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Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation

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1 The role of nitric oxide (NO) derived from constitutive and inducible nitric oxide synthase (cNOS and iNOS) and its relationship to oxygen-derived free radicals and prostaglandins (PG) was investigated in a carrageenan-induced model of acute hindpaw inflammation.

2 The intraplantar injection of carrageenan elicited an inflammatory response that was characterized by a time-dependent increase in paw oedema, neutrophil infiltration, and increased levels of nitrite/nitrate (NO_2^-/NO_3^-) and prostaglandin E_2(PGE_2) in the paw exudate.

3 Paw oedema was maximal by 6 h and remained elevated for 10 h following carrageenan administration. The non-selective cNOS/iNOS inhibitors, N^G- monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) given intravenously (30–300 mg kg^{-1}) 1 h before or after carrageenan administration, inhibited paw oedema at all time points.

4 The selective iNOS inhibitors, N-iminoethyl-L-lysine (L-NIL) or aminoguanidine (AG), failed to inhibit carrageenan-induced paw oedema during the first 4 h following carrageenan administration, but inhibited paw oedema at subsequent time points (from 5–10 h). iNOS mRNA was detected between 3 to 10 h following carrageenan administration using ribonuclease protection assays. iNOS protein was first detected 6 h and was maximal 10 h following carrageenan administration as shown by Western blot analysis. Administration of the iNOS inhibitors 5 h after carrageenan (a time point where iNOS was expressed) inhibited paw oedema at all subsequent time points. Infiltrating neutrophils were not the source of iNOS since pretreatment with colchicine (2 mg kg^{-1}) suppressed neutrophil infiltration, but did not inhibit the iNOS mRNA expression or the elevated NO_2^-/NO_3^- levels in the paw exudate.

5 Inhibition of paw oedema by the NOS inhibitors was associated with attenuation of both the NO_2^-/NO_3^- and PGE_2 levels in the paw exudate. These inhibitors also reduced the neutrophil infiltration at the site of inflammation.

6 Recombinant human Cu/Zn superoxide dismutase coupled to polyethyleneglycol (PEGrhSOD;12×10^3 u kg^{-1}), administered intravenously either 30 min prior to or 1 h after carrageenan injection, inhibited paw oedema and neutrophil infiltration, but had no effect on NO_2^-/NO_3^- or PGE_2 production in the paw exudate. The administration of catalase (40×10^3 u kg^{-1}), given intraperitoneally 30 min before carrageenan administration, had no effect on paw oedema. Treatment with desferrioxamine (300 mg kg^{-1}), given subcutaneously 1 h before carrageenan, inhibited paw oedema during the first 2 h after carrageenan administration, but not at later times.

7 These results suggest that the NO produced by cNOS is involved in the development of inflammation at early time points following carrageenan administration and that NO produced by iNOS is involved in the maintenance of the inflammatory response at later time points. The potential interactions of NO with superoxide anion and PG is discussed.

Keywords: Acute inflammation; nitric oxide synthase inhibitors; superoxide anion; peroxynitrite; carrageenan

Introduction

Carrageenan-induced paw oedema is a useful model to assess the contribution of mediators involved in vascular changes associated with acute inflammation. The development of oedema in the rat hindpaw following the injection of carrageenan has been described as a biphasic event in which various mediators operate in sequence to produce this inflammatory response (Vinegar *et al.*, 1969). The initial phase of oedema (0–1 h), which is not inhibited by non-steroidal anti-inflammatory drugs such as indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin (Di Rosa *et al.*, 1971). In contrast, the second accelerating phase of swelling (1–6 h), has been correlated with the elevated production of prostaglandins (PG, Di Rosa & Willoughby, 1971; Di Rosa *et al.*, 1971), and more recently has been attributed to the induction of inducible cyclo-oxygenase (COX-2) in the hindpaw (Seibert *et al.*, 1994). Local neutrophil infiltration and activation also contribute to this inflammatory response (Di Rosa & Sorrentino, 1968; Vinegar *et al.*, 1971; Boughton-Smith *et al.*, 1993) by producing, among other mediators, oxygen-derived free radicals such as superoxide anion (O_2^-) and hydroxyl radicals (see Fantone & Ward, 1982 for review).

Another important mediator in acute and chronic inflammation is nitric oxide (NO). NO is generated via the oxidation of the terminal guanidino nitrogen atom of L-arginine by the enzyme, nitric oxide synthase (NOS). Three major isoforms of NOS have been identified. Two expressed constitutively, are calcium/calmodulin-dependent and are classified together as constitutive NOS isoforms (cNOS). The third is a cytokine-inducible, calcium/calmodulin-independent isoform of NOS (iNOS) (see Moncada *et al.*, 1991; Moncada & Higgs, 1993 for reviews). NO is a potent vasodilator; its in-

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volvement during an inflammatory response may be related to its ability to increase vascular permeability and oedema through changes in local blood flow (see Moncada *et al.*, 1991; Moncada & Higgs, 1993 for reviews). Furthermore, NO has been shown to increase the production of pro-inflammatory prostaglandins in *in vitro* (Rettori *et al.*, 1992; Salvemini *et al.*, 1993; Inoue *et al.*, 1993; Corbett *et al.*, 1993; Davidge *et al.*, 1995), *ex vivo* (Salvemini *et al.*, 1994; Sautebin & Di Rosa, 1994) and *in vivo* studies (Salvemini *et al.*, 1995a,b; Sautebin *et al.*, 1995), potentially by S-nitrosation of cysteine residues in the catalytic domain of cyclo-oxygenase (COX) enzymes (Hajjar *et al.*, 1995).

In addition, NO can also react with superoxide anion to form peroxynitrite ($ONOO^-$), a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage (Beckman *et al.*, 1990; Radi *et al.*, 1991; Rubbo *et al.*, 1994). These findings suggest that NO has the ability to exert multiple cytotoxic effects during inflammatory responses including an increase PG production as well as the formation of $ONOO^-$.

The interactions between NO, free radicals and the COX pathway in acute inflammation are poorly defined. NO appears to be involved in the acute inflammatory response following the intraplantar injection of carrageenan into the rat hindpaw as non-selective cNOS/iNOS inhibitors such as N^G-monomethyl-L-arginine (L-NMMA) attenuate oedema at the early times (up to 3 h) following carrageenan administration (Ialenti *et al.*, 1992). However, the effects of NOS inhibitors at later times after the onset of the inflammatory response have not been investigated and the role of iNOS in this model of inflammation is not known. Therefore, we have used this model of acute inflammation induced by the injection of carrageenan into the rat hindpaw to assess the roles of constitutive and inducible nitric oxide synthase in the progression of the inflammatory response, as well as to study the relationship that may exist between NO, prostaglandins and oxygen-derived free radicals.

Methods

Carrageenan paw oedema

Male Sprague-Dawley rats (175–200 g, Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) were housed and cared for under the guidelines of the institutional animal care and use committee. They received a subplantar injection of carrageenan (0.1 ml of a 1% suspension in 0.85% saline) into the right hind paw. Paw volume was measured with a plethysmometer (Ugo-Basile, Varese, Italy) immediately prior to the injection of carrageenan and thereafter at hourly intervals for 10 h. Oedema was expressed as the increase in paw volume (ml) after carrageenan injection relative to the pre-injection value for each animal. Unless specified, drugs were administered intravenously in a volume of 2.5 ml kg, either 1 h before or 1 h after carrageenan injection.

