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DUAL ACTION OF NITRIC OXIDE IN PATHOGENESIS OF INDOMETHACIN-INDUCED SMALL INTESTINAL ULCERATION IN RATS

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We investigated the pathogenic role of nitric oxide (NO) in indomethacin-induced intestinal ulceration in rats. Nonfasting animals responded to a single administration of indomethacin (10 mg/kg, s.c.), resulting in multiple hemorrhagic lesions in the small intestine, mostly the jejunum and ileum. The damage was first observed 6 hr after indomethacin, the severity increasing progressively with time up to 24 hr later, accompanied with the gene expression of inducible NO synthase (iNOS) and the increase of nitrite and nitrate (NOx) contents in the mucosa. The ocurrence of damage was significantly prevented when iNOS induction was inhibited by dexamethasone given either once 0.5 hr before or twice 0.5 hr before and 6 hr after indomethacin. Likewise, aminoguanidine (a relatively selective iNOS inhibitor) reduced the severity of damage, irrespective whether given twice or as a single injection 6 hr after indomethacin. By contrast, the non-selective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) exhibited a biphasic effect, depending on the time of administration; the pre-administration worsened the damage, while the later administration reduced the severity of these lesions, yet both responses occureed in a L-arginine-sensitive manner. Pre-administration of L-NAME, but not aminoguanidine, significantly decreased NOx production in the intestinal mucosa of normal rats, while the increase of NOx production following indomethacin was significantly suppressed by the later administration of aminoguanidine as well as L-NAME. These results suggest that NO exerts a dual action in the pathogenesis of indomethacin-induced intestinal ulceration; NO generated by cNOS is protective against indomethacin, by maintaining the integrity of intestinal mucosa, while NO derived by iNOS plays a key pathogenic role in the ulcerogenic process.

Key words: *indomethacin, intestinal lesion, nitric oxide, iNOS, cNOS, L-NAME, rat*

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, even after short-term administration cause intestinal damage, including ulcers complicated by bleeding and performation in experimental animals and human

(1—4). Although the inhibition of cyclooxygenase, leading to depletion of endogenous prostaglandins (PG), is a major pathogenic factor, it is unlikely that PG deficiency alone is sufficient to initiate the process which ultimately results in intestinal ulceration (1, 5). Other factors such as bile and bacterial flora have also been considered as pathogenic elements in the intestinal ulceration (2, 6—8), yet the exact mechanism remains unknown. Recently, Whittle et al. (9) demonstrated that nitric oxide (NO) produced by inducible NO synthase (iNOS) plays a key pathogenic role in the occurrence of microvascular injury of small intestine following indomethacin.

In general, constitutively formed NO plays a key role in modulating microvascular tone and tissue integrity (10). By contrast, expression of iNOS can bring about tissue damage as well as microvascular injury (11). Kisse et al. (12) reported that pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME) augmented the rat colonic injury after intracolonic challenge with trinitrobenzene sulfonic acid (TNBS), whereas the delayed administration of L-NAME until 6 hr after TNBS challenge, when iNOS is detected, reduced tissue injury. Likewise, the microvascular injury induced in the rat jejunum by indomethacin was prevented by later administration of L-NAME, while the concurrect administration of L-NAME augmented the intestinal plasma leakage caused indomethacin (13). Thus, it is assumed that NOS inhibitors, depending upon the time of administration, exhibit beneficial or detrimental effects on gut inflammation, including indomethacin-induced intestinal damage.

In the present study, we examined the effects of NOS inhibitors on the intestinal ulcerogenic response to indomethacin in rats, by varying the time of administration, and demonstrated a dual action of NO in the pathogenesis of this lesion model.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (200～230 g, Nippon Charles River, Shizuoka, Japan) were used. The experiments were performed using non fasting 5～6 rats per group under unanesthetized conditions.

General Procedures

The animals were administered indomethacin subcutaneously in a dose of 10 mg/kg. The animals were killed on various time points, 0, 3, 6, 12 and 24 hrs after indomethacin, under deep

ether anesthesia, and the intestinal lesion score, NOS activity as well as nitrite/nitrate (NOx) contents were measured. In some cases, the animals were treated with L-NAME (a non-selective NOS inhibitor; 20 mg/kg), aminoguanidine (a relatively selective iNOS inhibitor; 20 mg/kg) (14) or dexamethasone (an inhibitor of iNOS induction; 3 mg/kg). These agents were given s.c. once either 0.5 hr before or 6 hr after indomethacin treatment. In the case of L-NAME, half the animals were administered L-arginine (500 mg/kg) s.c. 20 min before L-NAME.

