Nitric oxide donors retard wound healing in cultured rabbit gastric epithelial cell monolayers

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Kiviluoto, Tuula, Sumio Watanabe, Miyoko Hirose, Nobuhiro Sato, Harri Mustonen, Pauli Puolakkainen, Mikko Rönty, Tuula Ranta-Knuuttila, and Eero Kivilaaakso. Nitric oxide donors retard wound healing in cultured rabbit gastric epithelial cell monolayers. Am J Physiol Gastrointest Liver Physiol 281: G1151–G1157, 2001.—Effects of nitric oxide (NO) on gastric wound healing were investigated in primary rabbit gastric epithelial cell cultures. We analyzed the speed of cell migration, proliferation, and apoptosis after creating a round wound on the cell cultures. The monolayers were incubated with or without the NO donor sodium nitroprusside, oxatrazolimine 1,2,3,4-oxatrazolium, 5-amino-3-(3,4-dichlorophenylchloride), or the peroxynitrite generator 3-morpholinosydnomine-N-ethylcarbamide. The possible role of cGMP as a second messenger of NO was investigated with 8-bromo-cGMP. The role of O2·− was evaluated using diethyldithiocarbamate and pyrogallol. The effects of superoxide dismutase and allopurinol were also investigated. NO inhibited the speed of cell migration and proliferation and induced cell apoptosis in a dose- and time-dependent manner. The effects were augmented with O2·− generators and ameliorated by O2·− scavengers, whereas cGMP had no significant effect on wound healing. NO donors retard gastric wound healing by inhibiting migration and proliferation and inducing cell apoptosis. These effects do not seem to be mediated via cGMP, but O2·− or peroxynitrates may be involved.

gastric epithelium; cell migration; cell proliferation; apoptosis; peroxynitrite; hydroxyl radicals; superoxide; guanosine 3’,5’-cyclic monophosphate

NITRIC OXIDE (NO), a uniquely diffusible messenger molecule, participates in many physiological and pathophysiological processes in the gastrointestinal tract. In the stomach, the constitutively expressed calcium-dependent NO synthase (cNOS) has been identified at least in the surface epithelial and chief cells, submucosal arteriolar plexus, and somatostatin-releasing D cells. cNOS activity has also been identified in enteric ganglia and in their processes (8). NO exerts a protective action against various mucosal injuries by regulating regional blood flow and increasing epithelial mucus production (13, 29, 36). In addition, high levels of NO produced by inducible NOS (iNOS) play a potent role as a cytotoxic agent during infection and inflammation, thus promoting lethal injury to the victim cells. NO is also involved in certain immune responses in acute and chronic infections, e.g., by increasing permeability of intestinal epithelium (38). NO may also have some role in the development of certain neoplastic diseases in the gastrointestinal tract (4, 5, 23, 42).

In apoptosis NO seems to have a dual regulatory function. In certain cell types NO induces apoptosis, whereas in others it opposes this process (10, 17). It has been proposed that apoptosis caused by NO or its metabolites results from DNA damage and accumulation of tumor suppressor protein p53. Also, NO can reversibly bind to cytochrome a3 leading to inhibition of mitochondrial respiration and apoptosis (2). It has become increasingly evident that other reactive metabolites, e.g., peroxynitrite (PN), hydroxyl radicals, and superoxide radicals (O2·−), may play an important role as mediators of the cytotoxic effects of NO (22).

After superficial injury the gastric mucosa is rapidly restored. This is accomplished by migration of the surviving epithelial cells over the denuded mucosa (restitution) and, later, also by cellular proliferation. Some earlier reports suggested that NO somehow contributes to this healing process (1, 29). However, these studies were mostly performed with in vivo or ex vivo techniques, which provide multiple local targets and systemic interactions for the effects of NO, thus complicating the interpretation of the findings. The present study investigates the mechanisms by which NO affects wound repair in a primary monolayer cell culture with rabbit primary gastric oxyntic epithelial cells as the sole target for NO.

