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Beneficial effects of GW274150, a novel, potent and selective inhibitor of iNOS activity, in a rodent model of collagen-induced arthritis

Salvatore Cuzzocrea[a,*], Prabal K. Chatterjee[b], Emanuela Mazzon[c], Michelle C. McDonald[b], Laura Dugo[a], Rosanna Di Paola[a], Ivana Serraino[a], Domenico Britti[d], Achille P. Caputi[a], Christoph Thiemermann[b]

[a] *Institute of Pharmacology, School of Medicine, University of Messina, Torre Biologica, Policlinico Universitario Via C. Valeria, Gazzi, 98100 Messina, Italy*

[b] *Department of Experimental Medicine and Nephrology, The William Harvey Research Institute, Queen Mary, University of London, London, UK*

[c] *Department of Biomorphology, School of Medicine, University of Messina, Messina, Italy*

[d] *Department of Veterinary Medicine and Pharmacology, University of Messina, Messina, Italy*

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Abstract

The aim of this study was to investigate the role of inducible nitric oxide synthase (iNOS) on the modulation of the inflammatory response in mice subjected to collagen-induced arthritis. Collagen-induced arthritis was induced in wild-type mice (iNOS-WT) treated with GW274150, a novel, potent and selective inhibitor of iNOS activity, and in mice lacking the gene for iNOS (iNOS 'knock-out', iNOS-KO), by an intradermal injection of 100 μl of emulsion containing 100 μg of bovine type II collagen and complete Freund's adjuvant at the base of the tail. After 21 days, a second injection of type II collagen in complete Freund's adjuvant was administered. iNOS-WT mice developed erosive hind paw arthritis when immunised with type II collagen in complete Freund's adjuvant. Over a 35-day period, macroscopic clinical evidence of collagen-induced arthritis first appeared as periarticular erythema and oedema in the hind paws. By day 28, the incidence of collagen-induced arthritis was 100% in type II collagen-challenged iNOS-WT mice and the severity of collagen-induced arthritis progressed with radiographic evaluation revealing resorption of bone.

Histopathology of collagen-induced arthritis mice demonstrated erosion of the cartilage at the joint margins. iNOS-WT mice treated with GW274150 (5 mg/kg, i.p. daily) starting at the onset of arthritis (day 23), and iNOS-KO mice showed a delay of the development of the clinical signs at days 24–35 and an improvement of the histological status in the knee and paw. Immunohistochemical analysis for nitrotyrosine and for poly(ADP-ribose) polymerase revealed positive staining in inflamed joints from type II collagen-treated iNOS-WT mice. The degree of staining for nitrotyrosine and poly(ADP-ribose) polymerase were markedly reduced in tissue sections obtained from type II collagen-treated iNOS-WT mice, who had received GW274150 and from iNOS-KO mice. Furthermore, radiographic signs of protection against bone resorption were present in the joints of iNOS-WT mice treated with GW274150 as well as in the joint from iNOS-KO mice.

This study provides the first evidence that GW274150, a novel, potent and selective inhibitor of iNOS activity, attenuates the degree of chronic inflammation and tissue damage associated with collagen-induced arthritis in mice. Furthermore, these results suggest that the induction of iNOS and NO production are essential for the up-regulation of the inflammatory response during experimental collagen-induced arthritis.

Keywords: Arthritis, collagen-induced; GW274150; Nitric oxide (NO) synthase, inducible

1. Introduction

The free radical nitric oxide (NO) is synthesised from the guanidino group of L-arginine by a family of enzymes termed NO synthases (NOS). Three isoforms have been described and cloned, two of which are constitutively expressed (constitutive NOS, cNOS), and the other is inducible NOS (iNOS). Many inflammatory conditions are associated with production of comparatively large amounts of NO produced by iNOS, with consequent cytotoxic effects. Induction of iNOS can be inhibited by numerous agents, including glucocorticoids, thrombin, macrophage deactivation factor,

* Corresponding author. Tel.: +39-90-2213644; fax: +39-90-694951.
E-mail address: salvator@www.unime.it (S. Cuzzocrea).

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transforming growth factor-β, platelet-derived growth factor, interleukin-4, interleukin-10 and interleukin-13 (Moncada et al., 1991; Nathan, 1992).

Enhanced formation of NO following the induction of iNOS has been implicated in the pathogenesis of circulatory shock and inflammation (Thiemermann et al., 1995; Salvemini et al., 1996; Szabò, 1995).

Recent data suggest that some of the cytotoxic effects of NO be related to the production of peroxynitrite, a reactive oxidant formed by the rapid reaction of NO and superoxide (Brahn et al., 1998; Crow and Beckman, 1995; Cuzzocrea et al., 1997).

The biological activity and decomposition of peroxynitrite is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, and other factors), and these factors influence its toxic potential (Cuzzocrea et al., 1997, 1998a). In a number of pathophysiological conditions associated with inflammation or oxidant stress, peroxynitrite has been proposed to mediate cell damage (Brahn et al., 1998; Cuzzocrea et al., 1998b, 1999). Peroxynitrite is cytotoxic via a number of mechanisms including (i) the initiation of lipid peroxidation, (ii) the inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane pumps) (Brahn et al., 1998) and (iii) depletion of glutathione (Cuzzocrea et al., 2000a). Moreover, peroxynitrite can also cause DNA damage (Cuzzocrea et al., 2000b, 2001) resulting in the activation of the nuclear enzyme poly(ADP-ribose) synthetase (PARS), depletion of NAD and ATP and ultimately cell death (Eiserich et al., 1996). Interventions, which reduce the generation or the effects of reactive oxygen species or reactive nitrogen species, exert beneficial effects in a variety of models of inflammation including the model of collagen-induced arthritis used in this study. These therapeutic interventions include melatonin (Cuzzocrea et al., 1997), a vitamin E-like antioxidant (Cuzzocrea et al., 1999), a superoxide dismutase-mimetic (Oyanagui, 1994) and a peroxynitrite decomposition catalyst (Salvemini et al., 1998). Rheumatoid arthritis is an autoimmune disease characterised by the sequestration of various leukocyte subpopulations within both the developing pannus and synovial space. This chronic disease results in the inflammation of multiple joints with subsequent destruction of joint cartilage and erosion of bone. The pathogenesis of rheumatoid arthritis is not clearly understood (Harris, 1990). Type II collagen-induced arthritis in the mouse has proven to be a useful model of rheumatoid arthritis, as it possesses many of the cell and humoral immunity characteristics found in human rheumatoid arthritis (Stuart et al., 1984; Holmdahl et al., 1990). Models of collagen-induced arthritis have been widely employed to evaluate the efficacy of drugs in chronic inflammation.