Macroscopic Evaluation of Intestinal Lesions

Under deep ether anesthesia, both the jejunum and ileum were removed and treated with 2% formalin for fixation of the tissues. Then they were opened along the mesentric attachment and examined for lesions under a dissecting microscope with square grids ($\times 10$). The area (mm^2) of macroscopically visible lesions was measured, summed per small intestine, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals.

Determination of Nitrite and Nitrate (NOx) Contents

The NO generated in the mucosa was determined indirectly as nitrite/nitrate (NO_2^- and NO_3^-). Under deep ether anethesia, the animals were killed, and the small intestines were removed. After rinsing the intestine with cold saline, the mucosa (a 10 cm section starting 30 cm proximal to ileocecal junction) was scraped with glass slides and homogenized in 50 mM $KHPO_4$ buffer, followed by centrigugation for 10 min at 10.000 rpm at 4°C. The supernatant NOx levels were measured by the Griess reaction-dependent method described by Green et al. (15), after reduction of NO_3^- to NO_2^- with nitrate reductase. Nitrites were incubated with Griess reagent (0.1% naphthylene diamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4) for 10 min at room temperature, and the absorbance at 545 nm was measured. For the standard curve, sodium nitrite was used.

Analysis of iNOS and nNOS mRNA by RT-PCR

The animals were killed under deep ether anesthesia 6 hr after administration of indomethacin, and the small intestines were removed and then frozen in liquid nitrogen, stored at -80°C until use. Intestinal tissue samples were pooled from 2~3 rats for extraction of total RNA, which was prepared by a single-step acid phenol-chloroforum extraction procedure by use of TRIZOLE (GIBCO BRL, Gaithersburg, USA). Total RNA primed by random hexadeoxyribonucleotide was reverse-transcribed with SUPERSCRIPT Preamplification system (GIBCO BRL). The sequences of sense and antisense primers for the rat iNOS were 5′-ACAACAGGAA-CCTACCAGCTCA-3′ and 5′-GATGTTGTAGCGCTGTGTGTCA-3′, respectively, giving rise to a 651 bp PCR product (16). For the rat neuronal NOS (nNOS), the sequences of sense and antisense primers were 5′-GAATACCAGCCTGAT-CCATGGAA-3′ and 5′-TCCTCCAGGAGGGTGTCCACCGCATG-3′, respectively, giving rise to a 602 bp PCR product (17). For the rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a constisutively expressed gene, the sequences were 5′-GAACGGGAAGCTCAGGCATGGC-3′ for sense primer and 5′-TGAGGTCCACCACCCTGT-TGCTG-3′ for antisense primer, giving rise to a 310 bp PCR product (18). An aliquot of the RT reaction product served as a template in 35 cycles of PCR with 1 min of denaturation of 94°C, 0.5 min of annealing of 58°C and 1 min of eytension at 72°C on a thermal cycler. A portion of the PCR

mixture was electrophoresed in 1.8% agarose gel in TAE buffer (Tris buffer 40 mM, EDTA 2 mM and acetic acid 20 mM; pH 8.1), and gel was stained with ethidium bromide and photographed.

Preparation of Drugs

Drugs used were indomethacin, N^G-nitro-L-arginine methyl ester (L-NAME), aminoguanidine and L-arginine (Sigma Chemicals, St. Louis, MO, USA) and dexamethasone (Nacalai Tesque, Kyoto, Japan). Indomethacin were suspended in saline with a drop a Tween 80 (Wako), while dexamethasone were suspended in carboxymethylcellulose (CMC) solution. Other drugs were dissolved in saline. All drugs were prepared immediately before use and administered s.c. in a volume of 0.5 ml/100 g body weight.

Statistics

Data are presented as the means $\pm$ SE from 5 to 6 rats per group. Statistically analyses were performed using two-tailed Dunnett's multiple comparison test, and values of $P < 0.05$ were considered as significant.

RESULTS

Time Course Changes in Intestinal Ulceration and NOx Production After Indomethacin Administration

Macroscopic damage induced by 10 mg/kg of s.c. indomethacin appeared in the small intestine at the 6 th hour and progressively increased to reach a peak at the 24 th hour, the lesion score being 222.4 ± 13.5 mm^2 (*Fig. 1*). The damage was observed mostly in the jejunum and ileum, with an increase of mucosal microvascular permeability (data not included). Following administration of indomethacin, the amount of NOx generated in the intestinal mucosa was also found to significantly elevate already at 6 th hour (350.6 ± 53.5 nmol/g tissue), and further increased to reach a peak (505.6 ± 43.5 nmol/g tissue) at 12 th hour, which was about 3 fold greater than basal values (160.8 ± 22.5 nmol/g tissue) found in control animals without indomethacin.

