ROLE OF ENDOTHELIAL NITRIC OXIDE SYNTHASE IN AGGRAVATION OF INDOMETHACIN-INDUCED GASTRIC DAMAGE IN ADJUVANT ARTHRITIC RATS

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The role of nitric oxide synthase (NOS) isozymes in the aggravation of indomethacin-induced gastric damage in adjuvant arthritic rats was investigated. Two weeks after injection of Freund’s complete adjuvant, the animals were given indomethacin, and the stomach was examined for damage 4 h later. Indomethacin caused hemorrhagic lesions in the normal rat stomach, and these lesions were markedly aggravated in arthritic rats. Pretreatment with L-NAME (a nonselective inhibitor of NOS) and aminoguanidine (a relative selective inhibitor of iNOS) did not affect the ulcerogenic response in normal rats but dose-dependently prevented the aggravation of lesions in arthritic rats, but the effect of aminoguanidine was apparently less than that of L-NAME. The increased ulcerogenic response in arthritic rats was significantly suppressed by 1400 W (a selective inhibitor of iNOS) and L-NIO (a selective inhibitor of eNOS) but not by L-NPA (a selective inhibitor of nNOS). The concurrent administration of 1400 W and L-NIO almost totally abolished the aggravation of damage in arthritic rats. The expressions of eNOS and iNOS but not nNOS in the gastric mucosa were clearly enhanced in arthritic rats. Mucosal levels of non-protein sulphydryls were significantly lower in arthritic rats than those in normal rats. The aggravation of damage in arthritic rats was significantly prevented by glutathione. These results suggest that the increased ulcerogenic response to indomethacin in arthritic rat stomachs is mediated by NO derived from eNOS in addition to iNOS. It is assumed that eNOS/NO may act harmfully on the gastric mucosa of arthritic rats with mucosal SH deficiency.

Key words: adjuvant arthritis, indomethacin-induced gastric damage, nitric oxide, endothelial nitric oxide synthase, inducible nitric oxide synthase

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic disease characterized by persistent inflammation of multiple joints. Although non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used for the treatment of several inflammatory disorders including RA, these agents show gastrointestinal toxicity as a side effect, mainly due to the depletion of endogenous prostaglandins (PGs) (1, 2). It is known that patients with RA are more susceptible to NSAID-induced gastric lesions than other NSAID users. Indeed, we previously reported that the gastric ulcerogenic responses to NSAIDs, such as indomethacin and aspirin, were markedly aggravated in rats with experimentally-induced arthritis (3-5). We further showed that the aggravation of the gastric lesions in arthritic rats was attributable to the overproduction of nitric oxide (NO) derived from inducible isozyme of NO synthase (iNOS) (3).

NO plays an important role in the regulation of various cellular functions in cardiovascular, neuronal and immune systems as well as gastrointestinal tracts (6). The NOS that produces NO from the precursor L-arginine exists in three isozymes; neuronal NOS (nNOS/NOS1), endothelial NOS (eNOS/NOS3), and inducible NOS (iNOS/NOS2). The first two isozymes are expressed constitutively in various cells under normal conditions and are called constitutive NOS (cNOS), whereas iNOS is not expressed constitutively but induced by various factors such as cytokines and lipopolysaccharide (7). In the gastrointestinal tract, NO produced by cNOS is considered to be beneficial in maintaining the mucosal integrity (8). However, many studies indicate the involvement of iNOS in the pathogenesis of gastrointestinal inflammatory disorders such as inflammatory bowel disease, NSAID-induced intestinal ulceration and compound 48/80-induced gastric lesions, and it was suggested that NO produced by iNOS may play a detrimental role in such diseases (9-13). It was shown previously that the aggravation of NSAID-induced gastric lesions in arthritic rats was also significantly prevented by NO inhibitors, such as N6-nitro-L-arginine methyl ester (L-NAME: a nonselective inhibitor of NOS) and aminoguanidine (a relatively selective inhibitor of iNOS) (3). Interestingly, it was also found that the preventive effect of aminoguanidine on the increased ulcerogenic response in arthritic rats was apparently less than that of L-NAME. Thus, it is possible that other isozymes of NOS such as nNOS and eNOS may also be involved in the aggravation of NSAID-induced gastric lesions in arthritic rats.

