

Implication of Endogenous Nitric Oxide in Gastric Mucosal Protective Effect of T-593, a Novel Anti-ulcer Agent, in Rats

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ABSTRACT—The relationship of endogenous nitric oxide (NO) to the gastric mucosal protective effect of the novel anti-ulcer agent T-593, (\pm)-(E)-1-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-[2-[[[5-(methylamino)methyl-2-furyl]methyl]thio]ethyl]-2-(methylsulfonyl) guanidine, was investigated in rats. T-593 (3–30 mg/kg, p.o.) dose dependently prevented the formation of gastric mucosal lesions induced by oral administration of aspirin (200 mg/kg) in 0.15 N HCl (HCl-aspirin). Pretreatment with N^G -nitro-L-arginine methylester (L-NAME), a selective inhibitor of NO synthase (NOS), attenuated the mucosal protective effect of T-593. This effect of L-NAME was antagonized by pretreatment with L-arginine, a substrate of NOS, but not with D-arginine. Activity of total NOS composed of inducible and constitutive NOS in the gastric mucosa was decreased by HCl-aspirin, and T-593 inhibited this decrease. On the other hand, HCl-aspirin and T-593 did not affect inducible NOS activity in the gastric mucosa. Furthermore, we confirmed that T-593 inhibits the decrease in gastric mucosal blood flow (GMBF) induced by HCl-aspirin, and this effect is completely inhibited by pretreatment with L-NAME. These results suggest that the mucosal protective effect of T-593 is partly mediated by endogenous NO via improvement of GMBF and that a possible mechanism for the effect of T-593 is the maintenance of constitutive NOS activity in gastric mucosa.

Keywords: T-593, Anti-ulcer agent, Gastroprotection, Nitric oxide, Gastric mucosal blood flow

T-593, (\pm)-(E)-1-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-[2-[[[5-(methylamino)methyl-2-furyl]methyl]thio]ethyl]-2-(methylsulfonyl) guanidine, is a novel anti-ulcer agent that possesses both an acid anti-secretory activity by H_2 -receptor antagonism (1) and a gastroprotective faculty via improvement of the gastric mucosal protective factors such as mucosal blood flow (2) and mucus secretion (3). However, the mechanism for the improvement of the protective factors has not been fully elucidated.

Recently, it has been reported that nitric oxide (NO) (4–6) synthesized by two distinct types of NO synthase (NOS), the constitutive type whose activity is Ca^{2+} -dependent and the inducible type whose activity is Ca^{2+} -independent, is an important regulator of various physiological functions. With regards to the physiological function in gastric mucosa, it has been reported that NO regulates gastric mucosal blood flow (GMBF) (7, 8), mucus (9), acid (10, 11) and alkali secretion (12, 13). Furthermore, NO is thought to be an important mediator of gastroprotection, since both endogenous and exogenous NO protect gastric mucosa in various kinds of acute gastric mucosal lesion models in rats (14–19).

In the present study, we investigated the implication of endogenous NO in the gastric mucosal protection and the GMBF improvement induced by T-593 using the acute gastric mucosal lesion model produced in rats by oral administration of aspirin in 0.15 N HCl (HCl-aspirin).

MATERIALS AND METHODS

Animals

Male Wistar/ST rats (Nihon SLC, Hamamatsu) weighing 217–332 g were used. The animals were housed in a room at 20–24°C, air humidity of $60 \pm 10\%$, with a 12 hr light-dark cycle (light on 6:00 a.m.), and they were given food and water ad libitum. For 18 hr before the experiments, the animals were fasted, but allowed free access to water.

Reagents

T-593 was synthesized in our laboratories. Other agents used in the present study were as follows: famotidine (Sigma, St. Louis, MO, USA); aspirin (Mitsui Toatsu, Tokyo); N^G -nitro-L-arginine methylester (L-NAME),

L-arginine and D-arginine (Wako, Osaka); L-[^{14}C]arginine monohydrochloride (Amersham, Buckinghamshire, UK).

For studies on gastric mucosal protection, NOS activity in vivo and GMBF: T-593 and famotidine were suspended in 0.5% methylcellulose solution (0.5% MC). L-NAME was dissolved in saline. HCl-aspirin was prepared by suspension of aspirin in 0.5% carboxymethylcellulose sodium solution (0.5% CMC) containing 0.15 N HCl. L-Arginine and D-arginine were dissolved in saline.

