ELSEVIER

European Journal of Pharmacology 294 (1995) 47–54

ejp

Effect of a novel histamine H_2 receptor antagonist, IT-066, on acute gastric injury induced by ischemia-reperfusion in rats, and its antioxidative properties

Yuji Naito, Toshikazu Yoshikawa *, Kiichi Matsuyama, Nobuaki Yagi, Masahiro Arai, Yasunari Nakamura, Toshiro Kaneko, Norimasa Yoshida, Motoharu Kondo

First Department of Medicine, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602, Japan

Received 6 April 1995; revised 8 August 1995; accepted 11 August 1995

Abstract

The effect of a novel histamine H_2 receptor antagonist IT-066 (3-amino-4-[4-[4-(1-piperidinomethyl)-2-pyridyloxy]-*cis*-2-butenylamino]-3-cyclobutene-1,2-dione hydrochloride), on acute gastric mucosal injury induced by ischemia-reperfusion was investigated from the standpoint of oxygen radical-mediated lipid peroxidation in rats. Ischemia-reperfusion injury was produced in the rat stomach by applying a small vascular clamp to the celiac artery for 30 min and subsequent removal of the clamp for 60 min. The decrease in gastric mucosal blood flow was not influenced by treatment with IT-066. The antiulcer activity of IT-066 was demonstrated in this injury after intragastric ingestion as well as after intravenous injection. IT-066 significantly inhibited this injury in the presence of exogenous HCl. The mucosal protection by IT-066 was not reversed by pretreatment with indomethacin or nitric oxide synthase inhibitor. The increase in lipid peroxides in the gastric mucosa after ischemia-reperfusion was significantly inhibited by the intragastric treatment with IT-066 at doses of 1.0 and 3.0 mg/kg. The total area of erosions closely paralleled the accumulation of lipid peroxide with a significant correlation. A spin trapping method using 5,5-dimethyl-1-pyrroline-*N*-oxide showed that IT-066 scavenged superoxide radical and hydroxyl radical generated by the hypoxanthine-xanthine oxidase system and the hydrogen peroxide-ferrous iron system, respectively. IT-066 also significantly inhibited the in vitro increase of lipid peroxide in the gastric mucosal homogenates induced by a free radical initiator. These results suggest that the protective effect of IT-066 against ischemia/reperfusion-induced gastric mucosal injury may result in part from its antioxidative properties.

Keywords: IT-066; Ischemia-reperfusion injury; Gastric mucosal acute injury; Histamine H_2 receptor antagonist; Superoxide radical; Hydroxyl radical

1. Introduction

IT-066 (3-amino-4-[4-[4-(1-piperidinomethyl)-2-pyridyloxy]-*cis*-2-butenylamino]-3-cyclobutene-1,2-dione hydrochloride) is a novel histamine H_2 receptor antagonist that has potent and long lasting antisecretory and antiulcer effects (Isobe et al., 1990a; Muramatsu et al., 1990, 1991). The histamine H_2 receptor antagonistic activity of IT-066 following 30 min preincubation with parietal cells was shown to be 32 and 1560 times that of famotidine and of cimetidine, respectively (Isobe et al., 1990b). It has previously been shown that oxygen-derived free radicals, including superoxide radicals, play an important role in the pathogenesis of gastrointestinal injuries induced by ischemia-reperfusion (Granger et al., 1981; Itoh and Guth, 1985). We have described a novel model of gastric damage induced by ischemia-reperfusion in rats, in which gastric ischemia is induced by applying a vascular clamp to the celiac artery, followed by removal of the clamp (Yoshikawa et al., 1989). Using this model, it has been shown that oxygen radical-mediated lipid peroxidation is a crucial contributory factor in the development of gastric mucosal injury (Naito, 1993). Lipid peroxidation is believed to be an important cause of destruction and damage to cell membranes, because lipid peroxidation degraded

* Corresponding author. First Department of Medicine, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602, Japan. Tel.: 81-75-251-5505, fax: 81-75-252-3721.

