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Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase

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Abstract

Adjuvant-induced arthritis is a model of chronic inflammation that exhibits several pathological changes similar to those occurring in rheumatoid arthritis, an autoimmune disease in humans characterized by chronic inflammation of the joints. We have examined the role of inducible nitric oxide synthase in producing the pathological changes associated with adjuvant-induced arthritis. Plasma nitrite concentrations were maximally elevated 14 days following adjuvant administration compared to untreated control animals. Arthritic changes in the paw were first observed between days 10–12 and were maximally elevated 21 days following adjuvant administration. Inducible nitric oxide synthase immunoreactivity was found localized in the synovial tissue from adjuvant-treated rats, while untreated controls exhibited no inducible nitric oxide synthase staining. Two selective inducible nitric oxide synthase inhibitors, aminoguanidine and *N*-iminoethyl-L-lysine, suppressed the increase in plasma nitrite levels and joint inflammation associated with adjuvant-induced arthritis in a dose-dependent manner. *N*-Iminoethyl-L-lysine attenuated the inducible nitric oxide synthase immunoreactivity in adjuvant-treated rats. Blood pressure was not affected by the highest dose of *N*-iminoethyl-L-lysine administered in the drinking water, indicating a lack of inhibition of constitutive nitric oxide synthase.

Keywords: Inflammation; Nitric oxide (NO); Aminoguanidine; (Rat)

1. Introduction

Nitric oxide (NO) is an important physiological mediator synthesized by the enzyme nitric oxide synthase. Isoforms of nitric oxide synthase have been characterized, and these are distinguished by their Ca^{2+}/calmodulin dependence and by whether the enzyme is expressed constitutively (constitutive nitric oxide synthase) or is induced following exposure to cytokines or endotoxin (inducible nitric oxide synthase). Constitutive nitric oxide synthase, found in neurons and endothelial cells, transiently produces small amounts of NO, important in both intercellular and intracellular signalling (Bredt and Snyder, 1990; Moncada et al., 1991). Inducible nitric oxide synthase is induced in many cell types, including vascular smooth muscle cells, endothelial cells, hepatocytes, macrophages, neutrophils, chondrocytes, and synoviocytes (Charles et al., 1993; Palmer et al., 1993; Stadler et al., 1991; Stefanovic-Racic et al., 1992, 1993) in response to inflammatory and immunologic stimuli. Inducible nitric oxide synthase generates much larger quantities of NO over longer periods of time. The NO generated by macrophages activated by cytokines and endotoxin contributes to their cytotoxic and cytostatic properties against target cells (Moncada et al., 1991). Although NO generated by constitutive nitric oxide synthase appears to be beneficial in many physiological processes, the excess of NO generated by inducible nitric oxide synthase has been implicated in the pathogenesis of various inflammatory and immunologically mediated diseases, including graft-vs.-host disease (Langrehr et al., 1992), diabetes (Kolb et al., 1991; Corbett et al., 1991), viral infections (Zheng et al., 1993) and arthritis (Farrell et al., 1992; Jacob et al., 1992; Ialenti et al., 1993).

Rheumatoid arthritis is a systemic autoimmune disease in which the synovial membrane hypertrophies

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and becomes infiltrated with macrophages, neutrophils and lymphocytes. There is evidence for the release of cytokines as well as free radical generation within this inflamed synovium (Arend and Sayer, 1990; Dowling et al., 1990). It has been recently demonstrated that patients with rheumatoid arthritis have significantly elevated serum and synovial fluid nitrite concentrations (an oxidation product of NO) (Farrell et al., 1992; Jacob et al., 1992). Additionally, a non-selective inhibitor of nitric oxide synthase, N^G-methyl-L-arginine, has been found to reduce significantly the nitrite production, synovial inflammation and tissue damage associated with streptococcal cell wall-induced arthritis (McCartney-Francis et al., 1993) as well as the arthritis which develops spontaneously in the MRL-*lpr/lpr* mouse (Weinberg et al., 1994). Ialenti et al. (1993) have shown that arginine, the substrate for nitric oxide synthase, exacerbates, and that N^G-methyl-L-arginine, attenuates increases in paw volume, decreases in weight gain and increases in proliferation of T-lymphocytes which result from adjuvant-induced arthritis in the rat.

Adjuvant-induced arthritis is a model of chronic inflammation and associated joint pathology with many similarities to rheumatoid arthritis. The injection of mycobacteria in an oily base produces a systemic autoimmune response and subsequent chronic inflammation of the joints. We have examined the contribution of NO generated by inducible nitric oxide synthase in producing the pathological changes associated with this model. Here we present data demonstrating that (1) plasma nitrite/nitrate levels increased in the adjuvant-induced arthritis model and (2) two selective inhibitors of inducible nitric oxide synthase, aminoguandine and *N*-iminoethyl-L-lysine, suppressed the increase in plasma nitrite/nitrate levels as well as the inflammation and associated destruction within the joint.

2. Materials and methods

2.1. Materials

Male Lewis rats (150–200 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) and were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee and in accordance with NIH guidelines on laboratory animal welfare. *Mycobacteria tuberculosis H37 RA* was purchased from Difco Laboratories (Detroit, MI, USA). [^{3}H]Arginine was purchased from Dupont NEN (Boston, MA, USA). 2,3-Diaminonaphthalene was purchased from Aldrich (Milwaukee, WI, USA); Ultrafree-MC filter units from Millipore (Bedford, MA, USA); *Escherichia coli* lipopolysaccharide (0111:B4 serotype, Westphal Extract), nitrate reductase, N^G-monomethyl-L-arginine, aminoguandine, and all other chemicals and reagents were from Sigma (St. Louis, MO, USA). L-N^6-(1-Iminoethyl)lysine (*N*-iminoethyl-L-lysine) was synthesized in house.

