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Dietary Nitrate Inhibits Stress-induced Gastric Mucosal Injury in the Rat

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Dietary nitrate is reduced to nitrite by some oral bacteria and the resulting nitrite is converted to nitric oxide (NO) in acidic gastric juice. The aim of this study is to elucidate the pathophysiological role of dietary nitrate in the stomach.

Intragastric administration of nitrate rapidly increased nitrate and NO in plasma and the gastric headspace, respectively. Water-immersion-restraint stress (WIRS) increased myeloperoxidase (MPO) activity in gastric mucosa and induced hemorrhagic erosions by a nitrate-inhibitable mechanism. In animals that had received either cardiac ligation or oral treatment with povidone-iodine, a potent bactericidal agent, administration of nitrate failed to increase gastric levels of NO and to inhibit WIRS-induced mucosal injury. WIRS decreased gastric mucosal blood flow by a mechanism which was inhibited by administration of nitrate.

These data suggested that the enterosalivary cycle of nitrate and related metabolites consisted of gastrointestinal absorption and salivary secretion of nitrate, its conversion to nitrite by oral bacteria and then to NO in the stomach might play important roles in the protection of gastric mucosa from hazardous stress.

Keywords: Nitric oxide; Nitrate; Nitrite; Gastric mucosal injury; Stress ulcer

Abbreviations: NO, nitric oxide; NOx, nitrate and nitrite; WIRS, water-immersion-restraint stress; MPO, myeloperoxidase

INTRODUCTION

A major fraction of dietary nitrate is derived from green leafy vegetables.[1] Generally, vegetarians have larger intake of nitrate (~2 mmol/day) than do non-vegetarians (~0.6 mmol/day). About 25% of nitrate absorbed from gastrointestinal tracts is excreted in saliva.[2,3] Nitrate in plasma is secreted in saliva; the concentrations of nitrate in human saliva (~1 mM) are 10-times higher than those in plasma.[1,2] Healthy human subjects daily secrete ~1.5 l of saliva that contains ~1 mM nitrate.[3–5] Duncan *et al.*[6] reported that the tongue surface harbors facultative anaerobic bacteria that rapidly reduce nitrate to nitrite. Thus, a fraction of nitrate derived from food and saliva is converted to nitrite in the oral cavity. Because nitrite is chemically reduced to nitric oxide (NO) in acidic gastric juice,[7,8] various bacteria contaminating in food are effectively eliminated by the combined action of gastric acid and NO.

Various types of stress have been known to decrease blood flow and increase the infiltration of neutrophils in gastric mucosa, thereby inducing gastric mucosal injury.[9–11] We previously reported that site-directed superoxide dismutases (SOD) inhibited Water-immersion-restraint stress (WIRS)-induced gastric mucosal injury by promoting gastric microcirculation.[12,13] Depletion of the circulating neutrophils significantly suppressed the gastric mucosal injury caused by non-steroidal anti-inflammatory drugs.[14] Because NO inhibits the interaction of neutrophils with vascular endothelial cells, the decrease of this gaseous radical enhances the rolling, adhesion and tissue infiltration of neutrophils.[15,16] Although NO plays important roles in host defense,[17] role of nitrate, NO and related metabolites in the pathogenesis of gastric

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mucosal injury is not known. The present work describes the role of nitrate and related metabolites in the protection of gastric mucosa in the rat.

MATERIALS AND METHODS

Animal Experiments

Male Wistar rats, 200–230 g, were purchased from SLC, Co. (Shizuoka, Japan), fed laboratory chow and water *ad libitum* and used for experiments after overnight fasting. Animal experiments were approved by the Animal Care and Use Committee of Osaka City University Medical School.

One hour after intragastric administration of 0.2 mmol/kg of either KNO_3 or KCl, animals were placed in a wire-mesh cage and immersed in water (22 ± 0.5°C) to the depth of their xiphoid process to elicit WIRS-induced gastric mucosal injury.[18] At the indicated times, the stomach was excised, fixed with 10% formalin for 10 min, and washed with 50 ml of phosphate buffered saline (PBS). Gastric lesion were analyzed by using an NIH image 1.6 software. The ulcer index was expressed as the percentage of the injured area to the total mucosal area.