Determination of nitrite/nitrate and prostaglandin E_2 from carrageenan-injected rat paws

At specified times after the intraplantar injection of carrageenan, rats were killed and each paw was cut at the level of the calcaneus bone. Paws were gently centrifuged at 250 *g* for 20 min in order to recover a sample of the oedematous fluid. The volume of fluid that was recovered from each paw was measured. Blood was removed from the fluid sample by filtering through a 10,000 mol. wt. cut-off filter (Millipore, Bedford, MA, U.S.A.). Nitrite/nitrate (NO_2^-/NO_3^-) concentrations were measured by the diaminonapthalene (DAN) assay as described (Misko *et al.*, 1993b). Nitrate in the paw fluid samples (10 μl) was converted to nitrite by the incubation with nitrate reductase (14 mu) and the reduced form of nicotinamide adenine dinucleotide phosphate (1 nmol) for 10 min at room temperature. The reaction was terminated by dilution with water and addition of the DAN reagent. NO_2^-/NO_3^- concentrations were then determined fluorometrically (Misko *et al.*, 1993b). The PGE_2 concentration in each paw fluid sample was determined by specific ELISAs (Cayman Chemicals, Ann Arbor, MI, U.S.A.). All determinations were performed in duplicate. Total (T) NO_2^-/NO_3^- or PGE_2 present in the entire oedematous fluid of each paw was calculated as follows:

$$T = \frac{\text{pmol nitrate/nitrate or ng PGE2 in the sample} \times \text{paw oedema volume(ml)}}{\text{sample volume(ml)}}$$

Results are expressed as nmol NO_2^-/NO_3^- or ng PGE_2/paw.

Myeloperoxidase assay

Myeloperoxidase (MPO), a haemoprotein located in azurophil granules of neutrophils, has been used as a biochemical marker for neutrophil infiltration into tissues (Bradley *et al.*, 1982). In the present study, MPO was measured photometrically by a method similar to that described previously (Laight *et al.*, 1994). At the specified times following the intraplantar injection of carrageenan, tissue from the pads of the rat hindpaw was removed with a scalpel and 5 mm pieces were then obtained with a tissue punch (5 mm punch from Roboz, Rockville, MD, U.S.A.). Each piece of tissue was homogenized in 1 ml of 50 mM phosphate buffer, pH 6, containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The homogenized rat paw tissues were frozen (on dry ice) and thawed (immersion in warm water, 37°C) three times. Following centrifugation at 35,000 *g* for 20 min, 7 μl aliquots of each of the supernatants were mixed in wells of a 96 well plate with 200 μl of assay buffer (50 mM phosphate buffer, pH 6, containing 0.5% HTAB, 0.167 mg ml^{-1} *O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide). Changes in absorbance at 460 nm were measured spectrophotometrically over 5 min.

iNOS nuclease protection assay

At various times following the intraplantar injection of carrageenan, rats were killed and paws were removed, immediately frozen on dry ice and stored at −80°C. RNA was prepared from the paws by first pulverizing the frozen paws into a fine powder. The frozen powder was then partially solubilized in a denaturing solution containing 4 M guanidine thiocyanate (Fluka), 25 mM Na citrate, and 0.5% sarcosyl (Sigma, St. Louis, U.S.A.). The RNA was extracted by a series of phenol/chloroform extractions (Gibco BRL/Fisher, U.S.A.) and was then precipitated with ethanol. The purified RNA pellet was solubilized in water and stored at −80°C. RNA integrity was assessed by gel electrophoresis. A rat iNOS (riNOS) probe was prepared for use in the protection assay. Briefly, a 196 bp fragment corresponding to bases 3176 (Pst) to 3372 (EcoRV) of the rat iNOS DNA sequence (GenBank accession No. U03699.Gb_Ro) was amplified by RT-PCR using RNA isolated from inflamed rat granulomatous air pouch tissue expressing iNOS mRNA and protein (Salvemini *et al.*, 1995a). This fragment was purified and cloned into a pGEM 5Z transcription vector (Promega). A ^{32}P antisense RNA transcript was generated using Promega's Riboprobe Gemini System II Buffers to detect the riNOS mRNA isolated from the paw. The ribonuclease protection assay was performed using the RPA II kit (Ambion) essentially as described by the manufacturer. The radiolabelled riboprobe was purified on a 7 M urea, 8% polyacrylamide sequencing gel (Biorad). The gel slice containing the purified probe was eluted for 2 h at 37°C (buffer provided in the Ambion kit). Ten micrograms of paw RNA was evaporated to dryness in 1.5 ml microfuge tubes and reconstituted in 20 μl of the hybridization buffer provided. The labelled probe (200,000 c.p.m.) was then added and incubated at 85°C for 10 min, then overnight at 45°C. Following the overnight incubation, 200 μl of a 1:50 dilution of RNase A/T1

in RNase digestion buffer was added for 30 min in order to degrade all of the RNA which did not hybridize to the probe; 300 μl of the RNase inactivation/precipitation solution containing carrier was then added to each tube and allowed to precipitate for 30 min at −20°C. Following centrifugation, the protected RNA was mixed with 5 μl of gel loading buffer, heated to 90°C for 3 min, then separated on a 7 M urea, 8% polyacrylamide sequencing gel. The gel was then exposed to X-ray film for one to three days at −80°C.

Determination of iNOS protein by Western blot analysis

Soft tissue was removed from individual rat paws and homogenized in lysis buffer containing 10 mM N-[2-hydroxyethylpiperazine]-N'-[ethanesulphonic acid] (HEPES), pH 7.5, 100 μM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DDT), 2 μM tetrahydrobiopterin (THB), 2 μM flavin adenine nucleotide (FAD), 25 nM calmodulin, 0.5 mM phenymethylsulphonyl fluoride (PMSF), 10 μM leupeptin, 1.5 μM pepstatin A, and 10 μg ml^{-1} soybean trypsin inhibitor (SBTI). The homogenates were centrifuged at 12,000 *g* for 1 h at 4°C, then supernatants were fractionated on a 1.0 mm, 8% polyacrylamide Tris glycine gel (Novex, San Diego, CA U.S.A.). Proteins were transferred to Hybond ECL Nitrocellulose (Amersham, Arlington Heights, IL, U.S.A.) for 2 h at 100V at 4°C in a Bio-Rad Mini Trans-Blot cell in 25 mM Tris, 192 mM glycine (Novex, San Diego, CA, U.S.A.). The transfer buffer was replaced after 1 h. Following transfer, the membrane was blocked for 1 h at room temperature with 10% nonfat dry milk in Tris buffered saline tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). Polyclonal antisera 587 was obtained from New Zealand White rabbits immunized with a synthetic peptide corresponding to amino acids 1124–1144 of mouse iNOS conjugated to thyroglobulin. Primary antibody was diluted 1 : 2000 in 2.5% nonfat dry milk in TBST and incubated overnight with the membrane at 4°C. The blot was washed for 1–2 h with several changes of 2.5% nonfat dry milk in TBST, then incubated with a 1 : 2000 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Biorad, Melville, NY, U.S.A.) in 2.5% nonfat dry milk in TBST for 1 h at room temperature. The membrane was then washed twice for 15 min with 2.5% nonfat dry milk in TBST, then washed twice for 15 min with TBST (no milk, increasing the Tween concentration to 0.3%). The immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using Hyperfilm and ECL reagent (Amersham, Arlington Heights, IL, U.S.A.).