Gene Expression of nNOS and iNOS mRNA in Intestinal Mucosa after Administration of Indomethacin

RT-PCR analysis apparently revealed that iNOS mRNA was expressed in the intestinal mucosa at 6 th hour after administration of indomethacin, although it was not detected in the control mucosa (*Fig. 2*). On the other hand, the gene expression of nNOS mRNA remained unchanged before and after indomethacin treatment (data not shown).

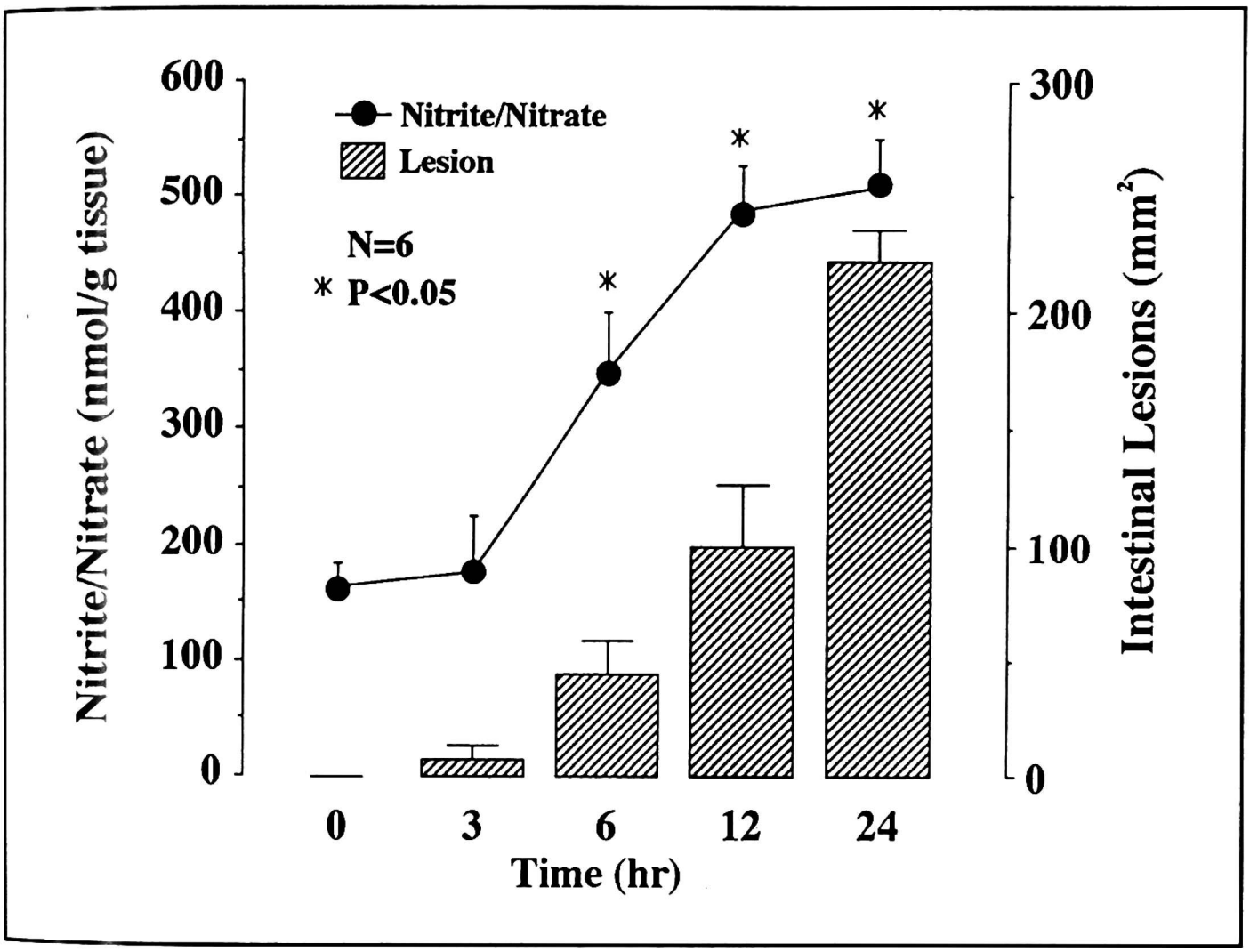

Fig. 1. The course changes in development of small intestinal lesions and increase of Nitrite and Nitrate (NOx) production in the intestinal mucosa following administration of indomethacin in rats. Animals were administered indomethacin s.c. in a dose of 10 mg/kg and killed 3, 6, 12 and 24 hr later. Data are presented as the means ± SE from 6 rats. * Significant difference from cintrol, at $P < 0.05$.