In this study, we hypothesise that enhanced NO formation by iNOS may contribute to tissue injury and pro-inflammatory signalling in experimental collagen-induced arthritis. To investigate this hypothesis, mice were subjected to collagen-induced arthritis and treated with either the novel, selective and potent inhibitor of iNOS, GW274150 [(*S*)-2-amino-(1-iminoethylamino)-5-thioheptanoic acid] or its vehicle (control) (Alderton et al., 1999). In a separate study, we have compared the degree of arthritis caused by collagen-induced arthritis in mice genetically deficient in the gene for iNOS (iNOS knockout mice, iNOS-KO) and their wild-type littermates (iNOS-WT).

2. Methods

2.1. Animals

Homozygous iNOS-KO, and iNOS-WT (wild-type C57B1/6 × 129/Sv), male mice (9 weeks; 20–25 g), kindly supplied by F.A.J. Van de Loo (Department of Rheumatology, University Hospital Nijmegen, Nijmegen, The Netherlands), were used to assess the role of iNOS in the pathogenesis of collagen-induced arthritis. A neocassette using homologous recombination as previously described (Alderton et al., 1999) replaced the first four exons of the NOS-2 gene. All animals were allowed access to food and water ad libitum. 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 E.C. regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Experimental groups

Animals were randomly divided into six groups ($n = 10$ for each group). The first group (iNOS-WT) was treated with vehicle (0.01 M acetic acid) and served as sham group. The second group (iNOS-WT) was treated with the emulsion (containing 100 μg of type II collagen). In the third and fourth groups, iNOS-KO mice received vehicle (0.01 M acetic acid) or the emulsion (containing 100 μg of type II collagen), respectively. In the fifth and sixth groups, iNOS-WT were treated with GW274150 (5 mg/kg, i.p. daily) starting from day 23 after collagen-induced arthritis induction or saline administration.

2.3. Induction of collagen-induced arthritis

Bovine type II collagen (type II collagen) was dissolved in 0.01 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4 °C and frozen at −70 °C until required. Complete Freund's adjuvant was prepared by the addition of *Mycobacterium tuberculosis* (MacMicking et al., 1995) at a concentration of 2 mg/ml. Before injection, type II collagen was emulsified with an equal volume of complete Freund's adjuvant. Collagen-induced arthritis was induced as previously described (Cuzzocrea et al., 2000a;

Szabò et al., 1998). Briefly, on day 1, mice were injected intradermally at the base of the tail with 100 μl of emulsion containing 100 μg of type II collagen. On day 21, a second injection of type II collagen in complete Freund's adjuvant was administered.

2.4. 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; 4, severe arthritis of the entire paw and digits (Szabò et al., 1998). The *arthritic index* for each mice was calculated by adding the four scores of individual paws. The Mean Arthritic Score (MAS) for each mice was calculated by dividing the total number of points scored by the group by the number of animals in the group. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile). The macroscopic score and the paw volume were considered by an investigator blinded for the treatment regime.

2.5. Assessment of collagen-induced arthritis-induced damage

At day 35, animals were sacrificed while under anaesthesia, and paws and knees were removed and fixed for histological examination, which was done by an investigator blinded for the treatment regime. The following morphological criteria were considered by an investigator blinded for the treatment regime: score 0, no damage; score 1, sloughing of the articular space oedema; score 2, inflammatory cell presence; score 3, bone resorption.

Histomorphometric analysis was carried out in the proximal tibia near the joint on 5-μm-thick sections, using a morphometry software, a computer with a digitizing board and a Nikon Labophot microscope equipped with both visible and UV light sources and a camera lucida attachment. Parameters for histomorphometry employed in this study, derived from Parfitt et al. (1987), have been approved by an ASBMR committee. To measure bone formation, osteoblast surface was quantified relative to bone surface (Ob/Bs). To measure bone resorption, eroded surface, osteoclast surface, were quantified relative to bone surface (ES/Bs, Oc.S/Bs).

2.6. Histological examination

For microscopic histological evaluation, paws and knees were removed and fixed in 10% formalin. The paws were then trimmed, placed in decalcifying solution for 24 h, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin/eosin and studied using light microscopy (Dialux 22, Leitz, Italy).

2.7. Radiography

Mice were anaesthetised 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 mice hind paws was performed using an X-ray machine (Philips X12, Germany) with a 40-kW exposition for 0.01 s. In each case, the *radiographic score* was calculated by an investigator blinded for the treatment regime. The following radiograph criteria were considered: score 0, no bone damage; score 1, tissue swelling and oedema; score 2, joint erosion; score 3, bone erosion.

2.8. Measurement of plasma nitrite/nitrate concentrations

Nitrite + nitrate (NOx) production, an indicator of NO synthesis, was measured in the supernatant samples as previously described (Cuzzocrea et al., 2000a). Briefly, the nitrate in the supernatant was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μM) at room temperature for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4; vol. 1:1) to 100-μl samples. The optical density at 550 nm (OD_{550}) was measured using enzyme-linked immunosorbent assay (ELISA) microplate reader (SLT-Labinstruments Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of DMEM (Dulbecco's modified Eagles medium).

2.9. Immunohistochemical localisation of nitrotyrosine and poly(ADP-ribose) polymerase

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or reactive oxygen species, was determined using immunohistochemistry as previously described (Cuzzocrea et al., 2000b; Szabò et al., 1998). Poly(ADP-ribose) polymerase activation was assessing by measuring the degree of poly(ADP-ribose) formation, which is secondary to the activation of the nuclear DNA repair enzyme (Heller et al., 1995; Küpper et al., 1996).

At day 35, joints were collected from mice, trimmed, placed in decalcifying solution for 24 h, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) H_2O_2 in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) for 20 min. Nonspecific 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 avidin and biotin. The sections were then incubated overnight

with primary anti-nitrotyrosine antibody (1:1000 dilution) or anti-poly(ADP-ribose) antibody (1:500 dilution) or with control solutions. Controls included buffer alone or non-specific purified rabbit immunoglobulin G. Specific labelling was detected with a biotin-conjugated goat anti-rabbit immunoglobulin G and avidin–biotin peroxidase complex.