MATERIALS AND METHODS

In this study isolated rabbit gastric mucosal monolayer cultures were used. All animals received humane care in

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compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 86–23, revised 1985). The authorization to perform this study was given by the Provincial Government of Uusimaa in accordance with Finnish legislation.

The process for the isolation of gastric mucosal cells from rabbit stomach was previously described in detail (41). Briefly, fasted male rabbits (weight 2.0–3.0 kg) were euthanized with an overdose of pentobarbital sodium. The stomach was quickly removed, opened along the greater curvature, and rinsed with ice-cold isotonic saline. The oxyntic mucosa was separated with a razor blade and minced into small pieces (2–3 mm²). The pieces were incubated in a medium containing 0.07% collagenase (type I; Sigma, St. Louis, MO), 130 mM NaCl, 12 mM NaHCO₃, 3 mM NaH₂PO₄, 2 mM MgSO₄, 1 mM CaCl₂, 0.1% bovine serum albumin, and 0.2% glucose for 15 min in a shaker bath at 37°C. After the incubation, the tissue was washed in Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (HBSS) with 1 mM EDTA. These procedures were repeated twice before the tissue was filtered through a metal mesh (mesh size 300 μm). The cells were then washed in Ca²⁺- and Mg²⁺-free HBSS containing 1 mM EDTA and 0.1% bovine serum albumin.

Cell Culture

Coon's modified Ham's F-12 medium supplemented with inactivated 10% fetal bovine serum, 10 x 10⁴ U/L penicillin, 100 mg/L streptomycin, and 0.25 mg/L amphotericin was used. The isolated gastric mucosal cells were grown on collagen (type I)-coated plastic culture dishes (diameter 60 mm; Corning Glass Works, Corning, NY) at a concentration of 5 x 10⁶ cells/dish. The cells were incubated in a humidified atmosphere containing 5% CO₂-95% air at 37°C.

Artificial Wounding and Restoration Assay of Monolayer

The cultured gastric epithelial cells formed a complete monolayer cell sheet within 48 h after cell inoculation. As reported earlier, >90% of the cells in the cell sheet were periodic acid-Schiff-positive mucous cells, 5% were succinic dehydrogenase-positive parietal cells, and 4% were Nile blue-positive chief cells (40). A round artificial wound with a cell-free area of constant size (3.0 mm²) was made in the center of the mucosal cell sheet using a modified pencil-type mixer with a rotating silicon tip without damaging the coated dish surface. After wounding, the total restoration assay including cell migration and cell proliferation was performed under fetal bovine serum-free conditions. The test agents, as described under Chemicals, were added immediately after the artificial wounding of the monolayer cell culture, and their effects were monitored by taking phase-contrast micrographs at 12–24 h intervals for 48 h. The cell-free area on the epithelial cell sheet was measured using an IBAS II image analyzer (Carl Zeiss, Oberkochen, Germany) and phase-contrast micrographs. The change in the cell-free area (round shaped) was converted to average migration speed (v) with the following formula

\[ v = \frac{A_1}{\pi t} - \frac{A_2}{\pi t} \]

where \( A_1 \) and \( A_2 \) are cell free-area measured before and after time interval \( t \).

Measurement of Nitrite/Nitrate Levels

NO production released by sodium nitroprusside (SNP) was assessed as combined accumulation of nitrite and nitrate into the culture medium. Total cumulative NO production over 24 and 48 h after artificial wounding of the monolayer was calculated from six different concentrations of SNP (10, 30, 100, 300, 500, and 1,000 μM) as described by Orpana et al. (26).

Determination of Cellular Disruption by Trypan Blue Exclusion

Trypan blue dye [100 μl of 1% (wt/vol) solution; Sigma] was added directly to the monolayer culture. After 5 min, the number of stained and nonstained cells was estimated in a random manner by counting 100–200 cells from each culture under a microscope at×40 magnification.