Immunohistochemical localization of iNOS and nitrotyrosine

Immunohistochemical staining for iNOS and nitrotyrosine was performed on 8 μm frozen sections of hindpaws obtained from rats perfused with Hanks balanced salt solution containing 20 mM HEPES and 1% formaldehyde. Tissue sections were air dried for 5 min and were post-fixed with 1% formaldehyde for 5 min at room temperature. For single staining, non-specific staining was blocked with 3% normal goat serum in 0.5 M Tris-HCl, pH 7.4, containing 1% Triton X-100, 1 h at room temperature. All subsequent incubations were carried out in this buffer. For detection of iNOS immunoreactivity, tissue sections were incubated for 16 h at 4°C with a 1/1000 dilution of either preimmune rabbit sera, the anti-iNOS antiserum 587 described above, or antiserum 587 preadsorbed with rat inducible iNOS protein purified from inflamed air pouch. Endogenous peroxidase activity was then reduced with periodic acid (Zymed Laboratories, Inc. San Francisco, CA, U.S.A.) for 45 s at room temperature followed by sequential incubations with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 2 h each. The reaction product was visualized using 3,3'-diaminobenzidine intensified with nickel chloride for 6 min. For detection of nitrotyrosine immunoreactivity, tissue sections were incubated for 16 h at 4°C with a 1/1000 dilution of either preimmune serum, an anti-nitrotyrosine polyclonal rabbit serum generated to nitrated keyhole limpet haemocyanin, or the anti-nitrotyrosine serum and excess nitrotyrosine (10 mM), followed by sequential incubations with biotinylated anti-rabbit IgG and avidin-biotin-glucose oxidase complex (ABC, Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 2 h each. The reaction product was visualized using tetranitroblue tetrazolium for 10 min. Sections were counterstained with Mayer's haematoxylin, mounted, and photographed using bright field microscopy. For double immunohistochemical localization of iNOS and nitrotyrosine, sections were post-fixed in 1% formaldehyde for 5 min and non-specific staining was blocked by incubating with PBS containing 10 mM tyrosine, 1% BSA, 0.2% powdered skim milk and 0.3% triton-X 100 (PBS-BB) for 30 min at room temperature. Sections were then incubated with a 1/500 dilution of antiserum 587 in PBS-BB at 4°C overnight. Staining for iNOS was localized using a 1/200 dilution of Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.) in PBS-BB. Next, sections were incubated with a 1/500 dilution of the anti-nitrotyrosine serum in PBS-BB and staining was localized using a 1/200 dilution of DTSF-conjugated-goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.) Specific staining was visualized with epifluorescence.

Materials

Male Sprague Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, U.S.A.) 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. 2,3-Diaminonaphthalene was purchased from Aldrich (Milwaukee, WI, U.S.A.). Human polymorphonuclear leukocyte myeloperoxidase was obtained from Calbiochem (La Jolla, CA, U.S.A.) and recombinant human Cu/Zn superoxide dismutase coupled to polyethyleneglycol (PEGrhSOD) was obtained from DDI Pharmaceuticals Inc. (Mountain View, CA, U.S.A.). All other chemicals and reagents were obtained from Sigma (St. Louis, MO, U.S.A.). N-iminoethyl-L-lysine (L-NIL) was synthesized in house as described previously (Connor *et al.*, 1995).

Statistical analysis

Results are expressed as mean ± s.e.mean for (*n*) rats. The results were analysed by Student's unpaired *t* test to determine the significant differences between means, or by a two-way ANOVA followed by a least significant procedure to determine the nature of this response. A P value of <0.05 was considered to be statistically significant.

Results

Time-dependent increase in paw volume, NO_2^-/NO_3^-, PGE_2 and neutrophil infiltration

The intraplantar injection of carrageenan in rats led to a time-dependent increase in paw volume that was maximal after 6 h and remained elevated thereafter for 10 h (Figure 1b). The increase in paw volume was associated with an elevated production of NO_2^-/NO_3^- in the paw exudates. This increase in NO_2^-/NO_3^- was observed within 30 min (from 0.5 ± 0.05 nmol/paw to 16.2 ± 4 nmol/paw, $n=6$), remained constant for the subsequent 3 h and then increased further at 6 and 10 h following carrageenan administration (Figure 2a). The increase in NO_2^-/NO_3^- at 6 h and thereafter is likely due to the activity of iNOS. iNOS mRNA was detected at 3 and 10 h by ribonuclease protection assays (Figure 1a) and iNOS protein was detected at 6 h and was maximal by 10 h following carrageenan administration by Western blot analysis (Figure 1a).

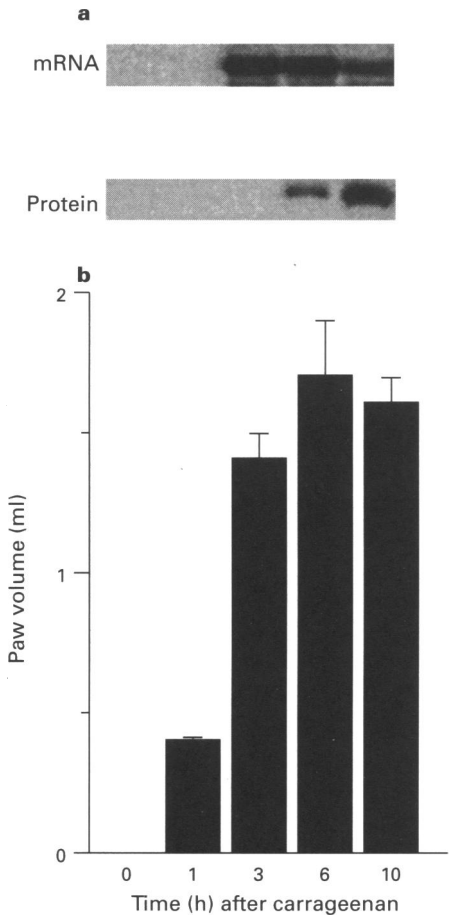

Figure 1 Time-dependent increase (0 to 10 h) in paw volume following carrageenan administration. The injection of carrageenan caused a time-dependent increase in paw volume that reached a maximum within 6–10 h (b; each point is the mean ± s.e.mean for 6 experiments). iNOS mRNA was expressed in the paw tissue within 3 h after carrageenan and reached a maximum by 10 h (a). iNOS protein was detected by Western blot analysis at 6 h and also reached a maximum by 10 h after carrageenan administration (a).

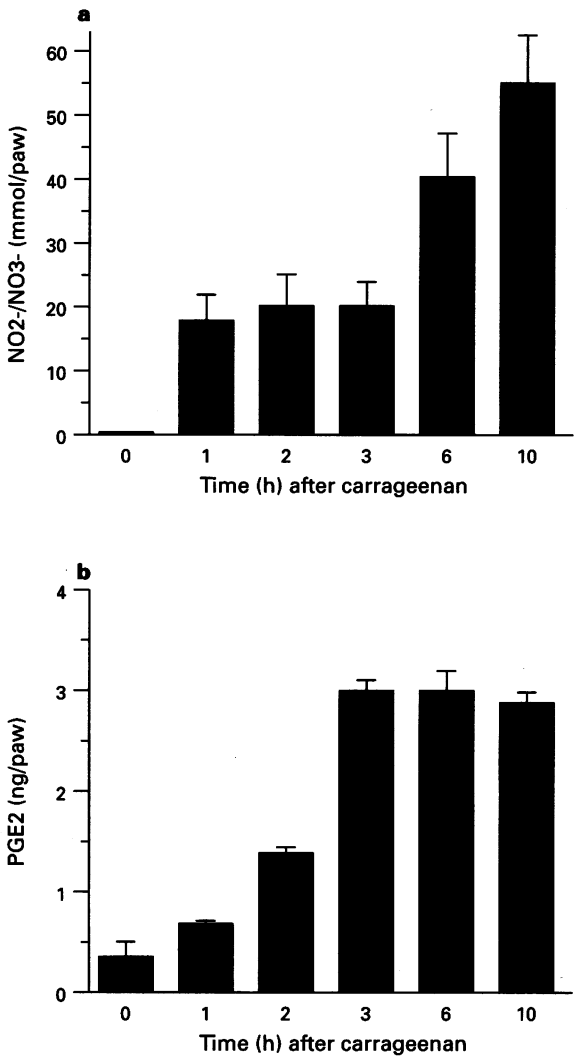

Figure 2 Time-dependent increase in NO_2^-/NO_3^- (a) and PGE_2 (b) following injection of carrageenan. Each point is the mean ± s.e.mean for $n=6$ animals.

