Participation of Nitric Oxide in the Mucosal Injury of Rat Intestine Induced by Ischemia-Reperfusion

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ABSTRACT

The dual role of nitric oxide as a cytoprotective or a cytotoxic free radical gas has been noted in various types of pathophysiological conditions, including the digestive system. The aim of this study was to examine the role of nitric oxide in the mucosal injury induced by ischemia-reperfusion in the rat small intestine. A transient intestinal ischemia was produced in the catheterized ileal segments of rats by occluding the anterior mesenteric artery for 60 min. Nitric oxide metabolites (NO₂⁻ and NO₃⁻) and lactate dehydrogenase activity in perfusates of the intestinal lumen were measured over 5 hr periods. The time-course of histological changes in small intestine was also observed. After ischemia-reperfusion, nitric oxide release in the intestinal lumen increased significantly and the dynamics of nitric oxide release correlated with that of lactate dehydrogenase leakage. The administration of N³-nitro-L-arginine methyl ester (1.0–2.5 mg/kg) inhibited this increased nitric oxide release and the lactate dehydrogenase leakage and afforded protection against the mucosal injury induced by ischemia-reperfusion. In conclusion, the nitric oxide production that was accelerated by ischemia-reperfusion of small intestine may possibly participate in the breakdown of intestinal mucosa after ischemia-reperfusion insult.

The small intestine is a most sensitive organ to ischemic insult, which causes barrier dysfunction and enhances bacterial translocation (Wells et al., 1989). There is some evidence that these ischemic injuries of the small intestine may be exacerbated significantly by reperfusion (Parks and Granger, 1986). Ischemia-reperfusion injury of the small intestine, associated with hemorrhage and other shock states, is characterized by a number of microvascular and mucosal alterations, including endothelial cell swelling, capillary plugging, a prolonged reduction in intestinal blood flow and mucosal barrier dysfunction (Schoenberg et al., 1984; Granger et al., 1986; Haglund et al., 1987). Nitric oxide (NO), initially identified as an endothelial-derived relaxing factor (Palmer et al., 1987), has been implicated in the pathogenesis of ischemia-related tissue damage (Moncada et al., 1991). The possible contribution of NO to the pathogenesis of ischemic small intestine has remained unproven. NO rapidly reacts with superoxide anion to form peroxynitrite anion (ONOO⁻), a highly reactive oxidizing agent capable of causing tissue damage. NO also contributes to the incidence of deamination-related genetic mutation (Wink et al., 1991). These events participate in the generation of NO cytotoxicity. On the other hand, under physiological conditions, NO, formed by calcium-dependent constitutive NO synthase, plays a crucial role in maintaining vascular integrity as well as mucosal barrier function (Whittle et al., 1990; Kubes, 1992; Kubes and Granger, 1992). There are data that NO reduces mucosal injury after ischemia-reperfusion of the intestine. NO generators significantly reduce mucosal injury after ischemia-reperfusion (Kurose et al., 1994) and L-NAME, a potent inhibitor of NO synthase, greatly exacerbates intestinal injury and increases mucosal barrier dysfunction associated with ischemia-reperfusion (Kubes, 1993).

In light of these conflicting observations, it seemed necessary to study the time course of NO release from small intestine during and after ischemic insult. Whether there is a correlation between the dynamics of NO production and the degree of the mucosal injury after ischemia-reperfusion of small intestine was thus examined. Effects of L-NAME on the development of tissue injury were also given attention.

Methods

Animals. Male Wistar rats weighing 275 to 325 g (Kyudo Co., Ltd., Kumamoto, Japan) were fed a standard rat chow (F-2; Funabashi Farm Co., Chiba, Japan) and tap water ad libitum. Groups of three...
or four rats were housed in a cage in an air-conditioned room at 21° ± 2°C, humidity of 55 ± 10%, with a 12-hr light-dark schedule (light on 7:00 a.m.). These rats were fasted for 24 hr before surgery and during the entire experimental periods, but free access to tap water was provided.

All animal studies were carried out in accordance with the National Institutes of Health “Guide for Care and Use of Laboratory Animals” and as approved by the Nagasaki University Animal Care and Use Committee.