SSDI 0014-2999(95)00512-9

the polyunsaturated fatty acids of cell membranes, with consequent disruption of membrane integrity (Fridovich, 1978; Niki, 1987). Recently, it has been reported that some antiulcer agents, including the histamine H_2 receptor antagonist, have antioxidative effects or free radical scavenging actions (Ching et al., 1993; Yoshikawa et al., 1991a,b, 1993a,b). These activities are partially responsible for their antiulcer effect. A previous report demonstrated that IT-066 was ineffective against ethanol-induced gastric damage (Konturek et al., 1992). Although topical ethanol is known to increase lipid peroxidation (Mizui and Doteuchi, 1986) and free radical scavengers have reduced this gastric injury (Terano et al., 1989; Kvietys et al., 1990), some additional factors maintaining gastric mucosal integrity have been involved (Trier et al., 1987; Oates and Hakkinen, 1988). The study using an ethanol-induced injury model did not allow the estimation of the antioxidative action of IT-066. The aim of the present study was to determine whether IT-066 can improve the acute gastric mucosal injury induced by ischemia-reperfusion in rats, and whether the agent can scavenge oxygen free radicals in vitro.

2. Materials and methods

2.1. Reagents

All chemicals were prepared immediately before use. IT-066 was a gift from Taisho Pharmaceutical Co. (Saitama, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), indomethacin, N^G-nitro-L-arginine (L-NNA) and xanthine oxidase were purchased from Sigma Chemical (St. Louis, MO, USA). Thiobarbituric acid, ferrous sulfate, hydrogen peroxides, 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), diethylenetriaminepentaacetic acid (DETAPAC) and hypoxanthine were obtained from Wako Pure Chemical (Osaka, Japan). 1,1,3,3-Tetramethoxy propane was obtained from Tokyo Kasei (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was procured from Labotec Co. (Tokyo, Japan). The recombinant human superoxide dismutase was a gift from Nippon Kayaku (Tokyo, Japan). All other chemicals used were of reagent grade.

2.2. Acute gastric mucosal injury induced by ischemia-reperfusion

Ischemia-reperfusion injury model

Male Sprague-Dawley rats weighing 180–200 g were obtained from Kearl Co. (Osaka), housed at 22°C in a controlled environment with 12 h of artificial light per day, and fed rat chow ad libitum. They were not fed for 18 h before the experiments, but were allowed free access to water. As in a previous report (Yoshikawa et al., 1989), ischemia was induced in animals given intraperitoneal urethane anesthesia (1000 mg/kg) by applying a small clamp to the celiac artery for 30 min, followed by the removal of the clamps for 60 min. The agent, IT-066, suspended in 5% arabic gum solution was given to the rats by gastric intubation 1 h before ischemia at doses of 0.3, 1.0, and 3.0 mg/kg. IT-066 dissolved in saline was administered to rats by intravenous injection 1 h before ischemia at doses of 0.01, 0.03, and 0.1 mg/kg. Control rats were given only vehicle solution. To study the role of endogenous nitric oxide, endogenous prostaglandins, and luminal acid in the effect of IT-066 against this injury, we assessed the effects of treatment with L-NNA, indomethacin, and exogenous HCl. The rats were allowed to drink L-NNA solution for 3 days (3 mg/kg/day) freely before ischemia. Indomethacin dissolved in saline was injected 1 h before ischemia at a dose of 5 mg/kg. 1 ml of 0.1 N HCl solution was administered by gastric intubation just before ischemia. The various treatments were conducted in a randomized order. We carried out the maintenance of animals and experimental procedures according to the guideline of the Japan Council on Animal Care.

Determination of gastric mucosal blood flow

Gastric mucosal blood flow was measured by laser Doppler flowmetry (ALF2100, Advance Co., Tokyo) under urethane anesthesia (1000 mg/kg) throughout the measurements. The probe was attached to the serosal side of the corpus mucosum and the effect of IT-066 at a dose of 3.0 mg/kg on the gastric mucosal blood flow was evaluated before, and 30 min after, ischemia, as well as 30 and 60 min after reperfusion.

Evaluation of gastric mucosal lesions

To estimate the severity of the gastric erosions induced by ischemia-reperfusion, the total area of the gastric lesions that appeared reddish was measured using a dissecting microscope with ×10 magnification by a person unfamiliar with the treatment. The extent of any gastric mucosal lesion was expressed in terms of the total area of the erosion.