2.2. Synthesis of N-iminoethyl-L-lysine

N-Iminoethyl-L-lysine was synthesized according to the method of Plapp and Kim (1974) with modifications. L-Lysine having the α-amino group protected (either via a Cu^{2+} complex or Boc protection) was allowed to react with ethylacetimidate-HCl in aqueous solution at pH 9 for 45 min. The solution was adjusted to pH 7.5 with 1 N HCl and allowed to stand at 25°C for 18 h. The Boc-protected product was isolated from a Dowex 50W (50–100 mesh) cation exchange resin column eluted with 10% aqueous pyridine; evaporation and deprotection with HCl yielded the *N*-iminoethyl-L-lysine-dihydrochloride salt. The Cu^{2+} complexed product was eluted from the Dowex column free of the copper with 1 N ammonium hydroxide; evaporation of the aqueous ammonium hydroxide solution yielded *N*-iminoethyl-L-lysine. The *N*-iminoethyl-L-lysine-hydrochloride salt was formed following adjustment of an aqueous solution of the *N*-iminoethyl-L-lysine to pH 6.2. *N*-Iminoethyl-D-lysine was prepared analogously from D-lysine using Boc as the α-amino protecting group.

2.3. Nitric oxide synthase activity

Nitric oxide synthase activity was measured by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as previously described (Misko et al., 1993). Inducible nitric oxide synthase was purified from lipopolysaccharide-induced RAW 264.7 cells and constitutive nitric oxide synthase from rat brain essentially as described by Bredt and Snyder (1990). The reaction mixture consisting of 50 mM Tris (pH 7.6), 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 2 mM $CaCl_2$, 10 μM FAD, 50 μM tetrahydrobiopterin, 30 μM L-arginine (containing L-[2,3-^{3}H]arginine at 300 cpm/pmol), 1 mM NADPH and purified constitutive nitric oxide synthase or inducible nitric oxide synthase in a final volume of 100 μl was incubated at 37°C for 15 min. An amount of nitric oxide synthase was used which yielded 30000 cpm for control activity measured in the absence of inhibitor. For constitutive nitric oxide synthase, 50 nM calmodulin was also added. The reaction was terminated by adding 300 μl of ice-cold buffer containing 10 mM EGTA, 100 mM Hepes, pH 5.5 and 1 mM citrulline. The [^{3}H]citrulline formed was then separated by chromatography on Dowex 50W X-8 cation exchange resin and the radioactivity determined in a liquid scintillation counter.

2.4. Nitrite / nitrate determination in plasma

Plasma nitrite/nitrate concentrations were determined using a fluorometric assay for the measurement of nitrite in biological samples as described by Misko et al. (1993). Plasma was filtered through a 10000 MW cut-off Ultrafree microcentrifuge filter unit (Millipore, Bedford, MA, USA) at 14000 rpm for 15 min. To convert nitrate to nitrite, 5–10 μl filtered plasma was incubated in 20 mM Tris, pH 7.6 containing 40 μM NADPH and 14 mU of nitrate reductase in a final volume of 50 μl. After incubation for 5 min at 20°C, the reaction was terminated by dilution with 50 μl of water, followed by addition of 10 μl of 0.05 mg/ml 2,3-diaminonaphthalene dissolved in 0.62 M HCl. After further incubation for 10 min at 20°C, the reaction was terminated with 10 μl 1.4 M NaOH. Fluorescence was measured at wavelengths of 365/450 nm excitation/emission using a fluorescence plate reader (IDEXX Laboratories, Westbrook, ME, USA).

2.5. Induction of adjuvant-induced arthritis

Arthritis was induced in pathogen-free, male Lewis rats weighing 150–200 g, by intradermal injection of 100 μl (total) of 20 mg/ml *Mycobacteria tuberculosis* suspended in squalene in three sites at the base of the tail. Each hind paw was scored visually on a scale of 0–4 for severity of arthritis to determine the severity index (0, no signs of inflammation; 1, moderate redness, first indication of swelling, joint flexible; 2, moderate redness, moderate swelling, joint still flexible; 3, redness, significant swelling and distortion of the paw, joint beginning to fuse; 4, redness, gross swelling and distortion of the paw, joint completely fused). Paw volume was determined using a water-displacement volumeter (Ugo-Basil, Varese, Italy). Nitric oxide synthase inhibitors were administered either by i.v. infusion or in the drinking water. For i.v. infusion of the inhibitors, aminoguanidine (3 M in buffered saline) or *N*-iminoethyl-L-lysine (1 M in buffered saline) was administered using an Alzet 2ML1 osmotic minipump (Alza Corp., Palo Alto, CA, USA) cannulated to the jugular vein beginning the same day as adjuvant administration. When added to the drinking water, aminoguandine was given at a dose of 1 mg/ml and *N*-iminoethyl-L-lysine was given at a dose range of 1–100 μg/ml beginning 3 days prior to adjuvant administration. At the end of the experiment, the left femoral artery was cannulated and systemic blood pressure was measured in conscious, restrained animals using a Grass Model 7E polygraph (Grass Medical Instruments, Quincy, MA, USA).

