Endothelial nitric oxide synthase modulates gastric ulcer healing in rats

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Received 18 January 2000; accepted in final form 7 March 2000

Ma, Li, and John L. Wallace. Endothelial nitric oxide synthase modulates gastric ulcer healing in rats. Am J Physiol Gastrointest Liver Physiol 279: G341–G346, 2000.—Nitric oxide has been shown to be beneficial for gastric ulcer healing. We determined the relative effects of endothelial and inducible nitric oxide synthases on gastric ulcer healing in rats. Ulcers were induced by serosal application of acetic acid. Ulcer severity, angiogenesis, and nitric oxide synthase expression were assessed 3–10 days later. The effects of inhibitors of nitric oxide synthase were also examined. Inducible nitric oxide synthase mRNA was only detected in ulcerated tissue (maximal at day 3), whereas the endothelial isoform mRNA was detected in normal tissue and increased during ulcer healing. Inducible nitric oxide synthase was expressed in inflammatory cells in the ulcer bed, whereas endothelial nitric oxide synthase was found in the vascular endothelium and in some mucosal cells in both normal and ulcerated tissues. Angiogenesis changed in parallel with endothelial nitric oxide synthase expression. $N^G$-(iminoethyl)-L-lysine did not affect angiogenesis or ulcer healing, while $N^G$-nitro-L-arginine methyl ester significantly reduced both. In conclusion, endothelial nitric oxide synthase, but not the inducible isoform, plays a significant role in gastric ulcer healing.

angiogenesis; wound healing; vascular endothelial growth factor

NITRIC OXIDE (NO) has been shown to be beneficial in gastric ulcer healing. Konturek et al. (11) demonstrated that NO synthase (NOS) inhibition by $N^G$-nitro-L-arginine (L-NNA) or $N^G$-monomethyl-L-arginine (L-NMMA) significantly delayed ulcer healing, reduced gastric blood flow around the ulcer, and impaired angiogenesis in the granulation tissue. On the other hand, administration of an NO donor (glyceryl trinitrate) or L-arginine, the substrate of NOS, significantly enhanced healing (8, 11) and reversed NOS inhibitor-induced delayed healing (7). In addition to stimulating mucus and bicarbonate secretion (2, 4) and maintaining gastric blood flow (17), NO also promotes angiogenesis in vivo and in vitro (25, 26).

Angiogenesis, a process of new blood vessel growth from preexisting vessels, requires a concerted interaction of a variety of cellular systems (21). It starts with the dissociation of basement membrane beneath endothelial cells, followed by endothelial migration, adhesion, proliferation, and tube differentiation. Ziche and co-workers (25, 26) have demonstrated that NO acts downstream of vascular endothelial growth factor (VEGF) and substance P in mediating angiogenesis in an in vivo corneal implant model in rabbits. NO has also been shown to stimulate angiogenesis in response to tissue ischemia in mice (15). NO donors promote endothelial cell proliferation and migration (26). Biosynthesis of NO is conducted by a family of isozymes, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). As the names suggest, nNOS and eNOS are primarily expressed (constitutively) in neurons and endothelial cells, respectively. In contrast, iNOS is not usually expressed in normal tissue but can be induced under various pathological situations (1). A role for NOS in tissue repair is supported by the findings that skin wound healing was impaired in both iNOS- and eNOS-deficient mice (12, 24). The roles of the different isoforms of NOS during ulcer healing are not well understood. We (8) previously observed that gastric ulcers in rats are rapidly colonized by a variety of bacteria (primarily Gram negative) and that these bacteria act to retard ulcer healing. Because bacterial endotoxin is a potent inducer of iNOS expression (1), we hypothesized that iNOS-derived NO produced in a setting of gastric ulceration may influence the healing of the ulcer. Therefore, in the present study, we have examined the relative contributions of eNOS and iNOS to gastric ulcer healing in rats.

MATERIALS AND METHODS

Animals and induction of gastric ulcer. All experiments described below were approved by the Animal Care Committee of the University of Calgary. Male Wistar rats weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, QC, Canada). They were fed a standard laboratory diet and tap water and kept in a room with controlled temperature (22 ± 1°C), humidity (65–70%), and a 12:12-h light-dark cycle. Food was withheld 18 h before ulcer induction. Gastric ulcers were produced by the method of...
Wang et al. (22) with slight modifications (8). Briefly, a laparotomy was performed, and the stomach was exposed in a halothane-anesthetized rat. Acetic acid (0.5 ml, 80% vol/vol) was instilled into a 3-ml syringe barrel, which had been cut and smoothed, and applied to the serosal surface of the stomach for 1 min. The acid was removed by aspiration, and the area was washed with sterile saline. The abdomen was sutured closed, and the rats were returned to the standard diet of laboratory chow and tap water. Control rats underwent a sham operation that involved performing a laparotomy and gently manipulating the stomach, but not applying acetic acid. Each group consisted of five to seven rats.

