Cytoprotective Action of L-Arginine against HCl-Induced Gastric Injury in Rats: Involvement of Nitric Oxide?

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ABSTRACT—We examined the cytoprotective effect of L-arginine on gastric damage induced by 0.6 N HCl in rats and investigated whether the mechanism of this action is related to the nitric oxide (NO)-mediated protection. The animals were given 0.6 N HCl by gavage and killed 1 hr later. L-Arginine (100, 300 and 750 mg/kg) given p.o. 30 min before HCl treatment prevented these lesions in a dose-dependent manner, but had no effect when given i.v. (200 mg/kg). Similar effects were observed by D-arginine but not by an equimolar dose of mannitol. This effect of L-arginine (p.o.) was attenuated significantly by prior administration of indomethacin (5 mg/kg, s.c.) but not by L-NAME (5 mg/kg, i.v.), the NO synthase inhibitor. Both L- and D-arginine produced a reduction in potential difference (PD), inhibition of gastric motility, and increases of luminal pH and mucosal blood flow when they were given intragastrically. Indomethacin significantly mitigated these changes induced by L-arginine except PD reduction, while L-NAME showed significant inhibition only against the increased pH response. We conclude that L-arginine given p.o. exhibits gastric cytoprotection against HCl-induced damage in rats, probably by acting as a mild irritant. The mechanism of this action may appear through "adaptive cytoprotection" mediated by endogenous prostaglandins and does not involve the NO-mediated protective pathway.

Keywords: Arginine, Gastric lesion, Cytoprotection, Nitric oxide, Prostaglandin

Nitric oxide (NO), synthesized from the semi-essential amino acid L-arginine by NO synthase, is now established to mediate various biological actions mainly through activating guanylyl cyclase under physiological conditions (1, 2). Recent studies showed that NO plays an important role in the modulation of the gastric mucosal integrity by interacting with sensory neuropeptides and endogenous prostaglandins (PGs) (3–5). It has been also shown that endogenous NO is involved in certain types of gastrointestinal protection as induced by capsaicin (6), carbenoxolone (7) and mild irritants (8). However, the effect of L-arginine, the precursor of NO, on the gastric mucosal integrity has not been much studied. Kitagawa et al. (9) reported that L-arginine given intravenously prevented gastric lesions caused by 0.6 N HCl or water-immersion stress, yet the mechanism remained unclarified. Since the intracellular levels of L-arginine are already high and the supply of L-arginine is not normally rate-limiting for the constitutive enzyme (2, 10), it is ambiguous whether the protective action of L-arginine is really mediated by the NO-dependent pathway.

In the present study, we thus investigated the effects of L-arginine on development of gastric lesions induced in rats by 0.6 N HCl in comparison with those of D-arginine, and determined whether the mechanism of this protection involves the NO-mediated pathway using Nω-nitro-L-arginine as the methyl ester (L-NAME), a potent inhibitor of NO biosynthesis.

MATERIALS AND METHODS

Male Sprague-Dawley rats (230–280 g; Charles River, Shizuoka, Japan), kept in individual cages with raised mesh bottoms, were deprived of food but allowed free access to tap water for 18 hr before the experiments. All studies were carried out with four to eight rats per group.

Induction of gastric lesions

Animals were given 1 ml of 0.6 N HCl by gavage and killed 1 hr later. L-Arginine was given p.o. (100, 300 and 750 mg/kg) 30 min or i.v. (200 mg/kg) 10 min before administration of HCl. For comparison, the effects of D-arginine and mannitol on HCl-induced gastric lesions were also examined. D-Arginine was given p.o. in doses of
100–750 mg/kg and i.v. in a dose of 200 mg/kg. Mannitol was given in a dose of 314.6 mg/kg, p.o. or 209.2 mg/kg, i.v., which is a dose equimolar to L-arginine at 300 mg/kg, p.o. or 200 mg/kg, i.v., respectively. In some cases, the effects of indomethacin, a cyclooxygenase inhibitor, and L-NAME, an inhibitor of NO biosynthesis (5), were examined on the mucosal protective action of L-arginine. Indomethacin (5 mg/kg) or L-NAME (5 mg/kg) was given s.c. 30 min or i.v. 10 min before administration of L-arginine (300 mg/kg, p.o.). The dose of indomethacin was selected to show over 80% inhibition of cyclooxygenase activity in the gastric mucosa (11), while the dose of L-NAME was selected to induce a marked increase of arterial blood pressure, which is reversed by L-arginine (200 mg/kg) given i.v. (Fig. 1). In each case, the animals were killed under deep ether anesthesia, and then their stomachs were removed, inflated by injecting 8 ml of 2% formalin, immersed in 2% formalin for 10 min to fix the tissue wall, and opened along the greater curvature. Then, the area (mm$^2$) of necrotic lesions was measured under a dissecting microscope with a square grid (×10). The person measuring the lesions did not know the treatment given to the animals.