Biochemical assay

The concentration of thiobarbituric acid-reactive substances was measured in the gastric mucosa using the method of Ohkawa et al. (1986), and in serum by the method of Yagi (1976) as an index of lipid peroxidation. In brief, the animals were killed by exsanguination from the abdominal aorta after ischemia-reperfusion, the stomachs were removed, the corpus mucosa was scraped off using two glass slides, and then homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl in a

Teflon Potter-Elvehjem homogenizer. The level of thiobarbituric acid-reactive substances in the mucosal homogenates was expressed as nmol of malondialdehyde per mg protein using 1,1,3,3-tetramethoxypropane as the standard. The total amount of protein in the tissue homogenates was measured by the method of Lowry et al. (1951).

2.3. Evaluation of antioxidative properties

Superoxide scavenging activity assay

Superoxide scavenging activity was measured by the electron paramagnetic resonance (EPR) spin trapping method. As in a previous report (Miyagawa et al., 1988), the superoxide generated by the hypoxanthine-xanthine oxidase enzyme system was trapped by DMPO, and the scavenging activity of the sample was measured by comparing the inhibition rate of the resulting DMPO-OOH signal intensity caused by the sample to the inhibition rate with a standard superoxide dismutase. For the actual measurements, 0.5 mM hypoxanthine, 0.1 mM DETAPAC, 0.1 M DMPO, and the sample or superoxide dismutase was added to a 100 mM phosphate buffer solution (pH 7.8). The reaction was started by the addition of xanthine oxidase. An aliquot of reaction mixture was then transferred into a quartz cell, and the EPR signal was measured 1 min after starting. The EPR instrument was a JEOL-JES-FR80 spectrometer (JEOL, Tokyo, Japan), and the measurements were carried out under the following conditions: magnetic field 335.8 ± 5 mT, microwave power 8.0 mW, frequency 9.420 GHz, modulation frequency 100 kHz, modulation amplitude 0.1 mT, sweep time 5 mT/min, response time 0.1 s, and receiver gain ×500. The second order rate constant ($k_{IT\text{-}066}$) for the reaction between IT-066 and the superoxide radical at pH 7.8 was determined by the kinetic competition of DMPO and IT-066 for superoxide radicals (Eq. 1):

$$\frac{k_{IT\text{-}066}}{k_{DMPO}} = \frac{F}{1-F} \times \frac{[DMPO]}{[IT\text{-}066]} \quad (1)$$

$k_{IT\text{-}066}$ = rate constant for reaction of superoxide radical with IT-066; k_{DMPO} = rate constant for reaction of superoxide radical with DMPO; [DMPO] = concentration of DMPO; [IT-066] = concentration of IT-066; F = inhibition rate of DMPO-OOH signal.

Hydroxyl radical scavenging assay

The Fenton system, containing DETAPAC, $FeSO_4$ and hydrogen peroxide was used as a hydroxyl radical generating system. As previously reported (Tanigawa, 1990), 50 μM $FeSO_4$, 0.125 mM DETAPAC, the test sample, and 1.0 mM DMPO were added to a 50 mM phosphate buffer solution (pH 7.8). The reaction was started by adding 1 mM hydrogen peroxide. An aliquot of the reaction mixture was then transferred into a quartz cell, and the EPR signal was measured 1 min after the start. Measurements were carried out under the following conditions: magnetic field 335.8 ± 5.0 mT, microwave power 6.0 mW, frequency 9.420 GHz, modulation frequency 100 kHz, modulation amplitude 0.1 mT, sweep time 10 mT/min, response time 0.1 s, receiver gain ×200, and temperature 18°C. The second order rate constant for the reaction between IT-066 and the hydroxyl radical at pH 7.8 was estimated with Eq. 2:

$$\frac{k'_{IT\text{-}066}}{k'_{DMPO}} = \frac{F'}{1-F'} \times \frac{[DMPO]}{[IT\text{-}066]} \quad (2)$$

$k'_{IT\text{-}066}$ = rate constant for reaction of hydroxyl radical with IT-066; k'_{DMPO} = rate constant for reaction of hydroxyl radical with DMPO; F' = inhibition rate of DMPO-OH signal.