**Assessment of gastric ulcer healing.** At 3, 6, and 10 days after ulcer induction, rats were killed and the ulcer areas were assessed (8). The stomachs were removed, opened along the greater curvature, and pinned out on a wax block. A paper grid with an area of 25 mm² was placed alongside the ulcer, which was then photographed. The ulcer area was determined by planimetry using ×5 enlargements of the photograph. The area of ulceration in pixels was converted to units of square millimeters using the paper grid as a reference. All planimetric determinations were performed using coded photographs so that the observer was blinded to the treatment the rats had received. A longitudinal section including the ulcer base and both sides of the ulcer margins was fixed in 4% neutral-buffered formalin at 4°C overnight and processed for immunohistochemical localization of iNOS, eNOS, or the von Willebrand factor. In addition, stomach samples (~150 mg, full thickness including ulcer base and ulcer margin) were taken for the isolation of eNOS and iNOS mRNA and determination of NOS activities.

**Drug treatment.** Rats were treated intraperitoneally with a selective iNOS inhibitor, N^6^-iminoethyl-L-lysine (L-NIL; Sigma Chemical, St. Louis, MO), or a nonselective NOS inhibitor, N^G^-nitro-L-arginine methyl ester (L-NAME; Sigma). L-NIL (3 mg/kg) was given twice daily from day 3 to day 5 after ulcer induction, whereas L-NAME (15 mg/kg) was given once daily from day 3 to day 9 after ulcer induction. These treatment regimens and doses were selected because in pilot studies they were found to produce the desired NOS inhibition and selectivity.

**Measurement of NOS activity.** Tissue samples (100–150 mg) were homogenized for 20 s in ice-cold Tris-·HCl solution (50 mM, pH 7.4; containing 320 mM sucrose, 0.1 mM EDTA, 1 mM diethiothreitol, 10 mg/ml soybean trypsin inhibitor, 10 mg/ml leupeptin, 2 mg/ml aprotinin, and 1 mg/ml phenylmethylsulfonyl fluoride). The homogenates were then centrifuged at 20,000 g for 30 min at 4°C. NOS activity was estimated by the conversion of L-[3H]arginine to the NO product L-[3H]citrulline as described by Brede and Snyder (3) with slight modifications. Briefly, supernatant (100 μl) was mixed with L-[3H]arginine (6.5 μCi/ml; NEN, Boston, MA) and Tris··HCl (150 μl, pH 7.4, 50 mM; containing 0.7 mM NADPH, 50 mM CaCl₂, and 7 mM L-valine to inhibit any arginase). EGTA (1 mM) and L-NAME (3 mM) were used to determine NOS activity and blank, respectively. The reaction mixture was incubated for 30 min at 37°C and terminated with ice-cold HEPES buffer (750 μl, pH 5.5; containing 1 mM EDTA and 1 mM EGTA), followed by 150 μl of HEPES buffer containing 3 mM L-arginine and 3 mM L-citrulline. The resulting mixture was then passed through a cation-exchange chromatographic column (0.6 g of Dowex AG50WX-8 resins; sodium form; Bio-Rad Laboratories, Hercules, CA) and eluted with HEPES buffer (1 ml) to separate L-[3H]citrulline from unreacted L-[3H]arginine. Thereafter, the radioactivity of the sample was counted using a liquid scintillation counter (Beckman LS 9800). The final result was expressed as picomoles of L-[3H]citrulline formed per minute per gram of protein. The protein concentration of the supernatant was measured using a protein assay kit (Bio-Rad).