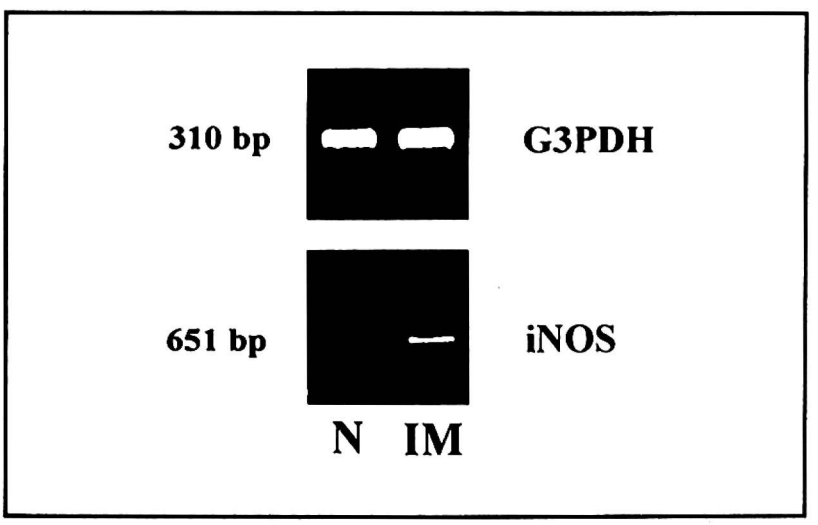

Fig. 2. Gene expression for iNOS in the intestinal mucosa following administration of indomethacin. Animals were administered indomethacin s.c. in a dose of 10 mg/kg and killed 6 hr later. Lane: N — normal; IM — indomethacin.

Effects of Various Drugs on Intestinal Ulcerogenic Response Induced by Indomethacin

Effects of L-NAME: Subcutaneously administered indomethacin at 10 mg/kg caused multiple hemorrhagic lesions in the small intestine 24 hr later; the lesion score was 212.5 ± 11.6 mm^2. Pretreatment of the animals with L-NAME 0.5 hr before indomethacin significantly worsened the severity of intestinal lesions, in a L-arginine-sensitive manner; the lesion score was 343.5 ± 42.1 mm^2 or 235.8 ± 29.2 mm^2, respectively, without or with L-arginine (*Fig. 3*). By contrast, later administration of L-NAME, 6 hr after indomethacin,