For study on NOS activity in vitro: T-593 was dissolved in 0.3 N HCl, neutralized with 0.1 N NaOH and successively diluted with total NOS activity assay buffer whose components are described below.

Each drug was prepared just before the experiment and given in a volume of 5 ml/kg (p.o.), 2 ml/kg (i.v., i.d.) or 1 ml/body (i.g.).

Studies on gastric mucosal protection

T-593 or famotidine (3–30 mg/kg, p.o., each) was administered 1 hr before oral administration of HCl-aspirin (200 mg/kg). Control animals received 0.5% MC. The animals were sacrificed 3 hr after the administration of HCl-aspirin, and the stomachs were removed and fixed in 1% formalin solution for 10 min. Then the stomachs were incised along the greater curvature, and the areas of each hemorrhagic lesion in the mucosa were measured under a stereoscopic microscope (JM, 10 \times ; Olympus Optical Co., Ltd., Tokyo). The sum of the lesion areas was used as the lesion index (mm 2).

In the experiments to examine the involvement of endogenous NO in the gastroprotective effect of T-593, L-NAME (10 mg/kg, i.v.) or saline was administered 30 min before administration of T-593 (30 mg/kg, p.o.) or 0.5% MC. Furthermore, L-arginine (250 mg/kg, i.v.), D-arginine (250 mg/kg, i.v.) or saline was administered 5 min before administration of L-NAME.

Studies on gastric mucosal NOS activity

Preparation of the NOS enzyme sample (20): T-593 (10 or 30 mg/kg, p.o.) or famotidine (30 mg/kg, p.o.) was administered 1 hr before oral administration of HCl-aspirin. Normal and control animals received 0.5% MC instead of drug, and normal animals received 0.5% CMC instead of HCl-aspirin. The animals were sacrificed 2 hr after the administration of HCl-aspirin, and the stomachs were removed and incised along the greater curvature. The gastric mucosa (approximately 100 mg) was separated from its underlying muscle layers and homogenized in 3 ml of ice-cold Tris/HCl buffer (50 mM, pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-mercaptoethanol, 2 μM leupeptin, 1 μM pepstatin A and 1 μM phenylmethylsulfonyl fluoride with a teflon homogenizer. Then the homogenates were sonicated and

centrifuged at 2500 rpm for 30 min. The supernatants were collected, and glycerol (10% v/v) was added. These NOS enzyme samples were stored at -80°C until determination of NOS activity.

For the study of the effect of T-593 alone on gastric mucosal NOS activity in normal rats, the gastric mucosal NOS enzyme samples were prepared as described above, 3 hr after oral administration of T-593 (30 mg/kg, p.o.) or 0.5% MC.

Determination of NOS activity: Total, i.e., inducible and constitutive, and inducible NOS activities were measured by monitoring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline according to the method of Bredt and Snyder (21). Briefly, for the determination of total NOS activity, 5 μl of each enzyme sample was added to a plastic tube holding 20 μl of total NOS activity assay buffer (50 mM HEPES buffer (pH 7.4) containing 1 mM NADPH, 1 mM EDTA, 1.25 mM CaCl_2 , 1 mM dithiothreitol and 10 $\mu\text{g}/\text{ml}$ calmodulin) containing 100 nM L-[^{14}C]arginine (0.05 μCi). These were incubated for 10 min at 22°C and the reaction was terminated by the addition of ice-cold 200 mM HEPES buffer (pH 5.5) containing 20 mM EGTA, 2 mM L-arginine and 2 mM L-citrulline. Then the reacted solutions were spotted onto TLC plates (Whatman silica gel 150A). The plates were developed in a solvent system (22) consisting of chloroform / methanol / ammonium hydroxide / water, 0.5:4.5:2.0:1.0 (vol/vol), over a distance of 16 cm. After drying the plates, autoradiographs were made to determine the positions of L-[^{14}C]arginine and L-[^{14}C]citrulline. The radioactivity at the silica gel positions containing L-[^{14}C]citrulline was measured using a liquid scintillation counter (Liquid Scintillation System LSC-3000; Aloka, Tokyo). For the determination of inducible NOS activity, we used the buffer modified by addition of 1 mM EGTA with no addition of CaCl_2 . Total and inducible NOS activities were expressed as the amount of L-[^{14}C]citrulline produced per 1 mg of protein of enzyme sample for 1 min (pmol/mg protein/min).