**NOS mRNA expression.** Total RNA was isolated from tissue samples using TRIzol reagent (GIBCO BRL, Gaithersburg, MD). Two micrograms of total RNA were used to generate the first strand of cDNA with an RT (GIBCO BRL) according to the manufacturer’s instructions. The cDNAs of iNOS, eNOS, and GAPDH were amplified by PCR using the sense and antisense primers as described previously (9). The amplification was conducted at 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 29/22 (iNOS/GAPDH) and 31/22 (eNOS/GAPDH) cycles, respectively. RT-PCR products were detected by electrophoresis on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

**Assessment of angiogenesis during ulcer healing.** Angiogenesis was assessed by counting the number of neangiocytes using immunohistochemistry for the von Willebrand factor (23) on formalin-fixed and paraffin-embedded sections. After deparaffination and rehydration, endogenous peroxidase activity in tissue was quenched with 0.3% hydrogen peroxide/methanol for 10 min at room temperature. Sections were incubated overnight at 4°C with rabbit anti-human von Willebrand factor (Dakopatts). The bound primary antibody was detected by the avidin-biotin-peroxidase method (Vector Laboratories, Burlingame, CA). Thereafter, the neomicrovessels were visualized after diamobenzidine reaction. They were counted under microscopy at ×200 magnification in a blind manner, as described previously (13). The final values were expressed as the number of angiogenic microvessels per square millimeter of tissue.

**Immunostaining of iNOS and eNOS.** After deparaffination, rehydration, and inactivation of the endogenous hydrogen peroxidase, antigen retrieval was performed by exposure to microwave radiation at 500 W for 10 min in citrate buffer (10 mM, pH 6.0). Sections were then incubated overnight (4°C) with primary antibodies against iNOS or eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, the procedures followed were the same as described above. The positive staining was absent when the primary antibodies were omitted.

**Statistical analysis.** All data are expressed as means ± SE for five to seven rats per group. Comparisons of data among groups were performed with one-way ANOVA followed by the Student-Newman-Keuls test. P < 0.05 was considered significant.

**RESULTS**

**Time course of angiogenesis and NOS expression.** Gastric ulcers were well established 3 days after serosal application of acetic acid. The damage spanned the full thickness of the mucosa and penetrated through the muscularis mucosae. In some cases, ulceration extended to the muscularis propria, but perforations were not observed during the course of study. The area of the ulcers decreased with time, as shown in Fig. 1A. Approximately 12 angiogenic microvessels/mm² were observed in normal submucosa (i.e., in the absence of an ulcer). Granulation tissue began to appear 3 days after ulcer induction. The number of angiogenic microvessels in the granulation tissue increased markedly after ulcer induction, reaching a maximum at day 6 (Fig. 1B).

As shown in Fig. 2, eNOS mRNA expression was detected in both normal and ulcerated gastric tissue.
The levels of expression increased after ulcer induction and peaked at day 6. In contrast, iNOS mRNA was not detectable in normal gastric tissue. In ulcerated tissue, iNOS mRNA expression was evident, with the highest levels of expression observed on day 3 and day 6 after ulcer induction (Fig. 3).

Expression of eNOS protein was prominent in both normal and ulcerated gastric tissues, consistent with the pattern of its mRNA expression. eNOS was mainly localized to the vascular endothelium and some mucosal cells (Fig. 4, A–C, G, and H). In contrast, iNOS protein was only detected in ulcerated stomachs and primarily expressed in inflammatory cells in the ulcer bed (Fig. 4, D–F). The profiles of eNOS and iNOS changed in parallel with their mRNA expression.

Effects of NOS inhibitors. To characterize the relative contributions of different isoforms of NOS to gastric ulcer healing, groups of five to seven rats were treated with vehicle, a selective iNOS inhibitor (L-NIL), or a nonselective NOS inhibitor (L-NAME) according to the protocol described in MATERIALS AND METHODS. Both iNOS and constitutive NOS (cNOS) activities were higher at day 6 than day 10 in the vehicle-treated group. This is consistent with the expression of NOS mRNA and protein (the assay for NOS activity does not distinguish between the two constitutive isoforms of NOS: eNOS and neuronal NOS). L-NIL significantly inhibited iNOS activity without affecting cNOS activity (Fig. 5). In pilot studies, we observed a loss of selectivity (inhibition of both iNOS and cNOS) when L-NIL was administered for 7 days, which is why we used the shorter treatment period in the present study.

L-NAME inhibited iNOS at day 6 in a similar fashion.
as L-NIL, but exhibited a stronger inhibition on eNOS at both day 6 and day 10 (Fig. 5).

Administration of L-NIL did not affect either angiogenesis or gastric ulcer healing, as indicated in Fig. 6. Neither the number of microvessels in the granulation tissue nor ulcer area was significantly affected by treatment with L-NIL, despite the marked inhibition of iNOS activity. On the other hand, L-NAME significantly suppressed angiogenesis and delayed ulcer healing.

