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Protective Mechanism of Nitric Oxide and Mucus against Ischemia/ Reperfusion-Induced Gastric Mucosal Injury

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This study investigated the role of nitric oxide on the oxidative damage in gastric mucosa of rats which received ischemia/reperfusion and its relation to mucus. Nitric oxide synthesis modulators such as L-arginine and N^G-nitro-L-arginine methyl ester, and sodium nitroprusside, a nitric oxide donor, were injected intraperitoneally to the rats 30 min prior to ischemia/reperfusion which was induced by clamping the celiac artery and the superior mesenteric artery for 30 min and reperfusion for 1 h. Lipid peroxide production, the contents of glutathione and mucus, and glutathione peroxidase activities of gastric mucosa were determined. Histological observation of gastric mucosa was performed by using hematoxylin-eosin staining and scanning electron microscopy. The result showed that ischemia/reperfusion increased lipid peroxide production and decreased the contents of glutathione and mucus as well as glutathione peroxidase activities of gastric mucosa. Ischemia/reperfusion induced gastric erosion and gross epithelial disruption of gastric mucosa. Pretreatment of L-arginine, a substrate for nitric oxide synthase, and sodium nitroprusside prevented ischemia/reperfusion-induced alterations of gastric mucosa. However, N^G-nitro- L-arginine methyl ester, a nitric oxide synthase inhibitor, deteriorated oxidative damage induced by ischemia/reperfusion. In conclusion, nitric oxide has an antioxidant defensive role on gastric mucosa by maintaining mucus, glutathione, and glutathione peroxidase of gastric mucosa.

Key Words: Nitric oxide, Mucus, Gastric mucosa, Ischemia/reperfusion

INTRODUCTION

Gastric ischemia is known to be associated with mucosal injury in several different clinical settings including trauma, major surgery (Lucas et al, 1971), and nonsteroidal antiinflammatory drug administration (Kitahora & Guth, 1987). More and more evidences suggest that majority of injury occurs during reperfusion (Perry et al, 1986; Perry & Wadhwa, 1988; Andrews et al, 1992), and this may be attributable to reactive oxygen metabolites initially generated at the level of the vasculature (Tsao et al, 1990). Sources of reactive oxygen metabolites in reperfusing tissues include the xanthine oxidase system, which produces superoxides and hydrogen peroxides during reperfusion (McCord 1985; Parks & Granger, 1986), and activated neutrophils, which infiltrate into the tissues and bind to the microvascular endothelium (Grisham et al, 1986; Hernandez et al, 1986).

Nitric oxide (NO) appears to play a protective role in the gastric mucosa as an endogenous vasodilator and its hyperemic response to damaging agents (Whittle et al, 1990; Lippe & Holzer, 1992). NO modulates gastric mucosal integrity by interacting with other protective mediators such as sensory neuropeptides and endogenous prostaglandins (Whittle et al, 1990; Pique et al, 1989; Tepperman & Whittl, 1989), and secretion of mucus or bicarbonate by a cyclic GMP-dependent process (Flemstrom & Turnberg, 1984). These act as a first line of mucosal defense against luminal aggressors in the stomach

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even if the precise role of NO in ischemia/reperfusion-induced gastric injury has not been clarified. In our previous study, we found that NO synthase activity was inhibited by hypoxia/reoxygenation, and thus NO production decreased in gastric cells exposed to hypoxia/reoxygenation (Kim & Kim, 1995, 1997), which may aggravate the oxidative damage of gastric mucosa. The possible protective mechanisms of NO on the gastric mucosa during ischemia/reperfusion injury are: 1) improvement of the tissue perfusion by vasodilation; 2) inhibition of platelet (Radomski et al, 1987) or polymorphonuclear leukocyte adherence to the endothelium (Kubes et al, 1991); and 3) scavenging of the superoxide radical (Gryglewski et al, 1986).

Gastric mucus forms a protective, continuous viscoelastic layer over the mucosa (Allen et al, 1989) and scavenges toxic reactive oxygen metabolites effectively (Grisham et al, 1987; Hiraishi et al, 1993). Thus, the focus of previous studies has been mostly on the role of mucus in maintaining the integrity of the gastric mucosa against luminal oxidants. NO donors, such as isosorbide dinitrate and S-nitroso-N-acetyl-penicillamine, increased mucus gel thickness in rat stomach (Flemstrom & Turnberg, 1984). These findings, along with the presence of NO synthase in the gastric mucosa (Brown et al, 1992), imply that NO is involved in gastric mucus release.