Six hours after carrageenan administration, the maximal increase in paw volume was also associated with neutrophil infiltration as measured by an increase in MPO activity in the paw tissue (from 214 ± 12 to 3636 ± 5 mu MPO/paw, $n=6$). Infiltrating neutrophils were found not to be the source of iNOS-derived NO. Treatment of rats with a subcutaneous injection of colchicine (2 mg kg^{-1}, 2 h before carrageenan administration; Boughton-Smith *et al.*, 1993), suppressed paw volume by at least 70% at all time points. Paw volume, measured 6 h after carrageenan administration, was decreased from 1.4 ± 0.07 ml to 0.3 ± 0.03 ml ($n=6$), and was associated with inhibition of neutrophil infiltration. MPO values decreased from 3636 ± 5 mu/paw to 4 ± 0.1 mu/paw ($n=6$). However, at the same time, ribonuclease protection assays demonstrated that the elevated expression of iNOS mRNA was unaltered by the colchicine treatment (not shown). In addition, this corresponded with a failure of colchicine to reduce the production of NO_2^-/NO_3^- in paw exudate (from 40 ± 7 to 41 ± 3 nmol/paw, $n=6$).

The paw tissues were also examined immunohistochemically for the presence of iNOS and nitrotyrosine (a marker for peroxynitrite formation). Immunohistochemical analysis of the paw tissues of control animals showed no iNOS or nitrotyrosine staining (Figure 3a and d). In contrast, 6 h following carrageenan administration, iNOS-like immunoreactivity was localized to discrete cells within the inflamed paw tissue (Figure 3b). This staining was specific for iNOS as it was completely eliminated by incubation of the iNOS antiserum with an excess of purified rat iNOS (Figure 3c). Based upon morphology and ED1 positive staining (not shown), the iNOS immunoreactivity was localized primarily to macrophage-like cells present in the inflamed paw tissue. Staining for nitrotyrosine was also found to be localized within discrete cells in the inflamed paw tissue (Figure 3e). This staining was almost completely eliminated by incubation of the primary anti-nitrotyrosine serum with 10 mM nitrotyrosine (Figure 3f). Using double immunofluorescence staining for iNOS (Figure 3g, red) and nitrotyrosine (Figure 3h, green), the staining for iNOS and nitrotyrosine was localized primarily to the same cells (Figure 3i, yellow-orange).

Carrageenan also elicited a time-dependent increase in PGE_2 production which increased after 2 h, reached a peak by 3 h and remained elevated thereafter (Figure 2b).

Effects of NOS inhibitors on paw volume, NO_2^-/NO_3^-, PGE_2 and neutrophil infiltration

The non-selective cNOS/iNOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (30–300 mg kg^{-1}) administered intravenously 1 h before carrageenan ($n=6$), inhibited the paw oedema at all time points (Figure 4a). Complete inhibition of oedema formation was observed during the first hour. Paw volume measured after 1 h decreased from 0.4 ± 0.01 ml in non-treated rats, to 0.3 ± 0.01 ml, 0.08 ± 0 ml and 0 ml in rats that received 30, 100 and 300 mg kg^{-1} L-NAME, respectively

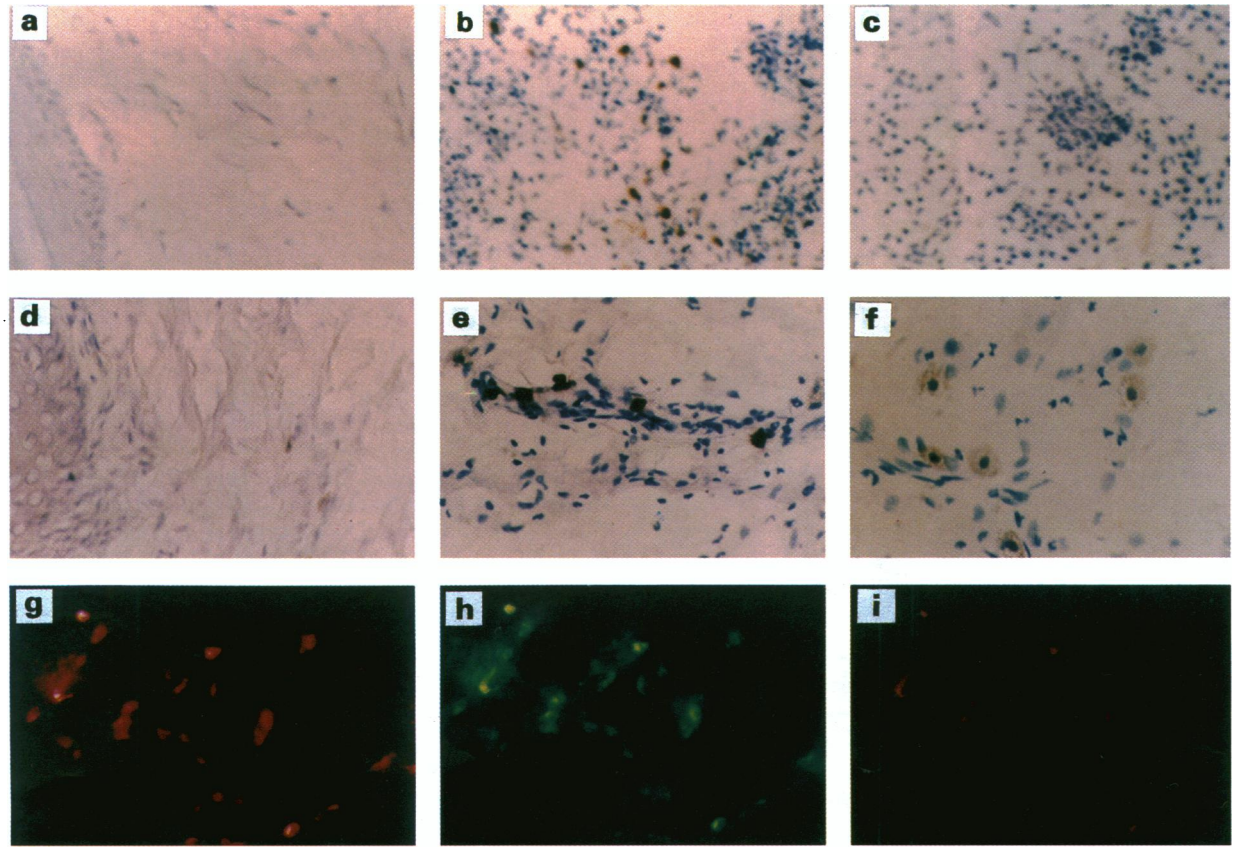

Figure 3 Immunohistochemical localization of iNOS and nitrotyrosine in the carrageenan-inflamed rat hindpaw. Control (a,d) and carrageenan-inflamed (all others) paw tissue sections were stained for iNOS or nitrotyrosine either using avidin-biotin-glucose oxidase (a–c), avidin-biotin-peroxidase (d–f) or indirect immunofluorescence (Cy3 or DFAP, g–i)-based detection. Six hours after the intraplantar injection of carrageenan, iNOS-like immunoreactivity was localized to discrete cells within the inflamed paw tissue (b,g). Based upon morphology and ED1 positive staining (not shown), iNOS was localized primarily to macrophage-like cells present in the inflamed paw tissue. Staining was absent in control tissue (a) or in inflamed tissue when the anti-iNOS antiserum was incubated with an excess of purified rat iNOS (c). Nitrotyrosine staining was also localized to discrete cells, similar in morphology to those expressing iNOS, within the inflamed paw tissue (e,h). Staining was absent in control paw tissue (d) or in inflamed tissue when the anti-initrotyrosine antiserum was incubated with 10 mM nitrotyrosine (f). Using double immunofluorescent staining, the iNOS (red, g) and nitrotyrosine (green, h) staining were co-localized primarily to the same cells as indicated by the yellow-orange colour (i) obtained when the photographs are double exposed.