The present study investigated the roles of NOS isozymes in the gastric ulcerogenic response to indomethacin in adjuvant-induced arthritic rats and demonstrated the pathogenic importance of eNOS/NO in the aggravation of these lesions in these rats.
MATERIALS AND METHODS

Animals

Male Dark Agouti (DA) rats (140-160 g, SLC, Shizuoka, Japan) were used. The animals were fed standard rat chow and tap water ad libitum. All experimental procedures were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

Induction of adjuvant arthritis

Adjuvant arthritis was induced by injection of 50 µl of Freund’s complete adjuvant (FCA; 10 mg/ml heat-killed Mycobacterium tuberculosis H37Ra was suspended in paraffin oil) into the plantar region of the right hindpaw. Normal rats were housed in the same manner for the same period of time, so that age- and batch-matched normal and arthritic rats were used in all of the subsequent experiments. The severity of arthritis was assessed by measuring the paw volume (edema) using a plethysmometer (Ugo-Basile, Comerio-Varese, Italy). Because paw edema in the left (uninjected) hindpaw was observed from 10 days, and reached a maximum at 14 days after the injection of FCA, animals were used at 14 days after the injection in all experiments as arthritic rats. Normal and arthritic rats were deprived of food but allowed free access to tap water for 18 h before the experiments.

Induction of gastric lesions by indomethacin

The animals were given indomethacin (30 mg/kg) orally and sacrificed under deep ether anesthesia 4 h later. Then, the stomachs were removed, inflated by injecting 7 ml of 2% formalin, immersed in 2% formalin for 10 min to fix the gastric wall, and opened along the greater curvature. The area (square millimeters) of each lesion developed in the glandular mucosa wall, and opened along the greater curvature. The area (square millimeters) of each lesion developed in the glandular mucosa was measured using a dissecting microscope with a square grid (x10), summed per stomach, and used as a lesion score. The investigator measuring the lesion sizes was blinded to the treatment given to the animals. The effects of the following drugs on the development of gastric lesions induced by indomethacin were examined in the age-matched normal and arthritic rats on day 14 after FCA injection; N G-nitro-L-arginine methyl ester (a non-selective inhibitor of NOS; L-NPA: 10 mg/kg), L-arginine (a selective inhibitor of nNOS; L-NPA: 10 mg/kg), L-arginine (a substrate of NOS, 300 mg/kg) and iminoethyl-L-ornitine (a selective inhibitor of eNOS; L-NIO: 30 mg/kg), L-arginine (a substrate of NOS, 300 mg/kg) and glutathione (GSH: 100 and 200 mg/kg) were given s.c. twice at 10 days, and reached a maximum at 14 days after the injection of FCA, animals were used at 14 days after the injection in all experiments as arthritic rats. Normal and arthritic rats were deprived of food but allowed free access to tap water for 18 h before the experiments.

RT-PCR analyses of NOS isozymes

Under ether anesthesia, the stomachs were removed, incised along the greater curvature, and the corpus mucosa was scraped using two glass slides, frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted from each sample using Sepasol RNA-I (Nacalai Tesque, Kyoto, Japan). Total RNA primed by random hexadecoxypribonucleotides was reverse-transcribed using ReverTra Ace-alpha (TOYOBO, Osaka, Japan). The sequences of sense and antisense primers for rat eNOS, iNOS, nNOS and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), which is constitutively expressed, are shown in Table 1. An aliquot of the reverse transcription reaction product served as a template in 35 cycles of PCR with 1 min of denaturation at 95°C and 1 min of extension at 68°C by Advantage 2 polymerase mix (Clontech, Mountain View, CA, USA) on a thermal cycler (PC806, Astec, Fukuoka, Japan). A portion of the PCR mixture was electrophoresed in a 1.8% agarose gel in Tris-EDTA-acetic acid buffer (40 mM Tris, 2 mM EDTA and 20 mM acetic acid, pH 8.1), and the gel was stained with ethidium bromide and photographed (BioDoc-it system, UV, Upland, CA, USA).