The present study aims to investigate the role of NO, using NO synthesis modulators such as L-arginine and N^G-nitro-L-arginine methyl ester (L-NAME) and an NO donor, sodium nitroprusside (SNP) on ischemia/reperfusion (I/R)-induced gastric mucosal injury and its relation to mucus by measuring LPO production, the contents of GSH and mucus, and GSH peroxidase activities of gastric mucosa. In addition, histological observation for gastric mucosa was performed by using hematoxylin-eosin staining and scanning electron microscopy.

METHODS

Materials

Chemicals used were GSH, GSH reductase, L-arginine, L-NAME, SNP (Sigma Chemicals, St Louis, MO, USA) and 2-thiobarbituric acid, and 1, 1, 3, 1-tetramethoxypropane (Fluka Chemicals, Switzerland). L-arginine, L-NAME, and SNP were dissolved in saline just before each experiment.

Animal preparation

Male Sprague-Dawley rats weighing 200~250 g were fasted overnight and allowed access to water *ad libitum*. NO synthesis modulators (L-arginine, 200 mg/kg; L-NAME, 10 mg/kg) or an NO donor (SNP, 100 μg/kg) were injected intraperitoneally to the rats. The doses of NO synthesis modulators and a NO donor was adapted from previous studies dealing with gastroprotection against necrotizing agents in rats (Andrews et al, 1994; Kitagawa et al, 1990). Control animals received an equivalent volume of saline. After 30 min, the animals were anesthetized with urethane (1 g/kg intraperitoneally), and the abdomen was opened through a midline laparotomy. The celiac artery and the superior mesenteric artery were clamped for 30 min and then reperfused for 1 h. The rats were sacrificed, and the stomach of each rat was removed. The stomach was opend along the lesser curvature and wash with phosphate buffered saline. Gastric mucosa was scraped from the stomach and homogenized with 0.1 M Tris-HCl buffer (pH 7.4) for the determinations of LPO, GSH, GSH peroxidase and protein. For the determination of mucus, gastric mucosa was weighed, and mucus was extracted with Alcian blue solution.

Determination of LPO

LPO was measured fluorometrically by adaptation of the method of Yagi (1976) using 1, 1, 3, 3-tetramethoxypropane as a standard. Briefly, 20% acetic acid, adjusted to pH 3.5, was added to tissue homogenates to achieve a final concentration of 15% and centrifuged at 3,000 g for 30 min. An aliquot of the clear supernatant (2 ml) was added to 0.15 ml of 8% sodium dodecyl sulfate and 0.15 ml of 0.8% 2-thiobarbituric acid. The mixture was vortexed and then boiled for 15 min at 95°C. The colored product was extracted by adding 2 ml of n-butanol and then, vortexing and centrifuging for 15 min. The fluorescence of upper layer was read in a Model SPF-500C spectrofluorometer (SLM Instruments, Urbana, IL, USA) at 515 nm excitation and 553 nm emission.

Determination of GSH

Tissue homogenates were deproteinated with equal

volume of 10% 5-sulfosalicylic acid and centrifuged at 12,500 g for 5 min at 4°C. An aliquot of the acid-soluble supernatant was 10-fold diluted with phosphate buffer (125 mM, pH 7.5) containing 6.3 mM EDTA. GSH reductase in a concentration of 5 units/ml and 6 mM 5, 5'-dithiobis-2-nitrobenzoic acid were added. The reaction was started with 0.3 mM NADPH. The change in absorbance was monitored spectrophotometrically at 412 nm (Griffith, 1980). Exogenous GSH was used as standard. Results were expressed as GSH in nmoles per mg protein. Protein content was determined by the method of Lowry et al (1951) with bovine serum albumin as standard.