In the experiments to evaluate the action of T-593 on NOS activity in vitro, the NOS enzyme samples isolated from gastric mucosa in normal rats were preincubated with T-593 (10^{-6} – 10^{-4} M) for 5 min. Subsequently, total NOS activity was determined in duplicate experiments.

Determination of protein content: Protein content was determined using a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA, USA).

Measurement of GMBF

Under urethane anesthesia, GMBF was measured by the electrochemically generated hydrogen-gas clearance method (DHM-3001; M.T. Giken, Tokyo) as reported by Kosu et al. (23). Briefly, a needle-type platinum elec-

trode was inserted into the corpus mucosa through the serosal membrane, and a reference electrode was placed in the abdomen. Measurements were taken at 15 min intervals by recording on a chart recorder. GMBF was expressed as % of the initial blood flow rate. Using a glass syringe with injection needle, HCl-aspirin was directly administered into stomach through the corpus mucosa at a site sufficiently far from the portion inserted with the electrode. T-593 (30 mg/kg, i.d.) or 0.5% MC was administered 1 hr before intragastric administration of HCl-aspirin. To examine the involvement of NO in the effect of T-593, L-NAME (2 mg/kg, i.v.) was administered 30 min before administration of T-593.

Statistical analyses

Data are presented as the mean \pm S.E.M. Dunnett's multiple comparison test or Student's *t*-test was used to determine statistically significant difference, and a *P* value of less than 0.05 was regarded as indicative of significant difference.

RESULTS

Effects on gastric mucosal lesions

Administration of HCl-aspirin produced hemorrhagic band-like lesions in the corpus mucosa. T-593 prevented the formation of gastric mucosal lesions in a dose-dependent manner, and the effects in doses of 10 and 30 mg/kg were statistically significant (Fig. 1). Famotidine prevented it in the same manner too. The ED₅₀ values of the effects of T-593 and famotidine are 6.4 and 13.4 mg/kg, respectively. The gastric mucosal lesions induced by HCl-

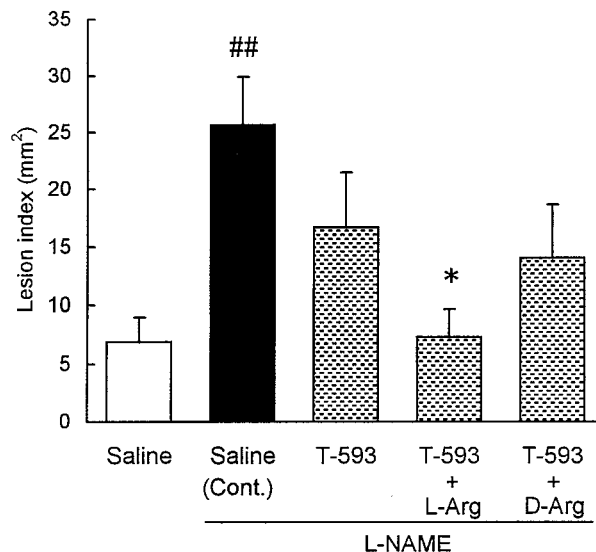


Fig. 2. Effects of L-NAME, L-arginine (L-Arg) and D-arginine (D-Arg) on gastroprotection by T-593 against HCl-aspirin in rats. T-593 (30 mg/kg) was administered orally 1 hr before oral administration of HCl-aspirin, and mucosal lesions were assessed 3 hr later. L-NAME (10 mg/kg, i.v.) was administered 30 min before administration of T-593, and L-arginine or D-arginine (250 mg/kg, i.v., each) was administered 5 min before administration of L-NAME. Each column and bar represents the mean \pm S.E.M. of 7 animals. ^{##}*P* < 0.01, significant difference from the animals not treated with L-NAME by Student's *t*-test, ^{*}*P* < 0.05, significant difference from the control by the Dunnett's test.