Western blot analyses for NOS isozymes

Under deep ether anesthesia, the stomachs were removed and incised along the greater curvature. Then, the corpus mucosa was scraped using two glass slides, frozen in liquid nitrogen, and stored at −80°C until use. Each sample was homogenized in lysis buffer (pH 7.4) containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 50 mmol/L dithiothreitol, 1 mmol/L EDTA, a protease inhibitor cocktail tablet (Complete mini, Roche, Penzberg, Germany), and 1% Triton X-100. After centrifugation at 20,000 g for 30 min at 4°C, the protein concentration in the supernatants was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). An appropriate volume of the sample was mixed with an equal volume of sample buffer (pH 6.8, 100 mM Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.02% bromophenol blue), subjected to SDS-PAGE (50 µg per 10 µl in each lane) using 7.5% acrylamide gels, and transferred electrophoretically to PVDF membranes. For immunoblot analysis, samples of the total eNOS, iNOS, and eNOS were heated at 95°C for 5 min before electrophoresis, while those of the dimeric form of eNOS were not heated and the temperature of the gel was maintained below 15°C during electrophoresis (low-temperature SDS-PAGE) (19, 20). The membranes were incubated with goat polyclonal anti-human eNOS (NOS 3) antibody (Santa Cruz Biotecnology, Santa Cruz, CA, USA), rabbit polyclonal mouse iNOS (NOS 2) antibody (BD Transduction Laboratory, Franklin Lakes, NJ, USA), goat polyclonal anti-human nNOS (NOS 2) antibody (Santa Cruz), or mouse monoclonal anti-β-actin antibody (Sigma Chemicals, St. Louis, MO, USA) and then treated with horseradish peroxidase-conjugated rabbit polyclonal anti-goat

Table 1. PCR primer sequences and product sizes

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<th>Molecule</th>
<th>Primer sequence</th>
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<tr>
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<td>antisense 5'-TCCTCACAGGATCAGGATGGCT-3'</td>
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</tr>
<tr>
<td>iNOS</td>
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<td>780 bp</td>
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<td></td>
<td>antisense 5'-CGGTGTTCCCGGAGTGGTGAAG-3'</td>
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<tr>
<td>nNOS</td>
<td>sense 5'-GAATACCAACAGGGACAGTGGCAG-3'</td>
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<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense 5'-GAACGGGAGAAGCTACTGCGATGCCC-3'</td>
<td>310 bp</td>
</tr>
<tr>
<td></td>
<td>antisense 5'-TGAGGTCCACCACCTGTGCTG-3'</td>
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</table>
Measurement of nitrite plus nitrate production in the stomach

Under ether anesthesia, the abdomen was opened, the pylorus was ligated, and the animals were allowed to recover from anesthesia. Four hours later, the animals were sacrificed by deep ether anesthesia, the stomachs were removed and the gastric contents were collected. After centrifugation for 10 min at 3000 r.p.m., the volume of each sample was measured. The concentration of nitrite plus nitrate, the metabolites of NO, in gastric contents was determined by the modified method of Griess reaction (21). Briefly, each sample was incubated with 0.05 units/ml nitrate reductase (from Aspergillus, Sigma), converting nitrate to nitrite in the presence of 0.1% NADPH for 1 hr at 37°C. Then, the samples containing nitrates were incubated with Griess reagent (0.1% naphthylene diamine dihydrochloride and 1% sulfanilamide in 2.5% H3PO4) for 10 min at 3000 r.p.m., the volume of each sample was measured. The integrated density of the bands was quantified using ImageJ 1.37v analysis software (National Institutes of Health, Bethesda, MD, USA). Absorbance was measured at 550 nm and the results were expressed as nanomoles per hour. Sodium nitrate was used to generate a standard curve.

Immunohistochemistry for eNOS and iNOS

Under deep ether anesthesia, the stomachs were removed and washed in phosphate-buffered saline (PBS). Then, each sample was embedded in O.C.T. compound (Miles, Elkhart, IN, USA), and frozen rapidly in carbon dioxide gas. Cryostat sections (CM1510, Leica, Wetzlar, Germany) cut serially at a thickness of 10 µm were mounted on MAS-coated slides (Matsunami, Osaka, Japan) and treated with 4% paraformaldehyde. The slides were incubated with goat polyclonal anti-human eNOS (NOS 3) antibody (Santa Cruz) or rabbit polyclonal mouse iNOS (NOS 2) antibody (BD Transduction Laboratory) and then treated with Alexa Fluor 546-labeled donkey polyclonal anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) or Alexa Fluor 488-labeled donkey polyclonal anti-rabbit IgG antibody (Molecular Probes), respectively. Immunofluorescence was observed using a confocal laser scanning microscope (LSM-510, Carl Zeiss Micromiae Inc., Thornwood, NY, USA).

