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Selective Inhibition of Inducible Nitric Oxide Synthase Exacerbates Erosive Joint Disease

Nancy L. McCartney-Francis,1 Xiao-yu Song, Diane E. Mizel, and Sharon M. Wahl

NO is an essential cytotoxic agent in host defense, yet can be autotoxic if overproduced, as evidenced in inflammatory lesions and tissue destruction in experimental arthritis models. Treatment of streptococcal cell wall-induced arthritis in rats with N\textsubscript{G}-monomethyl-L-arginine (L-NMMA), a competitive nonspecific inhibitor of both constitutive and inducible isoforms of NO synthase (NOS), prevents intraarticular accumulation of leukocytes, joint swelling, and bone erosion. Because increased inducible NOS (iNOS) expression and NO generation are associated with chronic inflammation, we investigated whether a selective inhibitor of iNOS, N\textsubscript{G}-iminooethyl-L-lysine (L-NIL), would have more directed anti-arthritis properties. Whereas both L-NMMA and L-NIL inhibited nitrite production by streptococcal cell wall-stimulated rat mononuclear cells in vitro and systemic treatment of arthritic rats with L-NMMA ablated synovitis, surprisingly L-NIL did not mediate resolution of inflammatory joint lesions. On the contrary, daily administration of L-NIL failed to reduce the acute response and exacerbated the chronic inflammatory response, as reflected by profound tissue destruction and loss of bone and cartilage. Although the number of iNOS-positive cells within the synovium decreased after treatment with L-NIL, immunohistochemical analyses revealed a distinct pattern of endothelial and neuronal NOS expression in the arthritic synovium that was unaffected by the isoform-specific L-NIL treatment. These studies uncover a contribution of the constitutive isoforms of NOS to the evolution of acute and chronic inflammation pathology which may be important in the design of therapeutic agents. The Journal of Immunology, 2001, 166: 2734–2740.

Materials and Methods

Arthritis induction

Pathogen-free Lewis (LEW/N) female rats (~100 g; Charles River Laboratories, Wilmington, MA) were injected i.p. with peptidoglycan-polysaccharide fragments (30 µg rhamnose/g body mass) prepared from group A SCW (Lee Laboratories, Grayson, GA). At indicated intervals, the arthritic response was quantified by scoring the four distal joints on a scale of 0–4 based on swelling, redness, and degree of distortion. The individual joint scores were summed to determine the articular index (AI), with a maximum possible score of 16. The indices for a group of animals were averaged and reported as the mean AI ± SEM. Statistical significance was determined using the nonparametric Mann-Whitney U test.
Administration of inhibitors

SCW-injected and control LEW/N rats were randomly selected for treatment. t-NIL was obtained from Searle (St. Louis, MO). t-NMMA was purchased from Calbiochem (San Diego, CA). Previous studies have demonstrated the effectiveness of oral delivery of t-NIL (10) and i.v. administration of NMMA (5) to inhibit NO production. In the present study, comparisons between oral, i.v., and i.p. delivery of inhibitors yielded similar results. When added to the drinking water, t-NIL was given at a dose of 100 μg/ml and the t-NIL-containing water was changed every 2–3 days. Alternatively, t-NIL in PBS was injected i.p. daily at a dose of 3 mg/kg body weight. In our hands, this dosage reduced plasma nitrite + nitrate levels in SCW-injected rats by 90%. t-NMMA was injected i.v. or i.p. daily at a dose of 30 mg/kg body weight (5). Control animals received an equal volume (1 ml) of PBS. Potential side effects of the t-NIL treatment were assessed by multiple parameters, including visual appearance as well as white blood cell counts, hematocrit levels, and body weights.

Histology and immunohistochemistry

Joint tissues from control and arthritic rats were excised and fixed in 10% buffered Formalin, decalcified in 10% EDTA, embedded in paraffin, sectioned (6 μm), and stained with hematoxylin and eosin for histopathology. Immunohistochemical staining for NOS was performed by overnight incubation at 4°C with either the eNOS-specific primary antibody (iNOS, 1:500; Upstate Biotechnology, Lake Placid, NY), or anti-nNOS (2 μg/ml; Transduction Laboratories, Lexington, KY), or anti-nNOS (2 μg/ml; Transduction Laboratories). The nonspecificity of the NOS isoform Abs was confirmed by Western blot analysis of macrophage, endothelial cell, and pituitary lysates (Transduction Laboratories) (Fig. 1).