2.10. Malondialdehyde measurement

Plasma malondialdehyde levels were determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). An aliquot (100 μl) of the plasma collected at the specified time was added to a reaction mixture containing 200 μl of 8.1% (w/v) SDS (sodium dodecyl sulfate), 1500 μl of 20% (v/v) acetic acid (pH 3.5), 1500 μl of 0.8% (w/v) thiobarbituric acid and 700 μl distilled water. Samples were then heated for 1 h at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

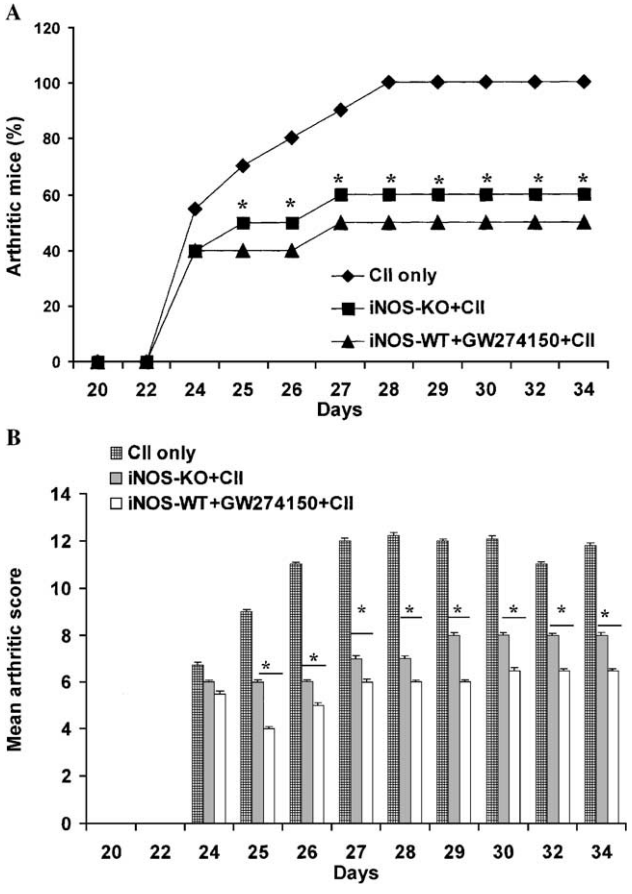

Fig. 1. Effect of absence or inhibition of iNOS on the onset of collagen-induced arthritis. (A) The percentage of arthritic mice showing clinical scores of arthritis. (B) Median arthritic score during collagen-induced arthritis. Data are mean ± S.E.M. *$P<0.01$ vs. wild-type mice subjected to collagen-induced arthritis only.

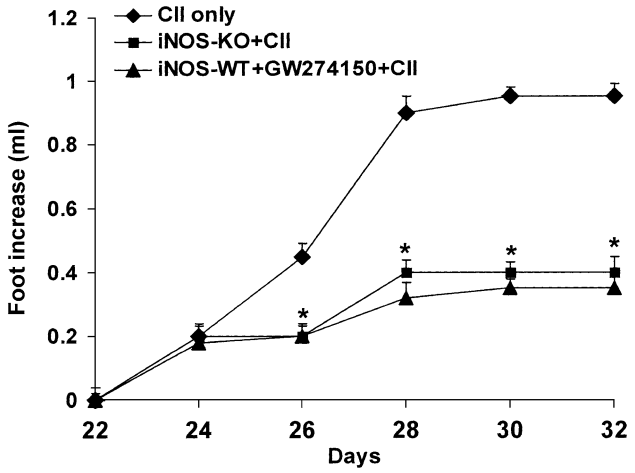

Fig. 2. Effect of absence or inhibition of iNOS on the paw edema formation during collagen-induced arthritis. Swelling in hind paws over time was measured at 2 days intervals. Data are mean ± S.E.M. *$P<0.01$ vs. wild-type mice subjected to collagen-induced arthritis only.

2.11. Myeloperoxidase assay

Polymorphonuclear leukocytes infiltration into the inflamed joints was indirectly quantitated using a myeloperoxidase assay, as previously described (Goldblum et al., 1985). Tissues were prepared as described above and placed in a 50 mM phosphate buffer (pH 6.0) with 5% hexadecyltrimethylammonium bromide. Joint tissues were homogenised, sonicated and centrifuged at 12,000 × *g* for 15 min at 4 °C. Supernatants were assayed for myeloperoxidase activity using a spectrophotometric reaction with *O*-dianisidine hydrochloride at 460 nm.

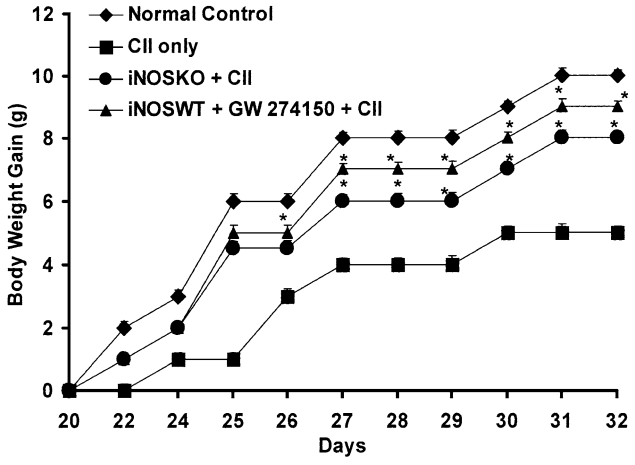

Fig. 3. Effect of absence or inhibition of iNOS on body weight gain during collagen-induced arthritis. Beginning on day 25, type II collagen-treated mice gained significantly less weight than the normal mice, and this trend continued through to day 35. Absence or inhibition of iNOS had a positive effect on the weight gain of type II collagen-treated mice. Data are mean ± S.E.M. *$P<0.01$ vs. wild-type mice subjected to collagen-induced arthritis only.