Determination of NO in Gastric Gas

Under urethane anesthesia and laparotomy, gastric gas was collected by a polyethylene tubing inserted into the frontal part of the stomach. In some cases, the cardia of the stomach was ligated without giving neuronal damage. The oral cavity of other groups was sterilized with 0.1 ml of 2% povidone-iodine to eliminate oral bacteria. At the indicated times after intragastric administration of either KNO_3 or KCl, gastric gas was collected and analyzed by using a gas-phase chemiluminescence NO analyzer NOA™280 (Sievers Instruments, Inc., Boulder, CO).[7]

Biochemical Analysis

At the indicated time, 0.2 ml of venous blood was collected in heparinized tubes and centrifuged at 12,000*g* for 5 min. The plasma was added equi-volume of methanol and centrifuged at 12,000*g* for 5 min. Nitrate and nitrite in the supernatants were analyzed by using a NOx analyzer ENO-20 (Eicom Co, Kyoto).[19]

Measurement of Myeloperoxidase Activity

At the end of each study, the gastric mucosal specimens were homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide and centrifuged at 12,000*g* and 4°C for 5 min. Myeloperoxidase (MPO) activity in the supernatant was determined by the *o*-dianisidine/H_2O_2 method using a Hitachi U-2000 spectrophotometer.[20]

Measurement of Mucosal Blood Flow

Animals were intragastrically administered with 0.2 mmol/kg of either KCl or KNO_3 solution at 1 h prior to giving WIRS. The blood flow in gastric mucosa was measured using a laser-Doppler flowmeter FLO-N1 (OMEGAWAVE, Inc., Tokyo) as described previously.[21] To avoid the effect of stress-induced hemorrhage, mucosal blood flow was measured after 2 h of WIRS. Under urethane anesthesia, a probe of the flowmeter was inserted through a frontal wall and placed on the mucosal surface of the posterior wall of the stomach. Values of blood flow were expressed as the mean ± SE derived from six different sites of gastric mucosa.

Statistical Analysis

Unless otherwise stated, data are presented as mean ± SE. Statistical analysis was performed by using ANOVA with StatView-J 4.5 software (Abacus Concepts Inc., Berkeley, CA) for Fisher's PLSD of multiple comparisons test. The level of significance was defined as $P < 0.05$.

RESULTS

Effect of Nitrate on Stress-induced Gastric Mucosal Injury

After intragastric administration of either KNO_3 or KCl, animals were subjected to WIRS for 4 h and the extent of gastric mucosal injury was analyzed. WIRS induced mucosal erosions in the stomach by a mechanism that was suppressed by KNO_3 but not KCl (Figs. 1 and 2). The acidity of gastric juice remained unaffected after administration of KNO_3 and KCl (pH 1.4–1.8).

Changes in Plasma Nitrate and Nitrite

Plasma levels of nitrite (NOx) rapidly increased after intragastric administration of KNO_3 and remained high levels during the experiments (Fig. 3). Nitrate accounted for more than 95% of plasma NOx. Administration of KCl had no appreciable effect on the plasma levels of NOx. After giving WIRS for 4 h, plasma levels of NOx were also determined (Fig. 4). Administration of KNO_3 similarly increased plasma levels of NOx in control and WIRS-treated rats.

Effect of Nitrate on Gastric NO

The concentration of NO in the gastric gas increased gradually and reached high levels after intragastric

