Nitric Oxide Synthase Inhibitors Have Opposite Effects on Acute Inflammation Depending on Their Route of Administration

Mark J. Paul-Clark, Derek W. Gilroy, Dean Willis, Derek A. Willoughby and Annette Tomlinson

*J Immunol* 2001; 166:1169-1177; doi: 10.4049/jimmunol.166.2.1169

http://www.jimmunol.org/content/166/2/1169

**References**

This article cites 42 articles, 9 of which you can access for free at:

http://www.jimmunol.org/content/166/2/1169.full#ref-list-1

**Why The JI? Submit online.**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Nitric Oxide Synthase Inhibitors Have Opposite Effects on Acute Inflammation Depending on Their Route of Administration

Mark J. Paul-Clark, Derek W. Gilroy,2 Dean Willis, Derek A. Willoughby, and Annette Tomlinson

The bulk of published data has shown that NO is proinflammatory. However, there also exists the conflicting notion that NO may be protective during an inflammatory insult. In an attempt to resolve this issue, we have compared the effects on inflammation of a range of NO synthase (NOS) inhibitors given either directly to the site of the inflammatory lesion or systemically. It was found that in the carrageenin-induced pleurisy, a single intrapleural injection of the selective inducible NO inhibitors S-(2-aminoethyl) isothiourea (AE-ITU; 3 and 10 mg/kg) and N-(3-(aminomethyl)benzyl)acetamidine (1400W; 10 mg/kg) or the selective endothelial cell NOS inhibitor L-NAME (1-iminoethyl)-ornithine (10 mg/kg) not only exacerbated inflammation at the very early stages of the lesion (1–6 h), but also prevented inflammatory resolution. By contrast, administering NOS inhibitors systemically ameliorated the severity of inflammation throughout the reaction. To elucidate the mechanisms by which inhibition of NO synthesis locally worsened inflammation, we found an increase in histamine, cytokine-induced neutrophil chemoattractant, superoxide, and leukotriene B4 levels at the inflammatory site. In conclusion, this work shows that the local production of NO is protective by virtue of its ability to regulate the release of typical proinflammatory mediators and, importantly, that NOS inhibitors have differential anti-inflammatory effects depending on their route of administration. The Journal of Immunology, 2001; 166: 1169–1177.

The signaling molecule NO is produced by the enzyme NO synthase (NOS)3 after activation. NOS is constitutively expressed in endothelial (ecNOS) and neuronal cells (neuronal NOS), while a third isoform (iNOS) is induced in response to inflammatory-like stimuli and is capable of sustained production of high levels of NO that is predominant in inflammation (for review, see Ref. 1). Although the cytostatic/cytocidal activity of NO forms part of the host defense mechanism, the excessive or inappropriate production of NO can lead to tissue damage possibly through the formation of the potent oxidizing and nitrating agent, peroxynitrite, by a coupling of NO with superoxide (O$_{2}^{-}$) (2).

NO generated from ecNOS has been reported to have both pro- and anti-inflammatory properties. Under physiological conditions, NO released from the endothelium regulates vascular tone and maintains vessel patency by helping prevent platelet aggregation and down-regulating adhesion molecule expression (3). However, mediators released during the acute phase of inflammation, including histamine, 5-hydroxytryptamine, bradykinin, platelet-activating factor, and substance P, evoke the release of endothelial NO, causing vasodilatation and vascular permeability, thus facilitating edema formation and trafficking of inflammatory cells (4).

L-arginine analogues are the pharmacological agents most commonly used to inhibit NO production, but have poor selectivity between NOS isoforms, thereby inhibiting iNOS and ecNOS during inflammatory insult, the latter resulting in a reduction of basal blood flow. In the majority of experiments, L-arginine analogues were given systemically, resulting in an amelioration of inflammation (5–7), but in some cases these anti-inflammatory actions were reversed by vasodilators (8, 9). Therefore, we suspect that NOS inhibitors, administered systemically, will inhibit ecNOS, resulting in vasoconstriction, followed by a reduction in blood delivery to the inflamed site. This sequence of events may ultimately lead to reduced cellular diapedesis and exudation and thus be interpreted as anti-inflammatory.

To test the hypothesis that NOS inhibitors will have different effects on inflammation depending on their route of administration, we used the carrageenin-induced pleurisy. This is a well-characterized model of acute inflammation in which iNOS activity, protein expression, and nitrite production peak between 1 and 6 h after carrageenin injection (10). The present study was designed to investigate the effects on inflammation of NOS inhibitors administered locally to the inflammatory site and to compare their effects with NOS inhibitors given systemically. To this end, we used S-(2-aminoethyl) isothiourea (AE-ITU) (11) and N-(3-(aminomethyl)benzyl)acetamidine (1400W) (12), which are structurally different and the most selective iNOS inhibitors commercially available, as well as the more selective ecNOS inhibitor, L-NAME (1-iminoethyl)-ornithine (L-NAME) (13). These drugs were administered into the pleural or peritoneal cavity immediately before the establishment

Department of Experimental Pathology, The William Harvey Research Institute, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, London, United Kingdom

Received for publication June 28, 2000. Accepted for publication October 11, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The work presented in this paper was supported by the Arthritis Research Campaign, United Kingdom.