2.6. Histology

Hindlimbs from control and adjuvant-treated rats were removed, skinned and fixed in 10% neutral buffered formalin. Following decalcification in EDTA, tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

2.7. Immunohistochemistry

Immunohistochemical staining for inducible nitric oxide synthase was performed using 10 μm frozen sections of synovium dissected from the hindlimb joints of control and adjuvant-treated rats. Tissues were fixed with 1% paraformaldehyde, pH 7.2, 5 min, room temperature, followed by 100% ethanol, 5 min, 4°C, and non-specific binding was blocked with 3% normal goat

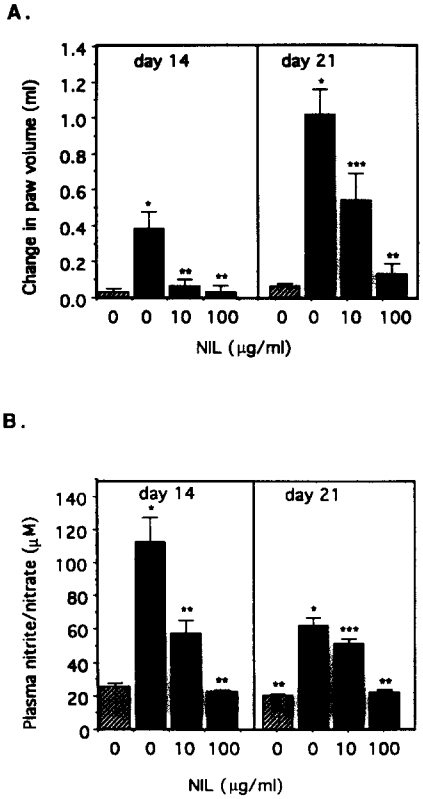

Fig. 1. Effect of *N*-iminoethyl-L-lysine on changes in hind paw volume and plasma nitrite/nitrate concentrations on days 14 and 21 following adjuvant administration. *N*-Iminoethyl-L-lysine was administered at doses of 10 and 100 μg/ml in the drinking water throughout the course of the experiment beginning 3 days prior to adjuvant administration. Adjuvant consisted of *M. tuberculosis* suspended in squalane injected intradermally into the base of the tail on day 0. (A) Change in paw volume from day 0 following adjuvant administration. Data are from a representative experiment in which meaurements of both hind paws were taken and expressed as a mean ± S.E.M. of 12–16 paws. (B) Concentration of nitrite/nitrate in plasma. Blood was collected into heparinized tubes and plasma was assayed for nitrite/nitrate content using the DAN fluorometric assay. Data are from a representative experiment of 4 experiments in which the individual means ± S.E.M. were determined from 6–8 rats (samples assayed in duplicate). * $P < 0.0005$ compared to normal control, ** $P < 0.0005$ compared to adjuvant control, *** $P < 0.05$ compared to adjuvant control.

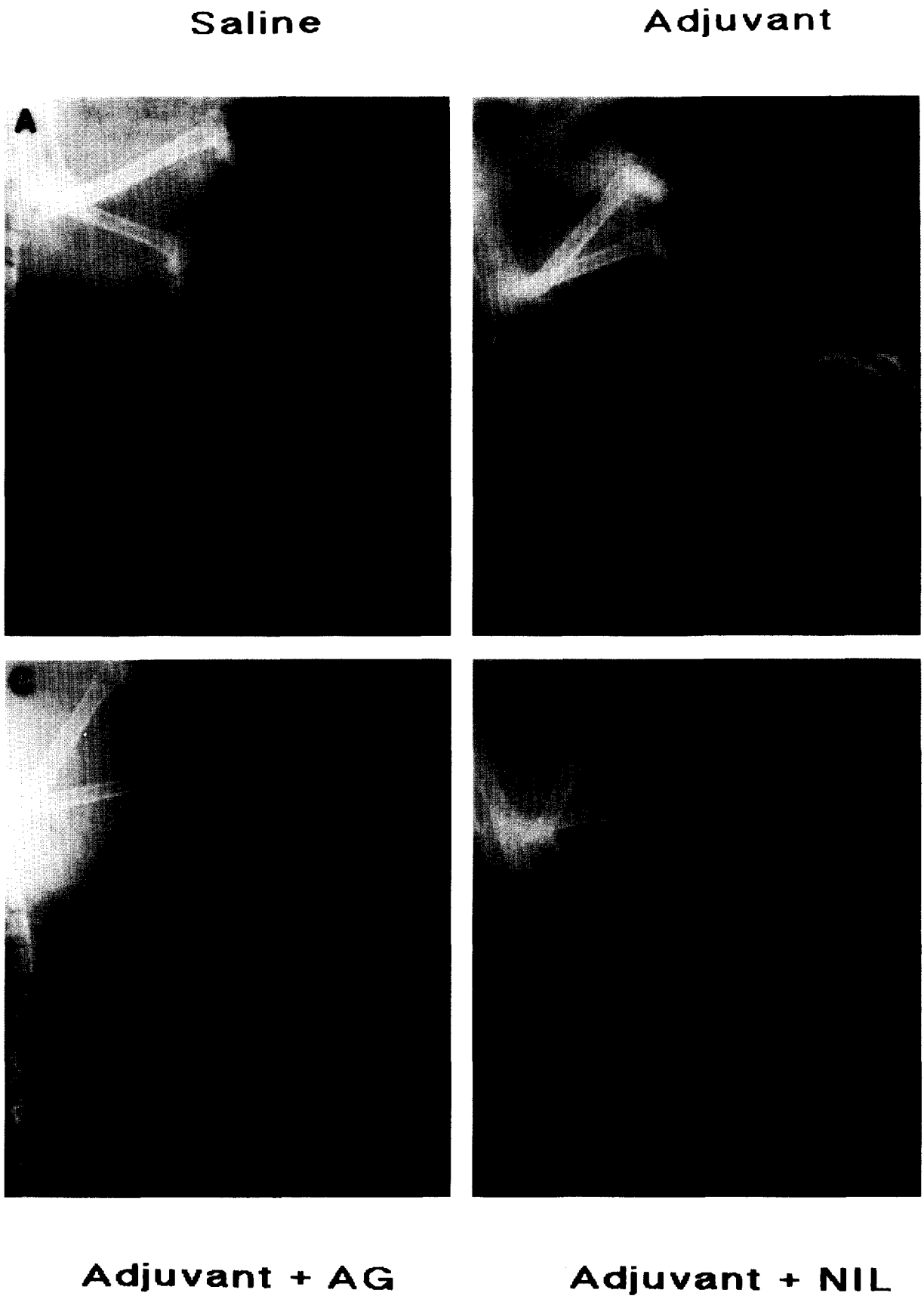
Saline
Adjuvant
A
Adjuvant + AG
Adjuvant + NIL