**Determination of potential difference, pH and mucosal blood flow**

Simultaneous measurement of gastric potential difference (PD), pH and mucosal blood flow (GMBF) was performed in the anesthetized rats according to the previously published method (12). Briefly, the animals were anesthetized with urethane (1.25 g/kg, i.p.) and the abdomens opened. Then, the stomach was exposed, mounted on the chamber (exposed area: 3.1 cm$^2$), and perfused with saline (154 mM NaCl) at the flow rate of 1 ml/min. The pH of fluid exiting from the chamber was measured by a pH glass electrode of the flow type (Horiba, Model 6901-25T, Kyoto, Japan), and the PD was determined using two agar bridges, one positioned in the chamber and the other in the abdominal cavity. Changes in both PD and pH were continuously monitored on a two channel recorder (Unicorder U-228, Nihon Densi Kagaku, Tokyo, Japan). On the other hand, GMBF was measured by laser Doppler flowmetry (Advance, Model ALF-2100, Tokyo, Japan) and by softly touching the probe (1 mm in diameter) on the surface of the corpus mucosa. After all parameters had well-stabilized, L-arginine was applied to the chamber for 30 min or given i.v. In the case of topical application, the perfusion was discontinued, the luminal solution was removed, and then the mucosa was exposed to 2 ml of L-arginine. Changes in PD, pH and GMBF were also investigated in the stomach before, during and after exposure to D-arginine (300 mg/kg) or mannitol (314.6 mg/kg). In some cases, indomethacin (5 mg/kg) or L-NAME (5 mg/kg) was given s.c. 30 min or i.v. 10 min before mucosal application of L-arginine (300 mg/kg). Some tissues were excised immediately after application of L-arginine and then immersed in 10% formalin for histological examination (H&E staining).

**Measurement of gastric motility**

Because previous studies showed that inhibition of gastric motility may be associated with the phenomenon of gastric cytoprotection (13, 14), the effect of L-arginine on gastric motility was examined using a balloon, according to a previously published method (13). Briefly, under ether anesthesia, a balloon (containing about 0.8 ml of water), the support catheter, and another catheter for intragastric administration of drugs, were placed in the glandular part of the stomach through an incision of the fore-stomach. The animals were then placed in Bollman cages, and the support catheter was connected to a pressure transducer and polygraph device (Nihon Kohden, Tokyo, Japan). Gastric motility was monitored as intraluminal pressure recordings after a complete recovery from anesthesia. Quantitative analysis of motility was performed by measuring the amplitude of each contraction.

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**Fig. 1.** Effect of L-NAME (5 mg/kg, i.v.) on arterial blood pressure in an urethane-anesthetized rat, and its reversal by L-arginine (200 mg/kg, i.v.). Blood pressure was monitored via a femoral artery using a pressure transducer and polygraph system (Nihon Kohden, RTA 1100M). Note that L-arginine given i.v. apparently reversed the pressor response induced by L-NAME.
(clear spike) over a 10-min period, determining the mean of a rat for this period from these values, and by calculating the mean±S.E. for each time period from five rats per group. L-Arginine was given i.v. (200 mg/kg) or intragastrically (300 and 750 mg/kg) after basal motility had become well-stabilized. The effects of i.g.-administration of D-arginine (300 mg/kg) and mannitol (314.6 mg/kg) on gastric motility were also examined. In some cases, indomethacin (5 mg/kg) or L-NAME (5 mg/kg) was given s.c. 30 min or i.v. 10 min before intragastric administration of L-arginine (300 mg/kg).

**Preparation of drugs**

Drugs used were L-arginine, D-arginine (Wako, Osaka, Japan), mannitol (Nacalai Tesque, Kyoto, Japan), indomethacin, Nω-nitro-L-arginine methyl ester (L-NAME: Sigma Chemicals, St. Louis, MO) and urethane (Tokyo Kasei, Tokyo, Japan). L-Arginine, D-arginine and mannitol were dissolved in distilled water. Indomethacin was suspended in saline with a drop of Tween 80 (Nacalai Tesque), while L-NAME was dissolved in saline. Each drug was prepared immediately before use and was given p.o., s.c. or intragastrically in a volume of 0.5 ml/100 g body wt. and i.v. in a volume of 0.2 ml/100 g body wt.