2 Address correspondence and reprint requests to Dr. Derek W. Gilroy, Department of Experimental Pathology, The William Harvey Research Institute, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1 M 6BQ, U.K. E-mail address: d.w.gilroy@mds.qmw.ac.uk

3 Abbreviations used in this paper: NOS, NO synthase; AE-ITU, S-(2-aminoethyl)isothiourea; CINC, cytokine-induced neutrophil chemoattractant; CMP 48/80, compound 48/80; COX, cyclooxygenase; DPTA NoNoate, 3,3′-(hydroxynitrosohydrazio)nobiS-l-propanamine; ecNOS, endothelial cell NOS; inNOS, inducible NOS; iNOS, inducible NOS; L-NAME, (1-iminoethyl)-ornithine (10 mg/kg) not only exacerbated inflammation at the very early stages of the lesion (1–6 h), but also prevented inflammatory resolution. By contrast, administering NOS inhibitors systemically ameliorated the severity of inflammation throughout the reaction. To elucidate the mechanisms by which inhibition of NO synthesis locally worsened inflammation, we found an increase in histamine, cytokine-induced neutrophil chemoattractant, superoxide, and leukotriene B4 levels at the inflammatory site. In conclusion, this work shows that the local production of NO is protective by virtue of its ability to regulate the release of typical proinflammatory mediators and, importantly, that NOS inhibitors have differential anti-inflammatory effects depending on their route of administration. The Journal of Immunology, 2001; 166: 1169–1177.

The signaling molecule NO is produced by the enzyme NO synthase (NOS)3 after activation. NOS is constitutively expressed in endothelial (ecNOS) and neuronal cells (neuronal NOS), while a third isoform (iNOS) is induced in response to inflammatory-like stimuli and is capable of sustained production of high levels of NO that is predominant in inflammation (for review, see Ref. 1). Although the cytostatic/cytocidal activity of NO forms part of the host defense mechanism, the excessive or inappropriate production of NO can lead to tissue damage possibly through the formation of the potent oxidizing and nitrating agent, peroxynitrite, by a coupling of NO with superoxide (O$_{2}^{-}$) (2).

NO generated from ecNOS has been reported to have both pro- and anti-inflammatory properties. Under physiological conditions, NO released from the endothelium regulates vascular tone and maintains vessel patency by helping prevent platelet aggregation and down-regulating adhesion molecule expression (3). However, mediators released during the acute phase of inflammation, including histamine, 5-hydroxytryptamine, bradykinin, platelet-activating factor, and substance P, evoke the release of endothelial NO, causing vasodilatation and vascular permeability, thus facilitating edema formation and trafficking of inflammatory cells (4).

L-arginine analogues are the pharmacological agents most commonly used to inhibit NO production, but have poor selectivity between NOS isoforms, thereby inhibiting iNOS and ecNOS during inflammatory insult, the latter resulting in a reduction of basal blood flow. In the majority of experiments, L-arginine analogues were given systemically, resulting in an amelioration of inflammation (5–7), but in some cases these anti-inflammatory actions were reversed by vasodilators (8, 9). Therefore, we suspect that NOS inhibitors, administered systemically, will inhibit ecNOS, resulting in vasoconstriction, followed by a reduction in blood delivery to the inflamed site. This sequence of events may ultimately lead to reduced cellular diapedesis and exudation and thus be interpreted as anti-inflammatory.

To test the hypothesis that NOS inhibitors will have different effects on inflammation depending on their route of administration, we used the carrageenin-induced pleurisy. This is a well-characterized model of acute inflammation in which iNOS activity, protein expression, and nitrite production peak between 1 and 6 h after carrageenin injection (10). The present study was designed to investigate the effects on inflammation of NOS inhibitors administered locally to the inflammatory site and to compare their effects with NOS inhibitors given systemically. To this end, we used S-(2-aminoethyl) isothiourea (AE-ITU) (11) and N-(3-(aminomethyl)benzyl)acetamidine (1400W) (12), which are structurally different and the most selective iNOS inhibitors commercially available, as well as the more selective ecNOS inhibitor, L-NAME (1-iminoethyl)-ornithine (L-NAME) (13). These drugs were administered into the pleural or peritoneal cavity immediately before the establishment

Department of Experimental Pathology, The William Harvey Research Institute, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, London, United Kingdom

Received for publication June 28, 2000. Accepted for publication October 11, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The work presented in this paper was supported by the Arthritis Research Campaign, United Kingdom.