DPPH radical scavenging assay

30 μmol of DPPH was dissolved in ethyl alcohol. The DPPH solution and IT-066 solution were mixed for 60 s and transferred to the flat cell for measurement of the concentration of DPPH by EPR spectroscopy. The EPR conditions were as follows: magnetic field 335.8 ± 5 mT, microwave power 8.0 mW, modulation frequency 100 kHz, modulation amplitude 0.1 mT, sweep time 5 mT/min, response time 0.3 s, and receiver gain ×200.

Lipid peroxidation assay

Lipid peroxidation in rat gastric mucosal homogenates induced by AAPH, a water-soluble free radical initiator, was monitored by measuring thiobarbituric acid-reactive substances, according to a previously described method (Hori et al., 1992). In brief, gastric mucosal tissues were obtained from decapitated male Sprague-Dawley rats and homogenized in ice-cold phosphate-buffered saline (10 mM, pH 7.4). The diluted homogenates were incubated with AAPH (50 mM) at 37°C for 60 min with or without IT-066. Thiobarbituric acid-reactive substances were measured by a previously described method (Ohkawa et al., 1986).

2.4. Statistics

The results are presented as means ± S.E.M. The data were compared by one-way analysis of variance (ANOVA) and differences were considered significant if the P value was less than 0.05 according to Scheffe's multiple comparison test or the Mann-Whitney test. The correlation coefficient was determined using simple regression analysis. All analyses were performed using the Stat View 4.0 program (Abacus Concepts) on a Macintosh computer.

3. Results

3.1. Effects of IT-066 on acute gastric mucosal injury induced by ischemia-reperfusion

Clamping of the celiac artery decreased gastric mucosal blood flow to 10% of that measured before clamping. Just after the removal of the clamp, the blood flow in the gastric mucosa recovered completely from its ischemic status. The decrease of blood flow during ischemia was not improved by the treatment with IT-066 at a dose of 3.0 mg/kg (data not shown).

Multiple erosions and bleeding were revealed on the glandular stomach after the reperfusion following 30 min of the gastric mucosal ischemia. The mean of the total area of gastric erosions at 60 min after reperfusion was 59.3 ± 8.3 mm^2 in rats receiving the 5% arabic gum intragastrically and 59.5 ± 6.8 mm^2 in rats receiving saline intravenously. The antiulcer activity of IT-066 was demonstrated in this injury by intragastric ingestion as well as by intravenous injection. A dose-dependent inhibition of lesion formation was found, ID_{50} being 0.53 mg/kg for intragastric administration and 0.016 mg/kg for intravenous injection (Tables 1 and 2). In addition, IT-066 at a dose of 3 mg/kg significantly ($P < 0.05$) reduced this injury in rats pretreated with L-NNA or indomethacin before ischemia (Table 3). The total area of erosions increased significantly in rats treated with exogenous HCl compared with those of rats in the absence of exogenous HCl. IT-066 at a dose of 3 mg/kg significantly reduced the area to 51.1% of the mean of the control group treated with exogenous HCl (Table 3).

Thiobarbituric acid-reactive substances in the gastric mucosa of animals receiving the 5% arabic gum increased significantly ($P < 0.05$) compared with those of rats undergoing sham operations 60 min after reperfusion (Table 4). The increase in thiobarbituric acid-reac-

Table 1
Effect of the intragastric administration of IT-066 1 h before ischemia on total area of gastric erosions induced by ischemia-reperfusion in rats

Treatment		n	Erosion area (mm^2)	Inhibition (%)	ID_{50} (mg/kg)
Sham operation		6	0.0 ± 0.0	–	
Control		6	59.3 ± 8.3	–	
IT-066	0.3 mg/kg	6	43.7 ± 9.2	26.3	
	1.0 mg/kg	6	12.3 ± 4.2 [a]	79.3	0.53
	3.0 mg/kg	6	4.7 ± 1.7 [a]	92.1	

Each value indicates the mean ± S.E.M. for 6 rats. IT-066 dissolved in 5% arabic gum solution was administered to rats by gastric intubation 1 h before ischemia. [a] $P < 0.01$ when compared with the value for control rats receiving 5% arabic gum solution.

