

# Dual Inhibition of Nitric Oxide and Prostaglandin Production Contributes to the Antiinflammatory Properties of Nitric Oxide Synthase Inhibitors

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## Abstract

We have recently put forward the hypothesis that the dual inhibition of proinflammatory nitric oxide (NO) and prostaglandins (PG) may contribute to the antiinflammatory properties of nitric oxide synthase (NOS) inhibitors. This hypothesis was tested in the present study. A rapid inflammatory response characterized by edema, high levels of nitrites ( $\text{NO}_2^-$ , a breakdown product of NO), PG, and cellular infiltration into a fluid exudate was induced by the administration of carrageenan into the subcutaneous rat air pouch. The time course of the induction of inducible nitric oxide synthase (iNOS) protein in the pouch tissue was found to coincide with the production of  $\text{NO}_2^-$ . Dexamethasone inhibited both iNOS protein expression and  $\text{NO}_2^-$  synthesis in the fluid exudate ( $\text{IC}_{50} = 0.16 \text{ mg/kg}$ ). Oral administration of *N*-iminoethyl-L-lysine (L-NIL) or *N*<sup>G</sup>-nitro-L-arginine methyl ester ( $\text{NO}_2\text{Arg}$ ) not only blocked nitrite accumulation in the pouch fluid in a dose-dependent fashion but also attenuated the elevated release of PG. Finally, carrageenan administration produced a time-dependent increase in cellular infiltration into the pouch exudate that was inhibited by dexamethasone and NOS inhibitors. At early times, i.e., 6 h, the cellular infiltrate is composed primarily of neutrophils (98%). Pretreatment with colchicine reduced both neutrophil infiltration and leukotriene  $\text{B}_4$  accumulation in the air pouch by 98% but did not affect either  $\text{NO}_2^-$  or PG levels.

In conclusion, the major findings of this paper are that (a) selective inhibitors of iNOS are clearly antiinflammatory agents by inhibiting not only NO but also PG and cellular infiltration and (b) that neutrophils are not responsible for high levels of NO and PG produced. (*J. Clin. Invest.* 1995, 96:301–308.) Key words: nitric oxide • *N*-iminoethyl-L-lysine • prostaglandin • dexamethasone • carrageenan

## Introduction

Nitric oxide (NO)<sup>1</sup> is formed from the terminal guanidino nitrogen atom of L-arginine by nitric oxide synthase (NOS). Two

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1. Abbreviations used in this paper: cNOS, constitutive NOS; COX, cyclooxygenase; iNOS, inducible NOS; L-NIL, *N*-iminoethyl-L-lysine; NO, nitric oxide;  $\text{NO}_2\text{Arg}$ , *N*<sup>G</sup>-nitro-L-arginine-methyl ester; NOS, nitric oxide synthase.

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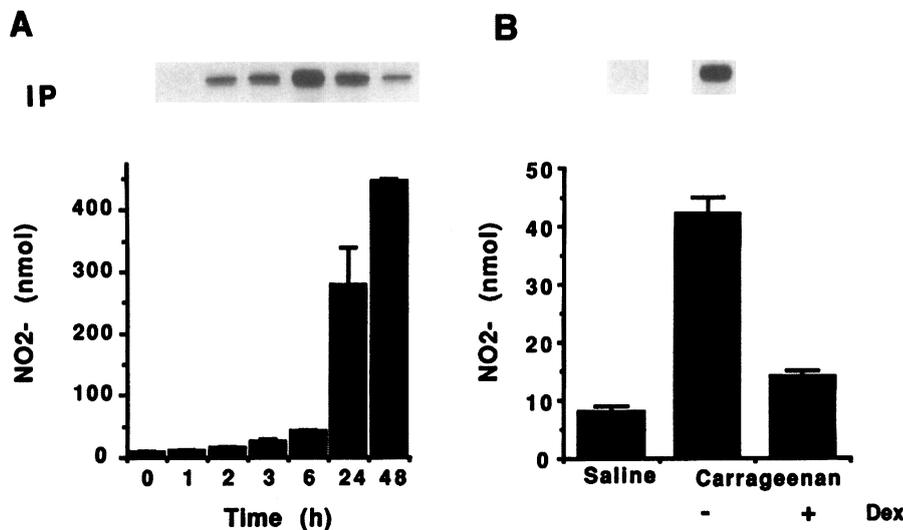
major forms of NOS have been identified (for review see reference 1). Under normal physiological conditions, the constitutive, calcium-dependent NOS isoform (cNOS) is present in numerous cells including endothelium and neurons (for review see reference 1). In an inflammatory setting or in the presence of endotoxin and cytokines, the inducible, calcium-independent NOS isoform (iNOS) is expressed in numerous cell types including endothelial cells, smooth muscle cells, and macrophages (for reviews see references 1 and 2). Production of low levels of NO from cNOS functions to regulate a number of homeostatic processes, whereas generation of larger quantities of NO from iNOS accounts for its proinflammatory and cytotoxic effects (for reviews see references 1 and 2).

Cyclooxygenase (COX; the enzyme that converts arachidonic acid to prostaglandins [PG]) is another critical enzyme in many inflammatory diseases. Two forms of cyclooxygenases have been identified (for reviews see references 3–5). The constitutive isoform (COX-1) is present in tissues such as the stomach, gut, or kidney, where PG production plays a cytoprotective role in maintaining normal physiological processes (for review see reference 3). In inflammatory processes, the inducible isoform of cyclooxygenase (COX-2) is expressed in many cells including fibroblasts and macrophages (6–9) and accounts for the release of large quantities of proinflammatory PG at the site of inflammation (for review see references 4 and 5). Production of high levels of proinflammatory PGs can be augmented further in the presence of NO. This effect of NO is probably due to its ability to activate the COX enzyme by an unknown mechanism (10, 11). In contrast, the inhibition of high levels of NO production by NOS inhibitors has been found to decrease the production of PG in *in vitro* and *ex vivo* models (10–15). These earlier findings allowed us to propose the hypothesis that dual inhibition of proinflammatory NO and PG may contribute to the antiinflammatory properties of NOS inhibitors. The rat air pouch is a convenient model to study acute and chronic inflammation (16, 17). This model was therefore used to test our hypothesis.