aspirin were aggravated by pretreatment with L-NAME (10 mg/kg, i.v.; Fig. 2). The inhibitory effect of T-593 (30 mg/kg, p.o.) on the formation of gastric mucosal lesions was attenuated by the pretreatment with L-NAME (10

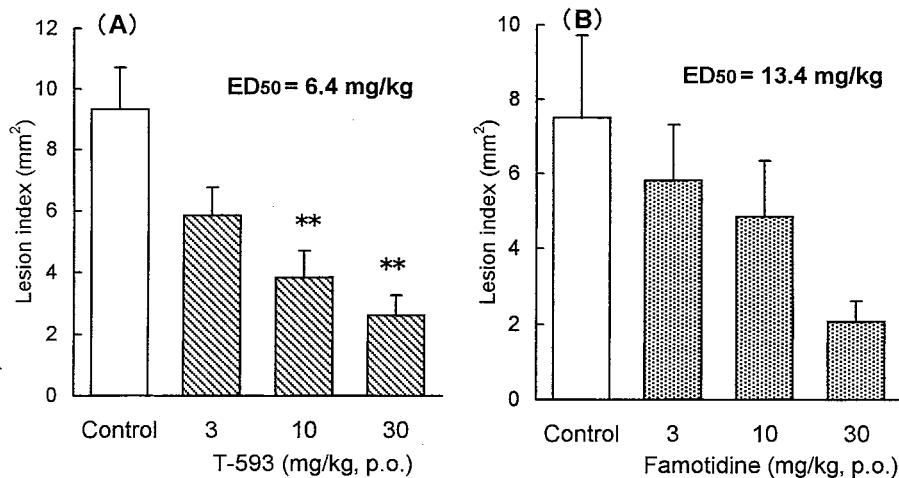


Fig. 1. Effects of T-593 (A) and famotidine (B) on gastric mucosal lesions induced by HCl-aspirin in rats. T-593 or famotidine was administered orally 1 hr before oral administration of HCl-aspirin, and mucosal lesions were assessed 3 hr later. Each column and bar represents the mean \pm S.E.M. of 7 animals. ^{**}*P* < 0.01, significant difference from the control by the Dunnett's test.

mg/kg, i.v.), and the effect of T-593 was not significantly different from the control in this case. This attenuation induced by L-NAME was antagonized by pretreatment with L-arginine (250 mg/kg, i.v.), but not with D-arginine (250 mg/kg, i.v.). Neither L-arginine nor D-arginine (250 mg/kg, i.v., each) alone affected gastric mucosal lesions caused by HCl-aspirin and L-NAME (Fig. 3).

Effects on gastric mucosal total and inducible NOS activities

Total NOS activity in gastric mucosa was significantly decreased by administration of HCl-aspirin (Fig. 4). T-593 (30 mg/kg, p.o.) significantly inhibited this decrease in total NOS activity. However, famotidine (30 mg/kg, p.o.) did not affect it.

In contrast, inducible NOS activity in gastric mucosa was not affected by HCl-aspirin (Fig. 5). Furthermore, neither T-593 (10 and 30 mg/kg, p.o.) nor famotidine (30 mg/kg, p.o.) administered before HCl-aspirin affected the inducible NOS activity. T-593 (30 mg/kg, p.o.) alone had no significant effect on gastric mucosal total NOS activity in normal rats (Table 1). T-593 (10^{-6} – 10^{-4} M) slightly inhibited total NOS activity in vitro (% of control: 10^{-6} M, 91.1%; 10^{-5} M, 91.1%; 10^{-4} M, 93.6%).