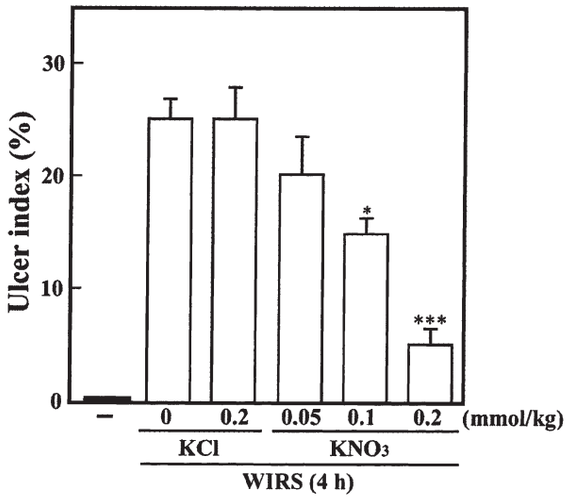

FIGURE 1 Effect of intragastric administration of nitrate on the stress-induced gastric mucosal injury. Animals were administered 0.5 ml of either KCl or KNO_3 solution (0.05–0.2 mmol/kg) 1 h before subjected to WIRS. After 4 h, the ulcer index of the stomach was determined as described in the text. Data show mean ± SE derived from 4 to 6 animals. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with KCl-administered group.

administration of KNO_3 (Fig. 5). To test whether nitrate is directly converted to NO in the stomach, we also measured gastric levels of NO in cardia-ligated animals. Gastric generation of NO was inhibited significantly by cardiac ligation even after intragastric administration of KNO_3. In contrast, gastric NO levels rapidly increased after intragastric administration of KNO_2.

To test the possible involvement of oral bacteria in the metabolism of nitrate, we measured gastric NO in animals whose oral cavity had been topically sterilized with povidone-iodine. Gastric generation of NO in KNO_3-administered rats was inhibited strongly in povidone-iodine-treated animals. Thus, intragastrically administered KNO_3 should have been transported into the circulation, secreted in the oral cavity, reduced to nitrite by some povidone-iodine-sensitive oral mechanism, and then transferred to the stomach to generate NO.

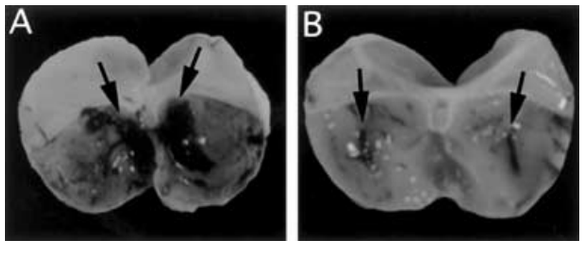

FIGURE 2 Suppression of stress-induced gastric mucosal injury by intragastric administration of nitrate. Animals were administered 0.5 ml of either KCl (A) or KNO_3 (B) solution (0.2 mmol/kg) 1 h before subjected to WIRS. After 4 h, the stomachs were excised, fixed with 10% formalin solution for 10 min, and washed with PBS. Arrows indicate the sites of mucosal injury associated with hemorrhage.

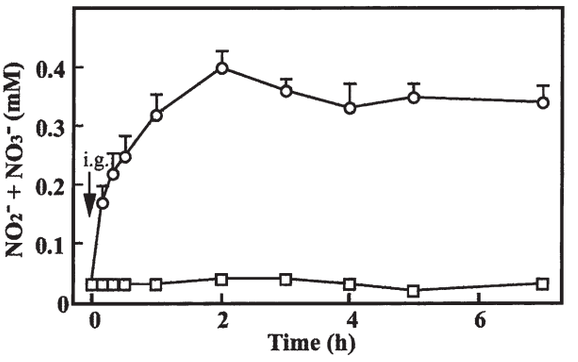

FIGURE 3 Effect of intragastric administration of nitrate on plasma levels of NOx. Animals were intragastrically administered (i.g.) 0.2 mmol/kg of either KCl (open squares) or KNO_3 solution (open circles). At the indicated times, plasma samples were collected and determined for NOx by a modified Griess reaction. Data shown mean ± SE derived from 5 to 10 animals.

Effect of Nitrate and its Metabolites on Gastric Injury

To test the effect of nitrate and its metabolites on stress-induced gastric mucosal injury, ulcer index of WIRS-treated rats was compared with that of povidone-iodine-treated animals. Administration of KNO_3 markedly suppressed WIRS-induced gastric mucosal injury by a povidone-iodine-inhibitable mechanism (Fig. 6).