Table 1
Comparison of IC_{50} values for inhibition of mouse inducible and rat brain constitutive nitric oxide synthase

	IC_{50} (μM)		
	Constitutive	Inducible	Selectivity
Aminoguanidine	555	21	26
N-Iminoethyl-L-lysine	100	3	33
N-Iminoethyl-D-lysine	N.D.	N.D.	

IC_{50} values were determined with mouse inducible nitric oxide synthase and rat brain constitutive nitric oxide synthase for aminoguanidine, *N*-iminoethyl-L-lysine and *N*-iminoethyl-D-lysine. Nitric oxide synthase activity was measured by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as described in Materials and methods. Selectivity is defined as the ratio of constitutive nitric oxide synthase to inducible nitric oxide synthase. N.D. = no detectable inhibition at 1 mM.

serum in 0.5 M Tris-HCl, pH 7.4 for 1 h at room temperature. All subsequent incubations were carried out in this buffer. Tissue sections were incubated with a 1/1000 dilution of either preimmune rabbit serum (normal rabbit serum) or an anti-inducible nitric oxide synthase antiserum generated in rabbits to a unique peptide sequence obtained from the carboxy terminal region of mouse inducible nitric oxide synthase (AVFSYGAKKGSALEEPKATRL), 16 h, 4°C. Endogenous peroxidase activity was reduced with periodic acid (Zymed Laboratories, San Francisco, CA, USA) for 45 s at room temperature, followed by sequential incubations with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA) for 2 h each. The reaction product was visualized using 3,3'-diaminobenzidine intensified with nickel chloride for 6 min. Sections were counterstained with Mayer's hematoxylin and mounted.

2.8. Statistical analysis

Data were evaluated using the Welch modified two sample *t*-test for samples of unequal variance.

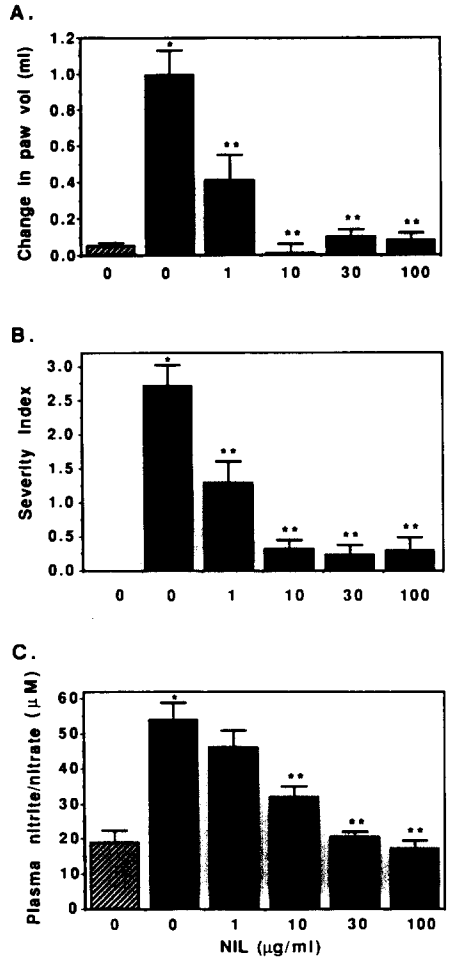

Fig. 3. Effect of increasing doses of *N*-iminoethyl-L-lysine on changes in hind paw volume, severity index and plasma nitrite/nitrate concentrations. *N*-Iminoethyl-L-lysine was administered at 1, 10, 30, and 100 μg/ml in the drinking water throughout the course of the experiment beginning 3 days prior to adjuvant administration. Arthritis was induced as described in the legend to Fig. 1. (A) Change in paw volume from day 0. Data are from a representative experiment in which meaurements were taken of both hind paws and expressed as a mean ± S.E.M. of 12–16 paws. (B) Severity index of inflammation. Each hind paw was assessed for visual signs of inflammation (redness, swelling) and joint movement and scored on a scale of 0–4. Data are from a representative experiment in which measurements were taken of both hind paws and expressed as a mean ± S.E.M. of 12–16 paws. (C) Concentration of nitrite/nitrate in plasma. Data are from a representative experiment of 4 experiments in which the individual means ± S.E.M. were determined from 6–8 rats (samples assayed in duplicate). * $P < 0.0005$ compared to normal control, ** $P < 0.0005$ compared to adjuvant control.