2 Address correspondence and reprint requests to Dr. Derek W. Gilroy, Department of Experimental Pathology, The William Harvey Research Institute, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1 M 6BQ, U.K. E-mail address: d.w.gilroy@mds.qmw.ac.uk

3 Abbreviations used in this paper: NOS, NO synthase; AE-ITU, S-(2-aminoethyl)isothiourea; CINC, cytokine-induced neutrophil chemoattractant; CMP 48/80, compound 48/80; COX, cyclooxygenase; DPTA NoNoate, 3,3′-(hydroxynitrosohydrazino)bis-l-propanamine; ecNOS, endothelial cell NOS; inNOS, inducible NOS; iNOS, inducible NOS; L-NAME, (1-iminoethyl)-ornithine; L-NMMA, (S)-monomethyl-L-arginine; LTB4, leukotriene B4; O$_{2}^{-}$, superoxide; PMN, polymorphonuclear cell; SSA, superoxide-scavenging activity; TAOS, total antioxidant status; 1400W, N-(3-(aminomethyl)-benzyl)acetamidine.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00
of a carrageen-induced pleurisy. Edema formation, infiltrating inflammatory cell numbers, and nitrite levels in exudates were measured at various times throughout the inflammatory process.

It was found that NOS inhibitors administered locally exacerbated inflammation and prolonged resolution as a result of an increase in the proinflammatory mediators histamine, leukotriene B4 (LTB4), O2−, and cytokine-induced neutrophil chemoattractant (CINC), suggesting that the local production of NO is protective. By contrast, however, giving NOS inhibitors systemically ameliorated inflammation, thereby showing differential anti-inflammatory properties of NOS inhibitors depending on their route of administration.

Materials and Methods

Induction of carrageenin-induced pleurisy and measurement of inflammatory parameters

Male Wistar rats (180 ± 20 g; T. Tuck and Sons, Battles Bridge, U.K.) were housed with a 12-h light-dark cycle and allowed access to food and water ad libitum. Animals were anesthetized with halothane and injected with 0.15 ml 1% carrageenin in saline into the pleural cavity. Pleural exudates were collected with or without lavage (0.5 ml of 3.15% tri-sodium citrate in saline) at various time points postcarrageenin injection. Blood-contaminated exudates were rejected. Exudate volumes were quantified and inflammatory cells counted (Dmm coulter-counter; Coulter Electronics, Luton, U.K.). Samples were centrifuged (800 × g, 10 min, 4°C) to separate inflammatory cells from exudate, both of which were then stored at −70°C for subsequent procedures.

Drug administration

Animals (n = 6–8 per group) were injected intrapleurally with either 3,3′-hydroxynitrosohydrazino-bis-l-propanamine (DPTA) NoNoate, AE-ITU, 1400W, t-NIO, or saline immediately before intrapleural injection of carrageenin. For experiments examining the systemic effects of NOS inhibitors, AE-ITU or N,N′-monomethyl-L-arginine (t-NMMA) was injected into the peritoneal cavity immediately before carrageenin injection into the pleural cavity.

Mast cell depletion

Rats were depleted of mast cells using compound 48/80 CMP 48/80, as previously described (14). Briefly, CMP 48/80 (0.1% w/v) was injected i.p. twice daily for 4 days (0.6 mg/kg for the first six injections and 1.2 mg/kg for the final two). Carrageenin-induced pleurisy was induced 6 h after the last injection of CMP 48/80.

Western blot analysis

Cell pellets were sonicated on ice in protease-inhibitory buffer (0.05 M Tris, 1 mM PMSF, 1.5 mM pepstatin A, 0.2 mM leupeptin) and centrifuged (4000 × g, 5 min, 4°C). Protein concentrations of the supernatants were determined by the Bradford assay. Samples were equilibrated for protein (1 mg/ml), mixed 1:1 with 2× Laemmli buffer (125 mM Tris base, 2 mM EDTA, 10% mercaptoethanol, 4% SDS, 20% glycerol, and 0.1% Coomassie brilliant blue, pH 6.8), and boiled for 5 min. Samples (30 μg/lane) and molecular weight markers were resolved by gel electrophoresis on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. iNOS protein was detected using a specific polyclonal rabbit anti-mouse Ab (1:5000), which does not cross-react with either eNOS or neuronal NOS (15). The signal was amplified with a HRP-linked goat anti-rabbit polyclonal Ab and visualized on x-ray film using ECL chemiluminescence reagents.