2.12. Reagents

GW274150 and primary anti-poly(ADP-ribose) antibody were obtained from Alexis Biochemicals (Milan, Italy). Primary anti-nitrotyrosine antibody was purchased from DBA (Milan, Italy). Biotin blocking kit, biotin-conjugated goat anti-rabbit immunoglobulin G and avidin–biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA, USA). All other reagents and compounds used were purchased from Sigma (Milan, Italy).

2.13. Data analysis

All values in the text and figures are expressed as mean ± standard error of the mean (S.E.M.) for n 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 (ANOVA), and individual group means were then compared with Student's unpaired t-test. For the arthritis studies, Mann–Whitney U-test (two-tailed,

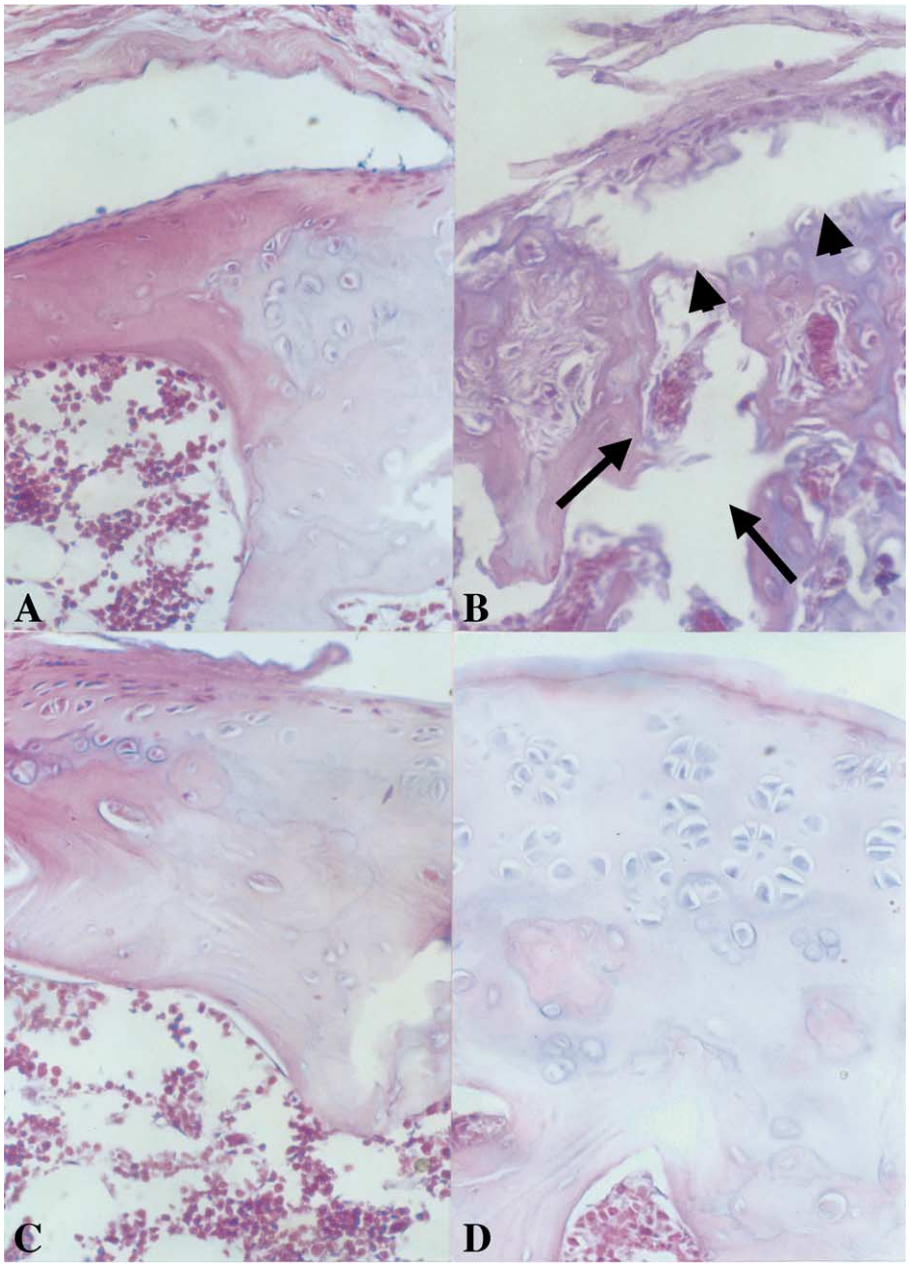

Fig. 4. Histological changes during collagen-induced arthritis. Representative hematoxylin/eosin-stained section of joint was examined by light microscopy in (A) sham-treated wild-type mice, (B) type II collagen-treated wild-type mice, (C) in type II collagen-treated iNOS-KO mice, and (D) in GW274150 and type II collagen-treated wild-type mice. Original magnification: × 180. The figure is representative of at least three experiments performed on different experimental days.

independent) was used to compare medians of the arthritic indices (Szabò et al., 1998). A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Day of onset and incidence of collagen-induced arthritis in mice

Collagen-induced arthritis developed rapidly in mice immunised with type II collagen and clinical signs (peri-articular erythema and oedema) of the disease first appeared in the hind paws between 24 and 26 days post-challenge (Fig. 1A). Furthermore, a 100% incidence of collagen-induced arthritis was observed by day 28 in type II collagen-immunised iNOS-WT mice. The maximum incidence of collagen-induced arthritis in the iNOS-WT mice which were treated with GW274150 and in iNOS-KO mice during the 35-day study period was significantly reduced (Fig. 1A) ($P<0.01$).

3.2. Clinical progression of collagen-induced arthritis in mice

Hind paw erythema and swelling increased in frequency and severity in a time-dependent mode with maximum arthritis indices of approximately 12 observed between 28 and 35 days post-immunisation (Fig. 1B). A significant suppression ($P<0.01$) of the arthritis index between days 25 and 35 post-immunisation was observed in iNOS-WT mice which were treated with GW274150 and in iNOS-KO mice (Fig. 1B). There was no macroscopic evidence of either hind paw erythema or oedema in the normal control mice (data not shown).