The results of our studies demonstrate that high levels of NO are produced after the induction of iNOS in the carrageenan-stimulated air pouch which contribute significantly to the inflammatory response as well as to the production of a portion of PGs and the cellular infiltration at the site of inflammation. Thus, inhibitors of NOS activity influence the course of inflammation by the dual inhibition of NO and PG and also by inhibiting cellular infiltration.

## Methods

*Air pouch model of inflammation.* Male Lewis rats weighing 175–200 grams were used throughout this study. Animals were fed normal chow and water *ad libitum*. Before experimental manipulation, the animals were anesthetized by inhalation of a mixture of  $\text{CO}_2/\text{O}_2$  (20:80). Air cavities were produced by the subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back. An additional 10 ml of air



**Figure 1.** Time-dependent accumulation of NO<sub>2</sub><sup>-</sup> in pouch fluid exudate after carrageenan administration and inhibition of NO<sub>2</sub><sup>-</sup> by dexamethasone. (A) The injection of carrageenan caused a time-dependent increase in NO<sub>2</sub><sup>-</sup> that reached a maximum within 2 d (bottom, each point is the mean ± SEM for n = 6 animals) which correlated with the appearance of iNOS protein as determined by immunoprecipitation (top). (B) The increase in NO<sub>2</sub><sup>-</sup> production observed at 6 h was completely abolished by dexamethasone (1 mg/kg orally, given 1 h before carrageenan; bottom, each point is the mean ± SEM for n = 6 animals). No immunoprecipitable iNOS protein was detected in pouch tissue taken from the dexamethasone-treated animals (top).

was injected into the cavity every 3 d to keep the space open. 7 d after the initial air injection, 2 ml of saline or of a 1% solution of carrageenan (Sigma Chemical Co., St. Louis, MO) dissolved in saline was injected directly into the pouch (17). Groups of six animals were killed at different time points (0, 3, 6, 24, and 48 h) after the injection of saline or carrageenan. The volume of exudate was measured, and the number of leukocytes present in the exudate was determined using a cell counter (Coulter Corp., Hialeah, FL). Differential cell counts were performed using Wright-Giemsa stain. The pouch exudates were assayed for PGE<sub>2</sub> by specific ELISAs (Cayman Chemical Co., Ann Arbor, MI) and for NO<sub>2</sub><sup>-</sup> using the diaminonaphthalene assay (18) as markers for COX and NOS activity, respectively. Data were expressed as total amounts of NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, or cells. The volume of the pouch fluid in saline-treated animals was ~1 ml and did not change over the 48-h time period; in the carrageenan-treated pouch this volume increased to 3 ml at 3 h and then remained fairly constant over the 48-h time period. When inhibitors were tested, compounds were administered twice daily by gavage (p.o., b.i.d.) with the initial treatment 2 h before the injection of carrageenan; six animals were used for each dose of drug tested.

**Colchicine treatment.** Animals received a single dose of colchicine (1.5 mg/kg, subcutaneously; references 17 and 19) 15 min before the injection of carrageenan. Rats were killed 6 h later, and the pouch fluid exudate was assayed for NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, leukotriene B<sub>4</sub>, and cellular infiltration. Colchicine treatment did not affect pouch fluid volume (3 ± 0.2 ml for carrageenan-injected rats and 3.1 ± 0.3 ml for carrageenan + colchicine-injected rats, n = 6).

**Metabolic labeling with [<sup>35</sup>S]methionine of cellular pouch exudates and determination of iNOS synthesis by immunoprecipitation.** At different time points after carrageenan administration, small pieces of pouch lining tissue were incubated for 4 h in methionine-free DME containing [<sup>35</sup>S]methionine (0.1 mCi/well, 10 mCi/ml; Amersham Corp., Arlington Heights, IL). Cells from the fluid exudate were also collected, separated by centrifugation, and plated into 35-mm plastic culture dishes. After a 30-min preincubation in methionine-free DME, the cells were incubated in the same medium containing [<sup>35</sup>S]methionine as described above. Solubilization and immunoprecipitation of the iNOS and COX-2 enzyme were performed as described previously using specific antibodies that recognized either iNOS or COX-2 (20, 21).

**Immunohistochemical localization of iNOS.** Immunohistochemical staining for iNOS was performed on cytospin preparations of cells (40,000 cells/slide) isolated from the pouch exudate and on 10-μm frozen sections of pouch lining tissues. Before preparing the cytospins, dead cells and debris were removed by centrifuging the cells at 300 g for 10 min through a layer of fetal bovine serum. Additionally, the cells obtained from the carrageenan pouch fluid were enriched for mononuclear cells from ~2–12% by separation using Ficoll-Hypaque. Cells and tissues were fixed with 1% paraformaldehyde, pH 7.2, for 5 min at

room temperature, followed by 100% ethanol, 5 min at 4°C. Nonspecific staining was blocked with 3% normal goat serum in 0.5 M Tris-HCl, pH 7.4, containing 1% Triton X-100 for 1 h at room temperature. All subsequent incubations were carried out in this buffer. Cells and tissue sections were incubated for 16 h at 4°C with a 1:1,000 dilution of either preimmune rabbit sera or the anti-iNOS antiserum described above. Endogenous peroxidase activity was then reduced with periodic acid (Zymed Laboratories, Inc., South San Francisco, CA) 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) for 2 h each. The reaction product was visualized using 3,3'-diaminobenzidine intensified with nickel chloride for 6 min. Cells and tissue sections were counterstained with Mayer's hematoxylin, mounted, and photographed using either Hoffman modulation optics or bright field microscopy.

