Direct measurement of nitric oxide release in gastric mucosa during ischemia-reperfusion in rats

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Wada, Kouichirou, Yoshinori Kamisaki, Tsuyoshi Ohkura, Gaku Kanda, Kentaro Nakamoto, Yosuke Kishimoto, Kumiyoko Ashida, and Tadao Itoh. Direct measurement of nitric oxide release in gastric mucosa during ischemia-reperfusion in rats. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G465–G471, 1998.—Nitric oxide (NO) generation in the rat gastric mucosa during ischemia-reperfusion was measured using an NO-sensitive electrode. Under pentobarbital sodium anesthesia, an electrode was inserted into the submucosa from the serous membrane side in the fundus. After steady-state baseline recording, the celiac artery was clamped for 30 min, and then ischemia-reperfusion was achieved by removing the clamp. The clamping of the celiac artery caused a decrease in blood flow and an increase in NO level in the gastric tissue. Just after the removal of the clamp, the NO level rapidly fell and returned to the baseline level. Administration of N\textsuperscript{G}-nitro-L-arginine methyl ester (an NO synthase inhibitor, 30 mg/kg ip) before ischemia significantly attenuated both the increase in NO level during ischemia and the formation of acute gastric mucosal lesions observed after 60-min reperfusion. Administration of superoxide dismutase (a superoxide radical scavenger, 10,000 U/kg iv) at the end of ischemia inhibited both the rapid decrease in NO level during the reperfusion and the gastric mucosal erosions. Because NO and superoxide radical produce a highly reactive peroxynitrite, it can be argued that NO has an important pathological role in acute gastric mucosal injury induced by ischemia-reperfusion. Our conclusion was strongly supported by immunohistochemical staining of nitrotyrosine residues, an indication of peroxynitrite formation.

This is the first report of direct measurement of NO release from the gastric mucosa during ischemia-reperfusion in vivo.

MATERIALS AND METHODS

Reagents. L-NAME was obtained from Wako Pure Chemical Industries (Tokyo, Japan). S-nitroso-N-acetyl-penicillamine (SNAP) was obtained from Dojindo Laboratories (Kumamoto, Japan). Superoxide dismutase (SOD) was obtained from Sigma Chemical (St. Louis, MO). Antinitrotyrosine mouse monoclonal antibody was obtained from Upstate Biotechnology (Lake Placid, NY). All chemicals were of reagent grade.

Acute gastric mucosal injury model. All animal experiments were performed in accordance with the guidelines for animal experimentation of the Faculty of Medicine, Tottori University. Male Wistar rats weighing 250–280 g from Japan SL C (Shizuoka, Japan) were fasted for 18 h before the experiments but were allowed free access to water. Acute gastric mucosal injury was produced by ischemia-reperfusion (31). Briefly, under pentobarbital sodium anesthesia (50 mg/kg), the celiac artery was clamped with a small clamp (Sugita standard aneurysm clip, holding force 145 g; Mizuho Ikakogyo, Tokyo, Japan) for 30 min. Reperfusion was achieved by removing the clamp (Fig. 1A). Sixty minutes after reperfusion, the rats were killed, and their stomachs were removed. Macroscopic gastric damage was assessed by the computer imaging analysis system and expressed as total area (mm\textsuperscript{2}) as described previously (31).

Measurement of NO in the rat stomach. NO generated in the gastric mucosa was measured with an NO monitor (model NO-501; Inter Medical, Nagoya, Japan) using a needle-type NO-sensitive electrode (NOE-47, 0.2 mm in outer diameter; see Ref. 9). Before the ischemia, the NO electrode was...
inserted into the submucosa (just below the muscularis mucosae) in the gastric fundus from the serous membrane side (Fig. 1, A and B). The counter electrode was inserted into the subcutaneous tissue. After a steady-state baseline recording, ischemia-reperfusion was initiated as described above. NO generation was measured and expressed in terms of a current in picoamperes. The cumulative NO production during ischemia and reperfusion was calculated as the area under the curve from 0 to 30 min and 30 to 60 min (Fig. 1C) and was expressed in nanoamperes times minutes. The amount of NO was determined using an NO calibration curve described by Ichimori et al. (9). Briefly, the current attributable to each concentration of SNAP in phosphate-buffered saline (PBS) at 37°C was measured to obtain a relationship between concentration and the current in the electrode. A 1,000-pA current was approximately equal to an NO concentration of 1 µM (17).

**Measurement of blood flow**. Blood flow was measured using a laser-Doppler flowmeter (BRL-100; Bio Research, Nagoya, Japan) according to a previously published method (31). While each rat was anesthetized, the probe of the laser-Doppler flowmeter was attached to the serosal membrane side of the stomach. The time course of blood flow to the whole stomach was measured before, during, and after the ischemic period (celiac artery clamping).