Fig. 2. Radiograph of hind limbs of rats 24 days following adjuvant administration. (A) Hind limbs of untreated control rats. (B) Hind limbs of adjuvant-induced rats exhibiting the deterioration of the joint and marked soft tissue swelling. (C) Hind limbs of adjuvant-inducded rats treated with aminoguanidine demonstrating partial protection of the joint and slight tissue swelling. (D) Hind limbs of adjuvant-induced rats treated with *N*-iminoethyl-L-lysine demonstrating the protective effect of *N*-iminoethyl-L-lysine on the joints and the absence of soft tissue swelling. Aminoguanidine or *N*-iminoethyl-L-lysine was administered as an i.v. infusion beginning the same day as adjuvant administration. Radiograph taken directly on Kodak X-OMAT AR Readypak film.

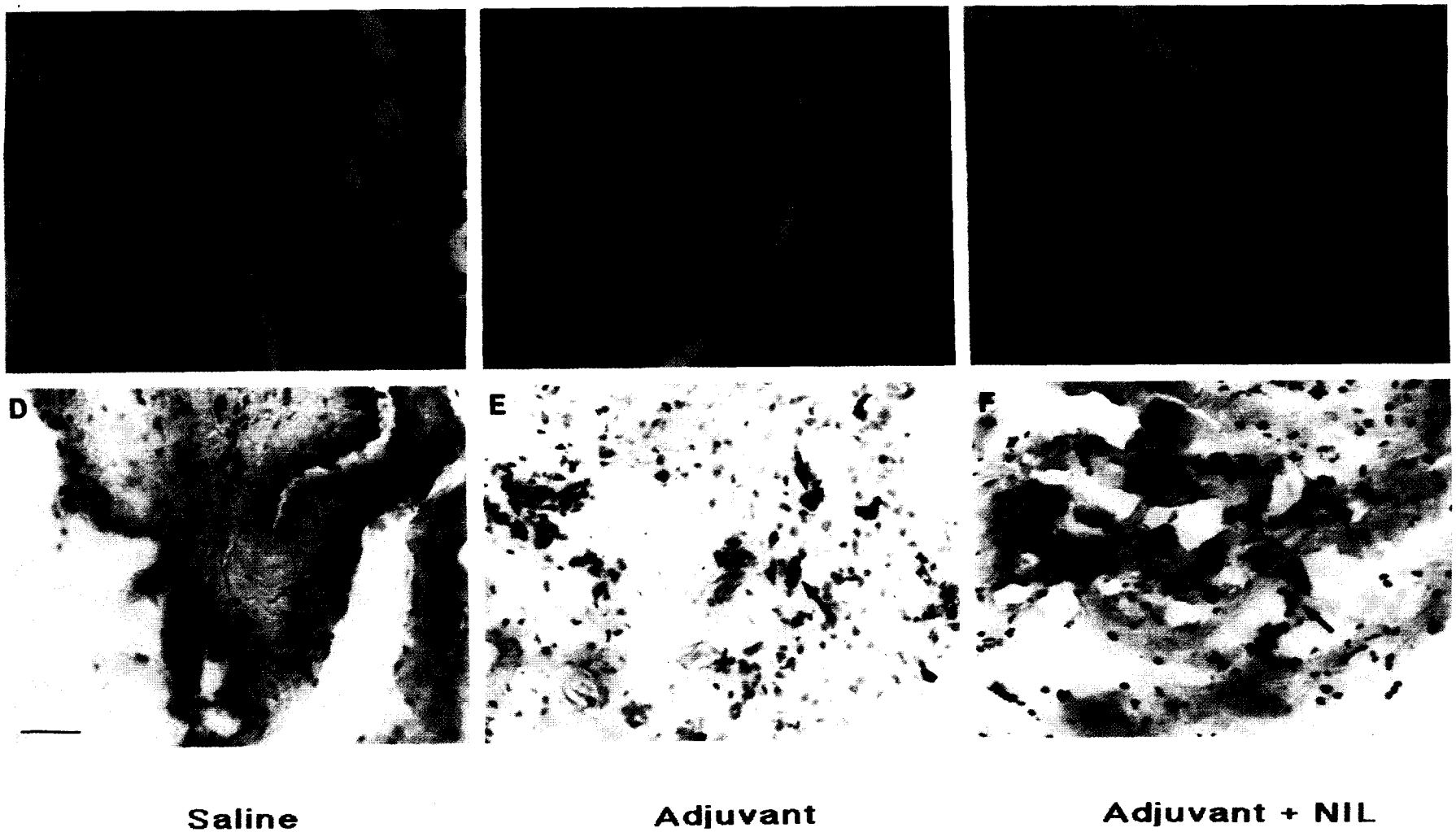

Fig. 4. Inhibition of histopathological changes by *N*-iminoethyl-L-lysine. (A–C) 10 μm paraffin sections stained with hematoxylin and eosin, bar = 20 μm; (D–E) Immunohistochemical staining of inducible nitric oxide synthase using avidin-biotin-peroxidase detection in 10 μm frozen sections of synovial tissue, counterstained with Mayer's hematoxylin, bar = 5 μm. (A) Talus-tarsal joint from saline control animal exhibiting normal histology. (B) Talus-tarsal joint from adjuvant control rat exhibiting marked synovitis, synovial hyperplasia, a narrowing of the synovial space and severe destruction of the cartilage and subcondral bone. (C) Talus-tarsal joint from adjuvant rat treated with 100 μg/ml *N*-iminoethyl-L-lysine exhibiting minimal inflammatory changes. (D) Synovial tissue from saline control showing no detectable inducible nitric oxide synthase staining. (E) Hypertrophied synovial tissue from adjuvant control exhibiting inducible nitric oxide synthase immunoreactivity (visualized as a dark brown reaction product) in discrete macrophage-like cells. (F) Synovial tissue from adjuvant/*N*-iminoethyl-L-lysine treated rats exhibiting only occasional and weak inducible nitric oxide synthase immunoreactive cells.

3. Results

3.1. Effect of aminoguanidine and N-iminoethyl-L-lysine on purified rodent constitutive nitric oxide synthase vs. inducible nitric oxide synthase

Prior to testing nitric oxide synthase inhibitors in vivo, the selectivity of inhibition by aminoguanidine and *N*-iminoethyl-L-lysine of constitutive nitric oxide synthase vs. inducible nitric oxide synthase was determined in vitro. Purified rat brain constitutive and mouse macrophage inducible enzymatic activity were measured using the conversion of [^{3}H]arginine to [^{3}H]citrulline. As shown in Table 1, aminoguanidine was 26-fold more selective for inducible nitric oxide synthase than for constitutive nitric oxide synthase, with IC_{50} values of 21 and 555 μM, respectively. *N*-Iminoethyl-L-lysine was approximately 10-fold more potent against inducible nitric oxide synthase than aminoguanidine and 6-fold more potent against constitutive nitric oxide synthase ($IC_{50} = 3$ and 100 μM, respectively) resulting in a 33-fold selectivity for the inducible form of the enzyme. *N*-Iminoethyl-D-lysine, the enantiomeric isomer of *N*-iminoethyl-L-lysine, had no inhibitory activity on inducible nitric oxide synthase or constitutive nitric oxide synthase up to 1 mM.