Surgical manipulations. After 1 week of acclimatization, the rats were anesthetized with halothane and a laparotomy was done under sterile conditions. The ileum was ligated with a silk thread and divided at the points of 10 and 15 cm proximal to the ileocecal junction. Then, 6F vinyl chloride tubes (ATOM Medical Co., Tokyo, Japan) were connected each side of the isolated ileum to build a ileal segment of 5-cm length. The catheters were led s.c. to the inter scapular region of animal’s neck. The externalized catheters were placed through a spring tether, and the animal was put into a mesh jacket to permit limited movement within the cage and was allowed free access to water. The mesenteric vessels of the ileal segment were left intact. A transient ischemia was produced in the catheterized rats by occluding the anterior mesenteric artery for 60 min with a aneurysmal clip, then the abdomen was closed. An hour after, the rat was reanesthetized with halothane, and the clip was removed to allow reperfusion of the blood flow. Another group, as sham controls, underwent the same operation with the exception of the occlusion of the anterior mesenteric artery. In yet another group, rats with a permanently occluded anterior mesenteric artery were maintained throughout the experiment.

Release of NO. In conscious and freely moving rats, 1 ml of saline prewarmed at 37°C was put into the ileal segment through the catheter, left there for 30 min and collected through the other side of the catheter. This procedure was done every 30 min during the experimental periods. Prior to assay, the perfusate samples were passed through a .22 µm membrane filter (MILLEX-GV; Millipore). Detection of NO2 and NO3 was performed by means of an automated NO detector-HPLC system (ENO-10, Eicom) (Arima et al., 1996; Yamada and Nabeshima, 1997). Briefly, NO2 and NO3 in the perfusate were separated by a reverse-phase separation column packed with a polystyrene polymer, and NO3 was reduced to NO2 in a reduction column packed with copper-plated cadmium filings. Griess reagent was added on-line, and absorbance at 540 nm was measured using a flow-through spectrophotometer (NOD-10, Eicom).

LDH leakage. Using the same sample used for detection of NO2 and NO3, LDH activity was determined according to the method of Cabaud and Woblenweki (1958). In brief, the reaction mixture contained 300 µl of 330 µM NADH, 70 µl of 6.4 mM sodium pyruvate and 10 µl of sample. The kinetics of NADH decrease was measured at 340 nm.

L-NAME pretreatment. L-NAME (Sigma Chemical, St. Louis, MO) was dissolved in sterile saline. To evaluate the effects of L-NAME on the release of NO and LDH leakage induced by ischemia-reperfusion, 1.0 mg/kg or 2.5 mg/kg L-NAME was administered subcutaneously 30 min before the induction of ischemia. Animals in the control group were given 1 ml/kg saline instead of L-NAME.

Tissue preparation for histology. To assess the level of mucosal injury to the small intestine after ischemia-reperfusion, another group of rats (n = 3 in each group) was prepared. Tissue samples were taken for histological assessment, during sham control, immediately after 1 hr ischemia, 1 hr after ischemia-reperfusion and 3 hr after ischemia-reperfusion. Tissues from rats induced ischemia-reperfusion with pretreatment of L-NAME (2.5 mg/kg; s.c.) were also taken. The samples were immediately fixed in 10% neutral buffered formalin, then were dehydrated, embedded in paraffin, cut at 4 µm, and stained with hematoxylin and eosin for standard microscopic evaluation. The glass slides were coded so as to allow for “blind” evaluation of tissue injury.

Statistical analysis. All data are expressed as group mean ± S.E.M., and n indicates the number of rats in each group. Nonparametric independent group comparisons were made. For multiple comparisons, the Kruskal-Wallis and Dunnett multiple comparison tests were performed. Statistical significance was inferred from a P value of <.05.

Results

Release of NO. Figure 1 summarizes the time course of NO release (measured as nitrite and nitrate levels) from the ileal segment in control (without vascular occlusion), ischemia-reperfused and permanently occluded rats. The basal luminal NO release after ileocolonic stable was stable at the level of 4 to 5 nmol/ml in average. In control ileal segments, the level of NO release slightly increased in a time-dependent manner. By the procedure of 1-hr ischemia and reperfusion, NO release was significantly increased from 30 min after reperfusion, with a peak level at 1 hr after reperfusion. This increase in NO release was observed during 3 hr after reperfusion, in comparison to control animals. In the ileal segment with a permanent occlusion, NO release was not different from that of the control group; however, NO release decreased significantly after 6 hr of occlusion, in comparison to ischemia-reperfused rats.