Determination of GSH peroxidase

GSH peroxidase activity was determined according to the method of Lawrence et al (1976). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 EU/ml oxidized glutathione (GSSG) reductase, 1 mM GSH, and 0.25 mM H_2O_2 in a total volume of 1 ml. The ingredients, except the enzyme source and peroxide, were combined at the beginning of each assay. Samples (0.1 ml) were added to 0.8 ml of the above mixture and incubated for 5 min at 25°C before initiating the reaction with the addition of 0.1 ml of peroxide solution. A sample of supernatant fluid with 10% homogenate solution and 1.15% KCl was made by centrifugation at 4,000 g for 10 min at 4°C. The absorbance at 340 nm was recorded for 5 min. The activity was then calculated from the slope of the lines as μmol of NADPH oxidized per min. The blank datum (the enzyme was replaced with distilled water) was subtracted from each value.

Evaluation of gastric mucus

The measurement of mucus levels bound to the epithelial surface was performed according to the method of Corne et al (1974). Briefly, gastric mucosa was weighed and immersed for 2 h in 0.1% Alcian blue in a 0.16 M sucrose solution buffered with 0.05 M sodium acetate (pH adjusted to 5.8 with 1N HCl). The unbound dye was then removed by 2 subsequent washings of 15 and 45 min in 0.25 M sucrose solution, and the mucus-bound dye was eluted by immersing the gastric mucosa in a 0.5 M $MgCl_2$ solution (pH 6.0) for 2 h. Then, the solution obtained was shaken with diethyl ether. The optical density of the aqueous phase was read at 605 nm with a Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, England). The amount of Alcian blue of gastric mucosa was expressed as absorbance unit (AU, optical density) per g wet weight.

Histological observation

For histological confirmation of the nature of the mucosal injury, the gastric sections (5 mm^2) were processed by routine techniques before being embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin and examined uner a light microscope (40 x).

For scanning electron microscopic observation, gastric sections were placed in a glutaraldehyde fixative (3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) at 4°C. Then, they were rinsed in buffer and postfixed in 1% osmium tetraoxide in the same buffer for 2 h at 4°C. Following dehydration through a series of graded ethanol, the tissues were displaced into isoamylacetate and dehydrated with CO_2 (critical point dry). Thin sections were mounted with silver paste, coated with gold ion (300 thickness) and examined in an electron microscope (Model S800, Hitachi, Ltd., Tokyo, Japan).

Statistics

Results were presented as mean SE of 6 animals. The statistical difference in experimental treatments was determined by an analysis of variance followed by the Newman-Keuls test (1984). *P* value of <0.05 was considered statistically significant.

RESULTS

Effect of I/R on LPO production, GSH content, GSH peroxidase activity, and mucus content in gastric mucosa of rats

As shown in Fig. 1 and 2, I/R significantly increased LPO production and decreased GSH content, GSH peroxidase activity and mucus content of gastric mucosa. The contents of LPO (nmole/mg protein) and GSH (nmole/mg protein) in gastric mucosa of control rats were 0.6 ± 0.04 and 37.9 ± 2.0 while those in rats received I/R were 1.2 ± 0.1 and 25.0 ± 2.0, respective-