3.2. Production of NO in adjuvant-induced arthritis

Initially, we determined whether NO levels were elevated following the induction of adjuvant-induced arthritis. Susceptible rats were injected with adjuvant and the concentration of nitrite and nitrate in plasma (as a measure of NO production) and paw volume were monitored (Fig. 1). Plasma nitrite/nitrate levels were significantly higher at day 14 than at day 21 (a 6-fold increase over normal controls at day 14 vs. a 3-fold increase at day 21), while paw volume continued to increase up to day 21 (2-fold higher at day 21 vs. day 14), suggesting the possible involvement of NO in producing tissue damage within the joint (Fig. 1A and B).

3.3. Suppression of adjuvant-induced arthritis by selective inhibitors of inducible nitric oxide synthase

Having established the selectivity of aminoguanidine and *N*-iminoethyl-L-lysine in vitro as well as the enhanced production of nitrite/nitrate in adjuvant-induced arthritis, we tested the effects of these selective inhibitors on the development of adjuvant-induced arthritis. In initial experiments aminoguanidine administered as an i.v. infusion rate of 3.7 mg/h (approximately equivalent to 450 mg/kg/day) using an Alzet pump cannulated to the jugular vein inhibited the increase in plasma nitrite/nitrate levels by 52%. Adjuvant-induced increases in paw volume were similarly inhibited (46%). In the same experiment, i.v. administration of 2.2 mg/h (approximately 270 mg/kg/day) of *N*-iminoethyl-L-lysine completely suppressed the increase in plasma nitrite/nitrate levels and the inflammatory changes in the paw (data not shown). Radiographic analysis of the joints confirmed the ability of *N*-iminoethyl-L-lysine to protect the integrity of the tarsal, talus and calcaneus bones as well as the soft tissue surrounding the joint, while adjuvant controls exhibited severe deterioration of these bones and dramatic soft tissue swelling. Aminoguanidine provided partial protection from the adjuvant-induced changes in the joint (Fig. 2).

To further characterize and compare these two inhibitors, the oral efficacy of *N*-iminoethyl-L-lysine or aminoguanidine was evaluated by inhibition of the increase in plasma nitrite/nitrate levels resulting from inducible nitric oxide synthase induction by a low dose of endotoxin (10 mg/kg lipopolysaccharide i.p.). Aminoguanidine administered at a dose of 1 mg/ml in the drinking water for 7 days prior to lipopolysaccharide had no effect on the increases in plasma nitrite/nitrate levels (data not shown). In contrast, *N*-iminoethyl-L-lysine administered orally (either at a dose of 100 μg/ml in the drinking water for 3 days prior to lipopolysaccharide or at a dose of 100 mg/kg by gavage immediately prior to lipopolysaccharide) significantly inhibited the increase in plasma nitrite/nitrate levels measured 6 h after lipopolysaccharide administration. Plasma nitrite/nitrate concentrations were elevated 30-fold following lipopolysaccharide treatment (575 μM vs. 15–20 μM for saline control) and *N*-iminoethyl-L-lysine administration reduced this response by $> 90\%$, indicating either increased oral bioavailability and/or potency compared to aminoguanidine.