**Statistics**

Data are presented as the mean±S.E. from 4–8 rats per group. Statistical analyses were performed using a two-tailed Dunnett’s multiple comparison test, and values of P<0.05 were regarded as significant.

**RESULTS**

**Effects of L-arginine on HCl-induced gastric lesions**

Oral administration of 0.6 N HCl (1 ml) induced hemorrhagic lesions in the glandular stomach, the lesion score being 118.3±10.2 mm². L-Arginine given p.o. (100, 300 and 750 mg/kg) significantly prevented the development of gastric lesions caused by HCl in a dose-related manner; the lesion score at 300 and 750 mg/kg was 40.2±17.3 mm² and 12.3±3.4 mm², respectively, which is significantly lower than that observed in the control animals (Fig. 2). In contrast, this agent given i.v. (200 mg/kg) did not significantly affect the induction of gastric lesions in response to HCl. Similar protection against HCl-induced gastric lesions was observed dose-dependently with D-arginine given p.o. (100, 300 and 750 mg/kg) but not given i.v. (200 mg/kg), and the degree of protection at 300 mg/kg, p.o. was 62.3%, which is equivalent to that obtained by L-arginine, p.o. at 300 mg/kg (Fig. 3). Mannitol given either p.o. or i.v. at the equimolar concentration with L-arginine (300 mg/kg, p.o. or 200 mg/kg, i.v.) was also ineffective for reducing the severity of HCl-induced gastric lesions.

Pretreatment of the animals with L-NAME (5 mg/kg) did not significantly modify the protective activity of L-arginine (300 mg/kg, p.o.) against HCl-induced gastric lesions, and L-arginine exhibited over 80% reduction in the severity of these lesions (Fig. 4). On the other hand, the protective effect of L-arginine on HCl-induced gastric lesions was significantly attenuated by prior administration of indomethacin (5 mg/kg). The degree of the mucosal protection afforded by L-arginine (300 mg/kg, p.o.) was only 12.3% in the presence of indomethacin, which is significantly lower than that (68.3%) observed in the absence of this agent. Neither L-NAME nor indomethacin alone had any significant influence on the development of gastric lesions in response to HCl, although the former showed a propensity to aggravate these lesions.

**Effects of L-arginine on PD, pH and GMBF in the stomach**

The stomach mounted on a chamber generated a PD of about -30 to -35 mV (mucosa negative), secreted acid to keep the luminal pH at 3.2 to 3.6 and maintained a GMBF of 10 to 15 ml/min/100 g. These values remained relatively constant during the 2-hr test period and were not changed significantly after exposure of the mucosa to saline. Mucosal application of L-arginine (100–750 mg/kg) caused a dose-dependent reduction of PD and increases of pH and GMBF: ΔPD, ΔpH and ΔGMBF (% increase) at 300 mg/kg were 19.2±1.2 mV, 2.3±0.4 and 35.5±5.5%, respectively (Figs. 5 and 6). L-Arginine given i.v. (200 mg/kg) did not produce any change in either of
Fig. 3. Effects of D-arginine and mannitol on gastric lesions induced by 0.6 N HCl in rats. The animals were given 1 ml of 0.6 N HCl p.o. and killed 1 hr later. D-Arginine and mannitol were given either p.o. 30 min or i.v. 10 min before HCl treatment, respectively. The doses of mannitol represent the equimolar concentration of L-arginine at the dose of 300 mg/kg, p.o. or 200 mg/kg, i.v. *Statistically significant difference from the control, at P<0.05. N: 6-8.

these parameters. On the other hand, D-arginine (300 mg/kg) applied to the mucosa caused PD reduction followed by an increase of pH and GMBF, and the degree of these changes was equivalent with those induced by the same dose of L-arginine. However, either of these changes

Fig. 4. Effects of L-NAME and indomethacin on the protective action of L-arginine against 0.6 N HCl-induced gastric lesions in rats. L-NAME (3 mg/kg, i.v.) or indomethacin (5 mg/kg, s.c.) was given 10 min or 30 min before administration of L-arginine (300 mg/kg, p.o.). *Statistically significant difference from the control, at P<0.05. N: 8.