3.3. Clinical severity of collagen-induced arthritis in mice

The data in Fig. 2 demonstrate a time-dependent increase in hind paw (each value represents the mean values of both hind paws) volume (in ml) in iNOS-WT mice immunised with type II collagen (Fig. 2). Maximum paw volume was observed at day 28 in the type II collagen-immunised iNOS-WT mice. Pharmacological (GW274150-treated iNOS-WT mice) and genetic inhibition of iNOS (iNOS-KO mice) significantly suppressed hind paw swelling from days 26 to 35 post-immunisation ($P<0.01$, Fig. 2). A maximal reduction in response hind paw swelling of 70% and 60%, respectively, was observed from days 28 to 35. No increase in hind paw volume over time was observed in normal mice (data not shown).

3.4. Effect of collagen-induced arthritis on body weight of mice

The rate and the absolute gain in body weight were comparable in normal mice and type II collagen-immunised iNOS-WT mice for the first week (Fig. 3). Beginning on day 25, the type II collagen-challenged iNOS-WT mice gained significantly less weight than normal mice, and this trend continued through day 35 (Fig. 3). After administration of GW274150, type II collagen-immunised iNOS-WT mice as well as type II collagen-immunised iNOS-KO mice exhibited a significant increase in weight gain when compared with the respective control group ($P<0.01$, Fig. 3).

3.5. Histopathological analysis of the effects of inhibition or absence of iNOS on collagen-induced arthritis

At day 35, histological evaluation of the paws in iNOS-WT subjected to collagen-induced arthritis mice revealed signs of severe arthritis, with cartilage damage (see arrowheads, Figs. 4B and 5A; Table 1). In addition, severe or moderate necrosis and sloughing of the synovium associated with a trabecular erosion were observed (see arrows, Fig. 4B). In GW274150-treated type II collagen-immunised

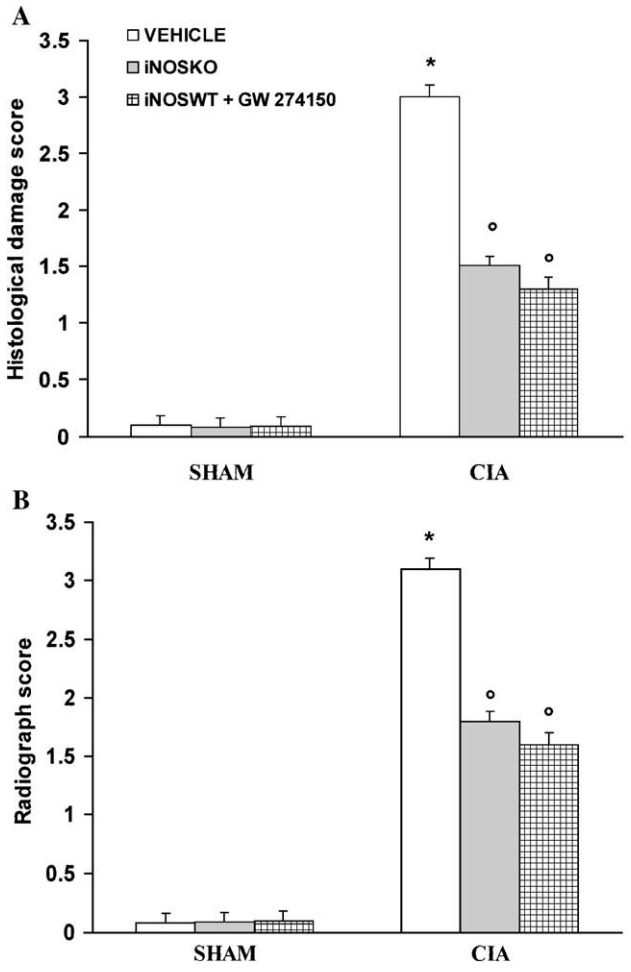

Fig. 5. Effect of absence or inhibition of iNOS on (A) histological damage score and (B) radiograph score. Data are mean ± S.E.M. *$P<0.01$ vs. normal (sham) animals, °$P<0.01$ vs. wild-type mice subjected to collagen-induced arthritis only.

Table 1
Histomorphometry data in proximal tibia

	Ob.S/BS (%)	ES/BS (%)	Oc.S/BS (%)
iNOSWT + Vehicle	1.01 ± 0.992	22.16 ± 1.32	1.06 ± 0.52
iNOSKO + Vehicle	1.11 ± 076	20.11 ± 1.32	1.16 ± 0.88
iNOSWTCIA + Vehicle	6 ± 0.75 *	30.12 ± 1.12 *	7.02 ± 0.72 *
iNOSKOCIA + Vehicle	2.9 ± 0.99°	27.98 ± 2.1°	3.11 ± 0.89°
iNOSWTCIA + GW274150	2.9 ± 1°	28.12 ± 2.9°	3.19 ± 1.03°

Data are expressed as the mean value ± S.E.M.
Ob.S/BS, osteoblast surface; ES/BS, eroded surface; Oc.S/BS, osteoclast surface.
* $P < 0.01$ vs. sham.
° $P < 0.01$ vs. CIA.

Table 2
Effect of absence or inhibition of iNOS on plasma nitrite/nitrate levels, myeloperoxidase activity (MPO) and malondialdehyde levels (MDA) during collagen-induced arthritis

	Ob.S/BS (%)	ES/BS (%)	Oc.S/BS (%)
iNOSWT + Vehicle	1.01 ± 0.992	22.16 ± 1.32	1.06 ± 0.52
iNOSKO + Vehicle	1.11 ± 076	20.11 ± 1.32	1.16 ± 0.88
iNOSWTCIA + Vehicle	6 ± 0.75 *	30.12 ± 1.12 *	7.02 ± 0.72 *
iNOSKOCIA + Vehicle	2.9 ± 0.99°	27.98 ± 2.1°	3.11 ± 0.89°
iNOSWTCIA + GW274150	2.9 ± 1°	28.12 ± 2.9°	3.19 ± 1.03°

Data are mean ± S.E.M.
* $P < 0.01$ vs. normal (sham) animals.
° $P < 0.01$ vs. wild-type mice subjected to collagen-induced arthritis only.

iNOS-WT mice as well as type II collagen-immunised iNOS-KO, the degree of arthritis was significantly reduced: a moderate infiltration into several of the larger joints comprised primarily of neutrophils, coupled with mild articular cartilage and bone erosion, was observed (Fig. 4C,D; Table 1). The beneficial effects of either inhibition or absence of iNOS in mice subjected to collagen-induced arthritis was reflected in histological damage scores taken from each group ($P < 0.01$, Fig. 5A). No signs of histological damage was present in sham treated wild-type mice (Figs. 4A and 5A).

