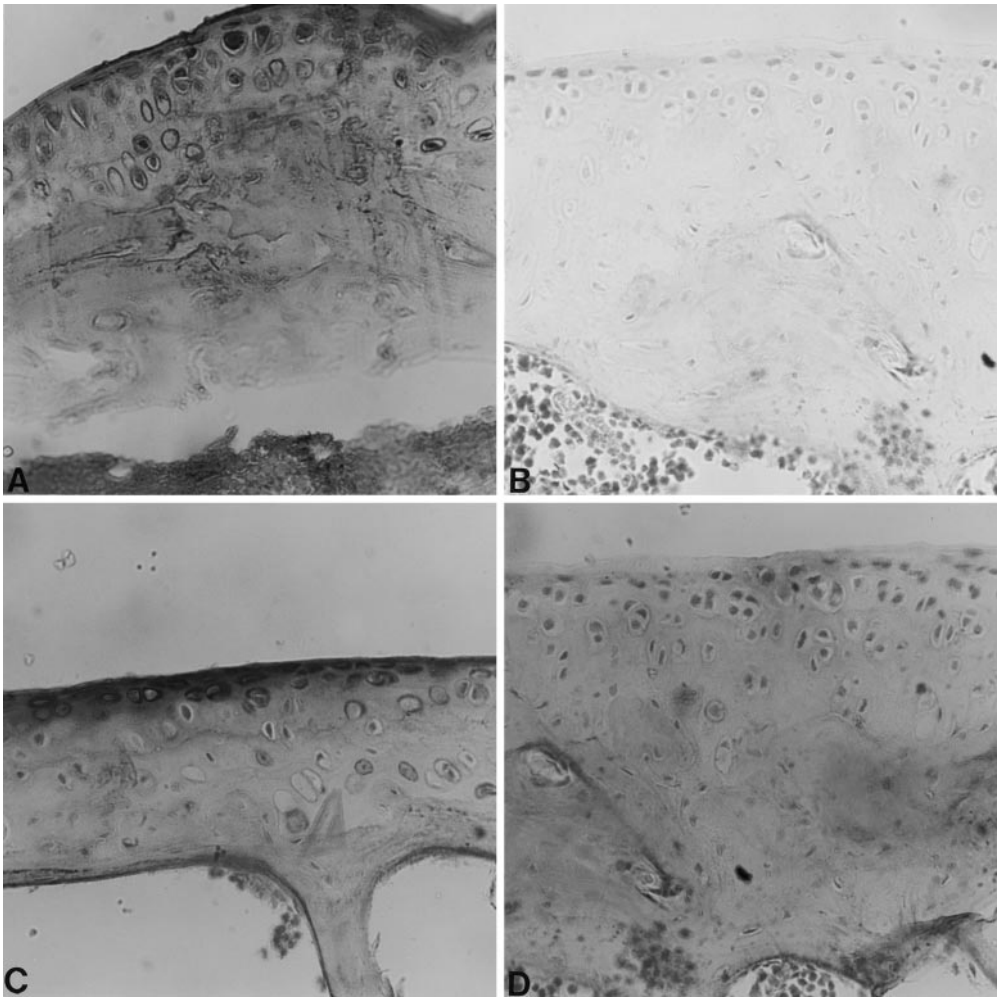

Fig. 11. Effect of 15*d*-PGJ_2 on iNOS and COX-2 immunostaining. iNOS and COX-2 immunostaining in the paw of a mouse after 35 days of CIA (A and C). A marked increase in iNOS and COX-2 staining is evident in the paws in arthritic mice. There was a marked reduction in the immunostaining in the paw of 15*d*-PGJ_2-treated mice (B and D). Original magnification, 125×. This figure is representative of at least three experiments performed on different experimental days.

eenan-treated mice and in the joints from mice treated with collagen. Our finding of reduced NO production by 15*d*-PGJ_2 in vitro is also in accordance with reports that 15*d*-PGJ_2 inhibits the expression of iNOS in vitro (see the introduction). Thus, the reduction of the expression of iNOS by 15*d*-PGJ_2 may contribute to the attenuation by this agent of the formation of nitrotyrosine in the lung from carrageenan-treated mice and in the joints from collagen-treated mice. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation "footprint" of peroxynitrite (Beckman, 1996). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich et al., 1998). Increased nitrotyrosine staining is considered, therefore, an indication of "increased nitrosative stress" rather than a specific marker of the generation of peroxynitrite. Thus, we propose that the reduction of the expression of iNOS protein and activity caused by 15*d*-PGJ_2 contributes to the reduction by this agent of the organ injury caused by acute and chronic inflammation in the rat.

ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms, including peroxidation of membrane lipids, protein denaturation, and DNA damage. ROS produce strand breaks in DNA that triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARP resulting in the depletion of its substrate NAD^+ in vitro and a reduction in the rate of glycolysis. Because 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 PARP Suicide Hypothesis". There is recent evidence that the activation of PARP may also play an important role in inflammation (Szabó et al., 1997, 1998; Cuzzocrea et al., 1998a,b). We demonstrate here that 15*d*-PGJ_2 attenuates the increase in PARP activity in the lung from carrageenan-treated mice and in the joints from collagen-treated mice.

In conclusion, our results indicate that 15*d*-PGJ_2 has strong anti-inflammatory properties resulting in reduced cytokine production, reduced PMN infiltration, reduced expression of iNOS and COX-2 protein and activity, and ultimately reduced degree of peroxynitrite formation and tissue injury. However, it is unclear whether 15*d*-PGJ_2 elicits these anti-inflammatory effects in a PPAP-γ–dependent or -independent manner. Although the exact mode of action of 15*d*-PGJ_2 remains to be determined, we speculate that 15*d*-PGJ_2 may be useful in conditions associated with acute and chronic inflammation.

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