Measurement of iNOS activity in inflammatory cells

iNOS activity was measured as the ability of inflammatory cells to catalytically convert [3H]-arginine to [3H]-citrulline. The reaction mixture consisted of NADPH (1 mM), tetrahydrobiopterin (5 μM), calmodulin (300 U/ml), EGTA (1 mM), and valine (1 mM). Samples were homogenized and added to each reaction tube. The reaction was initiated by the addition of 10 μl of t-arginine/[3H]-arginine at a final concentration of 10 μM and at a ratio of 1000:3 pmol of t-arginine/[3H]-arginine. Samples were incubated at 37°C for 30 min, and the reaction was terminated by the addition of ice-cold stop buffer (2 mM EGTA, 2 mM EDTA in 20 mM HEPES, pH 5.5). Samples were eluted through Dowex cationic exchange resin columns with stop buffer and collected in scintillation vials. Scintillation fluid was added to each vial, and the amount of radioactive t-citrulline was determined in a liquid scintillation counter. The protein concentration of samples was measured and NOS activity expressed as pmol t-citrulline/mg protein/30 min.

Cyclooxygenase (COX) activity in inflammatory cells

Cell pellets were sonicated on ice in protease-inhibitory buffer and incubated (37°C, 30 min) in the presence of arachidonic acid (30 μM), glutathione (5 mM), and adrenalin (5 mM). The reaction was terminated by boiling, and samples were then centrifuged (10,000 × g, 30 min). PGE2 in the supernatant was measured by RIA, and the results were expressed as ng PGE2/mg protein/30 min.

Measurement of PGE2 and LTB4 in cell-free exudates

PGE2 and LTB4 were measured by enzyme immunoassay and 6-keto PGF1α (as the stable breakdown product of PGI2) by RIA. Results were expressed as quantity (pg) of each eicosanoid in the total volume of cell-free exudate. Histamine levels were measured by RIA and results expressed as total histamine (ng) in the total volume of exudate. CINC levels were measured using a mouse Kc ELISA that had cross-reactivity with rat rCINC-1 protein, and results expressed as total amount of CINC (pg) in total volume of exudate.
Nitrite levels in cell-free exudates

Nitrite formation was measured by a modification of the Greiss reaction. Briefly, 10 μl of NADPH (10 μM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.16 U), and PBS (10 mM) were added to cell-free exudate in a 96-well plate. Nitrate was converted to nitrite by addition of 10 μl (0.08 U) nitrate reductase and incubated for 45 min. A total of 200 μl of Greiss reagent (equal volumes of 1% w/v sulfanilamide in 5% H₃PO₄ and 0.1% w/v N-(1-napthyl)ethylenediamine) was added and incubated for an additional 10 min. Nitrite concentrations were determined at 570 nm with a reference filter at 620 nm and results expressed as μM nitrite in cell-free exudate.

FIGURE 2. Effects of NOS inhibitors injected locally on inflammation at 1 h. Either AE-ITU, 1400W, or L-NIO was injected directly into the pleural cavity of rats immediately before carrageenin injection. Thereafter, their effects on exudate volume (a), cell number (b), and nitrite (c) in pleural exudates were determined 1 h after carrageenin injection. Data are expressed as mean ± SEM (n = 6 – 8 per group). *p ≤ 0.05 in comparison with saline controls.

Total antioxidant status in cells and cell-free exudate

Exudate and plasma total antioxidant status (TAOS) was measured as previously described (16). Briefly, the reaction mixture consisted of (final concentration): 20 μM 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS⁺) accumulation, was determined using an Anthos Labtech type plate reader (Austria). Experiments were conducted in triplicate on both cell-free inflammatory exudate as well as inflammatory cells. For the latter determinant, values were adjusted for protein concentration.

Superoxide-scavenging activity in cells and cell-free exudate

Superoxide-scavenging activity (SSA) was measured as previously described (17). Briefly, the reaction mixture consisted of (final concentration): 50 μM ferricyctochrome c (50 μM), 10 μM xanthine oxidase (20 μM/ml), 20 μM xanthine (100 μM), and 5–10 μl sample with the volume being made up to 100 μl with PBS (pH 7.4). The reaction was initiated by the addition of xanthine and conducted at 37°C. The increase in absorbance at 550 nm was measured over a 3-min period at 30-s intervals using an Anthos Labtech type plate reader (Salzburg, Austria). All determinants of initial reaction rates were made with and without sample at least in triplicate.
Materials

Carrageenin (Viscarin) was obtained from FMC (Rockland, ME); LTB₄, PGE₂, enzyme immunoassay kits, [³H]PGE₂, [³H]6-keto PGF₁α, and [³H]l-arginine were purchased from Amersham International (Buckinghamshire, U.K.). Mouse K₂商量 Quantikine immunoassay kit was obtained from R&D Systems (Oxon, U.K.). AE-ITU, 1400W, and l-NIO were purchased from Alexis (Nottingham, U.K). iNOS Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma (Poole, U.K.).