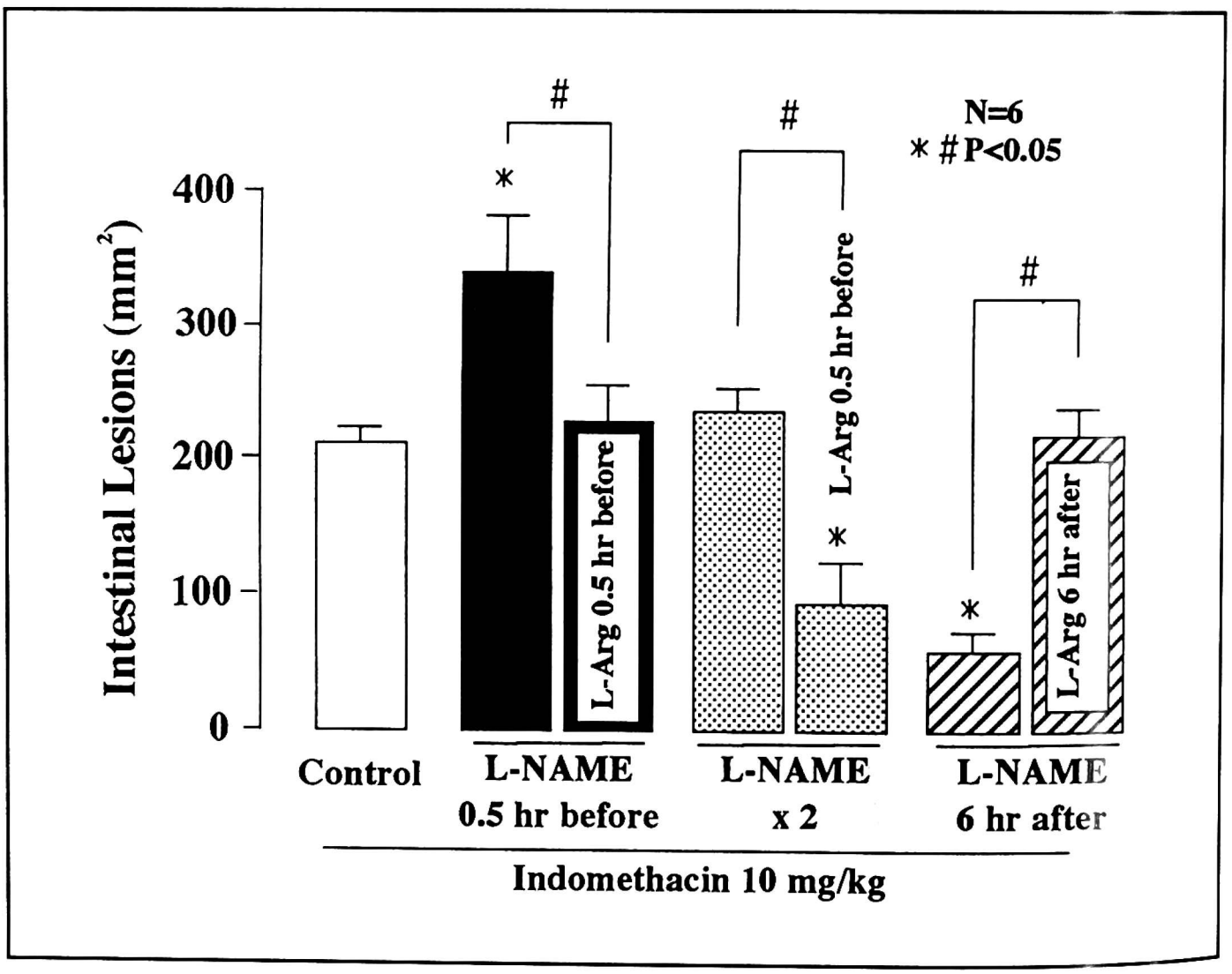

Fig. 3. Effects of L-NAME and L-arginine on indomethacin-induced intestinal ulceration in rats. Animals were administered indomethacin s.c. in a dose of 10 mg/kg and killed 24 hr later. L-NAME (20 mg/kg, s.c.) was administered 0.5 hr before or 6 hr after indomethacin, with or without co-administration of L-arginine (500 mg/kg s.c.). Data are presented as the means $\pm$ SE from 6 rats. Significant difference from control at $P < 0.05$; *from control; # L-NAME alone.

markedly suppressed the development of intestinal lesions, and this effect was also antagonized by co-administration of L-arginine, the lesion score being 59.3 ± 14.2 mm^2 after L-NAME alone and 219.0 ± 20.6 mm^2 after L-arginine plus L-NAME. However, when the aninals were treated with L-NAME twice,

0.5 hr before and 6 hr after indomethacin, the severity of intestinal lesions remained unchanged; the lesion score was 236.4 ± 18.3 mm^3, which was not significantly different from that (212.5 ± 11.6 mm^2) observed in control rats. In this group of rats, however, when L-arginine was co-administered with the first injection of L-NAME, the severity of intestinal lesions was significantly suppressed as compared to both the control group and the group treated with L-NAME alone.

Effects of aminoguanidine and dexamethasone: Aminoguanidine was effective in significantly reducing the severity of intestinal lesions in response to indomethacin, irrespective of whether the agent was given as a single injection 6 hr after indomethacin or twice at 0.5 hr before and 6 hr after indomethacin, the degree of inhibition being similar, 74.8% or 81.2%, respectively (*Fig. 4*).