LDH leakage. LDH leakage into the small intestinal lumen induced by ischemia-reperfusion was also investigated as an index of cell injury (fig. 2). In the control and ischemic group rats, a transient increase in LDH leakage was observed after the operation. With 1 hr of ischemia and reperfusion, LDH leakage was significantly increased during 2 hr after reperfusion, then was gradually reduced to the control level at 5 hr after reperfusion. In ileal segments of permanent vascular occlusion, LDH leakage gradually increased from 3 to 5 hr after the onset of occlusion.

Effects of L-NAME administration. As shown in figure 3, the NO release induced by ischemia-reperfusion into the small intestinal lumen decreased dose-dependently after systemic administration of L-NAME given 30 min before the ischemia. The administration of 1.0 mg/kg L-NAME moder-
ately reduced the ischemia-reperfusion-induced increase of NO release. Treatment with 2.5 mg/kg L-NAME significantly inhibited the increase of NO release from the reperfusion of blood flow during 3 hr. However, this dose of L-NAME did not inhibit the basal release of NO in the small intestinal lumen. Figure 4 shows the effect of L-NAME on LDH leakage into the small intestinal lumen. The LDH leakage induced by ischemia-reperfusion was also decreased dose-dependently by the pretreatment of L-NAME. While the administration of 1.0 mg/kg L-NAME significantly decreased the LDH leakage only at 4 hr after the reperfusion, the dose of 2.5 mg/kg L-NAME produced more potent decrease of the LDH leakage with significance at 30 min after and 4 hr after the reperfusion of blood-flow.

**Histology.** Just after 1-hr ischemia, there were no significant differences in pathological findings of small intestine between sham control and occluded rats (fig. 5, A and B). Two thirds of the entire layer of the intestinal mucosa were necrotic and hemorrhagic at 1 hr after reperfusion (fig. 5C), which corresponds to grades 5 to 6 on the grading scale described by Park et al. (1990). On the other hand, treatment with L-NAME (2.5 mg/kg) obviously reduced the degree of intestinal tissue injury at 1 hr after ischemia-reperfusion (fig. 5D), compared with findings in vehicle-treated rats (fig. 5C). The injury induced by ischemia-reperfusion was limited to one third of the mucosa in L-NAME-treated rats. At 3 hr, the injury in the mucosa remained in both vehicle-treated (fig. 5E) and L-NAME-treated rats (fig. 5F), although the number of infiltrating cells decreased in the L-NAME-treated rats.

**Discussion**

The relationship among increases in NO release into the lumen, LDH leakage and mucosal injury, was positive at 1 hr after ischemia-reperfusion in the rat small intestine. The amount of intraluminal NO$\textsubscript{2}$ and NO$\textsubscript{3}$ significantly increased at 1 hr after ischemia-reperfusion. NO is a free radical gas synthesized from L-arginine by NO synthase (Knowles et al., 1990) and is readily oxidized by O$\textsubscript{2}$ to NO$\textsubscript{2}$ and NO$\textsubscript{3}$ with a half-life of only seconds (Beckman et al., 1990). The mean diffusion distance of NO gas is predicted to be 200 to 600 μm (Saperas et al., 1995). Therefore, measurement of intraluminal NO$\textsubscript{2}$ and NO$\textsubscript{3}$, metabolites of NO, may reflect the dynamics of NO generation in the intestinal mucosa.