Data analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using a one-way Kruskal-Wallis nonparametric test, followed by a Mann-Whitney U test. A p value of ≤0.05 was considered to be statistically significant.

Results

Profile of iNOS expression and NO production in the carrageenin-induced pleurisy

In this model, polymorphonuclear cells (PMNs) are the principal cell type up to 12 h after carrageenin injection and are replaced by phagocytosing mononuclear cells that predominate up to resolution at 48 h. NO, as determined by measuring nitrite in the cell-free inflammatory exudate, was initially high 0.5–1 h after carrageenin injection (Fig. 1a). Thereafter, NO levels declined but increased again at 6 h in parallel with inflammatory cell iNOS enzyme activity. Neither NO nor iNOS activity was detectable during resolution at 48 h. In the inflammatory cells from the pleural cavity, there was a peak in iNOS protein expression at 6 h, mirroring iNOS activity (Fig. 1b), which gradually declined but increased again at inflammatory resolution at 48 h. This finding is similar to the recent report that COX 2 is also expressed in inflammatory mononuclear cells at resolution in this model (18), and further studies are being conducted to examine the relevance of this apparently enzymatically inactive iNOS during resolution.

Effects of administering NOS inhibitors into the pleural cavity at 1 h

As the initial peak in exudate nitrite levels was detectable at 1 h, this would suggest that the principal source of NO at this time was from the postcapillary endothelial cells lining the pleural cavity and not from an inducible source. For our first experiments, to determine the contribution of NO to the developing inflammatory response, we investigated the effects on inflammation of administering NOS inhibitors directly into the pleural cavity immediately before carrageenin injection. We found that AE-ITU at 3 and 10 mg/kg significantly increased exudate volume and inflammatory cell influx compared with untreated controls (Fig. 2, a and b). 1400W also significantly elevated exudate volume, but was without effect on cell numbers (Fig. 2, a and b). Both inhibitors reduced nitrite levels significantly at the higher dose used (Fig. 2c). Given their lack of selectivity, it is likely that AE-ITU and 1400W also inhibited ecNOS at the dosing levels used. Finally, in addition to reducing exudate nitrite levels, l-NIO (10 mg/kg) increased exudate volume compared with controls, whereas inflammatory cell numbers were unchanged (Fig. 2a–c).

FIGURE 4. Effects on pleural inflammation of administering an NO donor locally. DPTA NoNoate was injected directly into the pleural cavity of rats immediately before carrageenin. Six hours after carrageenin injection, the effects of this slow releasing NO donor were examined on exudate volume (a) and cell number (b) in pleural exudates. Data are expressed as mean ± SEM (n = 8 per group). *，p ≤ 0.05 in comparison with saline controls.

FIGURE 5. Effects of early NOS inhibition on inflammatory resolution in the rat carrageenin-induced pleurisy. AE-ITU was injected directly into the pleural cavity of rats immediately before carrageenin injection. Seventy-two hours later, at resolution, the consequence of early NOS inhibition was examined on exudate volume (a) and cell number (b). Data are expressed as mean ± SEM (n = 6 per group). *，p ≤ 0.05 in comparison with saline controls.
Effects of administering NOS inhibitors into the pleural cavity at 6 h

From these studies, it appears that the initial peak of NO generation at 1 h is anti-inflammatory. We therefore proceeded to examine whether the second peak of NO generation, apparently from iNOS, at 6 h has a similarly protective role. To this end, the effects of intrapleural injection of AE-ITU, 1400W, and L-NIO immediately before the establishment of a carrageenin pleurisy were determined. These experiments also showed that at doses that inhibited NO generation, NOS inhibitors significantly worsened inflammation by increasing pleural exudates and leukocyte influx into the pleural cavity in comparison with saline controls (Fig. 3, a–c). By contrast, in a parallel experiment, it was found that a slow-releasing NO donor (DPTA NOoate), administered locally at the time of carrageenin injection, reduced inflammatory cell influx (Fig. 4).

We then questioned whether this exacerbated inflammation, brought about as a consequence of NOS inhibition, represented a transient increase at this early phase or whether in fact it resulted in an eventual delay in inflammatory resolution. To discern this, AE-ITU was administered into the pleural cavity immediately before carrageenin injection (as above), and its effects on inflammation were determined at exudate volume (a), cell number (b), and nitrite (c) in pleural exudates (Fig. 5). Thus, from these experiments it appears that NO plays a critical role in determining the outcome of an inflammatory response.