Effect of Nitrate on Neutrophil Infiltration in WIRS-treated Rats

We also studied the effect of intragastrically administered KNO_3 on gastric infiltration of neutrophils. Although no detectable activity of MPO was

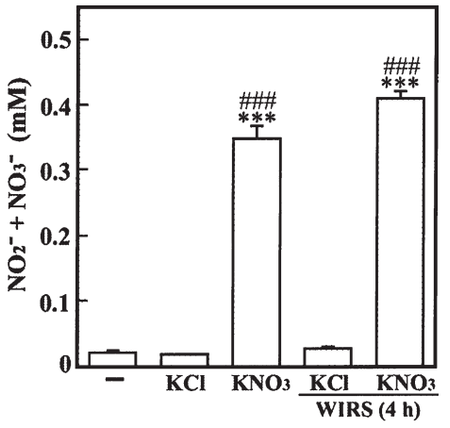

FIGURE 4 Effect of intragastric nitrate on plasma levels of nitrate and nitrite. Animals were intragastrically administered 0.5 ml of either KCl or KNO_3 (0.2 mmol/kg) 1 h before subjected to WIRS. After 4 h, plasma levels of NOx were measured. Data show mean ± SE derived from 4 to 14 animals. More than 95% of NOx in plasma was accounted for by nitrate. $^{***}P < 0.001$ compared with normal group. $^{###}P < 0.001$ compared with KCl-administered group.

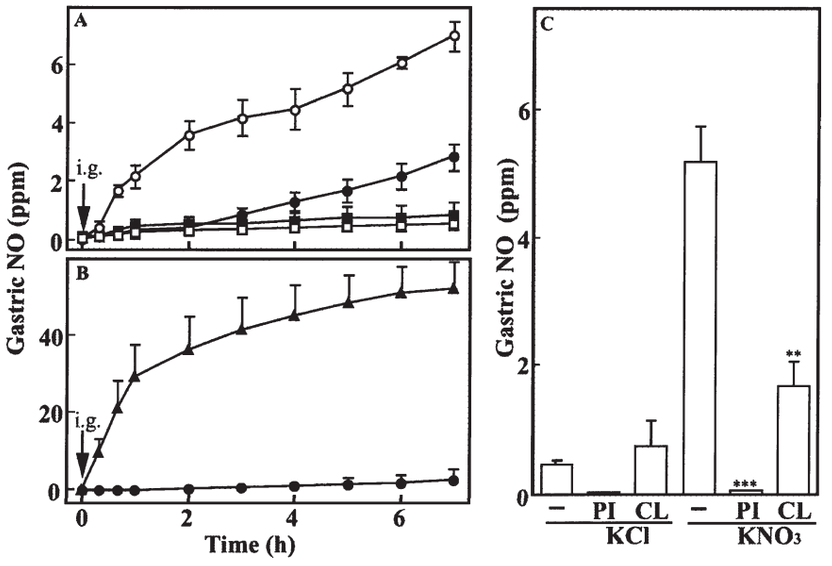

FIGURE 5 Effect of intragastric administration of nitrate on gastric NO levels. (A) Animals were administered 0.2 mmol/kg of either KCl (squares) or KNO_3 (circles) with (closed symbols) or without (open symbols) cardiac ligation. (B) After cardiac ligation, animals were intragastrically administered either 0.2 mmol/kg of KNO_3 (circles) or 2 μmol/kg of KNO_2 (triangles). At the indicated times, gastric headspace gas was collected and determined for NO. (C) After intragastric administration of either KCl or KNO_3, cardiac ligation (CL) or oral treatment with 0.1 ml of 2% povidone-iodine (PI) was carried out. After 5 h, gastric headspace gas was collected and determined for NO. Data show mean ± SE derived from four animals.$^{***}P < 0.001$ and $^{**}P < 0.01$ compared with KNO_3-administered group.

found in the gastric mucosa of control animals, WIRS significantly increased the enzyme activity by a mechanism that was inhibited by KNO_3 (Fig. 7). Administration of KCl had no effect on the WIRS-enhanced activity of MPO.