**Drug treatments**. SNAP (0.3–3 mg/kg), an NO donor, was dissolved in saline and administered intravenously at the indicated time to serve as a source of authentic NO. L-NAME (30 mg/kg ip), an NO synthase inhibitor, was dissolved in saline and administered 30 min before the ischemia. SOD (10,000 U/kg iv), a superoxide radical scavenger, was dissolved in saline and administered at the time of removing the clamp.

**Immunohistochemical staining of nitrotyrosine residues in the gastric tissue**. At the end of reperfusion, the rats were killed by exsanguination via the abdominal aorta, and the stomachs were removed and fixed with 3.7% formaldehyde-saline. After fixation, the stomach was horizontally sectioned at 3-mm intervals running across the glandular mucosa. Immunohistochemical staining of nitrotyrosine residues was performed by using a Dako catalyzed signal amplification kit (Carpinteria, CA). The visualization was performed by using 3-amino-9-ethylcarbazole as a chromogen.

**RESULTS**

**Relationship between SNAP concentration and current in the electrode**. Figure 2 shows a typical calibration line for the NO measuring system used in this study. It can be seen that the electrode current increased with the concentration of SNAP (3 µM-10 mM; r = 0.996). The electrode current was not affected by 100 mM of nitrite or nitrate. The electrode current was not affected by superoxide radical derived from the xanthine-xanthine oxidase system in PBS.

**Changes in gastric blood flow during ischemia-reperfusion**. The changes in gastric blood flow during ischemia-reperfusion are shown in Fig. 3. Clamping of the celiac artery decreased gastric blood flow to 20–25% of the preclamping level. Just after removal of the clamp, the blood flow recovered fully to the baseline level. There were no significant alterations in gastric blood flow during ischemia-reperfusion, when rats were treated with L-NAME (30 mg/kg ip). Administration of SOD (10,000 U/kg iv) did not affect the gastric blood flow (data not presented).
Effect of SNAP on NO level in gastric tissue. Typical recordings of the NO level in the gastric mucosa are shown in Fig. 4. In this system, stable baseline currents (from 2,000 to 3,000 pA) were observed in vivo. Treatment with SNAP (0.3–3 mg/kg) without ischemia showed a rapid and transient increase of NO level in the gastric mucosa (Fig. 4A). The NO level was dependent on the dose of SNAP.

Effect of L-NAME on NO levels during ischemia. With the clamping of the celiac artery, a continuous increase in NO level was observed during ischemia (Fig. 4B). However, just after the removal of the clamp, the NO level rapidly decreased to the baseline level. Until ischemia, administration of L-NAME (30 mg/kg ip) did not show a significant reduction in the NO level compared with the baseline level (Fig. 4C). The clamping of the celiac artery caused only a slight increase in NO level compared with the baseline value. The total amount of NO with or without L-NAME during the 30-min ischemia is shown in Fig. 5A. There was a significant difference between total amount of NO in rats treated with and without L-NAME.

NO levels during reperfusion with or without SOD. Just after the removal of the clamp, NO level rapidly fell to the baseline level (Fig. 4). The total amount of NO during the 30-min reperfusion phase is shown in Fig. 5B. Administration of SOD (10,000 U/kg iv) just after removal of the clamp significantly increased the amount of NO compared with that of control (Fig. 5B).

Effect of L-NAME or SOD on acute gastric mucosal injury. The total area of erosion on the gastric mucosa after ischemia (30 min)-reperfusion (60 min) was measured in each treated group. Administration of L-NAME (30 mg/kg ip) significantly decreased the total...
area of erosions, a morphological index of acute gastric mucosal injury induced by ischemia-reperfusion (Fig. 6). A good correlation between the total area of erosion and the total amount of NO during ischemia was observed \( (r = 0.733) \). This suggested that the decrease in erosion area in the presence of L-NAME was attributable to inhibition of NO synthase.

Administration of SOD (10,000 U/kg iv) just after the removal of the clamp produced a significant reduction in total erosion area compared with that of control (Fig. 6), without reducing NO level during reperfusion. Thus the scavenging of superoxide radical by SOD causes not only an increase in NO level during reperfusion but also a reduction in total erosion area, although no significant difference in the amount of NO level during ischemia between the control and the SOD-treated group was observed (Figs. 5 and 6).

Immunohistochemical staining of nitrotyrosine residues in the gastric tissue. Photomicrographs of immunohistochemical staining of nitrotyrosine residues, an indication of peroxynitrite formation in vivo, are shown in Fig. 7. No positive stainings against nitrotyrosine monoclonal antibodies were observed on the gastric mucosa from the stomach of normal rats (Fig. 7A). On the other hand, immunohistochemical stainings of nitrotyrosine residues were observed on the area of injured gastric mucosa (Fig. 7B) and on the endothelial cells (Fig. 7C) from the stomach of ischemia-reperfusion-treated rats. However, with the administration of SOD (Fig. 7D) or L-NAME (data not presented), these positive stainings against nitrotyrosine monoclonal antibodies were not observed on the gastric mucosa from ischemia-reperfusion-treated rats.