Cell Proliferation Assay

DNA synthesis was determined in the control, 300 μM SNP, and 1 mM N⁵-nitro-L-arginine methyl ester (L-NAME) monolayer cultures at different time intervals. DNA-synthesizing cells were detected by indirect immunohistochemistry using monoclonal anti-5-bromodeoxyuridine (BrdU) antibody (Sigma, St. Louis, MO) (18). BrdU was added at the time of wounding (0 h) and 12, 24, 36, and 48 h later, and incubation was continued for 1 h. Samples were processed and stained for BrdU by standard techniques. Microscopic pictures were taken with a phase-contrast microscope (Nikon, Tokyo, Japan) using Fujichrome 100 ASA film. Photomicrographs were taken at the edge of the wounded monolayer, and the BrdU labeling index (BrdU-positive cell number/total cell number × 100) was calculated in a randomly selected unit area (1.47 mm²) from the edge of the wound.

Apoptosis Assay

Gastric epithelial cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) with an in situ apoptosis detection kit (Apo-BrdU, no. 6576kk. PharMingen). The apoptosis assays were performed 24 and 48 h after wounding of the monolayer. The colored cells were analyzed using Becton-Dickinson FACScan flow cytometry and Compus Cyten laser scanning cytometry (LSC). Altogether 5,000–10,000 events/culture dish were collected by flow cytometry and analyzed using the Cell Quest program. Cell clusters and subcellular particles were removed by gating (i.e., only the single cells were analyzed). The measurements of LSC were performed by using CytoSpin preparation; the data were analyzed by using Win Cyte analysis. The labeling index for TUNEL-positive cells was calculated by the formula labeling index (%) = (positive cell number/total cell number) x 100.

Chemicals

Two kinds of NO donors in three different concentrations were used, SNP (100, 300, and 1,000 μM; Sigma) and 1,2,3,4-tetrazolium, 5-aminino-3-(3,4-dichlorophenyl)-3-nitro-L-arginine methyl ester (L-NAME) (Sigma, St. Louis, MO) (18). BrdU was added at the time of wounding (0 h) and 12, 24, 36, and 48 h later, and incubation was continued for 1 h. Samples were processed and stained for BrdU by standard techniques. Microscopic pictures were taken with a phase-contrast microscope (Nikon, Tokyo, Japan) using Fujichrome 100 ASA film. Photomicrographs were taken at the edge of the wounded monolayer, and the BrdU labeling index (BrdU-positive cell number/total cell number × 100) was calculated in a randomly selected unit area (1.47 mm²) from the edge of the wound.

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To investigate the role of endogenous production of NO in the cell monolayers NO synthesis was inhibited with a non-selective NOS inhibitor, l-NAME (ICN, Costa Mesa, CA), in three different concentrations (100, 300, and 1,000 μM).

To investigate the role of cGMP as the second messenger of NO, 8-bromo-cGMP (8-BrcGMP) (Sigma) was used in two concentrations (100 and 1,000 μM). To investigate whether the cytotoxic effects of NO are, at least in part, mediated by PN (a metabolite of NO and O2), the effects of increased amounts of O2· were studied with a superoxide dismutase (SOD) inhibitor, diethyldithiocarbamate (DDC, 10 μM), or an O2· generator, pyrogallol (PG, 500 μM), with or without the NO donor SNP. Xanthine oxidase (XO) is involved in the terminal oxidation of purines. It generates intracellular reactive oxygen metabolites such as O2·-. To eliminate the generated O2·-, an inhibitor of XO, allopurinol (AP, 2 mM) (Sigma) was used. In other experiments a O2·- scavenger, SOD (2,000 U/ml), was used, which enzymatically catalyzes the conversions of O2·- into H2O2, with or without the NO donor SNP. Because H2O2 itself is a potent biological oxidant, catalase (CAT, 100 U/ml) was used in these experiments to eliminate the generated H2O2. The control for each compound was the vehicle in its culture medium. All these chemicals were purchased from Sigma.

Statistics

The results are expressed as means ± SD. Student’s unpaired t-test for equal and unequal variances was used for statistical analysis of the raw data. General linear modeling was used in migration data to adjust for the possible differences in migration speed between different cultures. The SAS program GLM (SAS Institute, Cary, NC) was used to fit a model in which the dependent variable was the speed of migration and the independent variables were the group variable and a categorical variable for different cultures. A P value <0.05 was considered a statistically significant difference.