Leukocyte isolation

Peripheral blood was diluted in PBS (1:4), layered on Ficoll (Histopaque 1083; Sigma, St. Louis, MO), and centrifuged for 30 min. The mononuclear cells (PBMC) at the interface were collected and resuspended in DMEM containing gentamicin (10 μg/ml) and glutamine (2 mM). Polymorphonuclear cells (PMN) were isolated from peripheral blood by density gradient centrifugation (Polymorphprep diluted 5:1; Life Technologies, Rockville, MD). Mononuclear cells and neutrophils (5 × 10⁶/ml) were cultured for 36 h and supernatant fluids were collected and stored at −20°C. Nitrite assays

Nitrite content in cell culture supernatant fluids was measured by the colorimetric microplate method using Griess reagent (13). Plasma nitrite + nitrate concentrations were determined using a fluorometric assay (11, 14). Fluorescence was measured at a wavelength of 365/450 excitation/emission using a fluorescence plate reader (Idexx Laboratories, Westbrook, ME). Data were reported as mean concentration ± SEM. Statistical differences were determined using the unpaired two-tailed t test.

RT-PCR

Frozen hindleg joints were pulverized using a freezer/mill (Spex CertiPrep, Metuchen, NJ) and total cellular RNA was isolated using Trizol (Life Technologies, Gaithersburg, MD). RNA was reverse transcribed and cDNA levels were normalized based on an initial PCR amplification with GAPDH primers. iNOS and nNOS cDNA was amplified by semiquantitative RT-PCR as described previously (14). For analysis of eNOS, cDNA was amplified in the presence of a known amount of pretittered competitor DNA (mimic) which had an identical sequence to the eNOS cDNA except for a 50-bp deletion. The competitor DNA was generated by PCR amplification using the 5′ eNOS-specific primer and a modified 3′ competitor primer that contained an additional 20-base sequence located 50 bases upstream of the 3′ eNOS-specific primer. Conditions for PCR were as follows: 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min (GAPDH, 28 cycles; iNOS, 35 cycles; nNOS, 34 cycles; eNOS, 36 cycles). The primers (5′ to 3′) and their product sizes are as follows: GAPDH (555 bp): 5′-GTGAAAGTCTGGTCTCAACGAGTTT-3′, 3′-CAGACTCTCTGGATGGAATG-5′; iNOS (496 bp): 5′-CGGCCTTCGAAATTTGCGAAG-3′, 3′-GGTTGTCAGTCCATGCTTAA-5′; nNOS (328 bp): 5′-TGGAAAGGCAACGTTGTTGGTCCAGCAGGAGA-3′, 3′-GGTTGTCAGTCCATGCTTAA-5′; eNOS (189 bp): 5′-TGAAACCCCTTCCGGGATTCTGCAGGACCAGG-3′, 3′-GGTGGTCTTCTCAGGACTTGTGTCCAGTTGGGAGCATCGGC-5′.

PCR products were electrophoresed in a 1.5% agarose (iNOS, nNOS) or 2% NuSieve low-melting point agarose (FMC Bioproducts, Rockland, ME) (eNOS) gel and ethidium bromide-stained bands were scanned and quantitated by a fluorimager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Molecular Dynamics). The densitometric values of iNOS and nNOS products were normalized to GAPDH, and eNOS was compared with eNOS mimic. The ratios were then divided by that of the SCW-injected rat sample to assess relative RNA expression.