DISCUSSION

In the present study, using an NO-sensitive electrode, we have directly measured NO generated in the gastric tissue during ischemia-reperfusion. To our knowledge, this is the first report of direct monitoring of NO release in the gastric mucosa in vivo. The recording was not affected by nitrite, nitrate, or superoxide radical, suggesting that the electrode is specific for NO. This NO electrode has proved to have good sensitivity and specificity for detecting biologically released NO in vivo and can be used in real time monitoring in other organs (9, 17).

Our assertion that the current monitored by the NO electrode reflects NO release from the gastric tissue is based on three lines of evidence. First, the variations in NO levels during ischemia-reperfusion are corresponded to changes in blood flow; second, systemic administration of SNAP, an NO donor, produced a dose-dependent increase in NO level in gastric tissue without ischemia; third, administration of L-NAME, an NO synthase inhibitor, before ischemia significantly inhibited the release of NO during ischemia.

The major source of NO generation in the gastric tissue is believed to be blood vessels in the lower region of gastric glands and in the submucosa, because immunohistochemical study has shown that a constitutive isoform of NO synthase is located in the gastric glandular mucosa of the rat (21). Thus the gastric glandular mucosa may be a site of NO generation in the stomach.
It has been suggested that the increase in NO level during ischemia may be attributable to activation of the constitutive NO synthase, and not to the induction of the inducible form, because an increase was observed within 5 min after applying the clamp. It has been reported that shear stress induces NO generation in cultured endothelial cells in vitro (10, 19). Therefore, acute alteration of blood flow by clamping the celiac artery may cause activation of the constitutive NO synthase in the gastric mucosa.

Until ischemia, administration of L-NAME (30 mg/kg ip) did not show a significant reduction in the NO level compared with the baseline level. However, in our preliminary experiments, this dosage of L-NAME caused a continuous elevation of systemic blood pressure (10–20 mmHg). Therefore, differences of sensitivity against NO synthase inhibitor may exist.

On the other hand, it can be argued that a rapid fall in NO current just after removal of the clamp reflects quenching of NO by the superoxide radical generated upon reoxygenation rather than rapid diffusion of NO by recovered blood flow. This notion is supported by our earlier study (31) showing superoxide radical generation during reperfusion. The reaction of NO with superoxide radical to form the well-known oxidant peroxynitrite has been widely reported (2, 22, 23). Furthermore, in our present study, administration of SOD just before reperfusion caused a marked increase in NO current during reperfusion compared with a rapid reduction in NO level without SOD. These results suggest that removal of superoxide radical by SOD inhibits peroxynitrite formation and causes the accumulation of NO during the reperfusion period. Further investigations are necessary for a better understanding of peroxynitrite formation in vivo.

In this study, there was a positive correlation between the total erosion area and the amount of NO during ischemia; it is logical to assume that NO was responsible for the injurious effect of ischemia-reperfusion, perhaps via formation of peroxynitrite. However, administration of the NO donor SNAP without ischemia-reperfusion did not cause gastric mucosal injury, indicating the requirement for superoxide radical. Similarly, acute gastric mucosal injury was not observed by ischemia without the reperfusion, adding further support to the above notion that both NO and superoxide radical (generated upon reperfusion) are necessary for the injury caused by ischemia-reperfusion.

Peroxynitrite formed by the reaction between superoxide radical and NO has been reported to be strongly cytotoxic (2, 8, 23, 30) and may be involved in acute...
gastric mucosal injury. Therefore, in the present study, SOD treatment reduced both the rapid decrease in NO level during the reperfusion and the extent of gastric mucosal damage, attributable to the removal of superoxide radical.

Furthermore, using the immunohistochemical staining of nitrotyrosine, an indication of peroxynitrite formation, we have confirmed the formation of peroxynitrite on the injured gastric mucosa and on the endothelial cells in the stomach from the ischemia-reperfusion-treated rats. These data strongly supported our conclusions.

In conclusion, we have directly monitored NO release in gastric mucosa during ischemia-reperfusion in the rat using an NO-sensitive electrode in vivo. NO generation by the activation of constitutive NO synthase during ischemia and the subsequent formation of peroxynitrite during reperfusion are suggested to play important roles in acute gastric mucosal injury. Such studies can lead to a better understanding of the mechanisms involved in tissue injury by ischemia-reperfusion and may open the way for the development of preparations that can intercept the cytotoxic species.

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