3.6. Radiographic analysis of the effects of inhibition or absence of iNOS on collagen-induced arthritis

Radiographic analysis revealed no evidence of abnormal pathology in normal mice (data not shown). However, radiographic examination of hind paws from iNOS-WT mice at 35 days post type II collagen immunisation revealed bone matrix resorption and osteophyte formation at the joint margin (Fig. 6A). In GW274150-treated type II collagen-immunised iNOS-WT mice as well as type II collagen-immunised iNOS-KO, the degree of bone resorption, soft tissue swelling and osteophyte formation was markedly reduced in comparison with mice subjected to collagen-induced arthritis only (Fig. 6B,C). In GW274150-treated type II collagen-immunised iNOS-WT mice as well as type II collagen-immunised iNOS-KO, the radiaographic damage score was significantly reduced on comparison with mice subjected to collagen-induced arthritis only ($P < 0.01$, Figs. 5B and 6B).

In the proximal tibia the Ob.S/Bs, the ES/Bs and Oc.S/Bs were significantly increased at 35 days after type II collagen immunisation (Table 1). In GW274150-treated type II collagen-immunised iNOS-WT mice as well as type II collagen-immunised iNOS-KO, a markedly protected against bone resorption was observed (Table 1). There was no evidence of pathology in naive mice (Table 1).

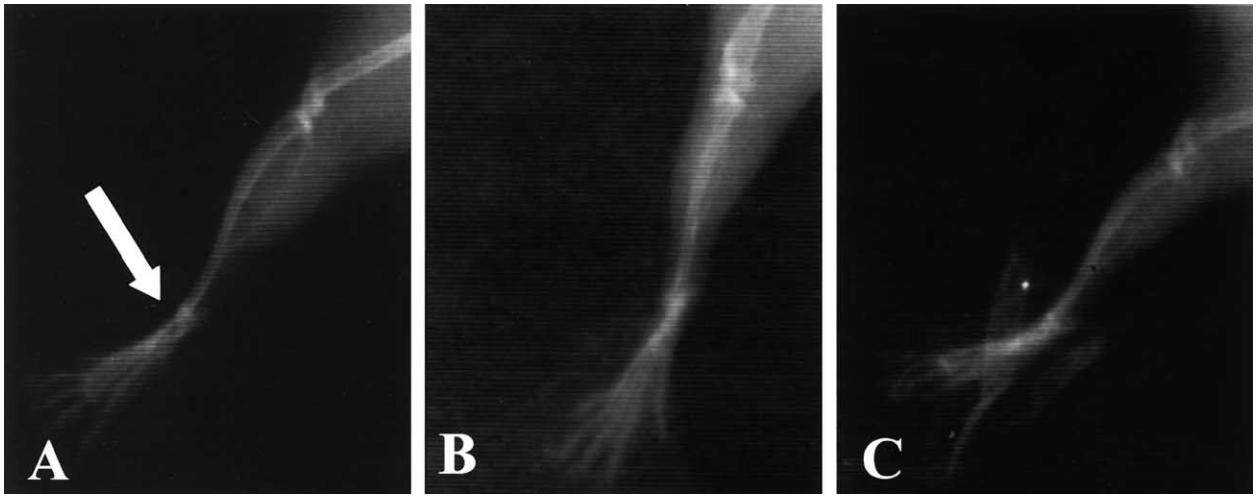

Fig. 6. Radiographic progression of collagen-induced arthritis in the tibiotarsal joint of mice with collagen-induced arthritis. No evidence of pathological alterations were observed in the tibiotarsal joints of normal mice (data not shown). (A) The hind paws from type II collagen-immunised wild-type mice demonstrated joint erosion (indicated by arrow). In GW274150-treated type II collagen-immunised wild-type mice (B) as well as in type II collagen-immunised iNOS-KO mice (C), the degree of bone resorption was markedly reduced. The figure is representative of at least three experiments performed on different experimental days.

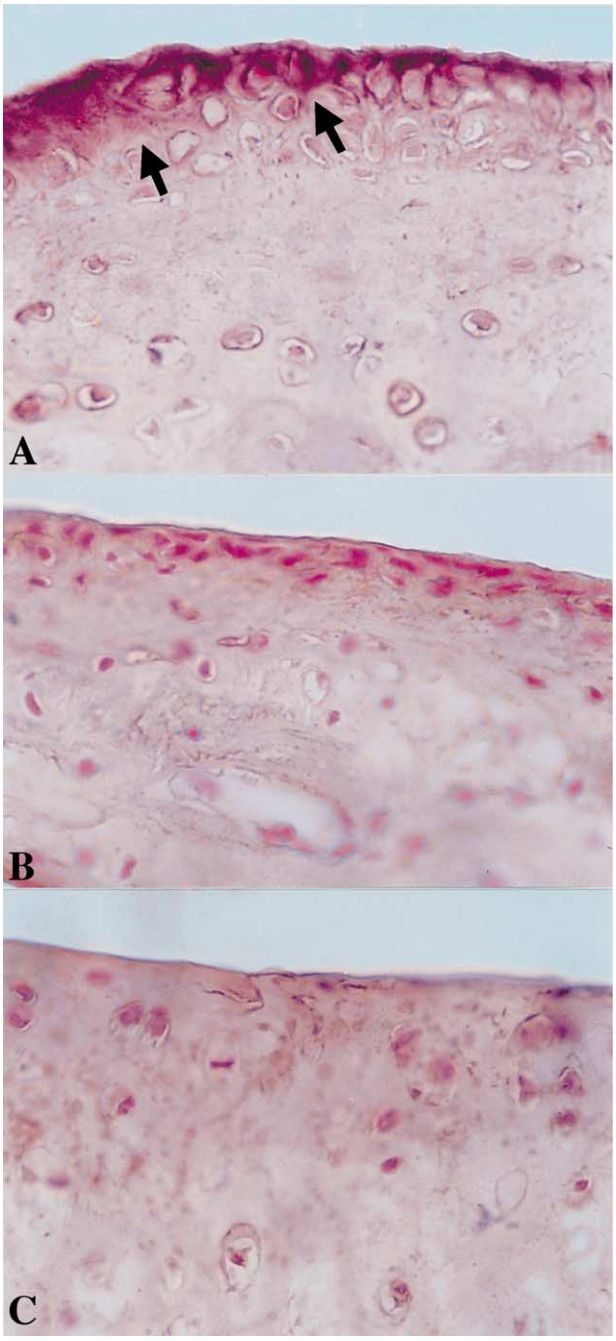

Fig. 7. Marked increase in nitrotyrosine staining mainly localized in the articular cartilage was evident in the paws of iNOS-WT mice subjected to collagen-induced arthritis (A). There was a marked reduction in nitrotyrosine immunostaining in the paws of GW274150-treated iNOS-WT mice (B) as well as in the paws of iNOS-KO mice (C). Original magnification: × 125. The figure is representative of at least three experiments performed on different experimental days.