Western blotting

Cell pellets (15 × 10⁶) or bone powder (100 mg) were suspended in lysis buffer containing 25 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1 mM PMSF, 1 mM Na₃VO₄, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml 4-(2-aminophenyl)-benzene sulfonylfluoride-HCl and placed on ice for 10 min. The resulting lysates were centrifuged at 14,000 rpm for 10 min and protein content of the supernatant was measured (Bio-Rad protein assay; Bio-Rad, Hercules, CA) using BSA as the standard. Lysate samples (100 μg) were boiled for 5 min in SDS sample buffer, electrophoresed in 7.5% SDS-acrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBST (100 mM Tris-HCl (pH 7.5), 154 mM NaCl, and 0.1% Tween 20) overnight at 4°C and incubated with isofrom-specific NOS Abs (iNOS, 1:1000; Upstate Biotechnology; eNOS, 1:1000; nNOS, 1:500; Alexis, San Diego, CA) or anti-actin (1:250; Sigma) for 3 h at room temperature or overnight at 4°C. The first antibody on each membrane was incubated with secondary Ab conjugated with HRP (donkey anti-rabbit IgG, 1:3000) for 1 h at room temperature, followed by three washes in TBST. The membranes were then developed with the ECL detection system (Renaissance; New England Nuclear, Boston, MA).

Results

Comparison of L-NMMA and L-NIL on NOS activity in vitro and in vivo

Based on the reported specificity and potency of L-NMMA, t-NIL, and AG for the NOS isozymes (10), we monitored their ability to suppress SCW-induced NO production in vitro. All three compounds inhibited, in a dose-dependent manner, nitrite production by SCW-stimulated rat mononuclear cells in vitro (Fig. 2). t-NIL was 10-fold more potent than L-NMMA (10 μM t-NIL, 72% inhibition vs 100 μM L-NMMA, 66% inhibition) and >50-fold more potent than AG (500 μM AG, 52% inhibition) in inhibiting NO production in this in vitro assay. Because both t-NIL and L-NMMA were more effective in vitro inhibitors of NO synthesis and represented both a nonspecific and an iNOS-specific inhibitor, we compared the in vivo efficacy of t-NIL with L-NMMA which we have previously shown to be effective in ameliorating SCW-induced tissue damage (5).

First, to document a suppressive effect in vivo, PBMC were obtained from animals receiving arthrogenic doses of SCW pep- tidoglycan-polysaccharide complexes which had been treated with either t-NMMA or t-NIL (Fig. 3A). Without further challenge in
vitro, PBMC from arthritic animals constitutively released substantial levels of NO. Similar to the in vitro data, nitrite production by PBMC from L-NIL-treated rats was reduced by 80% as compared with the untreated arthritic rats (SCW alone, \( p < 0.01 \)), whereas L-NMMA treatment resulted in 64–70% reduction in nitrite production (\( p = 0.05 \); Ref. 5). The systemic administration of NO inhibitors also influenced plasma levels of NO metabolites nitrite and nitrate (Fig. 3B). Plasma nitrite + nitrate concentrations were elevated \( >10 \)-fold during chronic arthritis following SCW treatment (248.6 ± 40.0 vs 18.03 ± 5.71 for PBS control, \( p < 0.01 \)). l-NMMA administered i.v. (or i.p.) at 30 mg/kg significantly reduced plasma nitrite + nitrate (\( p < 0.01 \)), whereas L-NIL reduced plasma nitrite + nitrate levels to control levels (\( p < 0.0001 \)). Inhibition by L-NIL was effective whether administered orally (at a dose of 100 \( \mu \)g/ml in the drinking water) or given i.p. daily (at 3 mg/kg). Thus, circulating levels of NO increase in association with the development of acute and chronic arthritis and the NO inhibitors, whether nonspecific or specific for iNOS, effectively diminish these systemic levels. At this level, the two different inhibitors appeared indistinguishable.

**Effect of selective inhibitors of iNOS on SCW-induced arthritis**

Having established that both nonspecific and selective NO inhibitors block peripheral leukocyte and plasma increases in NO metabolites in animals challenged with SCW, we next evaluated whether these inhibitors had comparable effects on the development of arthritis. As evident in Fig. 4, and as we previously reported (5), the daily administration of l-NMMA partially suppresses acute arthritis (days 3–5, 20–30% inhibition) and significantly inhibits the clinical manifestations of the chronic arthritis lesions (AI, 0.5 ± 0.29 vs 6.5 ± 0.93 for SCW alone, \( p = 0.007 \)). However, surprisingly, the continuous administration of l-NIL, a specific inhibitor of iNOS, failed to inhibit either acute or chronic synovial lesions. In fact, the daily delivery of l-NIL, whether orally or by i.p. injection, often exacerbated the arthritis (oral l-NIL: AI = 10.3 ± 0.5 vs 7.1 ± 0.6 for SCW alone, \( p < 0.012 \); i.p. l-NIL: AI = 9.5 ± 1.0 vs 6.5 ± 0.9 for SCW alone).