Effects on GMBF

GMBF was gradually decreased by intragastric administration of HCl-aspirin (Fig. 6). T-593 (30 mg/kg, i.d.) inhibited the decrease of GMBF, and a significant inhibition was observed from 120 min after administra-

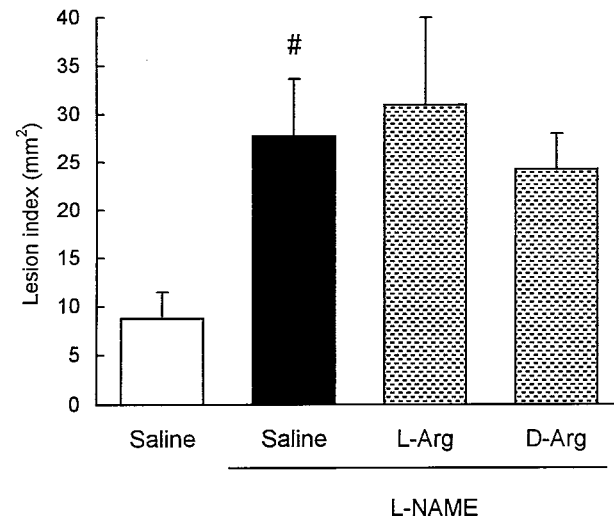


Fig. 3. Effects of L-arginine (L-Arg) and D-arginine (D-Arg) on mucosal lesions induced by HCl-aspirin and L-NAME in rats. Mucosal lesions were assessed 3 hr after oral administration of HCl-aspirin. L-NAME (10 mg/kg, i.v.) was administered 90 min before administration of HCl-aspirin, and L-arginine or D-arginine (250 mg/kg, i.v., each) was administered 5 min before administration of L-NAME. Each column and bar represents the mean \pm S.E.M. of 7 animals. [#] $P < 0.05$, significant difference from the animals not treated with L-NAME by Student's *t*-test.

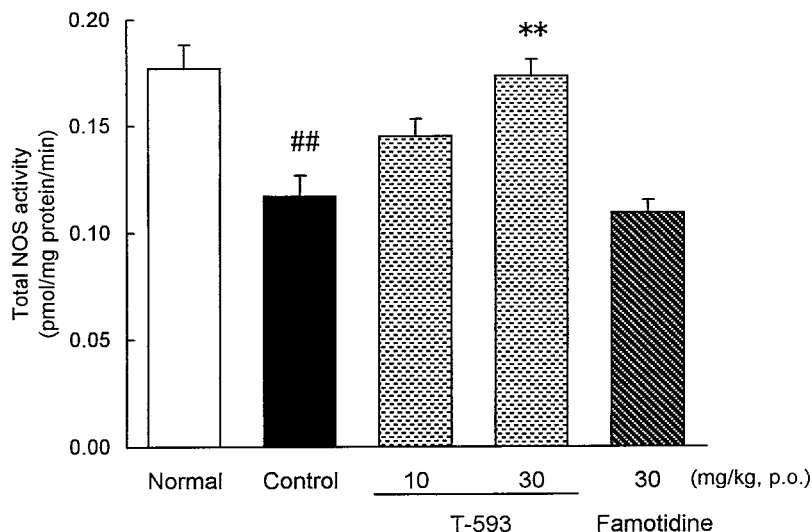


Fig. 4. Effects of T-593 and famotidine on decrease of gastric mucosal total NOS activity induced by HCl-aspirin in rats. T-593 or famotidine was administered orally 1 hr before oral administration of HCl-aspirin, and total NOS activity was measured 2 hr later. Normal animals received 0.5% CMC instead of HCl-aspirin. Each column and bar represents the mean \pm S.E.M. of 7 animals. ^{##} $P < 0.01$, significant difference from the normal animals by Student's *t*-test. ^{**} $P < 0.01$, significant difference from the control by the Dunnett's test.

tion of T-593. GMBF was transiently increased by administration of L-NAME (2 mg/kg, i.v.; Fig. 7). T-593 had no inhibitory effect on the GMBF decrease in rats pretreated with L-NAME.