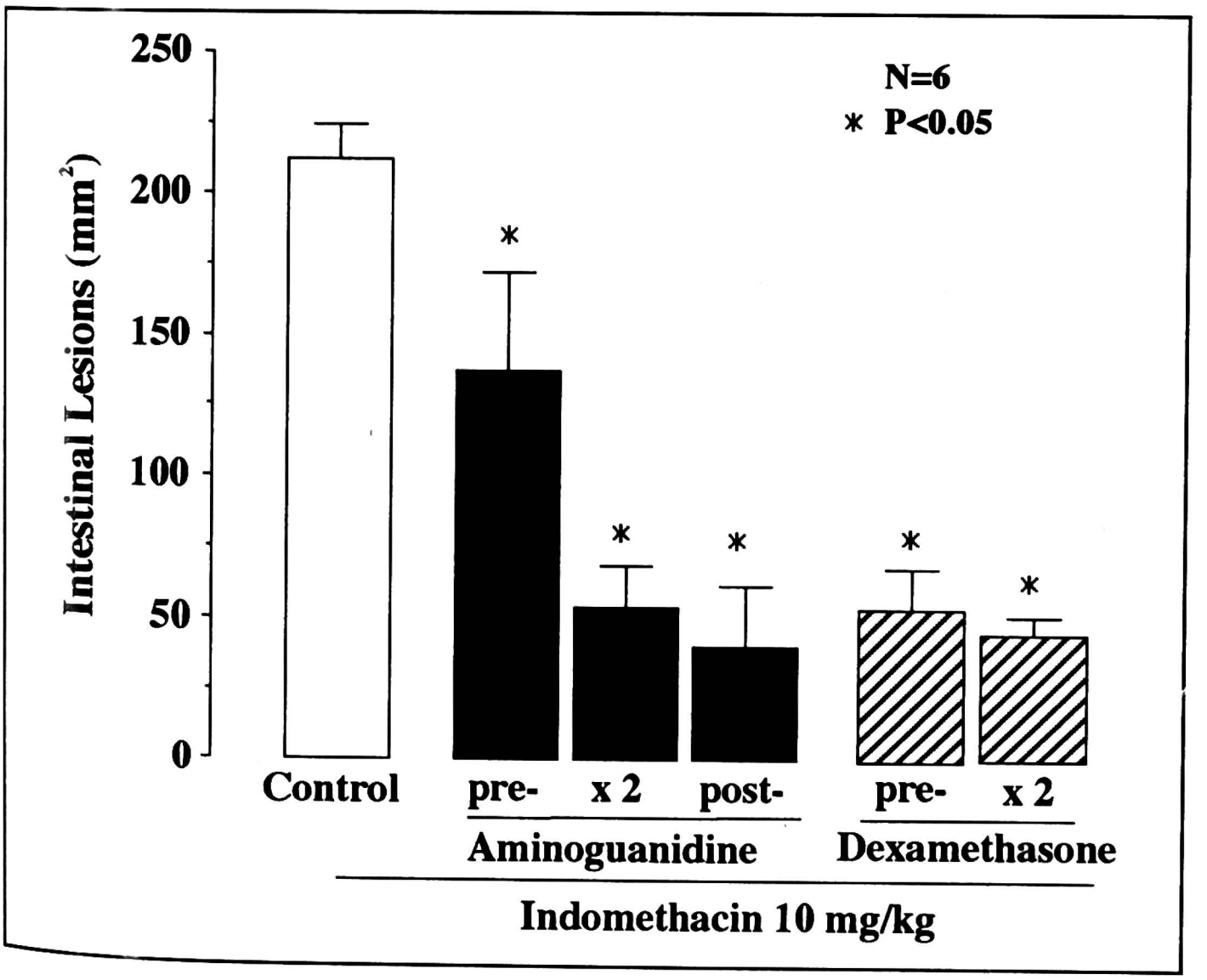

Fig. 4. Effects of aminoguanidine and dexamethasone on indomethacin-induced intestinal ulceration in rats. Animals were administered indomethacin s.c. in a dose of 10 mg/kg and killed 24 hr later. Aminoguanidine (20 mg/kg, s.c.) was administered one 0.5 hr or 6 hr after administration of indomethacin or twice 0.5 hr before and 6 hr after indomethacin treatment. Dexamethasone (3 mg/kg, s.c.) was given one 0.5 hr before indomethacin or twice 0.5 hr before and 6 hr after indomethacin treatment. Data are presented as the means $\pm$ SE from 6 rats. * Significant difference from control, at $P < 0.05$.

The severity of intestinal lesions was also significantly suppressed by a single pre-administration of aminoguanidine, although the degree of inhibition was 35.4%, less than that observed by twice or post administration. Likewise, dexamethasone given twice 0.5 hr before and 6 hr after indomethacin markedly reduced the development of intestinal lesions in response to indomethacin, the inhibition being 79.0% Likewise, this agent was also effective in significantly reducing the severity of intestinal ulceration (76.8%) when given one 0.5 hr before indomethacin treatment.