**FIGURE 2.** NOS inhibitors block NO synthesis in vitro. PEC (10^6/ml) from normal female Lewis rats (\( n = 2 \)) were cultured in duplicate for 36 h with SCW (5 \( \mu \)g/ml) and increasing micromolar amounts of NO inhibitors. Supernatant fluids were assayed in triplicate for nitrite content by the Griess reaction. Values represent mean ± SEM and are representative of three similar experiments. *\( p \leq 0.05 \) compared with SCW alone, unpaired two-tailed \( t \) test.

**FIGURE 3.** NOS inhibitors reduce NO production in the periphery of arthritic rats. A, PBMC were isolated on day 24 from arthritic rats treated with NO inhibitors and cultured (2 \( \times \) 10^6/ml) for 36 h without further stimulation. Supernatant fluids were assayed in triplicate for nitrite content by the Griess reaction. Values represent mean ± SEM and are representative of two similar experiments. B, Nitrite + nitrate concentrations in plasma were measured by a fluorescent microplate assay using 2,3-diaminonaphthalene. Data represent mean plasma nitrite + nitrate ± SEM from chronic phase rats treated with SCW alone (\( n = 7 \)), SCW + l-NIL (\( n = 10 \)), or SCW + l-NMMA (\( n = 4 \)). Control groups (PBS, l-NIL, or l-NMMA alone) consisted of three to four rats. *\( p \leq 0.05 \) compared with SCW + PBS, unpaired two-tailed \( t \) test.

(4A). The increased AIs reflected the histopathology of the joints obtained from these animals. Histological analysis of joints from rats treated only with SCW exhibited marked infiltration of inflammatory cells into the synovial and subsynovial tissue (Fig. 4B). Synovial hyperplasia, a narrowing of the synovial space, and severe deterioration of the cartilage and subchondral bone were also evident. By comparison, the tissues from the arthritic animals receiving l-NIL exhibited extreme tissue destruction including extensive loss of bone and cartilage (Fig. 4C). In contrast, joints from l-NMMA-treated animals exhibited sharply reduced inflammatory changes consonant with the decreased clinical inflammatory index (5).

**Systemic effects of specific iNOS inhibition**

To assess whether the l-NIL treatment was associated with systemic effects, we evaluated several parameters (Table I). When administered orally, l-NIL treatment partially reduced the leukocytosis normally observed in SCW-induced arthritis by 25% (24.6 \( \times \) 10^3/mm^3 vs 32.9 \( \times \) 10^3/mm^3 in untreated SCW-injected rats, \( p = 0.045 \)). In similar fashion, packed RBC volume as measured by hematocrit, which is typically reduced in arthritic animals, was increased following l-NIL treatment (43 ± 3 vs 36 ± 1.6 for SCW alone as compared with 47 ± 0.6 in PBS control rats).

\[0.012; i.p. L -NIL: AI = 10.3 ± 0.5 vs 7.1 ± 0.6 for SCW alone, \( p < 0.012 \); i.p. l-NIL: AI = 9.5 ± 1.0 vs 6.5 ± 0.9 for SCW alone)\]
although not fully restored to normal levels. Additionally, the characteristic weight loss in arthritic rats (148.3 ± 5.9 g for SCW-injected rats vs 206 ± 3.9 g for PBS rats, p < 0.05) was not reversed by l-NIL treatment (132.2 ± 4.2 g, p < 0.04 as compared with SCW alone). By comparison, the lack of toxicity of l-NMMA in the rat arthritis model was reflected in the normal hematocrit and body weights of l-NMMA-treated arthritic rats as reported previously (5). With regard to all of these parameters as well as physical appearance, rats treated with l-NIL alone (no SCW) were indistinguishable from rats treated with PBS alone.