**DISCUSSION**

Previous studies have documented the ability of NO donors and l-arginine (a precursor of NO synthesis) to accelerate the healing of experimental gastric ulcers (8, 11). Moreover, suppression of NO synthesis with a nonselective inhibitor of NOS was found to significantly delay ulcer healing (11). In the present study, we have confirmed this latter finding with a different, nonselective NOS inhibitor. Moreover, we provide evidence that it is the suppression of constitutively expressed NOS, rather than iNOS, that accounts for the impairment of ulcer healing. Thus, although iNOS is very strongly induced in ulcerated tissue in the stomach and iNOS activity is similarly upregulated, the NO derived from that isoform does not appear to play a role in modulating healing.

One of the mechanisms through which eNOS-derived NO might contribute to ulcer healing is by promoting angiogenesis, the formation of new microvessels from preexisting blood vessels. The expression of eNOS and the appearance of angiogenic microvessels increased in a parallel manner during ulcer healing, peaking at day 6. Angiogenesis plays a crucial role in
wound healing and tissue regeneration, and its importance in gastric ulcer healing has been addressed in a number of studies. For example, administration of fibroblast growth factor to rats with duodenal ulcers was shown to stimulate angiogenesis and accelerate ulcer healing (10). On the other hand, chronic administration of indomethacin resulted in inhibition of angiogenesis in granulation tissues and a significant retardation of gastric ulcer healing in rats (19). The presence of eNOS protein in the vascular endothelium of mucosa, submucosa, and especially granulation tissue suggests that eNOS might be involved in angiogenesis during ulcer healing. This is substantiated by the fact that angiogenesis and skin wound healing have been shown to be impaired in eNOS-deficient mice (12). Similarly, Murohara and colleagues (15) reported that angiogenesis in response to tissue ischemia was significantly suppressed in eNOS-deficient mice. VEGF, a potent angiogenic factor, failed to promote angiogenesis in eNOS-deficient mice (15), and the NOS inhibitor L-NAME has been shown to completely block the endothelial migration and proliferation stimulated by VEGF (20). Thus several studies are consistent with the hypothesis that eNOS-derived NO is a downstream signal for growth factor-induced angiogenesis.

One possible mechanism through which NO modulates angiogenesis is the suppression of protein kinase C-δ (PKC-δ). Indeed VEGF-triggered endothelial migration and proliferation has been shown to be a consequence of NO-dependent PKC-δ inhibition (20). L-NAME treatment reversed the inhibition of PKC-δ and thus slowed down the process of endothelial migration and proliferation. Moreover, NO donors, such as sodium nitroprusside, reduced PKC-δ activity and thereby promoted endothelial migration and proliferation (20). A further mechanism through which NO could modulate angiogenesis is by modifying adhesion molecule expression on endothelial cells. NO has been shown to maintain the functional expression of αvβ3-integrin, a mediator for endothelial migration, survival, and angiogenesis (14).

Relative to eNOS, iNOS produces large amounts of NO. Consistent with the findings of others (5, 18), we did not detect iNOS mRNA and protein in normal tissues. However, they were evident in ulcerated stomach and primarily localized in inflammatory cells at the ulcer base. iNOS mRNA and protein expression were greatest during the early phase of ulcer healing. We (8) have previously reported that gastric ulcers in rats are very rapidly colonized by a wide range of bacteria but primarily of the Gram-negative variety. It is possible that iNOS expression increases in response to this...
colonization by bacteria, since bacterial endotoxin is a very potent stimulus for iNOS induction. Moreover, iNOS may play a role in protecting the gastric mucosa from bacteria, since NO-derived reactive nitrogen species (e.g., peroxynitrite) have been shown to be cytotoxic to bacteria (6). However, if this were the case, one would have expected l-NIL, the selective inhibitor of iNOS, to delay ulcer healing.

In summary, the results of the present study confirm the importance of NO in the modulation of gastric ulcer healing. They further demonstrate that NO derived from iNOS does not play an important role in gastric ulcer healing. Although a role for neuronal NOS-derived NO cannot be excluded, the data presented herein suggest that eNOS-derived NO is the most important in terms of effects on the healing process, most likely through its effects on angiogenesis.

This work was supported by a grant from the Medical Research Council of Canada (MRC). J. Wallace is an MRC Senior Scientist and an Alberta Heritage Foundation for Medical Research Senior Scientist. L. Ma is supported by an MRC/AstraZeneca/Canadian Association of Gastroenterology Research Fellowship.

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