**Histology.** Pouch tissue lining from saline- and carrageenan-treated rats was dissected free of underlying tissue and fixed in 10% neutral buffered formalin. Tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

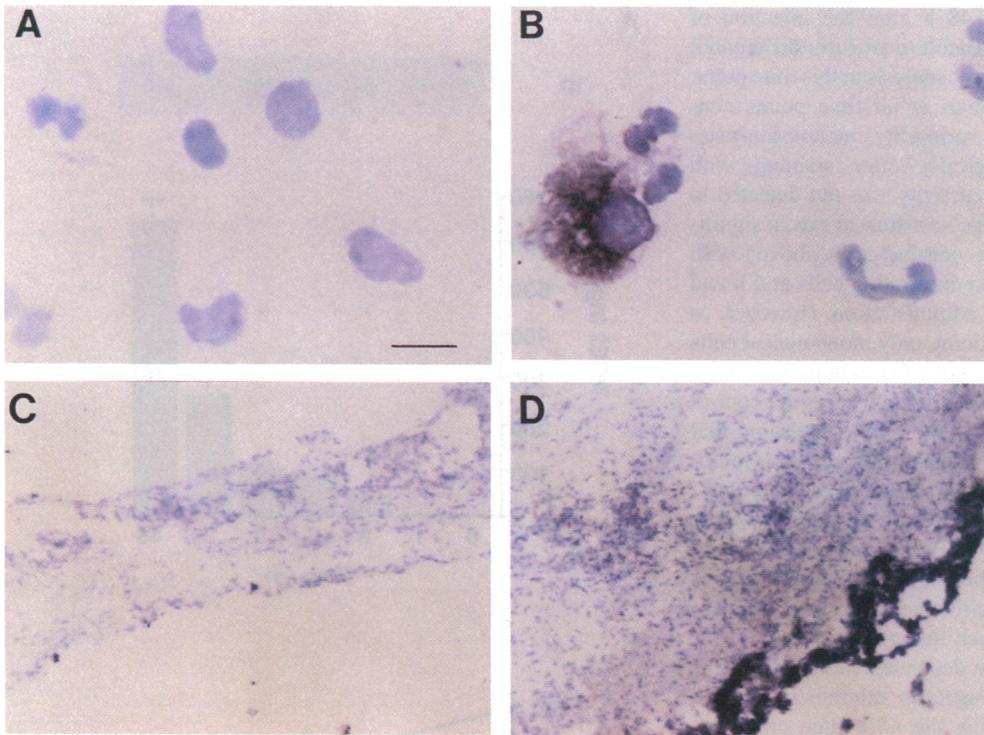
**Blood pressure measurements.** To determine the effects of the inhibitors on hemodynamic parameters, blood pressure measurements were performed both before and after drug administration using a IITC tail cuff blood pressure machine (Life Science Instruments, Woodland Hills, CA).

**Statistical analysis.** Results are expressed as mean ± SEM for (n) animals. The results were analyzed by Student's unpaired t test to determine the significant difference between means, or by a two-way ANOVA followed by a least significance procedure to determine the nature of this response. A P value of < 0.05 was considered significant.

**Materials.** Male Lewis rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). All animal protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines on laboratory animal welfare. 2,3-Diaminonaphthalene was purchased from Aldrich (Milwaukee, WI). All other chemicals and reagents were obtained from Sigma Chemical Co. N-iminoethyl-L-lysine (L-NIL) was synthesized in house as described previously (20).

## Results

**Time-dependent increase in NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cellular infiltration in the inflamed air pouch exudate.** Carrageenan induced a time-dependent increase in NO<sub>2</sub><sup>-</sup> in the pouch exudate fluid (Fig. 1 A); this effect was observed within 3 h and continued to increase during the 48-h time course studied. The increase in NO<sub>2</sub><sup>-</sup> accumulation in the carrageenan-treated pouch is likely

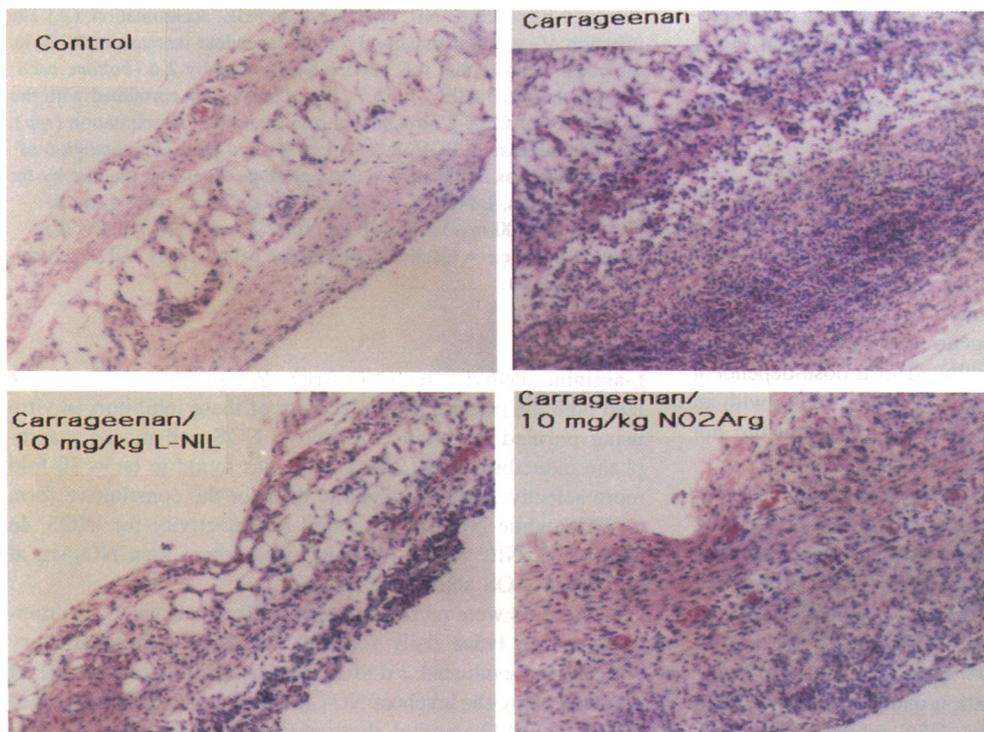


**Figure 2.** Immunohistochemical staining of iNOS in the carrageenan-induced rat air pouch. (A and B) Immunohistochemical staining of iNOS protein in cells from fluid exudates were obtained from control and carrageenan-treated rats. Cells were collected from the pouch exudates, centrifuged, fixed, and stained for iNOS using a specific antipeptide antibody. (A) Cells from a control pouch; (B) cells from carrageenan-treated rats; positive staining was found in macrophages (identified morphologically) but not in PMNs. (C and D) Staining of iNOS in pouch lining from control and carrageenan-treated rats. Frozen sections from the pouch linings were fixed and stained as above. (C) Control pouch; (D) carrageenan-treated pouch tissue with positive staining of iNOS in the inner layer of the tissue.

due to the activity of iNOS. Indeed, immunoprecipitation of pouch tissue with antibodies generated against iNOS indicated that iNOS protein was detected at 2 h after carrageenan, was maximal at 6 h, and then its synthesis tended to decline by 48 h (Fig. 1 A).  $\text{NO}_2^-$  synthesis in the pouch exudate from saline-treated animals did not change, remaining at a basal level of  $8 \pm 1 \text{ nmol}$  ( $n = 12$ ).