Identification of NOS isoenzymes in arthritic lesions

Based on the unanticipated effect of l-NIL on synovial pathology, we next focused on whether the l-NIL had any effect on localized synovial production of NO and whether it had any impact on tissue iNOS and/or the other NOS isoforms. Although no iNOS staining was detected in synovial tissue obtained from untreated control animals (data not shown), iNOS immunoreactivity was clearly evident within the hyperplastic synovial tissue from SCW-arthritic rats (Fig. 5A). The primary cells exhibiting specific iNOS immunoreactivity were morphologically consistent with macrophages. Synovial lining cells and some chondrocytes were also iNOS positive. After treatment with the iNOS-specific inhibitor l-NIL, the number of positive cells decreased, although some staining persisted proximal to the bone (Fig. 5B). Parallel staining with the Abs for eNOS and nNOS revealed an unanticipated distribution of these additional isoforms in the arthritic synovium, but not in normal synovium (PBS, l-NIL alone, data not shown). In addition to eNOS staining in the endothelial cells, multinucleated osteoclast-like cells residing on the borders of degrading bone were clearly eNOS positive (Fig. 5, C and D), but negative for iNOS and nNOS. Scattered cells, some of which were multinucleated, within the synovium and bone marrow stroma also were eNOS positive. nNOS staining, previously identified in bone (17), but not synovial tissues, was seen in the infiltrating leukocytes including macrophages and polymorphonuclear leukocytes (Fig. 5, E and F). These cells were negative for eNOS and did not stain in the absence of the primary Ab, indicating that staining was not due to a nonspecific peroxidase effect. More importantly, after treatment with l-NIL, even when the iNOS staining was reduced (Fig. 5B), neither eNOS (Fig. 5D) nor nNOS (Fig. 5F) staining was altered, documenting the specificity of the inhibitor, but suggesting that additional sources of NO were sustained even in the presence of l-NIL. In contrast, treatment with l-NMMA reduced both eNOS- and nNOS-positive cells as well as iNOS (data not shown), coincident with reduced inflammatory pathology (5).

Inflammatory cells as a source of iNOS and nNOS

To correlate the cellular distribution of the NOS isoforms identified by immunohistochemical analysis with cell-specific protein levels, mononuclear cells and neutrophils were isolated from rat

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Table 1. Effects of l-NIL treatment on circulating leukocytes in arthritic rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WBC (×10⁷/mm³)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>6.0 ± 0.8</td>
<td>206.3 ± 3.9</td>
</tr>
<tr>
<td>l-NIL</td>
<td>6.3 ± 1.1</td>
<td>191.8 ± 3.3</td>
</tr>
<tr>
<td>SCW</td>
<td>32.9 ± 1.6b</td>
<td>148.3 ± 5.9b</td>
</tr>
<tr>
<td>SCW + l-NIL</td>
<td>24.6 ± 1.8c</td>
<td>132.2 ± 4.2c</td>
</tr>
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*Values are the mean ± SEM. Rats were injected i.p. with PBS or SCW on day 1. l-NIL was administered in the drinking water (100 µg/ml) and rats were killed on day 37 (n = 2–8 rats for each group). 

b, p ≤ 0.05 in comparison with l-NIL control group.

c, p ≤ 0.05 in comparison with SCW treatment group using the Mann-Whitney U test.
peripheral blood, stimulated with SCW in vitro, and the cell extracts were monitored for iNOS, eNOS, and nNOS proteins using isoform-specific polyclonal Abs. As shown in Fig. 6, PBMC did not constitutively express iNOS, but produced the 130-kDa iNOS protein in response to SCW. Moreover, constitutive nNOS expression was evident which was elevated following exposure to SCW. eNOS levels were barely seen and minimally augmented by SCW. Neutrophils, on the other hand, were strongly positive for iNOS following exposure to SCW, but did not synthesize detectable levels of eNOS or nNOS protein before or after stimulation with SCW. In additional experiments, peritoneal macrophages stimulated by SCW were found to express low levels of both eNOS and nNOS proteins as well as substantially enhanced levels of iNOS. Thus, inflammatory cells triggered by SCW have the capacity to contribute to the overproduction of NO, not only through the expression of iNOS but also constitutive isoforms of NOS.