Fig. 1. Effect of the peroxynitrite generator 3-morpholinosydnonime-N-ethylcarbamide (SIN-1) and the nitric oxide (NO) donor 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichlorophenylchloride) (GEA-3162) on the speed of cell migration 24 h after artificial wounding of the monolayer culture (means ± SD; 9–13 cultures in each group, in each culture at least 12 wounds were examined; *P < 0.05 compared with controls). Note that SD is used instead of SE throughout. Comparisons were adjusted for possible differences in migration speed between different cultures.

Fig. 2. The production of NO metabolites NO2 and NO3 (means ± SD) caused by different concentrations of sodium nitroprusside (SNP) in the cell cultures (after 24- and 48-h incubation time). The control monolayers did not produce these metabolites in measurable amounts.

RESULTS

Effects of NO and PN Donors

GEA-3162. The highest concentration of the NO donor GEA-3162 (30 μM) caused a statistically significant (P < 0.01) retardation in the speed of cell migration and wound healing (21.4 ± 4.9 vs. 12.4 ± 8.9 μm/h), whereas the two lower concentrations of GEA-3162 had no significant effect on this process (Fig. 1).

SIN-1. The effects of SIN-1 were less pronounced than those of the NO donors. However, the two highest concentrations of SIN-1 (500 μM and 1 mM) had a significant inhibitory effect on the speed of cell migration and wound healing (Fig. 1).

Neither GEA-3162 nor SIN-1 caused any cellular damage in the monolayer cultures as judged from the trypan blue exclusion experiments.

Production of Nitrite/Nitrate by SNP

SNP produced nitrite and nitrate in a time- and concentration-dependent manner (Fig. 2). The most frequently used concentration of SNP (300 μM) produced 73.3 ± 7.7 μM nitrite/nitrate in 24 h. This concentration did not cause any destruction in the cell cultures as judged from the trypan blue exclusion experiments. In control monolayers (without SNP) there was no detectable nitrate/nitrite production.

Compared with the controls, SNP caused a significant inhibition of cell migration during the initial 24 h of wounding (Fig. 3). The highest concentration (1,000 μM) caused even diffuse cellular damage with cell death and detachment from the collagen matrix. The inhibition of the wound healing was, at least in part, reversible: when SNP was removed from the culture medium 24 h after its addition, the wound healing process was gradually normalized (Fig. 4).

BrdU staining displayed that SNP almost totally inhibited cellular proliferation at 24–36 h (Table 1). SNP also caused a statistically significant increase in apoptosis index. After 24 h the apoptosis index was 9.3 ± 2.9% in the control cultures and 24.4 ± 4.8% in SNP cultures (n = 12, P < 0.001; Table 2).
Effects of 8-BrcGMP

As compared with the controls, 8-BrcGMP had no significant effects on the speed of wound healing or cell migration (21.7 ± 6.3 vs. 22.7 ± 6.5 and 21.1 ± 5.6 vs. 21. ± 5.9 μm/h for controls and 100 and 1,000 μM 8-BrcGMP, respectively).

Effects of O₂⁻· Inductors

DDC. DDC is an agent capable of inhibiting endogenous SOD activity, which in turn leads to increased amounts of O₂⁻·. Reaction of O₂⁻· with NO results in enhanced formation of PN. To investigate the effects of increased amounts of PN, the monolayers were incubated in the presence of DDC with or without SNP. As shown in Table 2, incubation of the monolayers with SNP + DDC significantly inhibited cell migration compared with the control and SNP groups. The apoptosis index with SNP + DDC was increased compared with control.

PG. PG (which undergoes spontaneous autooxidation under physiological conditions, resulting in generation of O₂⁻·, with or without SNP, also significantly increased the inhibition of wound healing and the apoptosis index (Table 2).