Measurement of gastric mucosal non-protein sulphhydryl (SH) content

The amount of SH, such as GSH, was measured in the gastric mucosa according to a modified method (22). Under deep ether anesthesia, the stomachs were removed, incised along the greater curvature, and the corpus mucosa was scraped using two glass slides on ice. The mucosal scraping was weighed, homogenized in 2 ml of phosphate buffer (0.1 M NaH2PO4, 0.25 M sucrose, pH 7.4), and centrifuged at 4000 r.p.m. for 15 min at 4°C. An adequate volume of 25% trichloroacetic acid was added to the supernatant of each sample and then the sample was kept for 30 min on ice. After centrifugation at 3000 r.p.m. for 15 min, the supernatant was used for the determination of GSH using DTNB [5',5'-dithiobis (2-nitrobenzoic acid)] (Wako, Osaka, Japan). Absorbance was measured at 412 nm, and the results were expressed as micromoles per gram wet tissue weight. GSH (Nacalai Tesque) was used for preparing a standard curve.

Preparation of drugs

The drugs used were heat-killed Mycobacterium tuberculosis (H37Ra: Difco, Detroit, MI, USA), paraffin oil (Wako, Osaka, Japan), indomethacin, L-NAME, aminoguanidine (Sigma), glutathione (reduced form) (GSH; Nacalai Tesque), 1400 W, NPLA (Cayman Chemical, Ann Arbor, MI) and L-NIO (A.G. Science, San Diego, CA, USA). Indomethacin was suspended in carboxymethylcellulose (CMC; Nacalai Tesque), while other drugs were dissolved or suspended in saline. All drugs were prepared immediately before use and given in a volume of 1 ml/200 g body weight.

Statistical analysis

Data are presented as the mean±SE from 5-6 rats per group. Statistical analyses were performed using a two-tailed unpaired t-test and Dunnett's multiple comparison test, and values of P<0.05 were considered to be significant.

RESULTS

Effects of various NOS inhibitors and aminoguanidine on indomethacin-induced gastric lesions in normal and arthritic rats

Oral administration of indomethacin (30 mg/kg) produced hemorrhagic lesions in the gastric mucosa of both normal and arthritic rats. The severity of these lesions was markedly aggravated in arthritic rats when compared with normal rats, the
damage scores in normal and arthritic rats being 9.5±0.6 mm² and 73.5±5.0 mm², respectively (Fig. 1). In normal rats, the development of gastric lesions in response to indomethacin was not significantly affected by pretreatment with either L-NAME (30 mg/kg) or aminoguanidine (50 mg/kg). However, L-NAME (3-30 mg/kg) dose-dependently prevented the aggravation of indomethacin-induced gastric lesions in arthritic rats, the damage score at a maximal dose (30 mg/kg) being 11.0±1.5 mm², which was almost equivalent to that in normal rats (12.0±1.3 mm²). Likewise, aminoguanidine (10, 30 and 50 mg/kg) also significantly suppressed the aggravation of these lesions in arthritic rats, although the potency of the effect was less marked than that of L-NAME, the damage score at a maximal dose of aminoguanidine (50 mg/kg) being 47.2±5.3 mm², which was still significantly greater than that in normal rats.

On the other hand, the severity of gastric lesions induced in normal rats by indomethacin was not affected by pretreatment with L-arginine (300 mg/kg, p.o.) (Fig. 3). In contrast, L-arginine showed the tendency to further enhance the increased gastric ulcerogenic response to indomethacin in arthritic rats, the damage score being 83.0±5.5 mm², which was not statistically significantly different from that of the control (69.7±3.7 mm²).

Gene and protein expression levels of NOS isozymes in the stomachs of normal and arthritic rats

In RT-PCR analyses, the expression iNOS mRNA was evident in the gastric mucosa of arthritic rats, although this expression was barely detected in the normal rat stomach (Fig. 4).
In contrast, the gene expression of eNOS and nNOS was both observed in the gastric mucosa of both normal and arthritic rats. However, the expression level of eNOS mRNA was markedly up-regulated in the arthritic rat stomach, although the gene expression level of nNOS remained unchanged.