Effect of Nitrate on the Gastric Mucosal Blood Flow

Figure 8 showed the effect of nitrate on the gastric mucosal blood flow in control and WIRS-treated rats. Mucosal blood flow was decreased significantly by WIRS in KCl-administered group (51 ± 3.5% of sham group). Administration of KNO_3 markedly increased the blood flow of WIRS-treated rats.

DISCUSSION

The present work demonstrates that administration of KNO_3 effectively increased gastric NO levels and

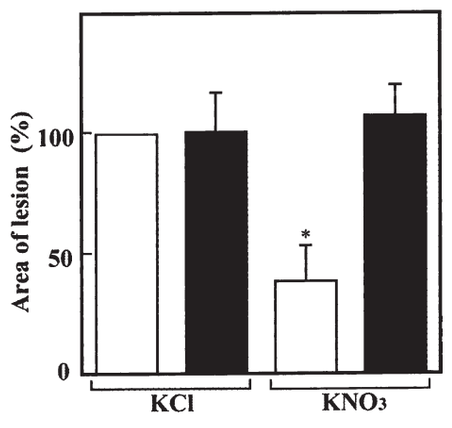

FIGURE 6 Effect of oral povidone-iodine on the stress-induced gastric mucosal injury. The oral cavity was topically administered with 0.1 ml of povidone-iodine (closed column) 1 h before giving WIRS. Then, 0.2 mmol/kg of either KCl or KNO_3 solution was administered intragastrically. After 4 h of WIRS, ulcer index of the stomach was determined as described in the text. Values for ulcer index are expressed as % of KCl-administered control group $n = 4–6$. $^{*}P < 0.05$ compared with KCl-administered group.

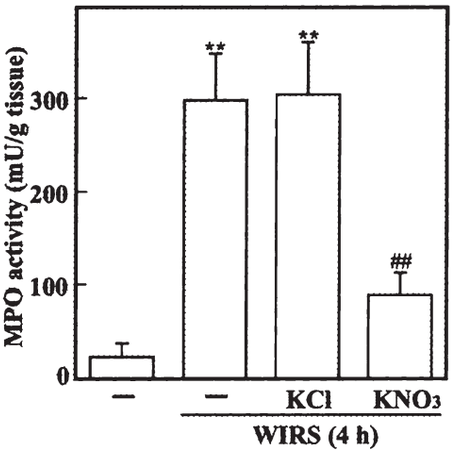

FIGURE 7 Effect of nitrate on the infiltration of neutrophils in the stomach. Animals were administered 0.2 mmol/kg of either KCl or KNO_3 solution 1 h before WIRS. After 4 h of WIRS, myeloperoxidase (MPO) activity in gastric mucosa was determined as described in the text. $^{**}P < 0.01$ and $^{\#\#}P < 0.01$ compared with normal and KCl-administered group, respectively.

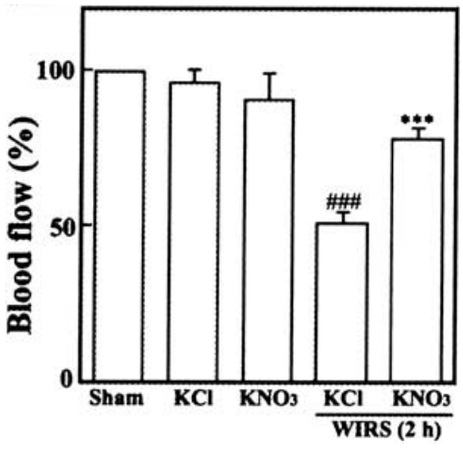

FIGURE 8 Effect of nitrate on gastric mucosal blood flow. Animals were administered 0.2 mmol/kg of either KCl or KNO_3 solution 1 h before giving WIRS. After 2 h of WIRS, gastric mucosal blood flow was measured as described in the text. Data show mean ± SE derived from 4 to 6 animals and expressed as % of sham-operated group. $^{###}P < 0.001$ compared with KCl-administered group; $^{***}P < 0.001$ compared with KCl- and WIRS-treated group.