($n=6$). At subsequent time points, L-NAME produced a partial, dose-dependent inhibition of paw oedema. In order to determine whether NO maintained the oedema following the injection of carrageenan, rats were given an intravenous injection of L-NAME 1 h after the intraplantar injection of carrageenan; paw swelling was assessed thereafter every hour for 10 h. Treatment with L-NAME (30–300 mg kg^{-1}, $n=6$) after the administration of carrageenan also reduced the increase in paw oedema in a dose-dependent manner (Figure 4b). Similar results were obtained with L-NMMA (30–300 mg kg^{-1}, $n=6$, Table 1A and B).

We next examined the role of iNOS by using two recently described selective iNOS inhibitors, L-NIL (Moore *et al.*, 1994; Connor *et al.*, 1995) and AG (Misko *et al.*, 1993a; Griffiths *et al.*, 1993; Wu *et al.*, 1995). We previously characterized both the potency and selectivity of these inhibitors *in vitro* using purified rat brain cNOS and mouse macrophage iNOS (Moore *et al.*, 1994). L-NIL and AG are approximately 30 fold more selective for the inducible than for the constitutive form of NOS, and L-NIL was approximately 10 fold more potent than AG at inhibiting iNOS activity (Moore *et al.*, 1994; Connor *et al.*, 1995). Neither L-NIL (30 mg kg^{-1}, i.v., $n=6$) nor AG (300 mg kg^{-1}, $n=6$) prevented the increase in paw volume observed from 0 to 4 h following carrageenan administration. However, these drugs inhibited the paw oedema observed at subsequent times (Figure 5). In addition, the administration of L-NIL (3–30 mg kg^{-1}, $n=6$) 5 h after the onset of inflammation, a time when iNOS was clearly expressed (Figure 1), inhibited paw oedema in a dose-dependent fashion (Figure 6). Similar results were obtained with AG, but higher doses were required to produce comparable effects (30–300 mg kg^{-1}, $n=6$, not shown).

The effects of both non-selective and selective NOS inhibitors on the production of NO_2^-/NO_3^- and PGE_2 in the paw exudate were also determined. Treatment with L-NMMA and L-NAME (300 mg kg^{-1}, $n=5$) inhibited the NO_2^-/NO_3^- and PGE_2 production in paw exudate at both 3 (Figure 7a and 8a) and 10 h (Figure 7b and 8b) after carrageenan administration. In contrast, L-NIL (30 mg kg^{-1}, $n=5$) and AG (300 mg kg^{-1}, $n=5$) inhibited NO_2^-/NO_3^- and PGE_2 release in paw exudate only at the 10 h time point (Figures 7b and 8b), but had no effect at 3 h following carrageenan administration (Figure 7a and 8a). Finally, treatment with the non-selective cyclo-oxygenase inhibitor, indomethacin (10 mg kg^{-1}, p.o., given 2 h before carrageenan, $n=6$), inhibited paw oedema at 2 h and at subsequent times ($n=6$, not shown), blocked PGE_2 production (Figure 8a and 8b), but had little effect on NO_2^-/NO_3^- production (Figure 7).

Both the non-selective cNOS/iNOS inhibitors as well as the more selective iNOS inhibitors attenuated neutrophil infiltration following carrageenan administration. Six hours after carrageenan treatment, MPO values decreased from 3636 ± 5 mu/paw in saline-treated rats to 150 ± 15 mu/paw, 138 ± 10 mu/paw, 500 ± 10 mu/paw and 570 ± 15 mu/paw in rats treated with L-NMMA, L-NAME (300 mg kg^{-1}, $n=6$), L-NIL (30 mg kg^{-1}, $n=6$) or AG (300 mg kg^{-1}, $n=6$), respectively.

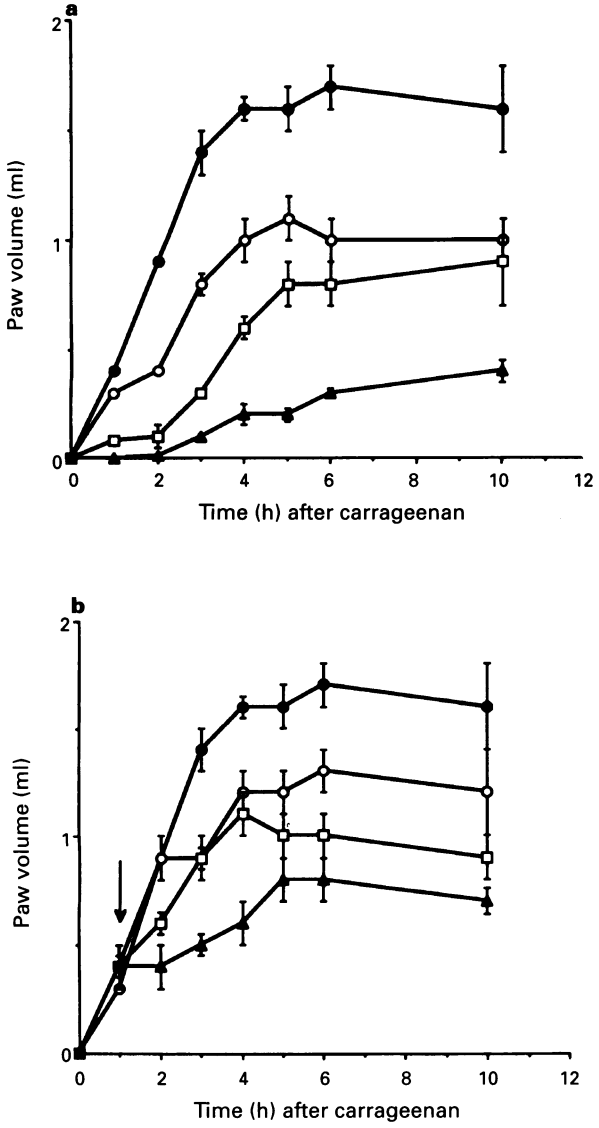

Figure 4 (a) Inhibition of carrageenan-induced paw oedema (●) by L-NAME at 30 (○), 100 (□) and 300 (▲) mg kg^{-1} when given 1 h before or (b) 1 h after as indicated by the arrow. Results are expressed as the increase in paw volume (ml). Each point is the mean ± s.e.mean for $n=6$ animals.