**Gene expression of NOS isoforms in arthritic joints is altered by NOS inhibitors**

Based on this surprising NOS isoform expression, we further assessed NOS gene expression in inflamed and treated joints at the RNA level. RNA was isolated from arthritic joint tissue and relative RNA levels were determined by RT-PCR. Increased expression of iNOS mRNA was observed in arthritic joints (Fig. 7, A and C), consistent with immunohistochemical data for iNOS protein and our previous data (5, 14). Interestingly, nNOS mRNA expression in control animals (PBS, l-NIL alone) was comparable to arthritic rats, even though immunohistochemical staining for
nNOS protein in control tissues was undetectable. In contrast to the high constitutive expression of nNOS mRNA, eNOS mRNA expression in joint tissue was not detectable by conventional RT-PCR and could only be observed in the presence of a mimic cDNA (competitive RT-PCR) (Fig. 7, B and C). After L-NIL treatment, iNOS mRNA expression, although somewhat variable, was consistently reduced (23–60% reduction as compared with SCW alone, \( p < 0.05 \)), whereas nNOS mRNA expression was relatively unchanged. On the other hand, eNOS mRNA expression, albeit low, was increased in L-NIL-treated arthritic rats.

Joint tissues were examined in parallel by Western blot analyses for the presence of the NOS proteins (Fig. 8). Consistent with the immunohistochemistry and RNA data, immunoreactive iNOS protein was elevated in arthritic joints and markedly reduced by L-NIL treatment. Both eNOS and nNOS proteins were expressed in joints of SCW-injected rats but not control rats and the levels of expression were unaffected by L-NIL treatment. In contrast, the nonspecific NOS inhibitor NMMA not only suppressed expression of iNOS protein but also the constitutive NOS isoforms eNOS and nNOS, likely reflecting, at least in part, the marked reduction in inflammatory infiltrate. These data are consistent with differential regulation of the three NOS isoforms during joint inflammation and following selective inhibition of one isoform.

**Discussion**

Inducible NOS has long been considered the villain responsible for excessive and autotoxic levels of NO in chronic pathogenic inflammatory lesions (3, 4). For these reasons, we considered that selective blockade of the iNOS pathway, without impinging on eNOS- or nNOS-dependent events, would provide an ideal strategy to disrupt only the presumptive harmful NO being produced in the synovium, and be a more effective anti-arthritis agent than L-NMMA (5). It was clear that excess NO contributed to the tissue pathology triggered by SCW deposition in the synovium. This dichotomy suggested that either iNOS-dependent NO was protective and/or that eNOS/nNOS were, in fact, also perpetrators of tissue injury. Although we cannot totally rule out a protective role for iNOS-derived NO (18), in these studies we have documented a contribution of the constitutive isoforms to the emerging arthropathy in the SCW model and that failure to concomitantly regulate the products of these pathways is detrimental. First, by immunohistochemical analysis, we demonstrate that all three NOS isoforms are found in inflamed synovium. eNOS is present not only in synovial endothelial cells, but also in bone-degrading cells, the osteoclasts, which express high levels of this isoenzyme in arthritic joints, as recently reported (17, 19). As expected, iNOS was expressed by inflammatory cells and synovial lining cells. Further analysis revealed that nNOS protein was also identified within infiltrated leukocytes in the inflamed synovium. Thus, all three isoenzymes were clearly represented but in a unique distribution in the arthritic synovium.

Second, to confirm the cellular distribution of the NOS seen by immunohistochemistry, isolated inflammatory cell populations were found to constitutively or inducibly express the isoenzyme proteins by Western blot analysis. Both mononuclear cells and polymophonuclear cells expressed substantial iNOS upon stimulation in vitro with SCW. PBMC and peritoneal macrophages also produced increased levels of nNOS protein and limited eNOS as detected in the cell extracts following challenge with the SCW. Although we were unable to detect nNOS protein by Western blot in PMNs, perhaps due to proteolytic degradation, recent studies have demonstrated constitutive expression of nNOS mRNA by rat circulating neutrophils which could be inhibited by the NOS inhibitor 7-nitroindazole but was not inhibited by L-NIL (20).