Carrageenan administration also caused a time-dependent

accumulation of leukocytes in the exudate (from  $0.9 \pm 0.03 \times 10^6$  leukocytes at time 0 to  $50 \pm 4$ ,  $67 \pm 3$ ,  $81 \pm 11$ , and  $342 \pm 51 \times 10^6$  leukocytes at 3, 6, 24, and 48 h after the administration of carrageenan). Cytospin preparations of cells obtained in the fluid exudate as well as frozen sections of pouch lining at 0 and 6 h after carrageenan injection were analyzed immunohistochemically to identify the cell type that expressed iNOS and COX-2. The high degree of cellular degranulation and cell death



**Figure 5.** Histology of the inflamed pouch after treatment with L-NIL or  $\text{NO}_2\text{Arg}$ . Pouch tissues were taken from saline-treated animals (Control), 2 d after carrageenan, 2 d after carrageenan in rats treated with L-NIL, and 2 d after carrageenan in rats treated with  $\text{NO}_2\text{Arg}$  (10 mg/kg).

observed in the fluid exudate at 48 h after the injection of carrageenan made it technically difficult to perform differential cell counts and immunohistochemical analysis at this time point. Exudates from saline-treated animals at all time points contained small numbers of cells, primarily monocytes/macrophages determined morphologically after staining with Wright's-Giemsa. iNOS immunoreactivity was not detected in these cells (Fig. 2 A). In the carrageenan-treated rats, a significant increase in cellular infiltrate occurred (see above) with PMNs accounting for > 98% of the infiltrating cells at 6 h and of 85% at 24 h after carrageenan administration. However, as shown in Fig. 2 B for the 6-h time point, only mononuclear cells in the exudate but not PMNs contained iNOS immunoreactivity. Immunohistochemical analysis of the pouch lining of control animals showed no iNOS staining (Fig. 2 C), suggesting that detectable iNOS enzyme was not induced during the development of the pouch tissue lining. In contrast, carrageenan induced the expression of iNOS-like immunoreactivity in cells of the inflamed pouch lining (Fig. 2 D). Based upon morphology and ED1 positive staining (not shown), iNOS induction was localized primarily to macrophage-like cells present in the superficial layer of the inflamed pouch lining.

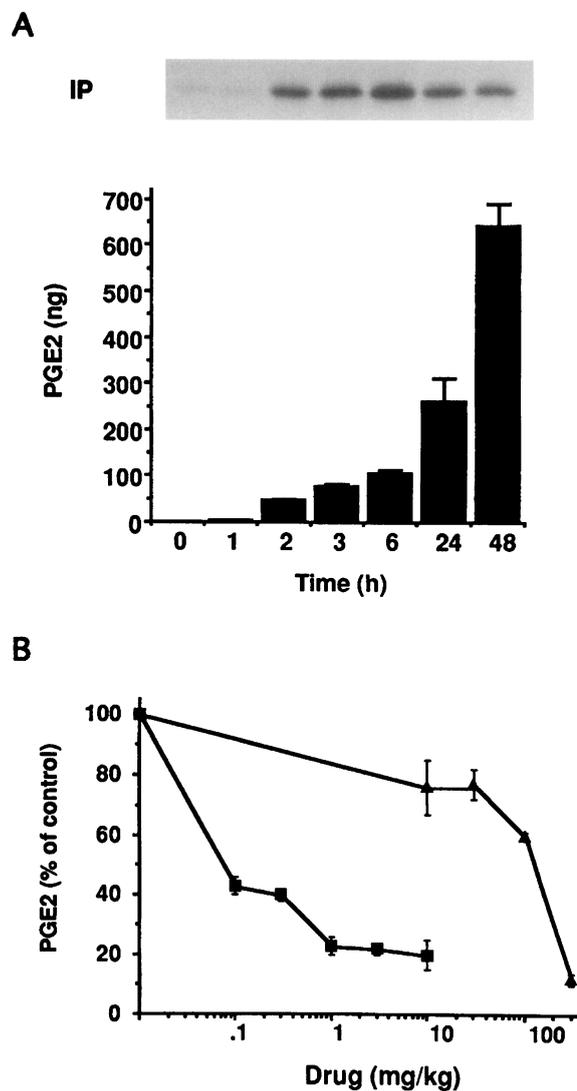
Carrageenan also elicited a time-dependent increase in PGE<sub>2</sub> production within 2 h after carrageenan administration and which continued to increase during the 48-h time course of the experiment (Fig. 3 A). COX-2 protein synthesis was also measurable by 2 h, was maximal after 6 h, and remained elevated for up to 48 h after carrageenan administration (Fig. 3 A).

**Effects of dexamethasone on NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cellular infiltration.** Glucocorticoids such as dexamethasone affect NO biosynthesis in vitro and in vivo by inhibiting the expression of iNOS without affecting cNOS synthesis or activity (22–24). The increase in NO<sub>2</sub><sup>-</sup> observed 6 h after carrageenan administration was inhibited by dexamethasone in a dose-dependent manner from 42±3 nmol NO<sub>2</sub><sup>-</sup> for carrageenan alone to 38±7, 20±1, and 14±1 nmol NO<sub>2</sub><sup>-</sup> for, respectively, carrageenan administered in combination with 0.01, 0.1, or 1 mg/kg dexamethasone, p.o., b.i.d. (*n* = 6 animals/group). As shown in Fig. 1 B, no immunoprecipitable iNOS protein was detected in pouch tissue from animals that received 1 mg/kg dexamethasone 1 h before carrageenan stimulation.