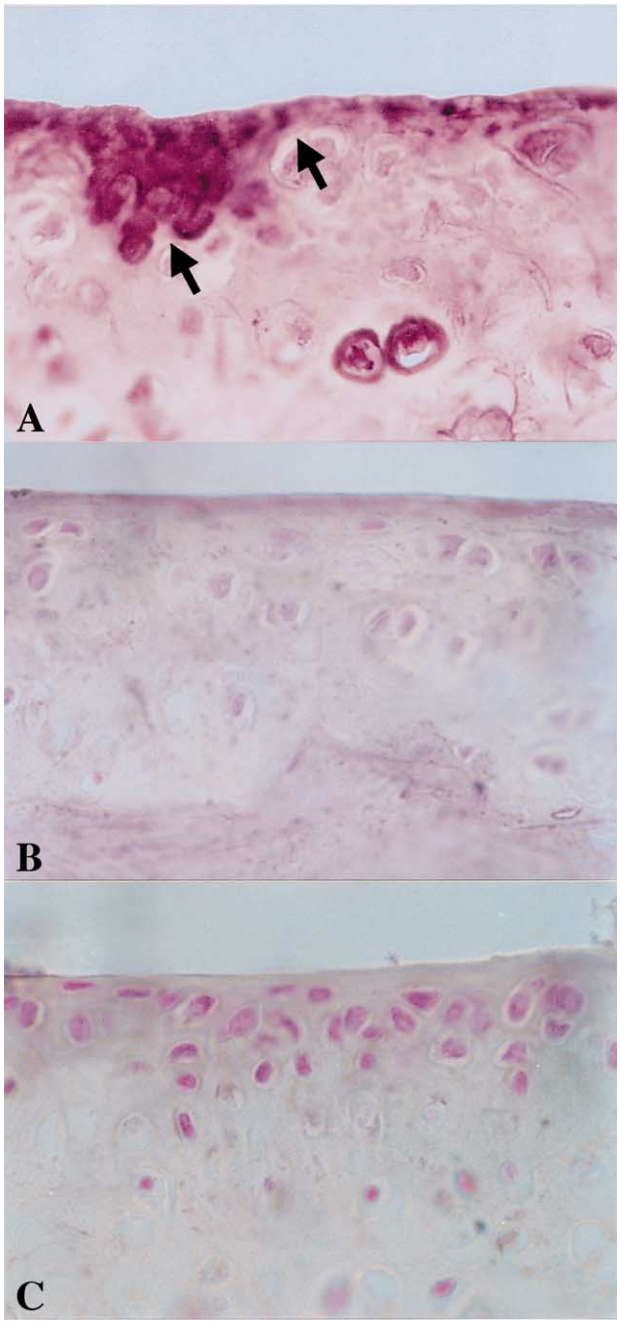

Fig. 8. Marked increase in poly(ADP-ribose) staining mainly localized in the articular cartilage was evident in the paws of iNOS-WT mice subjected to collagen-induced arthritis (A). Marked reduction in the poly(ADP-ribose) immunostaining was observed in the paws of GW274150-treated iNOS-WT mice (B) as well as in the paws of iNOS-KO mice (C). Original magnification: × 125. The figure is representative of at least three experiments performed on different experimental days.

3.7. Effect of inhibition or absence of iNOS on NO formation

Levels of nitrite/nitrate were significantly increased in plasma obtained from iNOS-WT mice subjected to collagen-induced arthritis ($P < 0.01$, Table 2). In contrast, nitrite/nitrate levels were significantly lower in the plasma of GW274150-treated iNOS-WT mice subjected to collagen-induced arthritis ($P < 0.01$, Fig. 7). No increases in plasma nitrite/nitrate levels in iNOS-KO mice were detected subsequent to type II collagen immunisation.

3.8. Effect of iNOS inhibition or absence on myeloperoxidase activity and malondialdehyde levels

The pattern of joint histopathology described above correlated with the influx of polymorphonuclear leukocytes into the joint, the joint space and surrounding tissue. Therefore, polymorphonuclear leukocyte infiltration was assessed in the inflamed joint tissue by measurement of the activity of myeloperoxidase, an enzyme specific to granulocyte lysosomes and, therefore, directly correlated to the number of polymorphonuclear leukocytes. In iNOS-WT mice, myeloperoxidase activity was significantly elevated 35 days after type II collagen immunisation ($P<0.01$, Table 2). In GW274150-treated type II collagen-immunised iNOS-WT mice as well as type II collagen-immunised iNOS-KO, myeloperoxidase activity was markedly reduced in comparison to those of iNOS-WT animals subjected to collagen-induced arthritis only ($P<0.01$, Table 2).

After 35 days, type II collagen immunisation caused a substantial increase in the plasma malondialdehyde levels in mice subjected to collagen-induced arthritis only ($P<0.01$, Table 2). In GW274150-treated type II collagen-immunised iNOS-WT mice as well as in type II collagen-immunised iNOS-KO mice, a significant reduction of the increase in malondialdehyde mediated by collagen-induced arthritis was observed ($P<0.01$, Table 2). On comparison with normal mice, no increases in plasma malondialdehyde levels were observed after administration of GW274150 or in plasma obtained from iNOS-KO mice (Table 2).