Effects of L-NAME and Aminoguanidine on NOx production in Intestinal Mucosa

The NOx contents in the intestinal mucosa increased from 160.8±22.5 nmol/g tissue to 505.6±43.6 nmol/g tissue at 24 th hour after indomethacin treatment (*Table 1*). Later administration (6 hr after indomethacin) of either L-NAME or aminoguanidine significantly decreased the NOx generation in the mucosa, the values being 111.5±19.3 nmol/g tissue or 199.3±42.8 nmol/g tissue, respectively. A single administration of L-NAME also significantly decreased the NOx contents in the intestinal mucosa of normal rats, from 166.5±7.5 nmol/g tissue to 97.3±4.5 nmol/g tissue, when determined 0.5 hr after administration. However, aminoguanidine had no effect on the NOx contents in the normal mucosa, the values being 168.4±9.1 nmol/g tissue.

Table 1. Effects of L-NAME and Aminoguanidine on Nitrite/Nitrate Contents in the Intestinal Mucosa

Treatment	No. of Rats	Nitrite/Nitrate Contents (nmol/g tissue)
Normal	5	166.5±7.5
+L-NAME	5	97.3±4.5 *
+Aminoguanidine	5	168.4±9.1
Normal	5	160.8±22.5
Indomethacin		
+Vehicle	5	505.6±43.5 *
+L-NAME	5	111.5±19.3 #
+Aminoguanidine	5	199.3±42.8 #

All values are presented as the means±SE from 5 rats per group. Animals were administered L-NAME (20 mg/kg, s.c.), or aminoguanidine (20 mg/kg, s.c.), killed 0.5 hr later, and NOx contents were measured by the Griess method. In some cases, indomethacin (10 mg/kg) was given s.c. 6 hr before administration of L-NAME or aminoguanidine, and the animals were killed 24 hr after indomethacin. Significamt difference at $P < 0.05$: * from normal; # from vehicle.

DISCUSSION

The present study showed that endogenous NO produced by iNOS plays a pathogenic role in the occurrence of intestinal ulceration following subcutaneous administration of indomethacin. These findings are in agreement with previous reports of Whittle and his group (9, 13), who showed that indomethacin increasee calcium-independent NO production, followed by occurrence of the microvascular injury in the rat jejunum. We further demonstrated in this study that NO plays a dual role in the pathogenesis of this ulcer model, a protective role by cNOS/NO and a proulcerogenic role by iNOS/NO. These data also support a recent finding by Laszlo and Whittle (13), who showed a protective role of NO formed by cNOS against the acute injurious actions of endogenously released platelet-activating factor (PAF) on microvascular integrity following indomethacin administration.

It is known that short-term and long-term administration of NSAIDs such as indomethacin can cause intestinal ulceration in human and laboratory animals (1-4, 9, 13, 19). Several factors have been postulated as the pathogenic element of intestinal ulceration induced by indomethacin, including PG deficiency, bile acid and bacterial flora (5—8), the exact mechanisms remain unexplored. Boughton-Smith et al. (20) first reported that bacterial endotoxin from E. coli, a predominant member of the rat gut bacterial flora, enhanced the intestinal permeability though increased expression of iNOS. Whittle et al. (9) later showed that the microvascular injury in the rat jejunum, which commened 18 hr following indomethacin, was associated with the expression of iNOS and was inhibited by the delayed administration of the NO synthase inhibitor L-NAME, suggesting a key pathogenic role of iNOS/NO in this phenomenon. Accordingly, it is considered that the release of bacterial products such as endotoxin may contribute to indomethacin-induced intestinal ulceration through overproduction of NO by iNOS in the mucosa. This contention was also supported by the present findings that indomethacin caused intestinal ulceration with concomitant increase of NOx and expression of iNOS mRNA in the mucosa and that the damage was prevented by the blockade of NO production by NO synthase inhibitors. NO interacts with the superoxide radicals to produce a cytotoxic peroxynitrite, which causes a deleterious influence on the gastrointestinal mucosal integrity (21). Rachimilewitz etal. (22) reported the tissue inflammation by applying the peroxynitrite generating system in the rat colonic mucosa, while Milleret al. (23) demonstrated the expression of iNOS and the formation of peroxynitrite in guinea pig ileitis. We have reported that the severity of indomethacin-induced intestinal ulceration was reduced by pretreatment with allopurinol a xanthine oxidase inhibitor as well as hydroxyurea a neutrophil reducing agent (19). Thus, it is assumed that a detrimental role of NO in indomethacin-induced

small intestinal ulceration may be explained by a cytotoxic effect of peroxynitrite produced from NO in the presence of superoxide radicals.