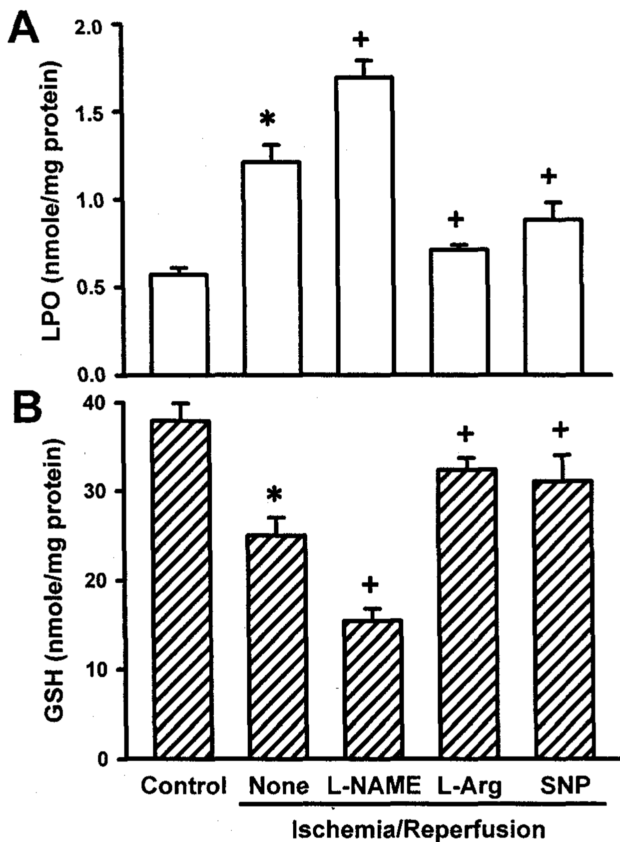

Fig. 1. Effects of NO synthesis modulators and SNP on the contents of LPO (A) and GSH (B) in gastric mucosa of rats received ischemia/reperfusion. NO synthesis modulators (L-arginine, 200 mg/kg; L-NAME, 10 mg/kg) or SNP (100 μg/kg) were injected intraperitoneally to the rats 30 min prior to ischemia/reperfusion, induced by clamping the celiac artery and the superior mesenteric artery for 30 min and reperfusion for 1 h. Control rats received saline instead of NO synthesis modulators or SNP. Values are means±SE of 6 animals. L-NAME, N^G-nitro-L-arginine methyl ester; L- Arg, L-arginine; SNP, sodium nitroprusside. *Statistically different from the control, $P<0.05$. $^+$Statistically different from the gastric mucosa of rats received I/R alone (none), $P<0.05$.

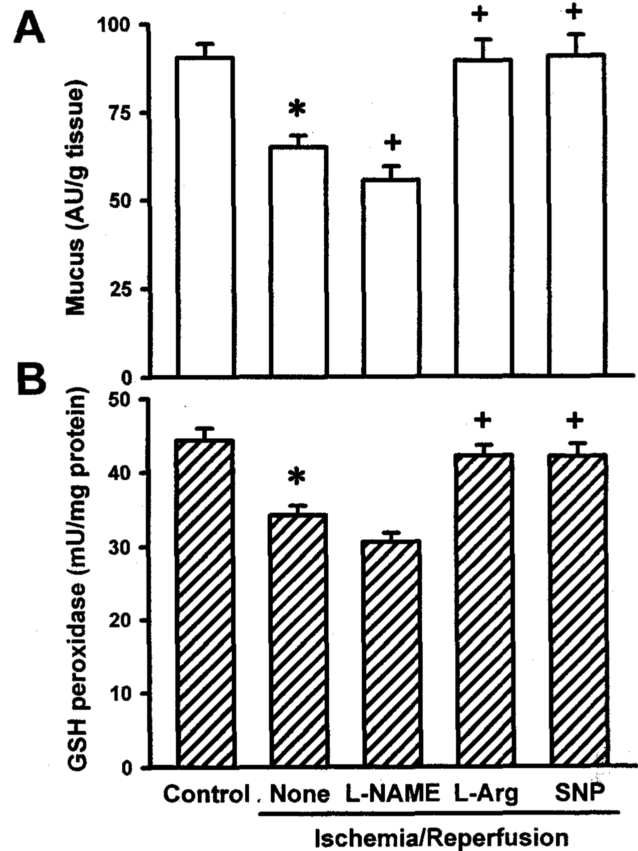

Fig. 2. Effects of NO synthesis modulators and SNP on mucus content (A) and GSH peroxidase activity (B) in gastric mucosa of rats received ischemia/reperfusion. NO synthesis modulators (L-arginine, 200 mg/kg; L-NAME, 10 mg/kg) or SNP (100 μg/kg) were injected intraperitoneally to the rats 30 min prior to ischemia/reperfusion, induced by clamping the celiac artery and the superior mesenteric artery for 30 min and reperfusion for 1 h. Control rats received saline instead of NO synthesis modulators or SNP. Values are means±SE of 6 animals. L-NAME, N^G-nitro-L-arginine methyl ester; L- Arg, L-arginine; SNP, sodium nitroprusside. *Statistically different from the control, $P<0.05$. $^+$Statistically different from the gastric mucosa of rats received I/R alone (none), $P<0.05$.

ly (Fig. 1). Mucus content (AU/g tissue) and GSH peroxidase activity (mU/mg protein) in gastric mucosa of control rats were 90.4±4.1 and 44.3±1.6, which were significantly decreased by I/R. Mucus content and GSH peroxidase activity in gastric mucosa of rats which received I/R were 65.0±3.3 and 34.1±1.3, respectively (Fig. 2).