Dexamethasone also inhibits the induction of COX-2 in vitro and in vivo without affecting COX-1 (for reviews see references 4 and 5). As expected, the increase in PGE<sub>2</sub> release was inhibited in a dose-dependent manner by dexamethasone (46±3, 53±5, and 99±2% inhibition with, respectively, 0.01, 0.1, and 1 mg/kg, p.o., *n* = 6). Finally and as shown in Table I, the cellular infiltration which occurred during the 2 d after carrageenan administration was inhibited in a dose-dependent manner by dexamethasone (0.01–1 mg/kg, p.o., b.i.d.) with an IC<sub>50</sub> value of 0.04 mg/kg. Dexamethasone treatment did not affect the basal levels of NO<sub>2</sub><sup>-</sup> and PGE<sub>2</sub> (not shown).

In summary, the results shown in Figs. 1–3 indicate that iNOS and COX-2 are expressed in the inflamed air pouch after the administration of carrageenan and subsequently accounted for the large production of NO<sub>2</sub><sup>-</sup> and PG observed in this model.

**Effects of NOS inhibitors (L-NIL and NO<sub>2</sub>Arg) on NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cellular infiltration.** We next examined the role of NO in producing the inflammatory changes occurring in the pouch after carrageenan administration using a selective iNOS inhibitor, L-NIL, and a nonselective NOS inhibitor, N<sup>G</sup>-nitro-



**Figure 3.** Effects of L-NIL or NO<sub>2</sub>Arg on PGE<sub>2</sub> accumulation. (A) The injection of carrageenan caused a time-dependent increase in PGE<sub>2</sub> in the pouch exudate that was maximally elevated by 2 d (bottom, each point is the mean±SEM for *n* = 12 animals); this correlated with the appearance of COX-2 protein analyzed by immunoprecipitation (top). (B) The increase in PGE<sub>2</sub> levels observed 2 d after administration of carrageenan was inhibited in a concentration-dependent manner by the NOS inhibitors, L-NIL (0.1–10 mg/kg, p.o., b.i.d., for 2 d, ■) or NO<sub>2</sub>Arg (1–300 mg/kg, p.o., b.i.d., for 2 d, ▲). Each point is the mean±SEM for *n* = 6 animals. Results are expressed as PGE<sub>2</sub> (percentage of control).

L-arginine-methyl ester (NO<sub>2</sub>Arg). We previously characterized both the potency and selectivity of these inhibitors in vitro using purified rat brain constitutive NOS and mouse macrophage inducible NOS (25). L-NIL was found to be ~30-fold more selective for the inducible than for the constitutive form of NOS while NO<sub>2</sub>Arg exhibited no selectivity for iNOS. In addition, L-NIL was ~6.5-fold more potent than NO<sub>2</sub>Arg at inhibiting iNOS activity (25).

Inhibitors were administered 2 h before carrageenan administration and twice daily for 2 d, and their antiinflammatory effects were evaluated 2 d after carrageenan administration. At this time point, the levels of NO<sub>2</sub><sup>-</sup> in the pouch exudate increased from 8±1 to 442±6 nmol, those of PGE<sub>2</sub> increased from 1±0.02

Table I. Effects of Dexamethasone and NOS Inhibitors on Cellular Infiltration in the Pouch Exudate after Carrageenan

Dose mg/kg	Percent cell accumulation		
	Dexamethasone	L-NIL	NO <sub>2</sub> Arg
0	100	100	100
0.01	85±3	100	
0.1	40±2	82±3	
0.3	ND	70±2	100
1	10±2	69±3	75±2
3	ND	53±2	57±3
10	7±1	26±3	46±2
30		15±2	34±2
100			30±2
300			

The large accumulation of leukocytes seen 2 d after carrageenan injection was inhibited in a dose-dependent fashion by dexamethasone, L-NIL, or NO<sub>2</sub>Arg (drugs were given orally for two consecutive days). Results are expressed as percent cell accumulation when compared with cell accumulation obtained in the presence of carrageenan alone. Each point is the mean±SEM for *n* = 6 animals.

to 642±50 ng, and the number of infiltrating leukocytes increased from 0.9±0.03 to 342±51 × 10<sup>6</sup> cells. Fig. 4 shows that this marked increase in NO<sub>2</sub><sup>-</sup> was inhibited in a dose-dependent fashion by L-NIL (IC<sub>50</sub> = 0.7 mg/kg, *n* = 6) or NO<sub>2</sub>Arg (IC<sub>50</sub> = 9.8 mg/kg, *n* = 6). Interestingly, accumulation of PGE<sub>2</sub> in the pouch exudate was also inhibited in a dose-dependent manner by L-NIL (IC<sub>50</sub> = 0.2 mg/kg) or NO<sub>2</sub>Arg (IC<sub>50</sub> = 1.8 mg/kg) (Fig. 3 B). Finally, cellular infiltration was also inhibited by the NOS inhibitors (IC<sub>50</sub> = 2.9 mg/kg for L-NIL and 8.5 mg/kg for NO<sub>2</sub>Arg) (Table I). NO<sub>2</sub>Arg or L-NIL had no effect on the volume of pouch fluid (3±0.4 ml for carrageenan-injected rats and 2.8±0.4 and 3±0.2 ml for carrageenan-injected rats that received either 30 or 10 mg/kg NO<sub>2</sub>Arg or L-NIL, p.o., b.i.d., respectively, *n* = 6).

To assess the antiinflammatory effects of the NOS inhibitors, the pouch lining was also examined histologically. As shown in Fig. 5, the noninflamed, control pouch lining tissue consists of a layer of flattened cells overlying loose vascular connective tissue. Macrophages and fibroblasts comprise the inner layer of flattened cells (16). 2 d after carrageenan administration, marked inflammatory changes were observed including pronounced cellular infiltration and edema (evidenced by the thickening of the tissue). Treatment with the selective iNOS inhibitor, L-NIL (10 mg/kg, p.o., b.i.d.), significantly reduced these changes such that minimal edema and cellular infiltration were seen. NO<sub>2</sub>Arg (10 mg/kg, p.o., b.i.d.) also reduced the inflammatory changes although to a lesser extent than L-NIL. The potency of these compounds at inhibiting the histological changes (at the highest doses used) correlated well with their ability to inhibit NO<sub>2</sub><sup>-</sup> and PGE<sub>2</sub> production and cellular infiltration. At these doses, L-NIL caused a 97, 80, and 93% reduction in NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cell infiltration, respectively (*n* = 6), whereas NO<sub>2</sub>Arg caused a 58, 26, and 54% reduction in NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cell infiltration, respectively, in the pouch fluid exudate (*n* = 6). Therefore, L-NIL appears to be a more potent antiinflammatory agent than NO<sub>2</sub>Arg as assessed in this inflammatory model.