3.9. Nitrotyrosine formation and poly(ADP-ribose) polymerase activation in collagen-induced arthritis

Immunohistochemical analysis of joint sections obtained from iNOS-WT mice subjected to collagen-induced arthritis revealed positive staining for nitrotyrosine (indicating peroxynitrite and/or reactive oxygen species production) and for poly(ADP-ribose) (indicating poly(ADP-ribose) polymerase activation) mainly localized in the articular cartilage (Figs. 7A and 8A). In contrast, no positive staining for nitrotyrosine or poly(ADP-ribose) was observed in joints obtained from GW274150-treated mice subjected to collagen-induced arthritis (Figs. 7B and 8B) as well as in type II collagen-immunised iNOS-KO mice (Figs. 7C and 8C). Staining for either nitrotyrosine or poly(ADP-ribose) was absent in joints obtained from normal and iNOS-KO mice and those administered GW274150 only (data not shown).

4. Discussion

The role of iNOS in the pathophysiology of arthritis is still controversial. For instance, increased concentrations of nitrite in serum and synovial fluid samples obtained from patients with rheumatoid arthritis and osteoarthritis were first reported in 1992 (Farrell et al., 1992).

Mice, in which the gene for iNOS has been deleted (iNOS knockout mice or iNOS-KO mice), have recently been used to investigate the role of this enzyme in various models of inflammation including experimental arthritis. In iNOS-KO mice, the cartilage proteoglycan loss caused by intraarticular injection of zymosan was dramatically reduced (Van de Loo et al., 1997).

Therefore, beneficial effects of NOS inhibitors in arthritis may include a reduction of the clinical symptoms of arthritis (Ialenti et al., 1993), prevention of weight loss (McCartney-Francis et al., 1993), reduction of bone erosion (determined by X-ray, radiographic score) and of the synovial expression of TNF-α (tumor necrosis factor-α) (Brahn et al., 1998). In addition, selective inhibition of iNOS activity with *N*-iminoethyl-L-lysine reduces the degree of arthritis (cartilage lesions and chondrocyte apoptosis) caused by sectioning the anterior cruciate ligament in dogs (Pelletier et al., 2000). All of these studies support a pro-inflammatory role of iNOS. There are, however, other studies that do not document a pro-inflammatory role of iNOS or even suggest that NO from iNOS may be an "anti-inflammatory" molecule. For instance, iNOS-KO mice subjected to septic arthritis induced by *Staphylococcus aureus* produced threefold more TNF-α and exhibited more articular destruction than did their WT controls. This study suggests that high output NO production is not a perquisite for severe articular damage and implies that NO from iNOS plays an important role in synovial defence (McInnes et al., 1998). In a model of streptococcal cell wall-induced arthritis in rats, daily administration of N^6-(1-iminoethyl)-L-lysine failed to reduce the acute response and exacerbated the chronic inflammatory response, as reflected by profound tissue destruction and loss of bone and cartilage (McCartney-Francis et al., 2001). Most notably, the swelling of knee joints as well as the degree of leukocyte infiltration (into the knee joint) associated with antigen-induced arthritis is significantly enhanced in iNOS-KO mice compared with WT mice (Veihelmann et al., 2001). Thus, it appears that the role of iNOS in arthritis depends on the stimulus and/or the experimental model used.

To determine if iNOS expression and NO production participate in pro-inflammatory signalling during collagen-induced arthritis, two approaches were undertaken in this study: (i) iNOS-WT mice subjected to collagen-induced arthritis were administered the highly specific and potent iNOS inhibitor GW274150, and (ii) the response of iNOS-KO mice to collagen-induced arthritis were compared with that of iNOS-WT mice.

(INOS CAR) Recently, it has been demonstrated that pharmacological inhibitors of NOS, and also ablation of the gene for iNOS reduce the development of the inflammatory response (Cuzzocrea et al., 1998a, 2000a; Ialenti et al., 1993; McCartney-Francis et al., 2001; McCord, 1993; McInnes et al., 1998; Salvemini et al., 1996). Here we demonstrate that the lack and the inhibition of iNOS gene reduces (i) the development of collagen-induced arthritis,

(ii) the infiltration of the joint with polymorphonuclear leukocytess (myeloperoxidase, histology), (iii) the degree of plasma lipid peroxidation, and (iv) the degree of joint injury (histology, radiography). All of these findings support the view that NO plays an important role in the degree of inflammation and joint injury caused by collagen-induced arthritis in the mice.

In addition, we demonstrate that the absence of the iNOS gene attenuates the nitrosilation of proteins in the joint of mice subjected to collagen-induced arthritis. 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; Oyanagui, 1994). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypoclorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Parfitt et al., 1987). Increased nitrotyrosine staining is considered, therefore, as an indication of ''increased nitrative stress'' rather than a specific marker of the generation of peroxynitrite. From the present data, we cannot determine the mechanism of tyrosine nitration: inhibition of NOS by NOS inhibitor would inhibit both NO formation (and thus, reduce the generation of peroxynitrite) as well as it would suppress nitrite formation (and thereby attenuate the peroxidase-dependent mechanisms of tyrosine nitration). Nevertheless, we can certainly conclude from the current data that the absence of iNOS does not abolish tyrosine nitration in vivo.

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 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 (Eiserich et al., 1998), NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed the *PARS Suicide Hypothesis* (Eiserich et al., 1996; Szabò and Dawson, 1998). There is recent evidence that the activation of PARS may also play an important role in inflammation (Cuzzocrea et al., 1998b; Novelli, 1992; Pelletier et al., 2000; Salvemini et al., 1996, 1998; Szabò and Dawson, 1998). We demonstrate here that absence and the inhibition of the iNOS gene attenuates the increase in PARS activity caused by collagen-induced arthritis carrageenan in the joint. Thus, we propose that iNOS induction is also important—at least in part—for the activation of PARS.

In conclusion, our data demonstrate that, on comparison with wild-type mice subjected to collagen-induced arthritis, mice with a targeted deletion of the iNOS gene (iNOS-KO) and iNOS-WT mice which were treated with GW274150, a novel, potent and selective inhibitor of iNOS activity, were significantly protected against the pathological changes of the joint associated with collagen-induced arthritis. These results suggest that the presence of a functional iNOS gene (and, hence, an enhanced formation of NO) plays a pivotal role in the pathophysiology collagen-induced arthritis. Furthermore, our data provide evidence that the degree of oxidative stress and the degree of poly(ADP-ribose) polymerase activation during collagen-induced arthritis are attenuated iNOS-KO mice and in mice in which iNOS activity was inhibited by GW274150.

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