Effects of NOS inhibitors on carrageenin-induced pleurisy when administered systemically

The fundamental question addressed in this study is whether there is a differential effect on inflammation by administering NOS inhibitors locally vs systemically. To determine this, we examined the effects on inflammation at 6 and 36 h of AE-ITU and L-NMMA when injected into the peritoneal cavity at the time of carrageenin injection. In keeping with the bulk of data published on the systemic effects of NOS inhibitors on inflammation, both drugs tested significantly reduced inflammation at 6 h (Fig. 6, a and b) at doses that inhibited inflammatory exudate nitrite levels (Fig. 6c) and at 36 h (Fig. 7, a and b), in which nitrite was undetectable. In contrast, AE-ITU and L-NMMA, when injected locally into the pleural cavity immediately before carrageenin injection, increased exudate volume and inflammatory cell number at 6 h (Fig. 2, a and b) and 36 h (Fig. 8, a and b). These findings present highly conflicting outcomes on inflammation depending on whether NOS inhibitors are administered either locally or systemically.

Effect of NOS inhibitors on pleural inflammation in the absence of carrageenin

To show that AE-ITU (3 mg/kg) and 1400W (10 mg/kg) did not exacerbate inflammation simply as a result of direct irritation, we injected these drugs, as well as saline, into the pleural cavity in the absence of carrageenin. Neither drug caused measurable exudate formation or an increase in cellular influx (data not shown). Moreover, cationic compounds, including NOS inhibitors, may cause nonspecific mast cell degranulation. Therefore, Nω-nitro-D-arginine-methyl ester, the inactive enantiomer of the nonspecific NOS

**FIGURE 6.** Effects of NOS inhibitors injected systemically on a rat carrageenin-induced pleurisy at 6 h.Either AE-ITU or L-NMMA was injected into the peritoneal cavity just before intrapleural carrageenin injection. Six hours later, the effects of these NOS inhibitors were determined on exudate volume (a), cell number (b), and nitrite (c) in pleural exudates. Data are expressed as mean ± SEM (n = 8 per group). *p ≤ 0.05 in comparison with saline controls.

**FIGURE 7.** Effects of NOS inhibitors injected systemically on a rat carrageenin-induced pleurisy at 36 h. Either AE-ITU or L-NMMA was injected into the peritoneal cavity just before intrapleural carrageenin injection. Thirty-six hours later, the effects of these NOS inhibitors were determined on exudate volume (a), cell number (b), and nitrite (c) in pleural exudates. Data are expressed as mean ± SEM (n = 8 per group). *p ≤ 0.05 in comparison with saline controls.
inhibitor L-NAME, was injected intrapleurally at the same molarity as AE-ITU (10 mg/kg) and caused no increase in inflammatory parameters (data not shown). As a final control experiment, using the MTT assay for the assessment of cell viability, we found that neither AE-ITU nor L-NMMA, when injected intrapleurally, caused toxicity to influxing inflammatory cells in the pleural cavity (data not shown).

Mechanisms by which NOS inhibition may exacerbate inflammation

In an attempt to elucidate the mechanisms by which inhibition of NO synthesis worsens inflammation, we measured levels of the most likely proinflammatory mediators after treatment with AE-ITU. The first candidate for investigation, histamine, was measured in cell-free exudates at 1 h, and levels were correlated with mast cell numbers (the cellular source of histamine) in pleural exudates. AE-ITU (10 mg/kg) significantly increased histamine (Fig. 9a), while reducing mast cell numbers by 72%, presumably as a consequence of increased cellular degranulation. Moreover, in animals previously depleted of mast cells using CMP 48/80, the increase in exudate volume observed at 1 h after treatment with AE-ITU was partially attenuated (compare Fig. 2a with 9b) at dosing levels that significantly reduced exudate nitrite levels (Fig. 9d). Although there was an expected reduction in exudate formation in animals treated with CMP 48/80 alone (Fig. 9b), there was no change in cell numbers (Fig. 9c). This observation is consistent with the fact that histamine mediates edema formation and not cell migration.

Another candidate for investigation was CINC, a potent neutrophil chemoattractant (19). Although in control animals levels peaked at 1 h (Fig. 10a), the intrapleural injection of AE-ITU (3, 10 mg/kg) caused a significant increase in CINC in cell-free pleural exudates (Fig. 10b). An additional explanation for exacerbated inflammation after NOS inhibition could be enhanced eicosanoid synthesis, particularly as NO has been suggested to modulate COX activity (for review, see Ref. 20). Thus, given the proinflammatory properties of eicosanoids and their abundance in the carrageenin-induced pleurisy up to 6 h (10), we examined the effects of NOS inhibition on inflammatory cell COX activity as well as cell-free exudate levels of PGE2, 6-keto PGF1α (Table I), and the neutrophil chemoattractant, LTB4. Surprisingly, AE-ITU (3 mg/kg) had no effect on either COX activity in inflammatory cell pellets or levels of PGE2 and 6-keto PGF1α. Interestingly, however, exudate levels of LTB4 were significantly increased in comparison with controls (Table I).