Table 2
Effect of the intravenous administration of IT-066 1 h before ischemia on total area of gastric erosions induced by ischemia-reperfusion in rats

Treatment		n	Erosion area (mm^2)	Inhibition (%)	ID_{50} (mg/kg)
Sham operation		6	0.5 ± 0.5	–	
Control		6	59.5 ± 6.8	–	
IT-066	0.01 mg/kg	6	34.8 ± 5.9 [a]	41.5	
	0.03 mg/kg	6	23.3 ± 4.3 [b]	60.8	0.016
	0.1 mg/kg	6	10.3 ± 3.5 [b]	82.7	

Each value indicates the mean ± S.E.M. for 6 rats. IT-066 dissolved in saline was administered to rats by intravenous injection 1 h before ischemia. [a] $P < 0.05$ and [b] $P < 0.01$ when compared with the value for control rats receiving saline.

Table 3
Effects of L-NNA, indomethacin, or exogenous HCl on the mucosal protective action of IT-066 against ischemia-reperfusion injury in rats

Group	Total area of erosions (mm^2)
Sham operation	0.0 ± 0.0
Ischemia/reperfusion (I/R)	47.2 ± 12.6
+ IT-066 [a]	11.2 ± 6.1 [e]
+ L-NNA [b]	60.2 ± 22.5
+ L-NNA + IT-066	7.6 ± 4.9 [f]
+ Indomethacin [c]	56.6 ± 13.1
+ Indomethacin + IT-066	22.0 ± 5.9 [g]
+ HCl [d]	125.2 ± 15.5
+ HCl + IT-066	64.0 ± 14.3 [h]

[a] IT-066 was given to the rats by gastric intubation 1 h before ischemia at a dose of 3.0 mg/kg. [b] Rats drank L-NNA solution freely for 3 days (3 mg/kg/day) before ischemia. [c] Indomethacin dissolved in saline was injected 1 h before ischemia at a dose of 5 mg/kg. [d] 1 ml of 0.1 N HCl solution was administered by gastric intubation just before ischemia. Each value indicates the mean ± S.E.M. for 5 rats. [e] $P < 0.05$, [f] $P < 0.05$, [g] $P < 0.05$ and [h] $P < 0.05$ when compared with the value for the I/R group, L-NNA group, indomethacin group and HCl group, respectively.

Table 4
Effect of IT-066 on the lipid peroxide levels in the gastric mucosa and serum after ischemia-reperfusion in rats

Treatment		n	Thiobarbituric acid-reactive substances	
			in gastric mucosa (nmol/mg protein)	in serum (nmol/ml)
Sham operation		6	0.333 ± 0.035	2.4 ± 0.7
5% arabic gum		6	0.543 ± 0.026 [a]	2.9 ± 0.2
IT-066	0.3 mg/kg	6	0.452 ± 0.056	2.3 ± 0.2
	1.0 mg/kg	6	0.318 ± 0.027 [b]	2.6 ± 0.7
	3.0 mg/kg	6	0.348 ± 0.031 [b]	2.2 ± 0.4

Each value indicates the mean ± S.E.M. for 6 rats. IT-066 dissolved in 5% arabic gum solution was administered to rats by gastric intubation 1 h before ischemia. [a] $P < 0.05$ when compared with the value for the sham-operated group. [b] $P < 0.01$ when compared with the value for control rats receiving 5% arabic gum solution.

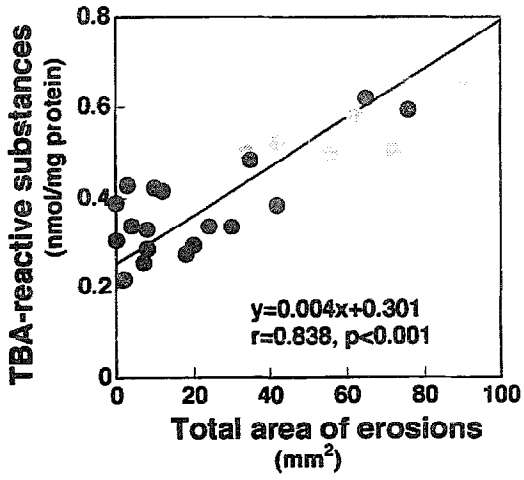

Fig. 1. Correlation between the total area of erosions and lipid peroxide contents in the stomach after ischemia-reperfusion. Thiobarbituric acid-reactive substances were measured in the gastric mucosa as an index of lipid peroxidation. Black circles: control group treated with 5% arabic gum solution; shaded circles: group treated with IT-066.