Fig. 5. Effects of L-arginine on PD, pH and GMBF responses of the chambered stomach of anesthetized rats. L-Arginine was applied topically to the chamber for 30 min or given i.v. Values indicate the maximal changes after treatment with L-arginine and represent the mean ± S.E. from 4-6 rats. *Statistically significant difference from the control (saline), at P<0.05.
was not observed by the equimolar concentration of d-mannitol applied to the mucosa. Although there was no macroscopic lesion in the mucosa immediately after application of L-arginine (300 mg/kg), histological examination showed apparent damage in the surface epithelial cells, including disruption of the apical membrane (not shown).

These changes in pH and GMBF induced by mucosal application of L-arginine (300 mg/kg) were significantly mitigated by prior administration of indomethacin (5 mg/kg), although the degree of PD reduction remained unchanged in the presence of this agent (Figs. 7 and 8). In these rats, the increased pH responses were similarly observed for the initial 15 min after L-arginine treatment, but became significantly smaller than those of the controls when examined at 30 min after the exposure. On the other hand, pretreatment of the animals with L-NAME (5 mg/kg) did not significantly affect both PD and GMBF responses induced by L-arginine, in addition to the increased pH response observed 15 min after the exposure. However, this treatment significantly shortened the duration of pH response, similar to indomethacin, and the values in pH observed 30 min after exposure to L-arginine were significantly lower in these animals when compared to the controls.

**Effects of L-arginine on gastric motility**

The stomachs of control animals contracted at a frequency of 11.3 ± 3.2/10 min with an amplitude of
18.6 ± 3.4 cmH2O. Administration of saline either intragastrically or i.v. did not affect motility in terms of either the frequency or amplitude. Gastric motility was inhibited by intragastric administration of L-arginine (Fig. 9), but not by this agent given i.v. (200 mg/kg) (not shown). The potency and duration of motility inhibition caused by intragastric administration of L-arginine were increased in a dose-dependent manner, and at 750 mg/kg, a significant effect persisted for up to 60 min after the administration. The motility inhibition caused by intragastric L-arginine (300 mg/kg) was not significantly affected by prior administration of L-NAME (5 mg/kg), but almost totally abolished by pretreatment of the animals with indomethacin (5 mg/kg). Gastric motility was also significantly inhibited by intragastric administration of D-arginine (300 mg/kg) but was not affected by the equimolar concentration of D-mannitol given intragastrically (not shown).

DISCUSSION

The endothelium-derived vasodilator NO plays an important role in the regulation of GMBF and in the modulation of the gastric mucosal integrity (3 – 5). However, the effect of L-arginine, the precursor of NO, on the gastric mucosal integrity has not been much studied. The present study was performed to examine whether L-arginine has any protective effect against HCl-induced gastric lesions and whether this mechanism is really mediated by endogenous NO.

We found that L-arginine exhibited potent cytoprotection against HCl-induced gastric lesions only when it had been given p.o. However, L-arginine given i.v. did not confer any protection against damage at the dose (200 mg/kg) that antagonized the elevated blood pressure response induced by the NO biosynthesis inhibitor L-NAME. This result disagreed with the observation by Kitagawa et al. (9) who showed significant protection by L-arginine (300 mg/kg, i.v.) against HCl-induced gastric damage, although the reason for these different results remains unexplained at present. In the present study, intragastric administration of L-arginine protected the stomach against HCl-induced damage in a dose-dependent manner. Yet this effect was mimicked by the enantiomer D-arginine and was not affected by prior administration of L-NAME but almost totally disappeared in the presence of indomethacin. These results suggest that the mucosal protective action of L-arginine (p.o.) may involve endogenous PGs in its mechanism but is unrelated to the NO-mediated pathway.

On the other hand, intragastric application of L-arginine produced a lowering of PD followed by increases of luminal pH and GMBF, in a dose-dependent manner. These functional effects were mimicked by D-arginine but not observed when L-arginine was given i.v. and were also significantly mitigated by pretreatment with indometh-
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Fig. 9. Representative recordings of gastric motility changes after intragastric administration of L-arginine (300 mg/kg) in the absence or presence of L-NAME and indomethacin in rats. L-NAME (5 mg/kg, i.v.) or indomethacin (5 mg/kg, s.c.) was given 10 min or 30 min before intragastric administration of L-arginine.

acrin, except for PD reduction. Such characteristic phenomena are commonly seen in the stomach after exposure to mild irritants such as hypertonic NaCl (11, 15). In fact, the histological study revealed damage in the surface epithelium of the mucosa immediately after exposure to L-arginine at 300 mg/kg or greater, suggesting that L-arginine acts on the stomach similar to mild irritants. The mechanism by which intragastric L-arginine caused PD reduction remains unclarified. In a preliminary study, we found a similar PD reduction when the mucosa was exposed to L-lysine but not to L-glycine or L-glutamine, indicating that this action is not commonly shared by amino acids. Certainly, the above effects of L-arginine were not mimicked by mucosal application of mannitol at the equimolar concentration. Thus, it may be assumed that L-arginine induces such changes because of its chemical property, probably shared by basic amino acids, but not simply due to its hyperosmolarity, especially at a high dose.