Consistent with the results of RT-PCR analysis, the protein expression level of iNOS analyzed by western blotting was barely detected in the normal rat stomach but was distinctly enhanced in the gastric mucosa of arthritic rats (Fig. 4B), the degree of expression in arthritic rats being significantly greater than that in normal rats (4.29-fold) (Fig. 4C). In addition, the protein expression of eNOS and nNOS was observed in the gastric mucosa of both normal and arthritic rats, but the expression level of eNOS but not nNOS was significantly enhanced in the arthritic rat stomach, as compared with normal rats (2.50-fold and 1.18-fold, respectively).

Immunohistochemical distribution of eNOS and iNOS in the gastric mucosa of normal and arthritic rats

In an immunohistochemical study, the expression of eNOS was observed mostly in the vasculature of the gastric mucosa of both normal and arthritic rats (x100) (Fig. 5). However, eNOS staining was dramatically enhanced in arthritic rats, especially in the vasculature around the surface of the gastric mucosa. On the other hand, little expression of iNOS was seen in the gastric mucosa of normal rats, whereas strong iNOS expression was found in the epithelial and inflammatory cells in the gastric mucosa of arthritic rats.

Expression of eNOS monomer and dimer in the gastric mucosa of normal and arthritic rats

The protein expression level of total eNOS (130 kDa) was apparently higher in arthritic rats than normal rats after conventional SDS-PAGE under reducing conditions (Fig. 6A). On the other hand, immunoblot analysis after low-temperature SDS-PAGE under non-reducing conditions revealed the expression of eNOS dimer (260 kDa) in the gastric mucosa of both normal and arthritic rats, yet the level of expression was higher in arthritic rats than normal rats. However, the expression of eNOS monomer (130 kDa) was not detected in the gastric mucosa of both normal and arthritic rats (Fig. 6B). In addition, the expression ratio of the dimer and monomer showed no difference between normal and arthritic rats, the values being 5.2±0.3 and 5.7±0.3, respectively (Fig. 6C).

Effects of L-NAME and aminoguanidine on NO production in the stomachs of normal and arthritic rats

The amount of nitrite plus nitrate, the metabolites of NO, in the stomach was significantly higher in arthritic rats than normal rats, the values being 139.5±22.7 nmol/h and 939.5±101.1 nmol/h, respectively (Table 2). The increase in NO production in arthritic rats was significantly suppressed by the prior administration of L-NAME (30 mg/kg), the inhibition being 77.9%, while this treatment only showed a tendency to decrease basal NO production in normal rats (inhibition: 29.3%). Likewise, aminoguanidine also significantly prevented the overproduction of NO in arthritic rats (inhibition: 39.7%), yet it had no effect on basal NO production in normal rats (inhibition: 10.8%).

Change in GSH in the gastric mucosa of normal and arthritic rats

The amount of GSH in the gastric mucosa was 1.80±0.09 µmol/g tissue in normal rats. However, the GSH content in the
The concentration of nitrite plus nitrate in the gastric mucosa was determined by the modified Griess method. Data are presented as the mean±SE from 6 rats. Significant difference at P<0.05; *from control (vehicle alone); #from the corresponding group in normal rats.

Arthritis was induced as the injection of Freund’s complete adjuvant (FCA) into the planter region of the right hindpaw, and the experiment was performed 14 days after FCA injection. L-NAME (30 mg/kg) and aminoguanidine (50 mg/kg) were administered s.c. twice, at 18 h before and 1 h before ligation. The concentration of nitrite plus nitrate in the gastric mucosa was determined by the modified Griess method. Data are presented as the mean±SE from 6 rats. Significant difference at P<0.05; *from control (vehicle alone); #from the corresponding group in normal rats.

**DISCUSSION**

The present study confirmed that the gastric ulcerogenic response to indomethacin was markedly aggravated in arthritic rats, and this aggravation was suppressed by pretreatment with L-NMMA and aminoguanidine. When the efficiency of their effects was compared, it was also confirmed that the effect of L-NMMA was much potent than that of aminoguanidine; L-NMMA at 30 mg/kg completely prevented the aggravation in arthritic rats, while aminoguanidine even at 50 mg/kg only partially prevented the aggravated response, and the lesions score was still significantly greater that in normal rats. Then, the effects of selective inhibitors of various NOS isozymes on the increased gastric ulcerogenic response to indomethacin were examined in arthritic rats. The exacerbation of these lesions in arthritic rats was significantly suppressed by 1400 W (a selective inhibitor of iNOS) and L-NIO (a selective inhibitor of eNOS) but not by L-NPA (a selective inhibitor of nNOS). It was also observed that the concurrent administration of 1400 W and L-NIO almost totally attenuated the aggravation of gastric lesions in arthritic rats. These findings strongly suggest that the increased gastric ulcerogenic response to indomethacin in arthritic rats is mediated by NO derived from not only iNOS but also eNOS.