Having established oral bioavailability and efficacy for *N*-iminoethyl-L-lysine, this dosing regimen was used in the adjuvant-induced arthritis model. The data from an initial experiment using *N*-iminoethyl-L-lysine are shown in Fig. 1. *N*-Iminoethyl-L-lysine, at a dose of 10 or 100 μg/ml in the drinking water beginning 3 days prior to adjuvant administration, partially or completely inhibited, respectively, the increase in plasma nitrite/nitrate concentrations and the inflammation as measured by changes in paw volume or visual assessment up to 21 days post-adjuvant. The hind paws and joints appeared completely normal with *N*-iminoethyl-L-lysine treatment.

As shown in Fig. 3, *N*-iminoethyl-L-lysine administered in the drinking water suppressed the increases in plasma nitrite/nitrate levels, paw volume and index of severity in a dose-dependent manner assessed 16 days post-adjuvant treatment. A corresponding dose-response relationship was also observed in the incidence of inflammation of the hind paws. At the 30 and 100

Table 2
Mean arterial blood pressure in adjuvant-arthritic rats following chronic treatment with *N*-iminoethyl-L-lysine

Treatment	Adjuvant	*N*-Iminoethyl-L-lysine	Mean blood pressure (mm Hg)
Saline control	–	–	115+2
Adjuvant control	+	–	105+2
Adjuvant/*N*-iminoethyl-L-lysine	+	+	113+3

The adjuvant/*N*-iminoethyl-L-lysine group received 100 μg/ml *N*-iminoethyl-L-lysine in their drinking water throughout the 21 days of the experiment beginning 3 days prior to adjuvant administration. At the end of the experiment, the femoral artery was cannulated and mean arterial blood pressure was measured. Each value represents the mean ± S.E.M. of 4 rats/group.

μg/ml dose, only 12.5% of the animals in each group exhibited any signs of inflammation, compared to 37.5% and 62.5% for animals receiving 10 and 1 μg/ml of *N*-iminoethyl-L-lysine, respectively. The incidence of hind paw inflammation was 100% for the adjuvant control group. At the end of these experiments, rats receiving the 100 μg/ml dose of *N*-iminoethyl-L-lysine were monitored for changes in blood pressure. As shown in Table 2, no changes in systemic blood pressure compared to saline-treated adjuvant controls were observed at the highest dose of *N*-iminoethyl-L-lysine, indicating a lack of inhibition of constitutive nitric oxide synthase. The slight decrease in blood pressue in adjuvant control rats may reflect the severity of the systemic disease that occurs in these animals. To further characterize the effects of *N*-iminoethyl-L-lysine in this model, white blood cell counts were also performed at the end of the experiments. As expected, the white blood cell count was elevated in the adjuvant control group compared to untreated controls (19010 ± 462 and 8366 ± 1402, respectively), indicative of a generalized inflammatory response. *N*-Iminoethyl-L-lysine had little effect on this response (18936 ± 158, 17169 ± 1872, 16988 ± 1084 and 15966 ± 519 for 1, 10, 30 and 100 μg/ml in the drinking water, respectively). Body weights were also monitored as an indication of the systemic condition. *N*-Iminoethyl-L-lysine treatment had no effect on the sub-normal weight gain (or even weight loss) characteristic of animals with adjuvant-induced arthritis (data not shown).

Histological analysis of the joints of the adjuvant control rats exhibited marked infiltration of inflammatory cells into the synovial and subsynovial tissue. Synovial hyperplasia, a narrowing of the synovial space and severe deterioration of the cartilage and subcondral bone were also evident (Fig. 4B). In contrast, the joints of the *N*-iminoethyl-L-lysine-treated animals appeared normal, exhibiting minimal inflammatory changes (Fig. 4A and C).

Due to the short half-life of NO, local production of NO within the joint would be necessary for NO to play a role in producing the pathological changes associated with adjuvant-induced arthritis. It was therefore determined whether cells containing inducible nitric oxide synthase immunoreactivity were present within synovial tissue obtained from the tarsal joint tissue using a rabbit polyclonal antiserum generated to a unique peptide sequence of mouse inducible nitric oxide synthase. The synovial tissue obtained from untreated control animals exhibited no inducible nitric oxide synthase staining. In contrast, inducible nitric oxide synthase immunoreactivity was localized to discrete cells within the hypertrophied synovial tissue obtained from adjuvant control rats (Fig. 4E). Based upon staining in adjacent sections using a rat anti-CD-4 antibody (W3/25, Accurate Corporation, Westbury, NY, USA; data not shown) as well as morphology, the cells exhibiting specific inducible nitric oxide synthase immunoreactivity appeared to be primarily macrophages. Due to the limited histopathological changes that occurred, the inducible nitric oxide synthase immunoreactivity was markedly reduced in adjuvant-treated rats which received the maximal doses of *N*-iminoethyl-L-lysine and only an occasional cell exhibited weak staining (Fig. 4D, E and F).