Effects of NOS inhibition on TAOS and SSA in inflammatory cells and cell-free exudates

In a series of final experiments, TAOS and SSA activity were measured as an indirect indication of O2− and other oxidant species formation. These assays are based on the principle that oxidants formed during inflammation (21) react with antioxidants such as glutathione, ascorbic acid, and α-tocopherol and reduce the
inflammatory cells and cell-free inflammatory exudate. Data are expressed as mean ± SEM (n = 6–8 per group). *p ≤ 0.05 in comparison to saline controls.

**Discussion**

In this study, we demonstrate that in acute inflammation the administration of NOS inhibitors directly into the inflamed site exacerbated the inflammatory response and prolonged resolution. Investigating the mechanisms that may account for this worsened inflammatory response, it was found that by inhibiting NO generation there was a corresponding increase in histamine, LTB₄, and reactive oxygen species, suggesting that during inflammation the local production of NO is protective by virtue of its ability to modulate levels of these proinflammatory mediators. By contrast, however, we found that administering NOS inhibitors systemically reduced inflammation, thereby demonstrating that NOS inhibitors have differential anti-inflammatory effects depending on their route of administration.

The vast majority of reports have shown that NOS inhibitors are considered anti-inflammatory in models of both acute and chronic inflammation. In a model of trinitrobenzene sulfonic acid-induced colitis in rats, L-NAME reduced neutrophil and macrophage influx (23), while in a carrageenin-induced pleurisy, L-NMMA also significantly reduced exudate formation and cellular influx (6). In these studies, however, NOS inhibitors were administered at high doses away from the site of inflammation, i.e., orally or i.p. Given their lack of selectivity, it is conceivable that systemic administration may also inhibit ecNOS remote from the inflammatory locus, resulting in vasoconstriction, reduced blood delivery to the inflamed site, and, thus, a reduction in inflammation. Indeed, it was shown that the anti-inflammatory effects of L-arginine analogues, given systemically, could be reversed by vasodilators (8, 9, 24). In agreement with these studies, we also found that when NOS inhibitors were injected i.p., immediately before intrapleural injection of carrageenin, pleural exudates were significantly reduced. Therefore, to bypass the systemic effects of NOS inhibitors, we administered AE-ITU, 1400W, and L-NIO locally and found them to exert a proinflammatory effect, without altering inflammatory cell viability. In support of our findings, others have also reported a protective role for NO. For instance, acetic acid-induced colitis in iNOS-deficient mice resulted in increased PMN-associated tissue damage in comparison with wild-type animals (25). A similar inflammatory cell accumulation in hepatic microvasculature was also observed in LPS-treated iNOS knockout mice (26). Thus, the protective effects of NO shown in these genetically modified animals may arise from the absence of only iNOS at the inflammatory site with ecNOS being functionally active, thereby avoiding systemic perturbations.

To elucidate the mechanism by which inhibition of NO generation exacerbates inflammation, we measured levels of a number of candidate proinflammatory mediators. As shown by others (27), mast cell-derived histamine peaked in this model between 0.5 and 1 h after carrageenin injection. Given that histamine mediates edema formation (27) and that NO stabilizes mast cells, thereby preventing histamine release (28), levels of this acute inflammatory mediator were measured after NOS inhibition. We found that

### Table I. Effects of local NOS inhibition on eicosanoid production in the rat carrageenin-induced pleurisy at 6 h³

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>AE-ITU</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX activity (ng/mg protein/30 min)</td>
<td>4.4 ± 1.4</td>
<td>5.8 ± 1</td>
</tr>
<tr>
<td>PGE₂ (pg)</td>
<td>637 ± 87</td>
<td>852 ± 77</td>
</tr>
<tr>
<td>6-ketoPGF₁α (pg)</td>
<td>1631 ± 364</td>
<td>1483 ± 328</td>
</tr>
<tr>
<td>LTB₄ (pg)</td>
<td>64 ± 7</td>
<td>91 ± 10*</td>
</tr>
</tbody>
</table>

³ AE-ITU (3 mg/kg) was injected into the pleural cavity of rats immediately prior to carrageenin injection. Six hours later, levels of eicosanoids were determined in the cell-free inflammatory exudate. Data are expressed as mean ± SEM (n = 6–8 per group). *p ≤ 0.05 in comparison to saline controls.