tive substances of the gastric mucosa 60 min after reperfusion was significantly inhibited by the intragastric administration of IT-066 at doses of 1.0 and 3.0 mg/kg ($P < 0.01$) as well as by the intravenous administration at a dose of 0.1 mg/kg (data not shown). Fig. 1 shows the correlation between the total area of erosions and thiobarbituric acid-reactive substances in the gastric mucosa of animals pretreated with or without IT-066 after ischemia-reperfusion. The total area of erosions closely paralleled the accumulation of thiobarbituric acid-reactive substances in the gastric mucosa with a significant correlation, the correlation coefficient being 0.838 ($P < 0.001$, Fig. 1). Serum thiobarbituric acid-reactive substances in the groups treated with or without IT-066 did not show any significant changes after ischemia-reperfusion when compared with those of rats undergoing sham operations.

3.2. Antioxidative properties of IT-066

Superoxide scavenging activity of IT-066

When xanthine oxidase was added to the complete system containing hypoxanthine and DMPO in phosphate buffer, the EPR signal was detected by an EPR spectrometer at room temperature. The 12 characteristic lines of the signal were observed 1 min after the addition of xanthine oxidase. The g value and hyperfine coupling constant were g = 2.006 G, aN = 1.41 mT, $aH\beta = 1.13$ mT, and $aH\gamma = 0.12$ mT, which could be assigned to a DMPO-OOH adduct (spin adduct trapping superoxide). IT-066 inhibited the DMPO-OOH signals in a dose-dependent manner ($IC_{50} = 0.081$ mM, Figs. 2 and 3). From the inhibition rate, $k_{IT\text{-}066}$

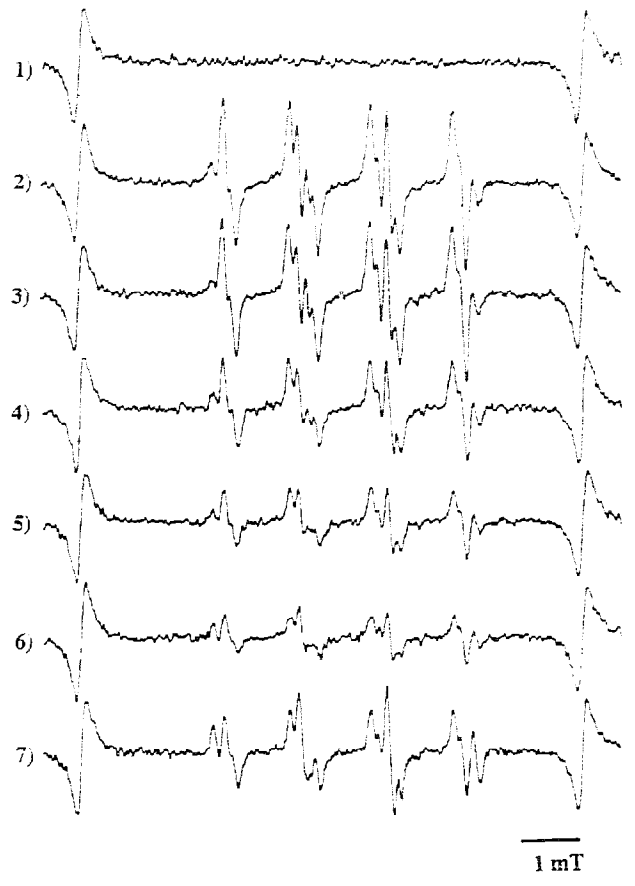

Fig. 2. Effect of IT-066 on the EPR signal DMPO-OOH generated from the hypoxanthine-xanthine oxidase system in the presence of 0.1 M DMPO. (1) DMPO only, (2) control, (3) IT-066, 10 μM, (4) IT-066, 50 μM, (5) IT-066, 100 μM, (6) IT-066, 500 μM, (7) superoxide dismutase 0.5 U/ml.

could be calculated to be 1.24×10^4 M^{-1} s^{-1} using $k_{DMPO} = 10$ M^{-1} s^{-1}, as previously reported (Finkelstein et al., 1980).