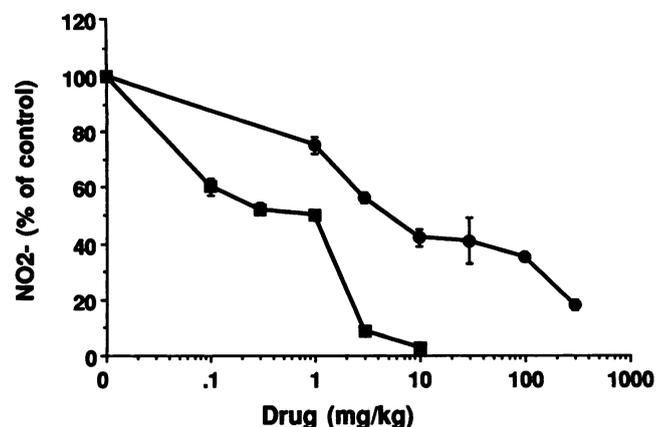


Figure 4. Effects of NOS inhibitors on nitrite accumulation in the air pouch. The increase in NO<sub>2</sub><sup>-</sup> levels observed in the air pouches after a 2-d treatment with carrageenan was inhibited in a concentration-dependent manner by the NOS inhibitors, L-NIL (0.1–10 mg/kg orally for 2 d, ■) or NO<sub>2</sub>Arg (1–300 mg/kg orally for 2 d, ●). Results are expressed as NO<sub>2</sub><sup>-</sup> (percentage of control). Each point is the mean±SEM for *n* = 6 animals.

**Effects of NS398 on NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cellular infiltration.** NS398, a selective COX-2 inhibitor (21, 26, 27), has been described recently as a potent antiinflammatory agent (21). Consistent with this finding, we found that the increase in PGE<sub>2</sub> production after carrageenan administration was inhibited by NS398 (by 78±6, 74±4, and 92±3% with 0.1, 1, and 10 mg/kg, p.o., b.i.d., respectively, *n* = 6). However, there was no correlation between the ability of NS398 to inhibit PGE<sub>2</sub> production and its ability to inhibit NO<sub>2</sub><sup>-</sup> levels and cellular infiltration. Thus, when compared with PGE<sub>2</sub> inhibition, NS398 produced only a small inhibition of NO<sub>2</sub><sup>-</sup> production (11±1, 16±8, and 28±8% with 0.1, 1, and 10 mg/kg, p.o., b.i.d., respectively, *n* = 6) and cellular infiltration (4±1, 1±0.4, and 10±6% with 0.1, 1, and 10 mg/kg, p.o., b.i.d., respectively, *n* = 6).

**Effects of colchicine on NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and cellular infiltration.** To determine whether the elevated NO<sub>2</sub><sup>-</sup> and PGE<sub>2</sub> concentrations in the pouch fluid exudate were dependent upon leukocyte infiltration, colchicine was used to prevent cellular infiltration into the exudate (17, 19). Analysis was performed only at the 6-h time point after carrageenan administration since at later time points colchicine was found to have toxic effects. Colchicine was given subcutaneously as a single dose (1.5 mg/kg) 15 min before carrageenan administration. Animals were killed 6 h later. Colchicine reduced cellular infiltration into the pouch fluid exudate by 98% (*n* = 6 animals), but did not inhibit either NO<sub>2</sub><sup>-</sup> or PGE<sub>2</sub> production (Fig. 6). In contrast, colchicine inhibited by at least 88% the increased production of leukotriene B<sub>4</sub> that occurred 6 h after the administration of carrageenan (from 0.06±0.008 to 0.8±0.03 and 0.1±0.01 ng/ml, *n* = 6, for, respectively, levels of leukotriene B<sub>4</sub> before carrageenan, 6 h after carrageenan, and 6 h after carrageenan in animals treated with colchicine).

**Hemodynamic effects.** The selective iNOS inhibitor, L-NIL, had no effect on blood pressure at the highest dose tested (30 mg/kg, p.o., b.i.d., for 2 d; from 101±3 to 97±14 mmHg, *n* = 4), indicating that it did not markedly affect the activity of constitutive NOS. In contrast and as expected, treatment with the nonselective NOS inhibitor, NO<sub>2</sub>Arg (300 mg/kg, p.o., b.i.d., for 2 d), caused a significant increase in mean arterial