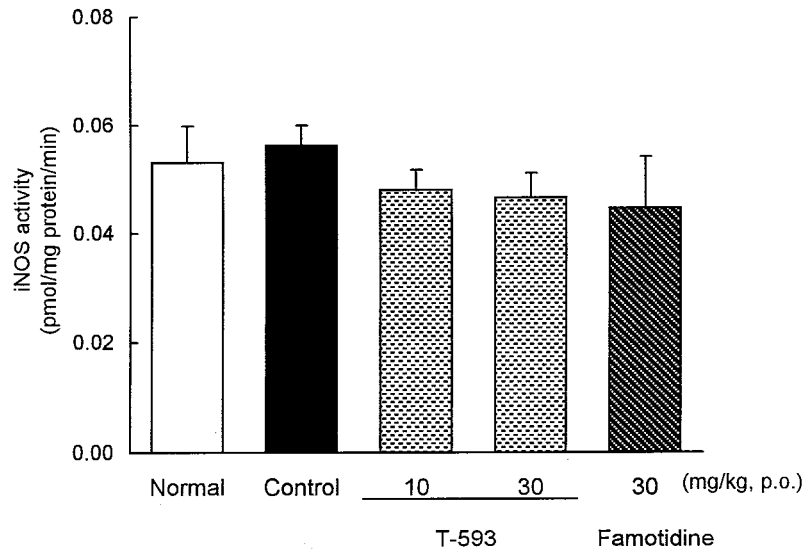


Fig. 5. Effects of T-593 and famotidine on gastric mucosal inducible NOS activity in rats treated with HCl-aspirin. T-593 or famotidine was administered orally 1 hr before oral administration of HCl-aspirin, and inducible NOS activity was measured 2 hr later. Normal animals received 0.5% CMC instead of HCl-aspirin. Each column and bar represents the mean \pm S.E.M. of 7 animals.

DISCUSSION

Much attention has recently been focused on the gastric mucosal protective action of NO. It has been reported that a lowering of endogenous NO production in the gastric mucosa augments gastric mucosal lesions via a decrease in GMBF, which is an important factor in mucosal protection (14, 15). Furthermore, exogenous NO derived from an NO donor has been demonstrated to protect the gastric mucosa against injury induced by ischemia-reperfusion (16) and administration of various kinds of noxious agents such as ethanol (17), hydrochloric acid (18) or endothelin (19).

It has been reported that T-593, a novel anti-ulcer agent, prevents the formation of gastric mucosal lesions by inhibition of gastric acid secretion via H_2 -receptor antagonism (1) and by improvement of gastric mucosal protective factors such as mucosal blood flow (2) and mucus secretion (3) in experimental models. This im-

provement of the protective factors is a predominant feature of T-593. However, the mechanism of this action is yet unclear.

Then, Kitahora and Guth (24) reported that HCl-aspirin causes gastric mucosal lesions accompanied by mucosal hemorrhage via an acid back-diffusion by the disruption of the mucosal barrier and a decrease of GMBF. In the present study, we investigated the implication of endogenous NO in the gastric mucosal protection and the GMBF improvement induced by T-593 using the acute gastric mucosal lesion model produced by HCl-aspirin in rats.

Both T-593 and famotidine prevented the formation of the gastric mucosal lesions induced by HCl-aspirin in a dose-dependent manner. We have already reported that the acid anti-secretory activity of T-593 is about six times less potent than that of famotidine (25). However, the mucosal protective effect of T-593 was about twice as strong as that of famotidine. These results suggest that the anti-secretory effect may contribute to the mucosal protective effect of famotidine against HCl-aspirin, but it does not mainly contribute to the effect of T-593.

The mucosal protective effect of T-593 was attenuated by pretreatment with L-NAME, which is a selective inhibitor of NOS. Furthermore, this counteractive effect of L-NAME was antagonized by pretreatment with L-arginine, a substrate of NOS, but not with D-arginine. Since pretreatment of L-NAME could not completely inhibit the effect of T-593, it is suggested that some effect that is not dependent on endogenous NO, e.g., anti-secretory

Table 1. Effect of T-593 on gastric mucosal total NOS activity in normal rats

Treatment	N	Total NOS activity (pmol/mg protein/min)	% of control
Control (0.5% MC)	7	0.114 \pm 0.012	100.0
T-593	7	0.108 \pm 0.013	94.7

T-593 or 0.5% MC was administered orally. Three hours later, gastric mucosal total NOS activity was measured. Total NOS activities are represented as the mean \pm S.E.M. of 7 animals.