inhibited the stress-induced gastric mucosal injury. Intragastrically administered nitrite but not nitrate selectively increased gastric NO levels in animals that received cardiac ligation, indicating that nitrate would have been transferred to the circulation, excreted in saliva, reduced to nitrite by oral bacteria, and then converted to NO in acidic gastric juice. Consistent with this notion is the findings that plasma levels of nitrate rapidly increased after administration of KNO_3. Such a gastrointestinal-salivary cycle for the metabolism and transport of nitrate and related compounds might play an important role in the maintenance of gastric NO levels, thereby protecting gastric mucosa from hazardous stress.

McKnight *et al.*[7] showed that nitrate levels in human plasma remained increased even at 6 h after oral administration of nitrate. Thus, dietary nitrate seems to participate in gastric generation of NO for relatively long time. It should be noted that, although cardiac ligation strongly inhibited the gastric generation of NO in KNO_3-administered rats, the gastric NO level was slightly higher than its basal level in KCl-treated animals. Furthermore, povidone-iodine completely inhibited gastric generation of NO both in KCl- and KNO_3-treated groups (Fig. 5C). Because the transfer of saliva to the stomach would have been inhibited completely by cardiac ligation, nitrite generated in the oral cavity could not participate in the generation of gastric NO. These observations suggest that a small fraction of gastric nitrate was also converted to NO in the stomach. In this context, Verdu *et al.*[22] reported the presence of nitrate reducing bacteria in human stomach. If bacteria having nitrate reducing activity also resided in rat stomach, some fraction of intragastrically administered KNO_3 would be reduced to nitrite and then to NO in acidic gastric juice in both control and cardia-ligated animals. The mechanism by which gastric generation of NO was inhibited partially by cardiac ligation and completely by povidone-iodine should be studied further.

NO has been known to inhibit the activation and endothelial adhesion of neutrophils.[16] Immunohistochemical analysis using specific antibodies revealed that administration of KNO_3 also inhibited the WIRS-induced expression of CD11b and infiltration of neutrophils in gastric mucosa (data not shown). Thus, gastric generation of NO might play an important role in the mechanism by which nitrate inhibited the WIRS-induced mucosal injury. This observation is consistent with the report that the enhanced generation of NO by endotoxin suppressed the WIRS-induced mucosal injury of the rat.[23] The present work also shows that continuous generation of NO via the gastrointestinal-salivary cycle of nitrate and related metabolites plays important roles in the suppression of both neutrophil infiltration and the decrease of gastric blood flow in WIRS-treated rats.

It has been reported that administration of endotoxin induced NO synthase in the stomach,[23] enhanced the generation of prostaglandin, and inhibited the gastric mucosal injury of ethanol-administered rats.[24] Thus, enhanced generation of prostaglandin may possibly underlie the mechanism by which gastric nitrate inhibited the WIRS-induced mucosal injury. However, the inhibitory effect of endotoxin was not affected by a loading dose of indomethacin, a potent inhibitor of prostaglandin synthesis.[24] Thus, prostaglandin does not seem to play a critical role in the inhibitory action of nitrate against WIRS-induced gastric mucosal injury of the rat.

Because NO is potent bactericidal compound,[25] its generation in gastrointestinal tracts is of critical importance in host defense. In this context, Benjamin *et al.*[8] reported that *Candida albicans* retained their viability in acidic medium but died in the presence of nitrite. Dykhuizen *et al.*[26] also reported that *Helicobacter pylori* died in nitrite-containing acidic medium but survived in nitrite-containing neutral medium. Thus, combination of gastric acid and NO might constitute a potent bactericidal system in the stomach. In the present experiments, we used 0.2 mmol KNO_3 per kg of body weight. This dose in human subjects corresponds to the amount of nitrate involved in about 300 g of dietary spinach. Thus, dietary intake of nitrate-enriched vegetables might play important roles in the protection of the stomach against hazardous pathogens and stress-induced mucosal injury via the gastrointestinal-salivary cycle of NO and related metabolites.

Acknowledgements

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