Effects of NO synthesis modulators and SNP on LPO production and GSH content in gastric mucosa of rats which received I/R

Pretreatment of L-arginine and SNP prevented the increase in LPO production and the decrease in GSH content of gastric mucosa induced by I/R (Fig. 1). L-NAME aggravated I/R-induced alterations in LPO production and GSH content of gastric mucosa. LPO contents (nmole/mg protein) in gastric mucosa of rats

which received I/R alone, pretreated with L-NAME, L-arginine and SNP were 1.2±0.1, 1.7±0.1, 0.7±0.03 and 0.9±0.1. GSH contents (nmole/mg protein) in gastric mucosa of rats which received I/R alone were 25.0±2.0, while those pretreated with L-NAME, L-arginine and SNP were 15.4±1.4, 32.3±1.4 and 31.0±3.0, respectively.

Effects of NO synthesis modulators and SNP on mucus content and GSH peroxidase activity in gastric mucosa of rats which received I/R

Pretreatment of L-arginine and SNP prevented the decreases in mucus content and GSH peroxidase activity of gastric mucosa induced by I/R. Mucus content (AU/g tissue) in gastric mucosa of rats which received I/R alone was 65.0±3.3, and those pretreated with L-arginine, SNP and L-NAME were 89.4±6.0, 90.7±6.0 and 55.5±4.0, respectively. L-NAME deteriorated I/R-induced mucus loss as shown

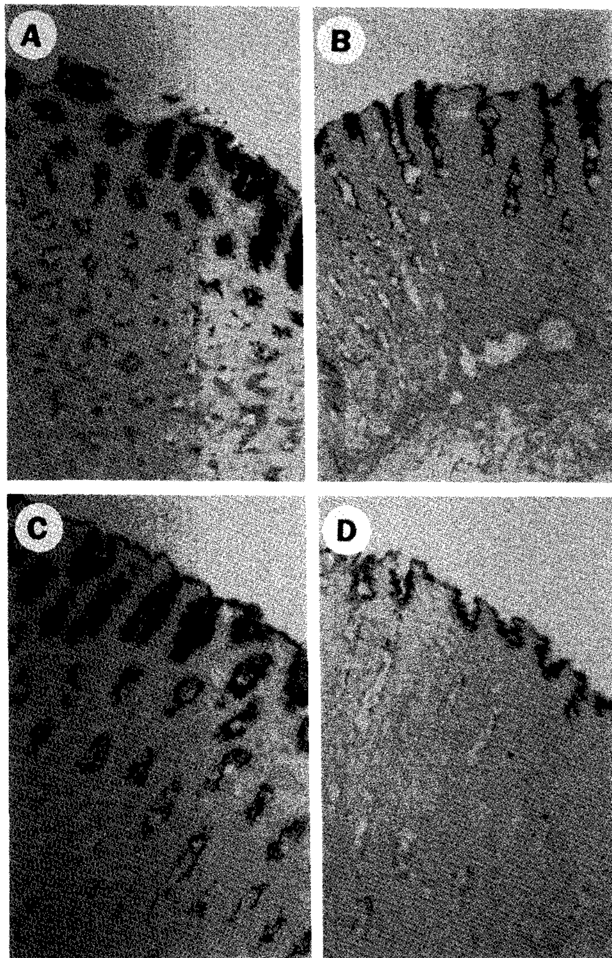

Fig. 3. Histological appearance of gastric mucosa in control rats (A), rats received I/R alone (B), pretreated with L-arginine (C) or L-NAME (D). NO synthesis modulators (L-arginine, 200 mg/kg; L-NAME, 10 mg/kg) were injected intraperitoneally to the rats 30 min prior to ischemia/reperfusion, induced by clamping the celiac artery and the superior mesenteric artery for 30 min and reperfusion for 1 h. Control rats received saline instead of NO synthesis modulators. Gastric sections were stained with hematoxylin and eosin (original magnification, 40 x). Gastric erosion and epithelial disruption were shown in gastric mucosa of rats which received I/R alone (B) and pretreated with L-NAME (D). L-NAME, N^G- nitro-L-arginine methyl ester.