4. Discussion

These studies demonstrate that two selective inhibitors of inducible nitric oxide synthase, aminoguanidine and *N*-iminoethyl-L-lysine, decreased inflammatory changes in the joint following the induction of adjuvant-induced arthritis supporting the hypothesis that inducible nitric oxide synthase plays a critical role in the pathophysiology of this model. Aminoguanidine attenuated but did not completely inhibit the increases in plasma nitrite/nitrate levels and inflammation of the joint associated with adjuvant-induced arthritis. However, *N*-iminoethyl-L-lysine, which is 10-fold more potent than aminoguanidine against inducible nitric oxide synthase and, unlike aminoguanidine, demonstrated oral efficacy in suppressing lipopolysaccharide-induced increases in plasma nitrite/nitrate concentrations, suppressed the associated changes in paw volume, severity index, incidence and joint erosion in a dose-dependent manner that correlated with the reduction of plasma nitrite/nitrate levels. Inhibition of nitric oxide synthesis was observed at doses which did not appear to inhibit constitutive nitric oxide synthase, as determined by a lack of effect on systemic blood pressure. These results in conjunction with the immunohistochemical localization of inducible nitric ox-

ide synthase within the inflamed synovium suggest that NO is involved in the joint inflammation associated with adjuvant-induced arthritis. *N*-Iminoethyl-L-lysine had no effect on either the elevated white blood cell counts or the reduction in body weight caused by adjuvant treatment, suggesting that the systemic inflammatory response, which appears to be induced by mediators other than NO, continued.

Examination of the timing of the changes in plasma nitrite/nitrate levels compared to swelling and inflammation in the paw suggests that the role for NO is early in the pathologic process of joint destruction. Maximal plasma nitrite/nitrate levels occurred one week before maximal paw swelling and inflammation were observed. Cannon et al. (1992) have reported similar results demonstrating that nitrite levels in urine are maximally elevated 14 days following adjuvant adminstration and then decline, while paw swelling was maximal 20–22 days following adjuvant administration. The increase in plasma nitrite/nitrate concentrations most likely reflects the systemic inflammatory condition as well as the inflammatory process occurring locally within the paw. The increases in plasma nitrite/nitrate concentrations found in rats with adjuvant-induced arthritis are consistent with previously reported data for patients with rheumatoid arthritis (Farrell et al., 1992). These patients were found to have elevated serum as well as synovial fluid levels of nitrite compared to normal controls. Assuming the increase in plasma nitrite/nitrate concentrations parallels increased NO production in the joint, it appears that excess NO must be generated over a period of time before gross pathological changes are observed in the paw. It has not yet been determined whether this is an indirect effect with NO acting as a signalling mechanism or a direct effect in which NO causes tissue damage. The latter possibility may explain the observation that at day 16 following adjuvant administration, the 10 μg/ml dose revealed no changes in paw volume even though the adjuvant-induced increase in plasma nitrite/nitrate concentrations were not completely inhibited. While there are several mechanisms which might be responsible for this observation, one explanation is that the damage resulting from the increased production of NO is a cumulative phenomenon with partial inhibition of NO production leading to a delay, but not a complete inhibition of pathological changes. There is evidence for cumulative effects of free radical damage in other disease states (Cross et al., 1987). The data in Fig. 1 suggest this is the case in the animals receiving the 10 μg/ml dose of *N*-iminoethyl-L-lysine.

The mechanism by which NO causes damage to the synovial joint is not known. It has been shown that NO can combine with superoxide forming peroxynitrite, which in turn yields the highly reactive hydroxyl radical, a species which has been shown to produce tissue damage (Beckman et al., 1990). In another rodent model of arthritis induced by injection of streptococcal cell wall fragments, superoxide dismutase has been shown to be protective (Skaleric et al., 1991). There is also preliminary evidence that NO causes the degradation of hyaluranon, a glycosaminoglycan found in cartilage and synovial fluid (Stefanovic-Racic et al., 1993). Hyaluranon has been shown to be sensitive to free radical damage and its degradation leads to a decrease in the viscosity of synovial fluid (Cross et al., 1987). NO has also been found to increase the activity of cyclooxygenase (Salvemini et al., 1993), which generates proinflammatory prostaglandins, mediators involved in the pathophysiology of rheumatoid arthritis and adjuvant-induced arthritis (Arend and Dayer, 1990). Although the precise mechanisms remain to be elucidated, it is clear that NO produced by inducible nitric oxide synthase plays a critical role in the pathology of adjuvant-induced arthritis and that the inhibition of inducible nitric oxide synthase prevented the joint destruction found in this model of rheumatoid arthritis.

The current treatments for rheumatoid arthritis are largely unsatisfactory. Non-steroidal antiinflammatory agents (NSAIDs) can cause gastric ulcers and, although they alleviate the pain associated with inflammation, do not affect the progression of the disease. Glucocorticoids are more effective than NSAIDs in the treatment of rheumatoid arthritis, but have numerous systemic side effects which often preclude their long-term use at therapeutic doses. Other therapies, including gold and methotrexate, exhibit inherent toxicities when administered at therapeutic doses (Luqmani et al., 1994; Stefanovic-racic et al., 1993). A selective inhibitor of inducible nitric oxide synthase may provide a novel therapeutic approach devoid of some of these side effects and may also prevent the progression of rheumatoid arthritis by limiting tissue and bone destruction within the joint. Additionally, considering the recent evidence on the regulation of cyclooxygenase activity by NO, a selective inhibitor of inducible nitric oxide synthase may also act as an anti-inflammatory agent by decreasing the production of proinflammatory prostaglandins. Thus, the evidence presented in the current study strongly suggests that nitric oxide produced by inducible nitric oxide synthase is injurious to the joint and that selective inhibition of this enzyme may represent an exciting breakthrough in the therapy of rheumatoid arthritis.

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