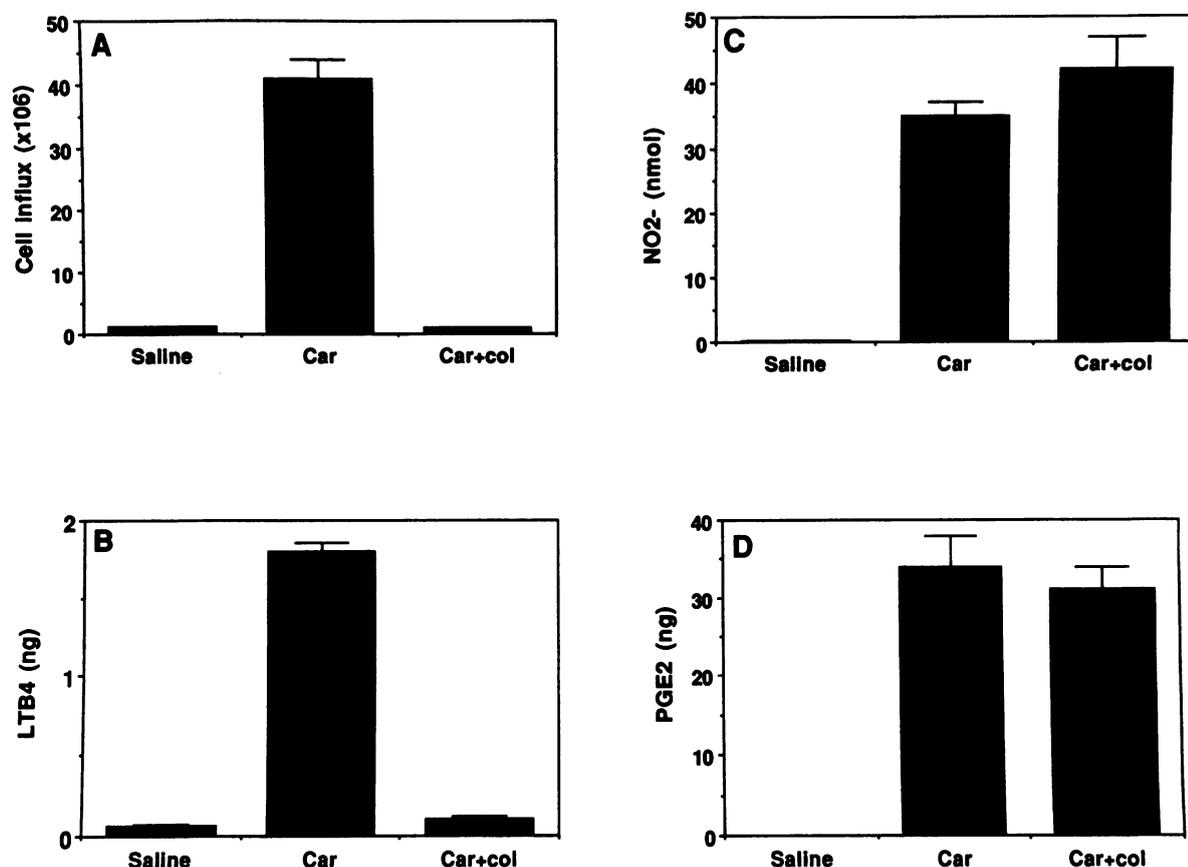


Figure 6. The effect of colchicine (*col*) on cell accumulation, NO<sub>2</sub><sup>-</sup>, PGE<sub>2</sub>, and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) elicited by the injection of carrageenan (*Car*) in the rat air pouch. Colchicine inhibited cell infiltration (A) and leukotriene B<sub>4</sub> (B) levels but failed to alter the concentration of NO<sub>2</sub><sup>-</sup> (C) and PGE<sub>2</sub> (D). Each point is the mean ± SEM for 6 animals.

blood pressure (from 101 ± 3 to 141 ± 5 mmHg, *n* = 4). Blood pressure was not affected by 2 d of treatment with the highest dose of NS398 (10 mg/kg, p.o., b.i.d., from 101 ± 3 to 114 ± 5 mmHg, *n* = 4) or dexamethasone (10 mg/kg, p.o., b.i.d., from 101 ± 3 to 100 ± 3 mmHg, *n* = 4).

## Discussion

The experiments performed in this study were designed to test the following hypothesis: does the dual inhibition of proinflammatory nitric oxide and prostaglandin contribute to the anti-inflammatory properties of NOS inhibitors?

A rapid induction of iNOS protein was observed in the inflamed pouch lining and cells from fluid exudates. Production of high levels of NO<sub>2</sub><sup>-</sup> in the pouch exudate fluid as a result of carrageenan induction coincided with the expression of iNOS protein. The contribution of iNOS in producing the inflammatory changes after carrageenan administration was further supported by the findings that (a) dexamethasone, a glucocorticoid that inhibits iNOS expression *in vitro* and *in vivo* without affecting cNOS (1, 22), blocked iNOS protein expression and completely suppressed NO<sub>2</sub><sup>-</sup> synthesis, and that (b) L-NIL, a recently described iNOS inhibitor (25), prevented the production of NO<sub>2</sub><sup>-</sup> elicited by carrageenan. In addition, iNOS-like immunoreactivity was localized to macrophages, but not to PMNs, suggesting that these cell types are the source of iNOS-derived NO<sub>2</sub><sup>-</sup>. Basal levels of NO<sub>2</sub><sup>-</sup> were very small and were not af-

ected by dexamethasone or L-NIL. Taken together these observations suggest that induction of iNOS, presumably in macrophages, is responsible for the large release of NO and that cNOS activity does not make a significant contribution to the NO formation in this model of inflammation. Induction of COX-2 in macrophages, but not infiltrating neutrophils, also appears to be responsible for the increased PG levels in this model (21).

We have recently reported in *in vitro* studies that NO activates COX-2, resulting in large production of PG (10, 12), a finding that has now been substantiated in a number of other systems (11, 13–15). As shown in this paper, induction of iNOS with the subsequent release of NO appears to enhance the production of PGE<sub>2</sub> at the site of inflammation. Indeed, when endogenous NO production was inhibited with L-NIL, the increased production of PGE<sub>2</sub> observed after the injection of carrageenan was markedly reduced. This effect is not due to a direct inhibition of the NOS inhibitor on COX activity or PG isomerase activity (10, 28). Therefore, modulation of the COX system by NO represents an important pathway that regulates the extent of the inflammatory process. In contrast, our results indicate that selective inhibition of COX-2 and hence PG production had only a minor effect on NO<sub>2</sub><sup>-</sup> production consistent with our previous observations *in vitro* (10, 12). The lack of significant inhibition of NO<sub>2</sub><sup>-</sup> production by a cyclooxygenase inhibitor supports the hypothesis that the overall inhibition of the inflammatory process by NOS inhibitors is not sufficient to explain their marked inhibitory effects on PG production.