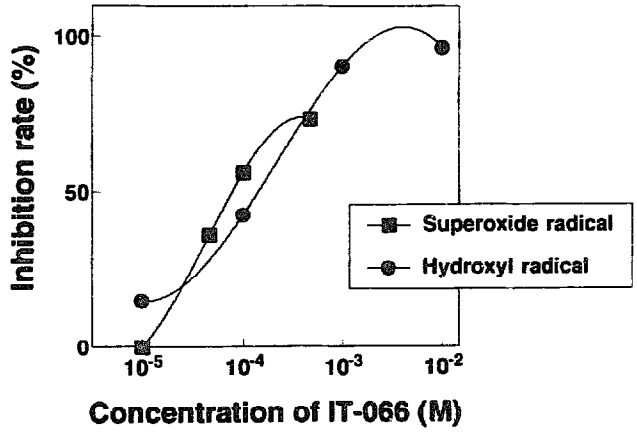

Fig. 3. Superoxide and hydroxyl radical scavenging activities of IT-066. Each point represents the mean of the inhibitory percentage of the EPR signal DMPO-OOH or DMPO-OH, from triplicate determinations.

Hydroxyl radical scavenging activity of IT-066

From the Fenton reaction mixture with DMPO, a four-line, 1:2:2:1, spectrum was detected with the EPR spectrometer at room temperature. The hyperfine coupling constants were aN = 1.49 mT and aH = 1.49 mT, which could be assigned to a DMPO-OH adduct (spin adduct trapping hydroxyl radical). IT-066 inhibited the DMPO-OH signal intensity in a dose-dependent manner (IC_{50} = 0.113 mM, Figs. 3 and 4). From the inhibition rate of DMPO-OH signal and the concentration of IT-066, $k'_{IT\text{-}066}$ could be calculated to be 1.86×10^{10} M^{-1} s^{-1} using $k'_{DMPO} = 2.1 \times 10^{9}$ M^{-1} s^{-1}, as previously reported (Finkelstein et al., 1980).

DPPH radical scavenging activity of IT-066

DPPH radical intensity was neither reduced nor increased by the addition of IT-066 at concentrations of 0.001, 0.01, 0.1, 1.0, or 10 mM.

Inhibition of lipid peroxidation by IT-066

AAPH increased the amount of thiobarbituric acid-reactive substances in the gastric mucosal homogenates after incubation at 37°C for 60 min. IT-066 caused a significant inhibition of the AAPH-induced increase in thiobarbituric acid-reactive substances in a concentration-dependent manner (Fig. 5).