Third, iNOS mRNA was up-regulated in joint tissue isolated from SCW-induced arthritic rats. nNOS mRNA was constitutively expressed without pronounced enhancement in inflamed tissues, whereas eNOS mRNA, although very low, was somewhat elevated in arthritic joints. Although we have not yet isolated sufficient osteoclasts from these inflamed joints to monitor mRNA expression for eNOS, the specific staining pattern of these cells with the eNOS Ab and recent identification of eNOS protein in cultured osteoclasts (17) predicts the presence of the eNOS gene. Based on our new findings, the failure of specific iNOS inhibitors to prevent SCW-induced synovial pathology may be due to the inability of these inhibitors to influence the NO generated by the eNOS and nNOS pathways, which at least by immunohistochemical staining as well as RNA and protein levels are relatively untouched by L-NIL therapy. Clearly, the L-NIL is active, since the circulating levels of nitrite and nitrate, the stable decomposition products of NO, are definitely reduced following treatment. Although the maximal levels of plasma nitrite + nitrate correspond with the peak inflammatory response, it is now less clear how these plasma NO metabolites relate to the pathology since bringing these levels to normal or near normal by inhibiting iNOS is not necessarily associated with a corresponding decrease in synovial disease. In either case, L-NIL or L-NMMA both suppress iNOS, resulting in decreased NO, but it may be the localized production of eNOS and nNOS along with iNOS which collectively drive the NO-mediated autotoxicity. However, the data are consistent with the idea that the enhanced levels of plasma nitrite + nitrate which occur during the development of arthritis result from the up-regulation of iNOS, with a lesser contribution of eNOS to systemic NO.

Whether the contribution of eNOS and nNOS to inflammatory pathology is unique to the synovium remains to be resolved. In this

**FIGURE 8.** Western blot analysis of arthritic joint tissue. Proteins from pulverized joints of treated (day 24 after SCW injection) and control (L-NIL alone) rats were separated on 7.5% SDS-acrylamide gels and immunoblotted with NOS isoform-specific Abs. Immunoreactive bands were detected with HRP-conjugated donkey anti-rabbit secondary Ab followed by an ECL detection system. Equal loading of protein per lane was verified with anti-actin Ab.
regard, the granulomatous response to SCW in the liver is reversed by L-NIL, demonstrating not only the bioactivity of the L-NIL but, for the first time, differential tissue responses (N. McCartney-Franccis and S. Wahl, manuscript in preparation). Thus, suppression of iNOS alone might be sufficient in soft tissues such as gut and liver (21) to block NO toxicity. Although L-NIL has been shown to suppress adjuvant-induced arthritis if administered prophylactically (10), it is not effective therapeutically (22). Similarly, prophylactic treatment with L-NIL reduced progression of experimental osteoarthritis in dogs by ~50% (12). Contrasted with the failure of L-NIL to prevent or reverse bacterial cell wall (SCW)-induced arthritis, these differential responses to L-NIL may reflect critical differences in clinical and immunological sequelae in these inflammatory models. In the arthritic joints of SCW-injected rats, both reactive nitrogen species and reactive oxygen species play pivotal roles in the pathologic process and therapeutic targeting of NO by L-NMMA (5) or superoxide and hydrogen peroxide by superoxide dismutase or catalase (23) is effective in blocking the inflammation and tissue destruction. However, the ineffectiveness of the iNOS specific inhibitor L-NIL in preventing inflammatory damage in the arthritic joint highlights the complex roles these reactive species play in the pathologic process. Deletion of the iNOS gene by homologous recombination in mutant mice renders these animals more susceptible to adjuvant (24) and septic (25) arthritis, consistent with our findings. Significantly, administration of a selective neuronal NOS inhibitor 7-nitroindazole reduced adjuvant-induced joint inflammation in rats (26), suggesting an inexplicable role for nNOS in arthritic pathology. These studies highlight the complexity of the pro- and anti-inflammatory activities of iNOS (27–29) and define a previously unrecognized role for the constitutively expressed NOS in the evolution of acute and chronic inflammatory synovial pathology. These isoenzymes must now also be considered in the design of NO-related therapeutic targets.

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