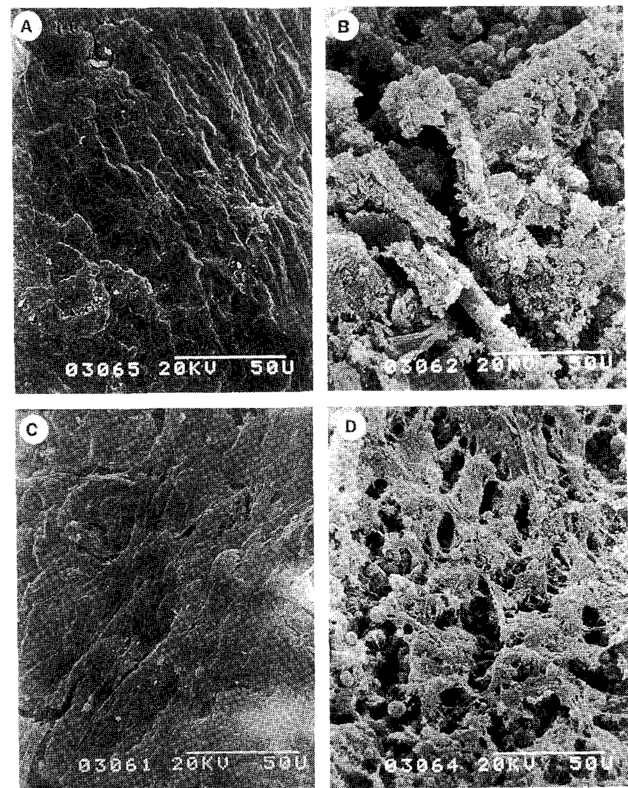

Fig. 4. Scanning electron microscopic appearance of gastric mucosa in control rats (A), rats received I/R alone (B), pretreated with L-arginine (C) or L-NAME (D). NO synthesis modulators (L-arginine, 200 mg/kg; L-NAME, 10 mg/kg) were injected intraperitoneally to the rats 30 min prior to ischemia/reperfusion, induced by clamping the celiac artery and the superior mesenteric artery for 30 min and reperfusion for 1 h. Control rats received saline instead of NO synthesis modulators. Marker bar represents 50 μm. Derangement and loss of gastric epithelium were observed in gastric mucosa of rats which received I/R alone (B) and pretreated with L-NAME (D). L- NAME, N^G-nitro-L-arginine methyl ester.

in its effect on the decreases in LPO production and GSH content induced by I/R. GSH peroxidase activity (mU/mg protein) in gastric mucosa of rats which received I/R alone was 34.1±1.3, and it increased when animals were pretreated with L-arginine (42.1 ±1.4) and SNP (42.0±1.7), but not with L-NAME (30.5±1.2).

Histologic evaluation of gastric mucosal damage

I/R induced the gross epithelial disruption and erosion (Fig. 3B) as compared with intact gastric mucosa of control rats (Fig. 3A). I/R-induced gastric damage was characterized by extensive areas of vasocongestion and sloughing of epithelium and focal sites of hemorrhage, which was prevented by pretreatment of L-arginine (Fig. 3C), but not by L-NAME (Fig. 3D). The protective effect of L-arginine on I/R-induced gastric mucosal damage was confirmed through histological evaluation by scanning electron microscopy (Fig. 4C). Scanning electron microscopic observation showed mucosal derangement and epithelical loss in gastric mucosa of rats which received I/R (Fig. 4B). Gastric mucosa of rats which received I/R with L-NAME (Fig. 4D) had similar epithelial disruption to those rats which received I/R alone (Fig. 4B).

DISCUSSION

We confirmed that both endogenous and exogenous NO has protective effect against I/R-induced gastric mucosal injury by maintaining tissue contents of GSH and mucus as well as GSH peroxidase activity and by inhibiting LPO production of gastric mucosa. The postulated action mechanism of NO is scavenging lipid radicals (peroxyl and alkoxyl radicals) and superoxides and thus, inhibiting GSH consumption in gastric mucosa since GSH acts both as a nucleophilic scavenger of superoxides and as a cofactor in the GSH peroxidase-mediated reduction of hydrogen peroxides (Meister & Anderson, 1983). It is reported that depletion to 20~30% of total GSH levels can readily impair the tissue's defense against oxidative damage from hydrogen peroxides, which may lead to irreversible tissue damage (Reed & Fariss, 1984). In the present study, I/R decreased both GSH content to 66% and GSH peroxidase activity to 77%, and increased LPO content of gastric mucosa to 200% of those shown in intact gastric mucosa of control rats. Thses results indicate that the maintenance of tissue GSH stores and GSH peroxidase activities as well as the inhibition on LPO production by NO could contribute to antioxidant defense mechanism of gastric cells, as suggested in the report by Kuo and Slivka (1994). They suggested that NO had a protective effect on acetaminophen-induced hepatotoxicity by modulating GSH homeostasis and that increased GSH stores in the setting of NO delivery might be explained by the provision of the substrate such as low-molecular-weight thiols for GSH synthesis.