Effect of PEGrhSOD, catalase and desferrioxamine on paw volume, NO_2^-/NO_3^-, PGE_2 and neutrophil infiltration

To evaluate the roles of superoxide anions, rats were treated with PEGrhSOD. Treatment with PEGrhSOD (given at 12×10^3 u kg^{-1}, 30 min before carrageenan, $n=6$) inhibited the increase in paw oedema at all time points by at least 60% (Table 2). This anti-inflammatory effect was associated with inhibition of neutrophil infiltration. MPO values decreased from 3636 ± 5 mu/paw in saline-treated rats to 597 ± 20 mu/paw in rats treated with PEGrhSOD at 6 h after carrageenan ($n=6$). However, it did not affect the NO_2^-/NO_3^- or PGE_2 production in the paw exudate ($n=6$, not shown). The anti-inflammatory effects that occurred with PEGrhSOD were also observed when the drug was given 1 h after carrageenan (Table 2) supporting a previous study by Boughton-Smith *et al.* (1993).

To evaluate the roles of hydrogen peroxide and hydroxyl radicals, rats were treated with catalase and desferrioxamine respectively. Treatment with catalase (40×10^3 u kg^{-1}, i.p. 30 min before carrageenan; Hirschelmann & Bekemeir, 1981) had no effect on the formation of oedema ($n=4$, not shown). Treatment with desferrioxamine (300 mg kg^{-1}, s.c.) 1 h before carrageenan, Boughton-Smith *et al.*, 1993) inhibited paw oedema after the first hour by approximately 60% ($n=5$) and after the second hour by 38% ($n=5$). However, no inhibition occurred at subsequent times (Table 2).

Discussion

The anti-inflammatory effects of NOS inhibitors in acute and chronic models of inflammation have been and continue to be characterized. In view of the complex nature of the inflammatory response, the determination of possible mechanisms by which NO modulates this response will aid in designing novel and more efficacious NOS inhibitors. Two important discoveries have been made recently that may help to explain potential mechanisms of action of NO in inflammatory conditions. The first observation is that NO stimulates COX activity resulting in the exaggerated production of pro-inflammatory PG (Salvemini *et al.*, 1993). The second is that NO can react with O_2^- to form the cytotoxic radical peroxynitrite (Beckman *et al.*, 1990).

In the studies described here, we examined the significance of these interactions in acute inflammation induced by the injection of carrageenan into the rat hindpaw. For clarity, the oedema occurring from 0–4 h is termed the 'early phase' and that occurring at subsequent time points (4–10 h) the 'sustained phase'. The non-selective cNOS/iNOS inhibitors (L-NAME and L-NMMA), whether given 1 h before or after the onset of inflammation, attenuated the oedema at all points indicating a role for NO in this inflammatory response. The degree of inhibition obtained during the first 2 h was remarkable, achieving approximately 90%. The oedema that occurs during the first hour results from the concomitant release of histamine, 5-HT and kinins and is markedly inhibited (70–80%) by treatment with a combination of histamine, 5-HT and bradykinin receptor antagonists (Di Rosa *et al.*, 1971). However, single administration of these receptor antagonists inhibits oedema by only 30–40% (Di Rosa *et al.*, 1971). Thus the inhibitory effects of the non-selective NOS inhibitors is comparable to that achieved with a mixture of histamine, 5-HT and bradykinin receptor antagonists.

In our present study using both molecular and pharmacological tools, we have demonstrated that iNOS is expressed in this model within 4 h after injection of carrageenan and that its induction and the subsequent production of NO is involved in maintaining the oedema during the sustained phase of the inflammatory response. Both AG and L-NIL, two recently described iNOS inhibitors (Misko *et al.*, 1993a; Griffiths *et al.*, 1993; Moore *et al.*, 1994; Connor *et al.*, 1995; Wu *et al.*, 1995) failed to inhibit carrageenan-induced paw oedema during the first 4 h of the response but inhibited paw oedema at all subsequent times (from 4 to 10 h, Figure 5). In addition, iNOS mRNA was detected between 3 and 10 h after carrageenan administration by ribonuclease protection assays and iNOS protein was detected at 6 h, and was maximal at 10 h by Western blot analysis. iNOS-like immunoreactivity was also localized to macrophage-like cells within the inflamed paw tissue, but not in neutrophils, suggesting that resident macrophages in inflamed paw tissue and/or infiltrating monocytes are the source of iNOS-derived NO. The therapeutic administration of the selective iNOS inhibitors, L-NIL or AG, at a time when iNOS activity was initially detected, prevented the subsequent increase in oedema indicating that NO generated by iNOS maintains oedema during the later stages of the inflammatory response. When compared to L-NIL, higher doses of aminoguanidine were required to exert anti-inflammatory effects, consistent with the fact that L-NIL is a more potent inhibitor of iNOS in *in vitro* studies (Moore *et al.*, 1994). In addition, the detection of nitrotyrosine immunoreactivity within the inflamed tissue, primarily localized to the same cells as those expressing iNOS, suggests that peroxynitrite is formed during this re-

Table 1 Inhibition of carrageenan-induced paw oedema by L-NMMA (30–300 mg kg^{-1}) given either 1 h before (A) or 1 h after (B) carrageenan injection

	L-NMMA (mg kg^{-1})			
	0	30	100	300
A				
Time (h)				
0	0	0	0	0
1	0.4±0.01	0.3±0.01	0.08±0*	0
2	0.9±0.02	0.4±0.01*	0.1±0.05*	0.01±0.5*
3	1.4±0.1	0.8±0.05*	0.3±0.01*	0.1±0.01*
4	1.6±0.2	1.0±0.1*	0.6±0.05*	0.2±0.05*
5	1.6±0.3	1.0±0.1*	0.8±0.1*	0.2±0.03*
6	1.7±0.2	1.0±0.1*	0.8±0.1*	0.3±0.02*
10	1.6±0.2	1.0±0.2*	0.7±0.1*	0.3±0.05*
B				
Time (h)				
0	0	0	0	0
1	0.4±0.01	0.3±0.01	0.4±0.1	0.4±0.05
2	0.9±0.02	0.9±0.1	0.6±0.05*	0.4±0.1*
3	1.4±0.1	0.9±0.05*	0.9±0.1*	0.5±0.05*
4	1.6±0.2	1.2±0.1*	1.1±0.1*	0.6±0.1*
5	1.6±0.3	1.2±0.1*	1±0.1*	0.8±0.1*
6	1.7±0.2	1.3±0.1*	1±0.1*	0.8±0.1*
10	1.6±0.2	1.2±0.1*	0.9±0.1*	0.7±0.05*

Results are expressed as the increase in paw volume (ml). Each point is the mean±s.e.mean for $n=6$ animals. *$P<0.05$ compared to the corresponding control value.

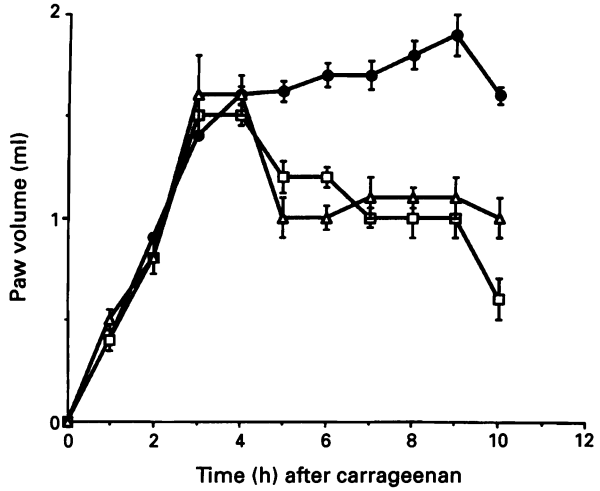

Figure 5 Inhibition of carrageenan-induced paw oedema (●) by L-NIL (30 mg kg^{-1}, □) or AG (300 mg kg^{-1}, △). Drugs were given i.v. 1 h before carrageenan. Results are expressed as the increase in paw volume (ml). Each point is the mean±s.e.mean for $n=6$ animals.