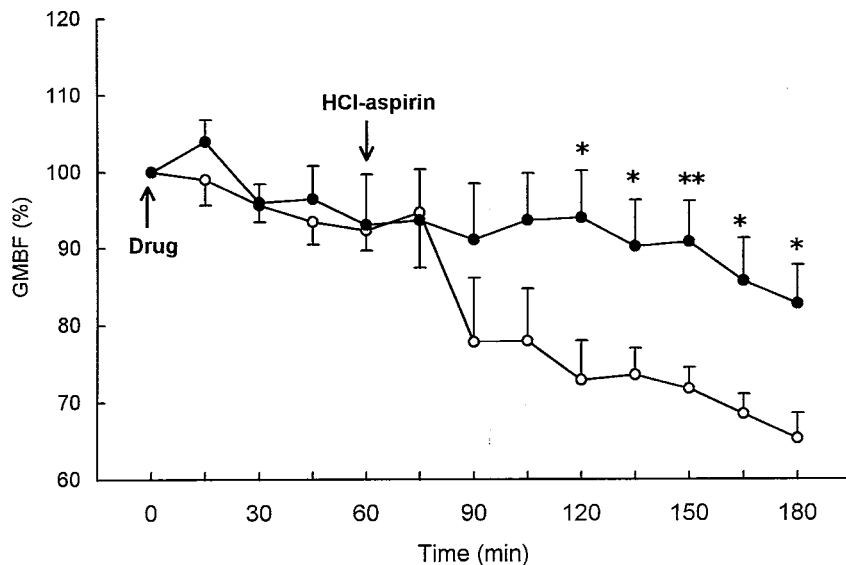


Fig. 6. Effects of T-593 on decrease of GMBF caused by HCl-aspirin in rats. Under urethane anesthesia, GMBF was measured by the hydrogen-gas clearance method. GMBF was expressed as % of the value before drug treatment. T-593 (30 mg/kg, i.d.) (●) or 0.5% MC (○) was administered 1 hr before administration of HCl-aspirin (i.g.). Each point and bar represents the mean \pm S.E.M. of 6 animals. * $P < 0.05$, ** $P < 0.01$, significant difference from 0.5% MC by Student's *t*-test.

effect by H_2 -receptor antagonism, may contribute to the effect of T-593. However, these results clearly indicate that endogenous NO participates in the mucosal protective effect of T-593.

To investigate further the implication of endogenous NO in the mucosal protective effect of T-593, we measured gastric mucosal total NOS activity composed of

inducible and constitutive NOS activity. In the present studies, the total NOS activity was significantly decreased by the administration of HCl-aspirin, and the decrease in total NOS activity was significantly inhibited by prior administration of T-593, but not by that of famotidine whose dose and mucosal protective effect is the same as T-593. The results described above suggest that the dis-

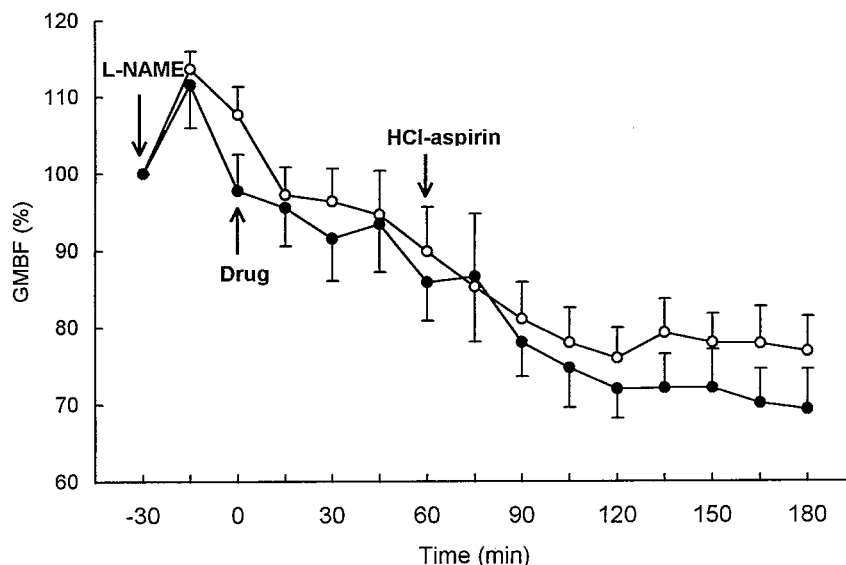


Fig. 7. Effects of T-593 on decrease of GMBF caused by HCl-aspirin in rats. Under urethane anesthesia, GMBF was measured by the hydrogen-gas clearance method. GMBF was expressed as % of the value before drug treatment. T-593 (30 mg/kg, i.d.) (●) or 0.5% MC (○) was administered 1 hr before administration of HCl-aspirin (i.g.). L-NAME (2 mg/kg, i.v.) was administered 30 min before administration of T-593. Each point and bar represents the mean \pm S.E.M. of 6 animals.