Upon exposure to excess H_2O_2, which may be generated during I/R and cannot be detoxified by tissues, H_2O_2 reacts with transition metals such as iron, presumably to form a more reactive peroxo complex (which may yield hydroxyl radical) (Halliwell, 1982; Sutton & Winterbourn, 1989), leading to tissue damage. Thus, as a possible protective mechanism of NO in I/R-induced gastric mucosal injury, NO may confer protection by chelating transition iron to form iron-nitrosyl compounds (Darley-Usmar & Halliwell, 1996; Sergent et al, 1997) without affecting endogenous antioxidant defenses. Other possible antioxidant role of NO is to terminate radical chain propagation by directly reacting with lipid radicals (peroxyl and alkoxyl radicals) (Rubo et al, 1994) and thus, sparing endogenous antioxidant, α-tocopherol (Hayaishi et al, 1995). Superoxide is generated within the cells, and interaction between NO and superoxide results in the generation of a comparatively stable peroxynitrite radical (Mccall & Vallance, 1992; Casini et al, 1997). Cells detoxify H_2O_2 by using endogenous catalase and GSH peroxidase acting on GSH (Yoshikawa et al, 1993). Thus, GSH and GSH peroxidase are consumed by the cells upon exposure to H_2O_2. If NO scavenges superoxide generated by I/R, relatively low amount of H_2O_2 could be produced, and cellular levels of GSH and GSh peroxidase shall be maintained. This may explain the present results: 1) the decreases in GSH and GSH peroxidase by I/R and 2) the maintenance of cellular GSH and GSH peroxidase by NO supply from L-arginine and SNP.

On the other hand, it has been proposed that gastric mucus has not only a protecting effect on gastric mucus, but also an effect of a physiologically important antioxidant or free radical scavenger (Cross et al, 1984) in addition to the fact that it has a protecting

effect on gastric mucosa from acid, pepsin, and pathogenic microorganisms as well as enzymatic and mechanical injury (Allen, 1984). This proposal is based on the fact that certain sugars such as glucose or mannitol are potent hydroxyl radical scavengers, and mucus contains very high concentrations of some similar sugars (N-acetyl-glucosamine, galactose, and fucose). Conversely, the mucus gel cover of the gastric mucosal surfaces was degraded to fragments, while viscosity and gel-forming properties were reduced after reaction with hydroxyl radicals and hydrogen peroxides (Greeth et al, 1983; Robertson et al, 1971). It was reported that this fragmentation with a decrease in the viscosity of gastric mucus accompanied by an increase in its thiobarbituric acid reactivity is indicative of oxidative damage to the tissues (Cross et al, 1984). In the present study, I/R-induced decrease in the content of mucus was related to the increase in LPO production. This suggested that decreased production of mucus could be one of the causes of I/R-induced oxidative damage which may be mediated by increased production of reactive oxygen metabolites and partially by decreased production of NO. Decrease in NO production by hypoxia/reoxygenation was observed in our previous studies using gastric cells (Kim & Kim, 1995, 1997). Together with possible scavenging effect of NO for lipid radicals, superoxides and transition metals, the maintenance of mucus production by NO could provide partial but significant protection against reactive oxygen metabolites-induced gastric mucosal damage.

In summary, endogenous antioxidant defense factors in gastric cells such as GSH, GSH peroxidase and mucus were decreased by I/R. These I/R-induced changes were prevented by NO. Thus, it is concluded that NO has an antioxidant defensive role on gastric mucosa by maintaining GSH, GSH peroxidase, and mucus, and provision of NO might protect gastric mucosa against I/R-induced injury.

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