### Table II. Effect of NOS inhibition on TAOS of inflammatory cells and cell-free exudates in the carrageenin-induced pleurisy at 1 h and 6 h⁴

<table>
<thead>
<tr>
<th></th>
<th>Cell (µM l-ascorbate equivalent antioxidant capacity/mg protein)</th>
<th>Exudate (µM l-ascorbate equivalent antioxidant capacity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Control</td>
<td>1997 ± 366</td>
<td>466 ± 57</td>
</tr>
<tr>
<td>AE-ITU (3 mg/kg)</td>
<td>288 ± 73*</td>
<td>73 ± 12*</td>
</tr>
<tr>
<td>AE-ITU (10 mg/kg)</td>
<td>578 ± 86*</td>
<td>169 ± 32*</td>
</tr>
</tbody>
</table>

⁴ AE-ITU was injected into the pleural cavity of rats immediately prior to carrageenin injection. One and 6 h later, total antioxidant status was determined in both inflammatory cells and cell-free inflammatory exudate. Data are expressed as mean ± SEM (n = 8–10 per group). *p ≤ 0.05 in comparison to saline controls.
while histamine was increased, mast cell numbers decreased in pleural exudates. Additional experiments showed that prior depletion of mast cells with CMP 48/80 attenuated plasma exudation observed with NOS inhibitors without altering the increase in inflammatory cells. In support of this observation, others have shown that inhibition of NO in mesenteric postcapillary venules increased inflammatory cell emigration, plasma extravasation, free radical-mediated tissue damage, mast cell degranulation, and histamine release (29).

As well as histamine, levels of CINC were also determined. CINC, which is induced through a NF-κB pathway in response to inflammatory stimulation (30), mediates PMN chemotaxis in the rat (19) by up-regulating PMN CD11/18 expression, thus facilitating PMN diapedesis (31). NOS inhibition increased levels of CINC in cell-free exudates and, in addition to histamine, may be a contributing factor to the increased inflammatory cell numbers recorded after NOS inhibition. Levels of lipid-derived mediators were also measured particularly as inhibition of NO synthesis increases endothelial PGL2 release (32) and PGE2. PGL2, and LT B4 facilitate edema formation and inflammatory cell influx. Treatment with NOS inhibitors locally at a time when iNOS protein expression and enzyme activity are maximal had no affect on either PGE2 or PGL2, but significantly increased LT B4. Previous experiments have also shown that NO donors dose dependently reduce LT B4 production from activated PMNs (33). Interestingly, differential cell counts revealed that AE-ITU at 6 h caused an increase in the proportion of PMNs, presumably as a result of an increase in CINC and LT B4. Collectively, these mechanistic studies demonstrate that NO generated at the inflammatory site critically regulates the severity of the inflammatory response by keeping in check levels of such potentially proinflammatory mediators as histamine, CINC, and LT B4.

As a final mechanism, we examined levels of O2− after NOS inhibition indirectly as a reduction in TAOS and SSA, and found that these indices of O2− and other oxidant species were reduced, suggesting that in the absence of NO, O2− generation was enhanced. O2− is produced by PMNs and macrophages from the enzyme activity of NADPH oxidase and xanthine oxidase at inflammatory sites. Both enzyme systems contain a heme prosthetic group with which NO can react to inhibit O2− release (34). Therefore, inhibiting NO removes the brakes on O2− production. In support of this notion, others have shown that NO generation reduces O2− levels, while its inhibition increases O2− production both in vitro and in vivo (35–37). As O2− has been associated with tissue damage and loss of function during inflammatory episodes (38), it is conceivable that one of the contributors to an enhanced inflammatory response, consequent to NOS inhibition, is O2− generation. Indeed, elevated levels of O2− increase histamine release from mast cells (39), as well as LT B4 and PMN accumulation in a model of pancreatitis through a platelet-activating factor-dependent mechanism (40). In addition, raised levels of O2− activate NF-κB (41), which may account for an elevated production of the chemokine CINC. Although no direct effect of O2− on the up-regulation of CINC has been demonstrated, an O2− scavenger N-acetylcysteine significantly reduced NF-κB DNA binding and CINC mRNA expression in inflamed lungs (42). Therefore, a disturbance in the balance between NO and O2− production may lead to an increase in proinflammatory mediators and provides a possible mechanism for the exacerbation of inflammation observed in this study.

In conclusion, in the rat carrageenin-induced pleurisy, inhibition of NO at the inflammatory site exacerbates inflammation and prolongs the pathology, suggesting a protective role for NO in this model. Moreover, NOS inhibitors appear to have differential effects on inflammation depending on their route of administration.

Acknowledgments

We thank P. T. Gunnarsson and M. J. Carrier for help with TAOS and SSA assays.

References