On the other hand, permanent occlusion induced a slight increase of NO release, whereas NO release was not significantly different from that seen in control animals. It has been reported that the increase of NO release by reperfusion after ischemia may be due to the resupply of oxygen and L-arginine and to the activation of constitutive NO synthase by intracellular calcium elevation during reperfusion (Kumura et al., 1994). Luminal LDH leakage, a marker of cell injury, also clearly increased at 1 hr after ischemia-reperfusion, but did not change at that time in control animals or permanent vascular occluded animals. The level of NO release decreased gradually from 5 hr after permanent vascular occlusion,
The potential cytotoxic effects of NO on the intestine (Boughton-Smith et al., 1993; Laszlo et al., 1994) and other organs are well documented (Knowles et al., 1990; Nathan, 1992; Zhang et al., 1993; Dawson et al., 1993; Moncada and Higgs, 1993) while the cytoprotective action of NO has been noted in digestive tissues, in which local applications of agents releasing NO reduce the severity of ethanol- or endothelin-induced gastric mucosal damage (MacNaughton et al., 1989; Lopez-Belmonte et al., 1993). In tissues exposed to ischemia-reperfusion, NO was seen to be cytoprotective (Ma et al., 1993; Kurose et al., 1994) and also injury producing (Zweier et al., 1987; Matheis et al., 1992; Nakashima et al., 1995). NO is synthesized by various isoforms of NOS. In which, inducible NOS (iNOS), rather than constitutive endothelial NOS or neuronal NOS, is thought to be the isoform that produces the large quantities of NO that can result in tissue damage or death (Gross and Wolin, 1995). The iNOS is expressed in cells only after several hours of exposure to cytokines and/or lipopolysaccharide (Forstermann et al., 1994). In our data, NO release was significantly increased from 30 min after reperfusion, with a peak level at 1 hr after reperfusion. Gross and Wolin (1995) are suggesting in their review that after tissue ischemia-reperfusion, a sustained elevation in intracellular calcium may cause the constitutive NOS isoforms to produce cytotoxic quantities of NO. Thus, the constitutive ones may contribute to the increase of NO after reperfusion in this studies.

The cytoprotective role of NO is based on findings that NO production of endothelial cells decreased after ischemia-reperfusion and NO donors attenuated the increased albumin leakage in mesenteric venules exposed to ischemia-reperfusion and reduced the ischemia-reperfusion-induced leukocyte adherence/emigration. Possible explanations for the different results are as follows. 1) We measured NO metabolites released in intraluminal segments instead of plasma. 2) We measured LDH leakage as an index of cell injury instead of albumin leakage as an index of barrier dysfunction. 3) We collected samples from ileal segments in conscious and free moving rats, whereas other workers collected samples from anesthetized animals. It is very likely that the results obtained under anesthetized conditions might be different from those obtained in unanesthetized animals.

Pretreatment with L-NAME inhibited increases in the release of NO and the LDH leakage induced by ischemia-reperfusion in a dose-dependent manner. Histologically, L-NAME treatment obviously reduced the degree of intestinal tissue injury compared with findings in non-L-NAME-treated rats. These results further support the notion that excessive amounts of NO are cytotoxic. There are data on the cytotoxic effects of NO synthase inhibitors on ischemia-reperfusion injury (Kanwar et al., 1994), in which epithelial permeability of the postischemic feline small intestine was further facilitated by L-NAME infusion. The dosage of L-NAME used in the present study (1 and 2.5 mg/kg s.c.) was lower than that used by other workers (~8 mg/hr infusion). Several lines of evidences indicate that relatively low doses of NO synthase inhibitors ameliorate postischemic brain damage (Nowicki et al., 1991; Buisson et al., 1992; Nagafuji et al., 1992); however, high doses exacerbate ischemic brain damage (Weissmann et al., 1992; Kuluz et al., 1993). In the present study, 2.5 mg/kg L-NAME treatment decreased only NO release induced by ischemia-reperfusion but did not inhibit the basal release of NO. This is important to take into account if the potential of NO to be cytoprotective or cytotoxic is considered.

NO released into the lumen of conscious and free moving rats was accelerated by ischemia-reperfusion of small intestine, and this excess and inappropriate NO may possibly lead

Fig. 5. Light micrographs of rat intestinal mucosa from control (without vascular occlusion) (A), 1 hr ischemia (B), 1 hr after ischemia-reperfusion (C), 1 hr after ischemia-reperfusion + L-NAME (2.5 mg/kg) (D), 3 hr after ischemia-reperfusion (E) and 3 hr after ischemia-reperfusion + L-NAME (2.5 mg/kg) (F). Lines and arrows in each panel indicate the injured area. (Hematoxylin and eosin stain, 200X.)
to a breakdown of mucosa in the small intestine after the ischemia-reperfusion insult.

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**References**


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