Consistent with the previous findings (3), the gene and protein expression of iNOS in the gastric mucosa was barely observed in normal rats but was markedly enhanced in arthritic rats. Although the gene and protein expression of eNOS and nNOS were observed in normal rat stomachs, the expression level of eNOS, but not nNOS, was markedly enhanced in arthritic rats. An immunohistochemical study showed that eNOS expression was mostly observed in the vasculature of the gastric mucosa in normal rats but up-regulated markedly in arthritic rats, especially in the vasculature around the surface of the gastric mucosa. These observations also suggest that the increased gastric ulcerogenic response in arthritic rats is accounted for by the up-regulation of eNOS/NO, in addition to iNOS/NO. While iNOS is a transcriptionally regulated enzyme, eNOS is considered to be a constitutively expressed protein. Recent studies showed that eNOS is also subjected to the regulation of expression (23). Indeed, the up-regulation of eNOS was reportedly observed in the gastric mucosa (24) and the colonic mucosa (25) during ulcer healing. Many studies showed that excessive production of NO mainly derived from iNOS contributes to the pathological aspects of inflammatory arthritis in humans and experimental animals (26-28). Haruna et al. (29) further showed that endothelial dysfunction observed during arthritis is attributable to upregulation and uncoupling of eNOS. Thus, it is assumed that upregulation of iNOS and eNOS may be involved in the progression of arthritis. At present, however, the mechanism underlying the upregulation of iNOS and eNOS expressions in the stomach during arthritis remains unknown.

Of interest in the present study is that eNOS/NO may act harmfully on the gastric mucosa in arthritic rats, similar to stomach was significantly decreased in the stomachs of arthritic rat (0.62±0.05 µmol/g tissue), which was approximately one third of the normal level (Fig. 7).

**Effect of exogenous GSH on indomethacin-induced gastric lesions in normal and arthritic rats**

The prior administration of exogenous GSH (100 and 200 mg/kg) significantly reduced the severity of indomethacin-induced gastric lesions in normal rats (inhibition: 58.4%) (Fig. 8). In addition, this treatment also prevented the aggravation of these lesions in arthritic rats (inhibition: 72.6%).

**Table 2. Effects of L-NAME and aminoguanidine on the production of nitrite plus nitrate in the stomachs of normal and arthritic rats**

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<th>Normal (nmol/h)</th>
<th>Arthritis (nmol/h)</th>
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<tr>
<td>Control</td>
<td>139.5±22.7</td>
<td>939.5±101.1*</td>
</tr>
<tr>
<td>L-NAME</td>
<td>98.6±7.9</td>
<td>275.6±51.5*#</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>124.4±33.5</td>
<td>607.2±67.9*#</td>
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Arthritis was induced by the injection of Freund’s complete adjuvant (FCA) into the planter region of the right hindpaw, and the experiment was performed 14 days after FCA injection. L-NAME (30 mg/kg) and aminoguanidine (50 mg/kg) were administered s.c. twice, at 18 h before and 1 h before ligation. The concentration of nitrite plus nitrate in the gastric mucosa was determined by the modified Griess method. Data are presented as the mean±SE from 6 rats. Significant difference at P<0.05; *from control (vehicle alone); #from the corresponding values of normal; *from the corresponding group in normal rats.

**Fig. 7.** Change in the amount of non-protein SH in the gastric mucosa of normal and arthritic rats. The experiment was performed 14 days after the injection of FCA. Data are presented as the mean±SE from 6 rats. *Significant difference at P<0.05 from normal rats.