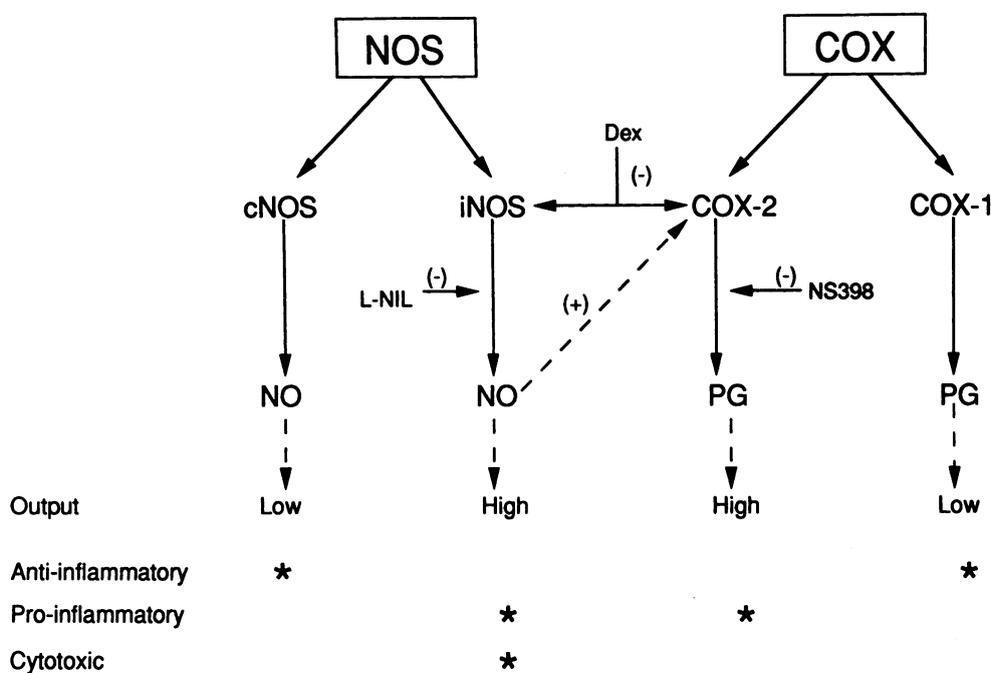


Figure 7. Proposed model of the interactions between the NO and COX pathway in inflammation.

The inflammatory response that occurs after the injection of carrageenan in the air pouch is also characterized by cellular infiltration (references 16 and 17 and this paper). Neutrophils account for > 98 and 85% of the infiltrating cells at 6 and 24 h, respectively, after carrageenan. In contrast, monocytes in the pouch fluid comprised 2 and 15% at 6 and 24 h, respectively, after carrageenan (16, 21). Table I shows that the large increase in cellular infiltration at 48 h after carrageenan was attenuated by either dexamethasone or L-NIL. Since we could not perform accurate cell differential at 48 h after carrageenan due to the cellular deterioration (see Methods), we could not determine whether dexamethasone or L-NIL affected the distribution of neutrophil/monocyte infiltration at this time point. Nevertheless, inhibition of cellular infiltration by dexamethasone or L-NIL may well contribute to the overall antiinflammatory effects of these drugs.

To determine whether the increasing  $\text{NO}_2^-$  and PG in the exudate was a consequence of neutrophils infiltration or was produced by resident pouch lining cells, colchicine was used to block the cellular influx into the exudate. Our results suggest that neutrophil infiltration does not significantly contribute to  $\text{NO}_2^-$  and  $\text{PGE}_2$  production in the fluid exudate early in the inflammatory response (6 h). Colchicine blocked neutrophil infiltration by 99%, but did not inhibit the 5- and 100-fold increases in  $\text{NO}_2^-$  or PG production which occurred after 6 h after carrageenan administration. This is substantiated further by the finding that iNOS-like immunoreactivity was not localized to neutrophils. In contrast, the infiltrating neutrophils are the source for the elevated leukotriene  $\text{B}_4$  levels in the fluid exudate, as the leukotriene  $\text{B}_4$  levels were markedly attenuated by the colchicine treatment. At the early time point,  $\text{NO}_2^-$  and  $\text{PGE}_2$  are most likely formed by cells comprising the pouch lining tissue. However, at the later stages of the inflammation (48 h), the elevated production of NO/PG may be produced not only by resident macrophages/fibroblasts in the pouch lining but also by infiltrating monocytes.

When the effects of L-NIL were compared with those of

the nonselective cNOS/iNOS inhibitor  $\text{NO}_2\text{Arg}$  (29), we found that  $\text{NO}_2\text{Arg}$  decreased  $\text{NO}_2^-$  and  $\text{PGE}_2$  production and cellular infiltration, but to a lesser extent than L-NIL. This is consistent with the fact that L-NIL is a more potent inhibitor of iNOS activity than  $\text{NO}_2\text{Arg}$  in vitro (25). This greater antiinflammatory effect of L-NIL versus  $\text{NO}_2\text{Arg}$  was also observed histologically. Thus, both the leukocytic infiltration into the tissue as well as the edema were reduced to a much greater extent in the L-NIL-treated animals as compared with those treated with  $\text{NO}_2\text{Arg}$ . Although infiltrating neutrophils are not responsible for the increased production of proinflammatory NO and PG, their inhibition at the inflamed site by the NOS inhibitors could very well contribute to the antiinflammatory effects of the NOS inhibitors. It is well known that neutrophils release a variety of mediators and enzymes endowed with proinflammatory properties.

The importance of our findings is highlighted in the model shown in Fig. 7. The induction of iNOS and COX-2 by mediators of inflammation such as endotoxin or cytokines occurs in a number of diseases and results in the elevated production of the proinflammatory mediators, NO and PG. Furthermore, the functional interaction between the NOS and COX pathways in inflammation suggests that NO may exacerbate the inflammatory process through the generation of additional proinflammatory PG. Regulation of COX by NO may also depend on the type and extent of the inflammatory process since it has also been reported that COX is inhibited by NO (30).

Besides its proinflammatory role, a feature of NO that is not shared by PG is its potent cytotoxic effect. This could explain why in arthritis a nonsteroidal antiinflammatory such as indomethacin, by blocking PG production but not NO, alleviates the symptoms associated with the inflammatory insult but does not modify the course of the disease (for review see reference 31). The production of low amounts of NO and PG from the constitutive enzymes appears to regulate a number of important physiological processes including the inhibition of platelet aggregation and white blood cell adhesion, regulation of blood

vessel tone, and cytoprotection in the kidney and intestinal mucosa. It is therefore imperative to preserve cNOS and COX-1 activity in an inflammatory setting. This can be achieved by the use of glucocorticoids. The potent antiinflammatory glucocorticoid dexamethasone is a perfect example of a dual iNOS (for reviews see references 1 and 2) and COX-2 (for reviews see references 4 and 5) synthesis inhibitor. Unfortunately, serious side effects of steroids, independent of their ability to block iNOS and COX-2 expression, limit their clinical use. Agents such as L-NIL may have the advantage of not only alleviating inflammatory symptoms through dual inhibition of NO and NO-driven COX-2 activation, but also, by removing the cytotoxic effects of NO, may prove to be important disease modifying drugs. Furthermore, the combination of a selective iNOS inhibitor together with a COX-2 selective inhibitor may be beneficial in the treatment of inflammatory disorders, suppressing most of the proinflammatory actions of NO and PG without affecting the physiological properties of these very important mediators.

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