Effects of O₂⁻· Scavengers

SOD + CAT. To further explore whether O₂⁻· scavengers could normalize wound healing, SOD together with CAT, with or without SNP was used. Compared with the effects of SNP alone, the combination of SNP and SOD + CAT significantly enhanced the speed of cell migration, but it did not significantly improve the apoptosis index (Table 2).

Effect of Inhibition of NO Synthesis: L-NAME

To examine the effects of total elimination of NO in the wound healing, three concentrations of L-NAME (n = 7 each) were used in the monolayer cultures. Compared with the controls, the speed of cell migration during the first 24 h of wound healing was essentially the same in all L-NAME groups (Fig. 5). Even with the highest concentration of L-NAME (1 mM) the number of BrdU-positive cells at different time intervals was essentially the same as in the controls. At 24 h BrdU-positive cells were identified only rarely, and at 36 h the number of BrdU-positive cells was at its maximum. The number of these cells decreased after complete healing of the wound (Table 1).

DISCUSSION

NO seems to have a protective action against gastric mucosal injury provoked by hypertonic salt solution (33), hydrochloric acid (31), ethanol (43), taurocholate (32, 34), ischemia-reperfusion (28), water immersion (11), or cold restraint-induced stress ulcer (6). Furthermore, NO seems to contribute to gastric adaptive cytoprotection induced by mild irritants (18). Inhibition of NOS with the nonselective NOS inhibitor L-NAME impairs the recovery of transmucosal electric resis-
wounds were examined). Comparisons were adjusted for possible administration of L-arginine (the substrate for NOS) with portal hypertension in the rat (25). Furthermore, development of gastric mucosal lesions in association with mucosa. For instance, NO seems to play a role in the experiments NO can also damage the gastrointestinal pathophysiological conditions. According to some other amounts of NO can act as cytotoxic agents in several flow, and neuroendocrine function, whereas excessive regulating gastrointestinal motility, regional blood amounts of NO act as classic neurotransmitters in sal blood flow (12, 14, 19, 21, 30).

protective mechanism of NO is improvement of mucus. It has been suggested that in in vivo conditions the most important protective mechanism of NO is improvement of mucosal blood flow (12, 14, 19, 21, 30).

The effects of NO seem to be “dose dependent.” Small amounts of NO act as classic neurotransmitters in regulating gastrointestinal motility, regional blood flow, and neuroendocrine function, whereas excessive amounts of NO can act as cytotoxic agents in several pathophysiological conditions. According to some other experiments NO can also damage the gastrointestinal mucosa. For instance, NO seems to play a role in the development of gastric mucosal lesions in association with portal hypertension in the rat (25). Furthermore, administration of L-arginine (the substrate for NOS) dose dependently enhanced gastric mucosal damage caused by 20% ethanol in the rat (9). Also, high levels of NO formed by iNOS seem to contribute to the damage associated with colon and small intestinal mucosal inflammation (35). However, there are also findings divergent from these results. Exogenous NO generated by NO donors [3-(cis-2,6-dimethyl piperidino)-sydnonimine (CAS 754), SIN-1] did not cause cellular, vascular, or mucosal dysfunction in normal or infectious small intestine in the cat (20).

Several reports suggest that an excessive amount of NO, whether endogenous or exogenous, deteriorates cellular viability and can be cytotoxic to gastrointestinal mucosa (4, 9). Brown and coworkers (4) showed that surface cells are more susceptible to damage by NO than other cells of the gastric epithelium. Furthermore, it has been shown that high levels of NO generated by bacterial endotoxin challenge in rats cause gastrointestinal cell injury both in vivo and in vitro in gastric mucosal cell suspensions. This response seems to be causally related to an increase in intracellular Ca\(^{2+}\) concentration and protein kinase C activation (3, 37).

In the present model of rabbit primary gastric epithelial cell monolayer cultures, exogenous NO retarded wound healing in a time- and concentration-dependent manner by inhibiting cell migration and proliferation and by inducing cell apoptosis. The inhibition of wound healing was reversible after the NO donor SNP was removed from the culture medium. The highest concentration of SNP completely and irreversibly inhibited wound healing, provoking cellular ruffling and detachment from the collagen matrix. It is possible that this concentration might have a direct toxic action on the cell cultures. For this reason, a smaller concentration of SNP was used as the standard concentration in subsequent experiments.