The involvement of endogenous PGs in the mucosal protection and the functional responses induced by mild irritants has been well-demonstrated by many investigators (11, 15–17). The increase of luminal pH in the stomach exposed to mild irritants is considered to be due to both inhibition of acid secretion and diffusion of HCO₃⁻ (17), while the GMBF response may be mediated by endogenous PGs and capsaicin-sensitive sensory neurons (11, 18). To our surprise, the increase of luminal pH after exposure to L-arginine was markedly inhibited by the NO biosynthesis inhibitor L-NAME, despite the fact that other parameters remained unaffected. It may be assumed that the increased pH response caused by L-arginine is mediated by NO, since L-NAME by itself did not affect luminal pH in the stomach without exposure to L-arginine. We previously showed that this pH response occurs as one of the consequential events following the disruption of the surface epithelial cells (11, 15–18). In general, there are two types of NO synthase, one is the constitutive NO synthase and the other is the inducible one (2). The functional consequences of the formation of NO by the inducible NO synthase have not been clearly established, but it is likely that it plays a role in pathological vasodilation and tissue damage (2, 10). Arginine analogues such as L-NAME are specific inhibitors of both the constitutive and inducible NO synthase (19). Thus, it might be possible to speculate that mild irritants increase the NO synthase activity, probably by damage in the surface cells, leading to formation of NO which may contribute in part to the increase of luminal pH. Since L-NAME did not significantly affect the cytoprotective effect of L-arginine, such pH responses may not be in-
volved in the protective mechanism of this agent.

The increased GMBF response induced by L-arginine was significantly attenuated by indomethacin but not by L-NAME. If the irritation by L-arginine of the stomach was associated with activation of the constitutive NO synthase or the expression of the inducible NO synthase, the GMBF response would be also be inhibited by L-NAME, similar to the pH response. In the present study, L-NAME alone caused a definite increase in blood pressure but did not reduce GMBF under urethane anesthetized conditions (see Fig. 7). Tepperman and Whittle (5) reported significant decrease of GMBF after administration of L-NAME in pentobarbital-anesthetized rats, while Lippe and Holzer (20) reported that inhibition of NO synthesis failed to alter basal GMBF in the rats anesthetized with urethane. It may be assumed that these different results may be due to different experimental conditions such as anesthesia. As in the present study, the mucosal application of L-arginine further increased GMBF even in the animals pretreated with L-NAME, it is obvious that GMBF was increased in response to topical application of L-arginine, mainly mediated by endogenous PGs, irrespective of whether the NO synthase had been inhibited by L-NAME or not.

We have proposed that inhibition of gastric motility may be closely associated with the phenomenon of gastric cytoprotection (12–14). Intragastric L-arginine also inhibited gastric motility at the doses which exhibited cytoprotection against HCl-induced lesions. This effect was significantly reversed by prior administration of indomethacin but not by L-NAME, providing further evidence for the close relationship between these two factors. These results are consistent with the previous observation that mild irritants inhibit gastric motility mediated by endogenous PGs, and that this action is certainly antagonized by pretreatment with indomethacin (14). Thus, the mechanism of cytoprotection by L-arginine (p.o.) may involve the motility inhibition in addition to the increase of GMBF, although at the present moment, it remains undefined which factor plays a determinant role in the mucosal protection.

Taken together, the present study showed that L-arginine given p.o. but not i.v. provides gastric protection against HCl-induced lesions in rats, probably by acting as a mild irritant. The mechanism of this action may appear through “adaptive cytoprotection” mediated by endogenous PGs, but is unrelated to the NO-mediated protective pathway. This study also showed that mild irritation to the gastric mucosa might activate the NO synthase, resulting in the release of NO, which may be involved in the regulation of luminal alkalinization in the stomach exposed to mild irritants. Although the present study failed to show the involvement of NO in adaptive cytoprotection, further studies will be needed to clarify the pathophysiological role of NO in the irritated mucosa.

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