It should be however, noted in the present study that time-course changes in NOx levels and the severity of damage following indomethacin treatment were different; the NOx levels in the intestinal mucosa elevated at 6 th hour after indomethacin administration and further increased, reaching a peak at 12 th hour, while the severity of damage, first observed at 6 th hour, progressively increased with time and reached a peak at 24 th hour. These results suggest that excessive levels of NO, formed by iNOS, may be involved in later extension process of the damage caused by indomethacin. Previous studies suggest a local action of PAF in acute intestinal lesions after ischemia or administration of indomethacin plus NO synthase inhibitors (13, 24). The underlying mechanism for the early damage caused by indomethacin remains unknown, but it may involve multiple factors including PG depletion, bile acids, food substances, microorganisms and smooth muscle contraction (5, 6, 8, 25, 26). In our study, apparent macroscopic damage was observed as early as 3 hr after dosing of indomethacin. This result was in agreement with the findings by Anthony et al. (26) who observed that early vascular damage occurred at 3 rd hour after indomethacin. In contrast, Whittle et al. (9) and Laszko and Whittle. (11) did not observe the vascular injury until 18 hour after indomethacin. Although the reason for these different results remains unknown, it may be due to different experimental conditions such as strain of rats and housing circumstances. Bertrand et al. (27) recently reported that TNF-α production linked to the toxicity of indomethacin in the small intestine, especially at the early stage of the ulcerogenic process. They showed that TNF-α induces iNOS activity, which then acts as the effector pathway to produce injury.

It is believed that NO formed by cNOS under physiological cirumstances is important for housekeeping function in the gastrointestinal mucosa (10). Laszlo et al. (13) recently demonstrated that following indomethacin administration, the early inhibition of NO synthase by L-NAME leads to acute microvascular injury in the rat intestine, indicating a protective role of NO formed by cNOS in the intestine. Indeed, we showed in the present study that intestinal ulcerogenic response to indomethacin was worsened or prevented by pre- or post-administration of L-NAME a non-selective NO synthase inhibitor, respectively. By contrast, aminoguanidine, considered to be a relatively selective inhibitor of iNOS (14), prevented the intestinal ulceration, irrespective of whether the agent was given twice (pre- and post-indomethacin) or as a single administration 6 hr after indomethacin. Certainly, the intestinal ulceration was reduced by an inhibitor of iNOS induction dexamethasone. A biphasic effect of L-NAME was also observed in the TNBS-induced rat

colonic ulcer model, depending whether the agent was given before or after induction of iNOS (12). These findings clearly suggest that non-selective NO synthase inhibitors such as L-NAME have a biphasic effect, depending on the time of administration. The worsening effect of L-NAME may be brought about by the blockade of NO production due to cNOS inhibition. The blockade of NO production by L-NAME may cause a decrease of mucus secretion and an increase of motility (28—30). The mucus layer might be a barrier for bacterial invasion to intestinal mucosa, which is responsible for excessive NOx production through induction of iNOS (23, 31). In addition, intestinal hyper-motility may cause the mucosal hypoxia and microvascular injury due to smooth muscle contraction, leading to neutrophil infiltration and release of various cytokines (26, 31). It is assumed that these functional changes may increase the mucosal susceptibility to initial insult following indomethacin, resulting in aggravation of intestinal ulceration.

At present, the source cells for iNOS expression in the intestinal mucosa remain unknown. We previously reported that indomethacin provoked intestinal ulceration, together with an increase of iNOS/NO production and myeloperoxidase (MPO) activity (19, 32), suggesting an involvement of leukocytes in the pathogenesis of these lesions. Other study also showed an involvement of TNF-α in the pathogenic mechanism of these lesions, especially in the early event preceding the elevations of both NO production and MPO activity as well as the lesion formation (27). It is known that TNF-α induces the expression of iNOS in various cells including leukocytes (33, 34). On the other hand, Mannaioni et al. (35) demonstrated that mast cells releases NO-like substances, suggesting the presence of NO producing system in the cell. We have previously shown the involvement of mast cells in the pathogenesis of indomethacin-induced intestinal lesions (19). Thus, it is possible that both leukocytes and mast cells may be the source cells for the expression of iNOS and NO production in the intestinal mucosa following administration of indomethacin.

In conclusion, the present together with previous results suggest that endogenous NO exerts a dual action in the pathogenesis of indomethacin-induced intestinal ulceration; NO generation by cNOS is protective against indomethacin, by maintaining the integrity of intestinal mucisa, while NO derived by iNOS plays a key pathogenic role in the ulcerogenic process. Accordingly, the non-selective NO synthase inhibitor L-NAME shows a dual effect on this lesion model, depending on the time of administration, worsening the damage by the pre-administration and preventing the damage by the later administration. It is thus considered that selective inhibitors of iNOS may be therapeutic value in intestinal ulceration in response to NSAIDs.

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