ruption of the mucosal barrier is caused by an impoverishment of the protective effects of NO, which is caused by lowering of total NOS activity, and that the maintenance of total NOS activity, i.e., the maintenance of NO production, in the gastric mucosa contributes to the mucosal protective effect of T-593.

It is well-known that NOS is divided into two distinct isozymes. One is constitutive NOS, whose activity is Ca^{2+} -dependent and which might modulate resting vascular tone, and the other is inducible NOS, whose activity is Ca^{2+} -independent and is enhanced following exposure of macrophages, smooth muscle cells and other cells to endotoxin or cytokines (5, 6). The administration of HCl-aspirin or both T-593 and HCl-aspirin did not affect inducible NOS activity in the gastric mucosa. On the other hand, it has been reported that constitutive NOS activity is regulated by intracellular Ca^{2+} concentration (5, 6). From that fact, it could be thought that T-593 modulates intracellular Ca^{2+} concentration and activates total NOS via an increase of constitutive NOS activity. However, we confirmed that T-593 alone has also no significant effect on gastric mucosal total NOS activity in normal rats and that T-593 slightly inhibits total NOS activity in vitro. These results suggest that the effect of T-593 on NOS activity in vivo is not due to direct action on the NOS enzyme and is instead due to the maintenance of constitutive NOS activity, but not inducible NOS activity.

It has been reported that the administration of HCl-aspirin decreases GMBF in rats (24, 26), and this event was confirmed in the present study. To clarify the inter-relationship of the gastroprotective effect of T-593, GMBF and endogenous NO, we examined the effect of T-593 on the decrease in GMBF induced by HCl-aspirin and the influence of pretreatment with L-NAME on the effect of T-593. T-593 inhibited the GMBF decrease induced by HCl-aspirin, and this effect of T-593 was completely inhibited by pretreatment with L-NAME. These results suggest that the improvement of GMBF by T-593 is mediated by endogenous NO.

Recently, Arai et al. (27) reported that T-593 inhibits gastric bleeding and lesions induced by indomethacin and water immersion stress with perfusion of 0.13 N HCl via inhibition of the decrease of GMBF in a process dependent on endogenous NO. The report also indicates that there is a relationship between the effect of T-593 and endogenous NO.

It has been reported that T-593 inhibited the decrease in mucus glycoprotein contents in the gastric mucosa injured by HCl-aspirin in rats (3) and that NO regulates not only mucosal blood flow (7, 8), but also mucus secretion (9). These findings suggest that the effect of T-593 on mucus secretion is also mediated by endogenous NO.

Recently, it has been reported that NO participates in various biological actions, not only in vasodilation (28), but also in the aggregation of platelets (29), leukocyte adhesion (30), angiogenesis (31) and immune surveillance system (32). Whittle et al. (33) reported that endogenous NO acts in concert with various biological mediators (e.g., prostaglandin, capsaicin-sensitive sensory neurons) for the modulation of gastric mucosal integrity. Furthermore, Konturek et al. (34) reported that endogenous NO plays an important role in the maintenance of blood flow around the ulcer, in the angiogenesis in the granulation tissue and, thus, in the healing of chronic gastric ulcers. Thereby, physiological role of NO has gradually become more clear, but much remains unknown. We must perform further studies to clarify the mechanism for the action of T-593 on the NO-system.

In conclusion, our results suggest that the HCl-aspirin-induced gastric lesion is caused by a decrease in NOS activity, which results in the lack of any NO-mediated protective effect. The present studies indicate that the mucosal protective effect of T-593 is partly attributed to the improvement of GMBF mediated by endogenous NO and that the maintenance of constitutive NOS activity, i.e., the maintenance of NO production, contributes to the effect of T-593.

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