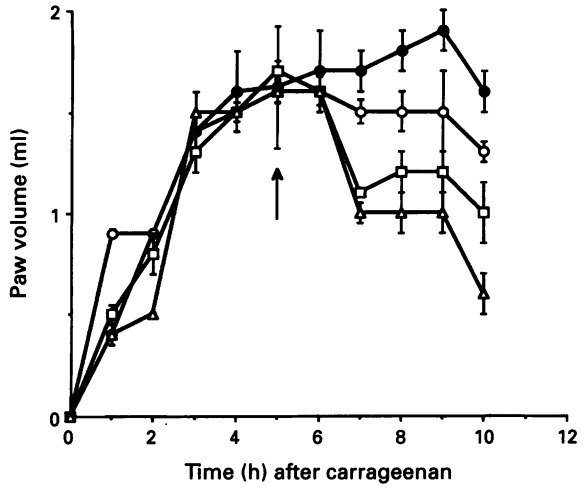

Figure 6 Inhibition of carrageenan-induced paw oedema (●) by L-NIL at 3 (○), 10 (□) or 30(△) mg kg^{-1}. L-NIL was given 5-h after the administration of carrageenan. Results are expressed as the increase in paw volume (ml). Each point is the mean±s.e.mean for $n=6$ animals.

sponse and may be responsible for inducing a portion of the tissue damage in the hindpaw. Importantly, the anti-inflammatory effects of the iNOS inhibitors were found to be independent of changes in arterial blood pressure. The non-selective NOS inhibitors on the other hand raise arterial blood pressure and this could account for some of their ability to attenuate the oedema response to carrageenan.

The involvement of cNOS in the early phase and of iNOS during the sustained phase of the oedematous response was also demonstrated by the differential ability of L-NMMA or L-NAME and L-NIL or AG to inhibit NO_2^-/NO_3^- production in the paw exudate. Three hours after carrageenan injection (a time point where iNOS was not expressed), NO_2^-/NO_3^- production was inhibited by L-NMMA or L-NAME, but not by L-NIL or AG suggesting that the NO generated in the early phase originated from cNOS activation. In contrast, the production of NO_2^-/NO_3^- in the paw exudate at the 10 h time point (when iNOS was expressed) was inhibited not only by L-NMMA or L-NAME, but also by L-NIL or AG, indicating that iNOS was responsible for the enhanced production of NO during the sustained phase of the response.

Prostaglandins are also important mediators of acute inflammation. Previous studies have demonstrated that although PGs do not contribute initially to the oedema occurring 1 h after carrageenan administration, their production does produce some of the inflammation within the second hour and at subsequent time points (Di Rosa *et al.*, 1971; Seibert *et al.*, 1994). The inducible isoform of cyclooxygenase (COX-2) appears to be responsible for PG production under these circumstances (Seibert *et al.*, 1994). Interestingly, as shown in Figure 8, the inhibition of NO generation by NOS inhibitors during both the early and sustained phase of oedema was associated with a reduction in PG production. We have recently reported that in both *in vitro* and *in vivo* studies, NO activates COX-1 and COX-2 resulting in an enhanced production of PG. In addition, NOS

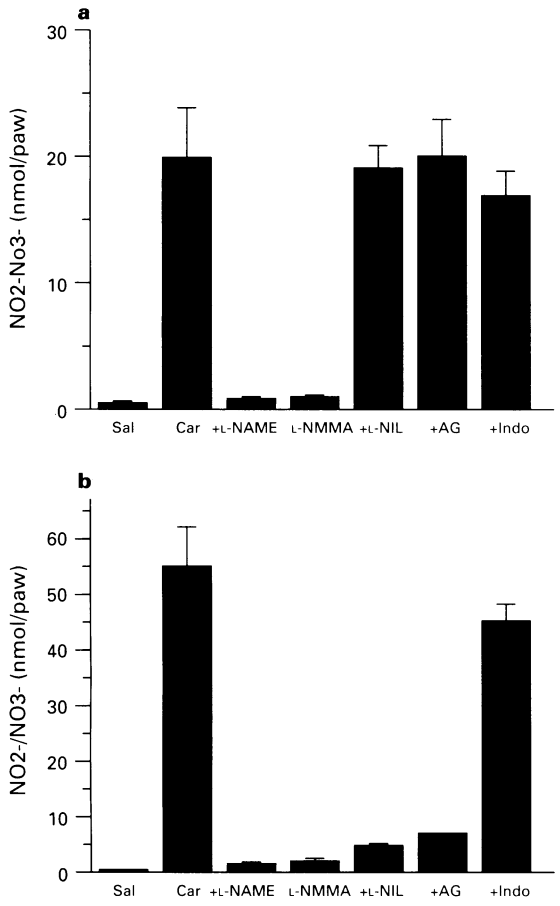

Figure 7 Effects of NOS inhibitors or indomethacin on NO_2^-/NO_3^- production at 3 (a) and 10 (b) h after carrageenan administration. The non-selective NOS inhibitors (L-NAME, L-NMMA) inhibited NO_2^-/NO_3^- production at 3 and 10 h (a and b), whereas the selective iNOS inhibitors (L-NIL or AG) inhibited NO_2^-/NO_3^- production only at the 10 h time point (b). Indomethacin had no effect (a and b). Each point is the mean ± s.e.mean for $n=6$ experiments.

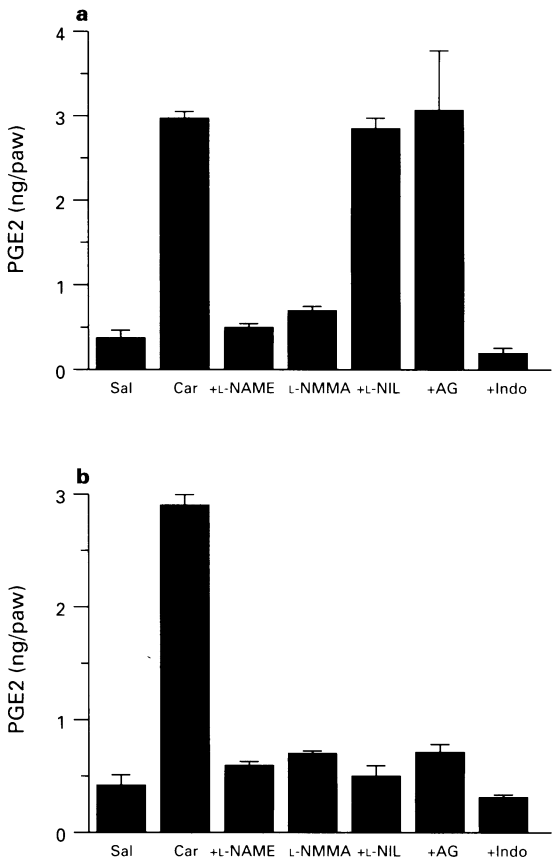

Figure 8 Effects of indomethacin or NOS inhibitors on PGE_2 production at 3(a) and 10(b) h after the administration of carrageenan. PGE_2 production at 3 and 10 h was completely inhibited by treatment with indomethacin (a and b). PGE_2 release at 3 and 10 h was also attenuated by the non-selective NOS inhibitors (L-NAME, L-NMMA), whereas the selective iNOS inhibitors (L-NIL, AG) attenuated PGE_2 production only at the 10 h time point (b). Each point is the mean ± s.e.mean for $n=6$ experiments.