**Fig. 8.** Effect of GSH on indomethacin-induced gastric lesions in normal and arthritic rats. The experiment was performed 14 days after the injection of FCA. After 18 h fasting, the animals were given indomethacin (30 mg/kg, p.o.) and sacrificed 4 h later. GSH (100 and 200 mg/kg) was given s.c. twice at 18 and 1 h before the administration of indomethacin. Data are presented as the mean±SE from 6 rats. Significant difference at P<0.05; *from control (vehicle alone); #from the corresponding group in normal rats.
indomethacin-induced gastric lesions in arthritic rats. Several studies have demonstrated that NO plays a dual role in the ulcerogenic response of the gastrointestinal mucosa depending on NOS isozymes; a protective effect of NO derived from eNOS such as nNOS and eNOS (6, 8), and a proulcerogenic effect of NO derived from iNOS (10, 30). Overproduction of NO, especially produced by iNOS, disrupts cellular functions and causes injuries in various cells and tissues (6, 31). Souza et al. (32) indeed showed that iNOS/NO plays a pathogenic role in indomethacin-induced gastric damage and granulocyte infiltration. This pathological situation, termed “nitrosative stress” (33), has been considered to be linked with oxidative stress due to reactive oxygen species (ROS) (33, 34). In particular, peroxynitrite (OONO-) generated from NO and superoxide (O2-) is a potent cytotoxic oxidant that irreversibly damages cells and tissues through the oxidation of free thiols and nitrination of tyrosine residues as well as lipid peroxidation (35-37). Lamarque and Whittle (38) reported that ROS were involved in the development of gastric damage induced by an NO donor in rats. Rachmilewitz et al. (39) also reported that a local application of an OONO- generating system caused colonic inflammation in rats. However, it is uncertain that a large amount of NO produced by eNOS, when up-regulated under certain conditions including arthritis, causes nitrosative stress in cells and tissues similar to iNOS. In the present study, we observed that the increased ulcerogenic response to indomethacin in arthritic rats was further enhanced by the administration of exogenous L-arginine, a substrate of NOS, though the effect was not statistically significant. These findings suggest that additional amounts of NO produced by eNOS in the gastric mucosa of arthritic rats showed a deleterious but not protective action and further supported an idea that NO even produced by eNOS, similar to iNOS, may also cause nitrosative stress to the gastric mucosa of arthritic rats. Since L-arginine exists abundantly even in the gastric mucosa to saturate NO production, it would be understandable that exogenous L-arginine did not cause a pronounced enhancement of the increased ulcerogenic response in arthritic rats. Indeed, we observed in the present study that the amount of NO production in the arthritic rat stomach was already increased nearly 7-fold compared with the normal rat stomach.

Endogenous non-protein sulhydryls (SH), mainly existing as intracellular glutathione (GSH), are known to play an important role in the regulation of various physiological functions in the body (40). Several reports demonstrated the protective role of SH, including hydrogen sulfide, in the stomach against various ulcerogenic stimuli (22, 41-44). It has also been reported that GSH reacts with NO to generate nitrosative stress in cells and tissues similar to iNOS. In the present study, we observed that the increased ulcerogenic response to indomethacin in arthritic rats was already increased nearly 7-fold compared with the normal rat stomach. This increase in NO production was observed in the arthritic rat stomach compared with normal rats. This increase in NO production was significantly suppressed by L-NAME or aminoguanidine, and the inhibitory effect of L-NAME was much more potent than that of aminoguanidine, together suggesting that eNOS does produce NO in the gastric mucosa under the present experimental conditions. Thus, it is unlikely that the increased ulcerogenic response to indomethacin in arthritic rats may be due to the overproduction of O2- generated by the uncoupling of eNOS.

We previously reported that the production of prostaglandins (PGs) was significantly increased in arthritic rat stomachs accompanied with upregulation of cyclooxygenase (COX)-2 (3, 5). PGs are known to cause a negative influence on the production of proinflammatory cytokines and iNOS mediated by EP2/EP4 receptors (55-57). In addition, we recently reported that PGs suppressed acid secretion through EP3 and IP receptors in the damaged stomach (58). It is possible that the upregulation of COX-2/PGs suppresses the increased production of NO and acid secretion during adjuvant arthritis. Therefore, the increased ulcerogenic response to indomethacin in arthritic rats may be, at least partly, brought about by further increase of NO production through inhibiting PG generation. Further study will be needed to elucidate the role of prostaglandins in this phenomenon.

Taken together, the present study showed that the aggravation of indomethacin-induced gastric lesions in adjuvant arthritic rats is accounted for by the up-regulation of eNOS/NO, in addition to iNOS/NO. It is assumed that eNOS/NO may act harmfully on the gastric mucosa in arthritic rats with the mucosal SH deficiency.

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Conflict of interests: None declared.

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