In the present study the gastric epithelial cell monolayer cultures did not produce detectable amounts of endogenous NO, and the nonselective NOS inhibitor L-NAME did not have any influence on the speed of wound healing. This finding is in accordance with ear-
lier reports suggesting that, for some reason, the cultured cells lose their ability to produce NO (26).

It has been assumed that cGMP acts as a second messenger of NO, and an increase of intracellular cGMP concentration has even been used as an indirect indicator of the amount of NO produced (45). However, this concept has been questioned, because in vitro even very high concentrations of NO (10- to 100-fold increase) have only a minor influence on the level of a cGMP analog, 8′-BrcGMP (which readily penetrates the cell membrane and is rather resistant against phosphodiesterases), was used to further elucidate these effects. The data demonstrate that excess of cGMP is not the cause of the inhibitory effects of NO on wound healing.

The present findings suggest that the toxicity of NO donors is mediated, at least in part, by its reaction with \( \text{O}_2^{-} \), yielding the potent oxidant PN (ONOO−). This view is supported by the findings that the XO inhibitor AP, which blocks the formation of \( \text{O}_2^{-} \), attenuated the inhibition of wound repair caused by SNP via an enzymatic pathway. Also, a potent \( \text{O}_2^{-} \)-scavenger, SOD, abolished the retardation of wound healing caused by SNP. On the other hand, agents that increase the availability of \( \text{O}_2^{-} \), such as DDC (an inhibitor of SOD) or PG (an exogenous \( \text{O}_2^{-} \)-generator), significantly potentiated the retardation of wound healing provoked by NO donors. In general, the defense against free radicals in cell cultures may be less effective than in vivo experiments.

NO also promotes apoptosis, which obviously contributes to the delayed wound healing provoked by NO. It seems that this action of NO requires simultaneous intracellular generation of \( \text{O}_2^{-} \). The data in Table 2 indicate that the cell membrane-permeant XO inhibitor AP, which blocks enzymatic endogenous intracellular generation of \( \text{O}_2^{-} \) from purines, completely abolishes the increase in apoptosis induced by the NO donor SNP. In contrast, the membrane-impermeant SOD (with catalase), which exerts its \( \text{O}_2^{-} \)-scavenging action extracellularly, has no such action. This suggests that \( \text{O}_2^{-} \) may act as a second messenger of NO or, more likely, that it produces together with NO their reactive metabolite, PN, as discussed above. However, it seems that \( \text{O}_2^{-} \) may also directly induce apoptosis, because the \( \text{O}_2^{-} \) donor PG (without NO) promotes an amount of apoptosis similar to that promoted by the NO donor SNP.

It was reported earlier that NO alone is almost nontoxic against cultured rat liver endothelial cells under low \( \text{O}_2 \) partial pressures but highly cytotoxic in the presence of nonphysiological high \( \text{O}_2 \) partial pressure (15). In the present experiments the \( \text{O}_2 \) partial pressure was kept under 20% as recommended by those authors. Thus our results are not explicable in terms of high \( \text{O}_2 \) partial pressure.

As yet, there are only a few reports on the effects of NO on cell proliferation in general (15) and, to our knowledge, none as regards gastric epithelial primary cultured nontransformed cells. The present finding of an inhibitory effect of NO on cellular proliferation is in accordance with that of Yang and coworkers (44), who demonstrated that exogenous NO inhibits the proliferation of cultured vascular endothelial cells. Furthermore, SIN-1, a donor of NO and \( \text{O}_2^{-} \), dose dependently inhibited growth of human keratinocytes without loss of viability (39).

In conclusion, NO donors retard gastric wound healing by inhibiting cell migration and proliferation and inducing cell apoptosis. These effects do not seem to be mediated via cGMP, but \( \text{O}_2^{-} \) or PNs may be involved.

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