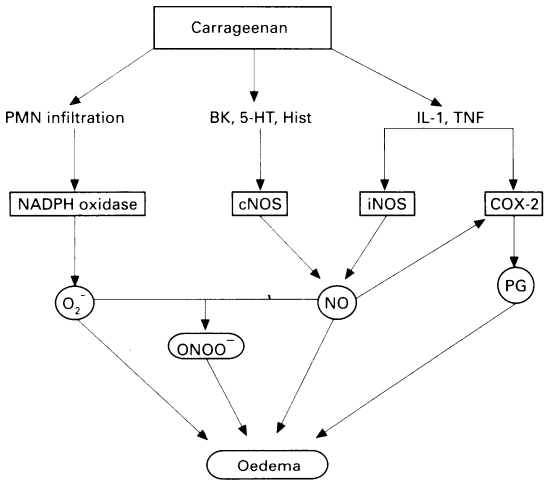

Figure 9 Proposed model of mechanism(s) of action of NO in carrageenan-induced acute inflammatory response in the rat hindpaw.

inhibitors inhibit PG formation (Salvemini *et al.*, 1993; 1995a,b), a finding that has now been confirmed in a number of other studies (Inoue *et al.*, 1993; Corbett *et al.*, 1993; Sautebin & Di Rosa, 1994; Sautebin *et al.*, 1995). The inhibition of PG production by NOS inhibitors does not appear to be due to the direct inhibition of COX activity or PG isomerase activity (Salvemini *et al.*, 1993; 1994). It appears likely that NO derived from cNOS enhances PG formation at the early time points and NO derived from iNOS enhances PG production at the later times.

Carrageenan-induced paw oedema is neutrophil-dependent (this study; Di Rosa & Sorrentino, 1968; Vinegar *et al.*, 1971; Boughton-Smith *et al.*, 1993). As was observed previously in the carrageenan-induced air pouch model of inflammation (Salvemini *et al.*, 1995a), it seems that early in the inflammatory response in the hindpaw, infiltrating neutrophils are not the source of iNOS-derived NO since the anti-inflammatory effects of colchicine were not associated with inhibition of NO. Activated neutrophils, are an excellent source of oxygen-derived free radicals which have been implicated in many models of acute inflammation in which there is a neutrophil-dependent increase in vascular permeability (see Fantone & Ward, 1982 for review). As previously reported by others (Hirschelmann & Beckemein, 1981; Boughton-Smith *et al.*, 1993), we found that the removal of O_2^- by PEGrhSOD significantly inhibited the paw oedema. Furthermore, PEGrhSOD also inhibited neutrophil infiltration, a result that is consistent with a role for O_2^- in mediating neutrophil adhesion and infiltration (Schraufstatter *et al.*, 1987; Warren *et al.*, 1990). PEGrhSOD failed to affect NO and PG production, providing further evidence that infiltrating neutrophils do not contribute to the production of these mediators. Removal of hydrogen peroxide by catalase had no effect and removal of hydroxyl radicals by desferrioxamine inhibited oedema weakly only at the first hour. These results suggest that O_2^- rather than hydrogen peroxide or hydroxyl radicals are key players in the inflammatory response.

The interactions between NO, PG and O_2^- are summarized

Table 2 Inhibition of carrageenan-induced paw oedema by PEGrhSOG when given 30 min before (−30) or 1 h (+1) after carrageenan administration (**A**) and lack of effect of catalase (Cat) or desferrioxamine (Des) (**B**)

	0	*−30*	*+1*
A			
Time (h)			
0	0	0	0
1	0.4±0.01	0.1±0.01*	0.4±0.05
2	0.9±0.02	0.5±0.07*	0.6±0.01*
3	1.4±0.1	0.7±0.01*	0.7±0.02*
4	1.6±0.2	0.9±0.05*	0.8±0.07*
5	1.6±0.3	0.9±0.05*	0.8±0.07*
6	1.7±0.2	1±0.1*	0.8±0.05*
10	1.6±0.2	1.2±0.2*	1±0.1*
B			
Time (h)	*0*	*Cat*	*Des*
0	0	0	0
1	0.4±0.01	0.5±0.01	0.05±0.1*
2	0.9±0.02	0.9±0.1	0.6±0.03*
3	1.4±0.1	1.3±0.2	1.3±0.01
4	1.6±0.2	1.5±0.2	1.5±0.03
5	1.6±0.3	1.6±0.2	1.6±0.1
6	1.7±0.2	1.7±0.2	1.8±0.03
10	1.6±0.2	1.8±0.2	1.7±0.1

Results are expressed as the increase in paw volume (ml). Each point is the mean±s.e.mean for $n=6$ animals. *$P<0.05$ compared to corresponding control value.

in the model shown in Figure 9. Within the first hour following carrageenan injection, oedema is induced by the release of mediators such as histamine, bradykinin and 5-HT, but not by PG. These mediators, following activation of their receptors on endothelial cells, trigger cNOS activation resulting in the generation of NO. Since treatment with non-selective NOS inhibitors produced virtually complete inhibition of oedema formation at this time, we propose that NO released as a consequence of cNOS activation may be the final common mediator responsible for the early phase of the inflammatory response to carrageenan. Whether the cNOS efects are endothelial or neuronal NOS-mediated remains to be investigated in future studies. As the inflammatory response progresses, iNOS is induced and generates larger quantities of NO which appear to maintain the oedema during the sustained phase. Although the mechanism(s) involved in the regulation of iNOS induction in this model are not known, it is likely that cytokines are involved. In mice following the intraplantar injection of carrageenan, TNF-α, IFN-γ as well as cytokines such as IL-1 and IL-2 are produced (Ianaro *et al.*, 1994). These molecules have been shown to induce iNOS in a variety of cells including macrophages, smooth muscle cells and endothelial cells (see Moncada & Higgs, 1993 for review). COX-2 is also induced within 2 h after carrageenan administration (Seibert *et al.*, 1994). The NOS and COX pathways appear to operate together to amplify the inflammatory response. This is achieved by a synergistic interaction between NO and PG on blood flow and microvascular permeability (Warren *et al.*, 1992), as well as by a NO-driven COX activation leading to the exaggerated production of PG (Salvemini *et al.*, 1993; 1995a,b). The dual inhibition of NO and PG obtained with NOS inhibitors could account for their marked anti-inflammatory effect.

Neutrophil infiltration in response to carrageenan and following NADPH oxidase activation generates an oxygen-respiratory burst giving rise to oxygen-derived free radicals such as O_2^-. O_2^- promote lipid peroxidation, increase vascular permeability, elicit cellular recruitment and produce tissue damage (see Fantone & Ward, 1982 for review). Furthermore, the generation of O_2^- in the presence of NO forms the cytotoxic radical $ONOO^-$ (Beckman *et al.*, 1990). $ONOO^-$ can in turn decompose to form additional toxic molecules including nitrogen dioxide and OH (Beckman *et al.*, 1990; Radi *et al.*, 1991). The localization of nitrotyrosine immunoreactivity, a marker of peroxynitrite formation, within the inflamed paw tissue during the late phase of carrageenan-induced hindpaw inflammation suggests that $ONOO^-$ was generated and could be responsible in part for the production of tissue damage in this model. The precise biochemical nature of the role of $ONOO^-$ remains to be elucidated, but it appears not to involve to any great extent the formation of OH.

In summary, we propose that possible mechanisms by which NOS inhibitors exert their anti-inflammatory effects include the inhibition of: neutrophil infiltration, PG and NO production, and the generation of peroxynitrite. These findings also suggest that the inhibition of the sustained phase of the paw oedema following the intraplantar injection of carrageenan is suitable for the *in vivo* assessment of the anti-inflammatory actions of novel inhibitors of the inducible form of nitric oxide synthase.

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