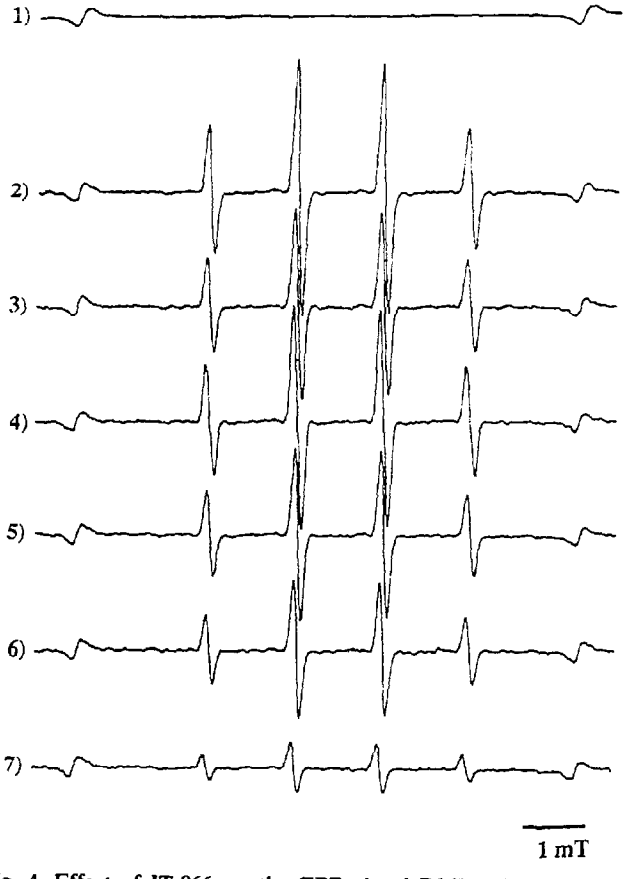

Fig. 4. Effect of IT-066 on the EPR signal DMPO-OH generated from the hydrogen peroxide-ferrous sulfate system in the presence of 1 mM DMPO. (1) DMPO only, (2) control, (3) IT-066, 0.1 μM, (4) IT-066, 1.0 μM, (5) IT-066, 10 μM, (6) IT-066, 100 μM, (7) IT-066, 500 μM.

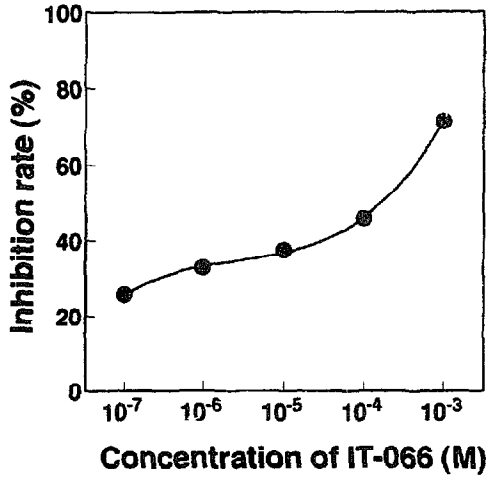

Fig. 5. Effect of IT-066 on free radical-induced lipid peroxidation of gastric mucosal homogenates. Each point indicates the mean of 4 experiments.

4. Discussion

IT-066 was shown to prevent the development of acute gastric mucosal lesions induced by ischemia-reperfusion in a dose-dependent manner without reversing the reduced gastric mucosal blood flow. The antiulcer activity of IT-066 was demonstrated after intragastric ingestion as well as after intravenous injection, suggesting that the pharmacological action of IT-066 to maintain gastric integrity should be considered to the exclusion of a topical, mild irritant action of the agent. Pretreatment with indomethacin or nitric oxide (NO) synthase inhibitor (L-NNA) did not reverse the protective effect of IT-066 against reperfusion-induced gastric mucosal injury. In addition, IT-066, at a dose of 3 mg/kg, significantly reduced reperfusion-induced gastric mucosal injury to 51.1% of the mean of the control group in the presence of exogenous HCl. These results indicate that IT-066 elicits its protective action on the gastric mucosa by some mechanism other than inhibition of acid secretion or histamine H_2 receptor antagonism, and that this action is not associated with endogenous prostaglandins or nitric oxide, mediators which have been reported to have a cytoprotective action against gastric mucosal injury in several experimental models (Lacy and Itoh, 1982; MacNaughton et al., 1989; Robert et al., 1979; Whittle et al., 1990).

Increases in thiobarbituric acid-reactive substances, an index of lipid peroxidation, in the gastric mucosa after ischemia-reperfusion were significantly inhibited by the treatment with IT-066. The possibility that oxidative stress due to excess formation of oxygen radi-

cals is closely involved in ischemia-reperfusion injury, has been the focus of much attention. However, it has been extremely difficult to establish a direct cause-and-effect relationship between oxygen radical formation and the actual lesions induced by ischemia-reperfusion. Lipid peroxidation mediated by oxygen radicals is believed to be an important cause of destruction and damage to cell membranes, because a single initiating event in which hydroxyl radical or metal ion-free radical complexes abstract methylene hydrogen atoms from polyunsaturated fatty acids, can result in conversion of hundreds of fatty acid side-chains into lipid peroxides, which alter the structural integrity and biochemical functions of membranes (Fridovich, 1978; Niki, 1987). A recent study using this model has demonstrated that lipid peroxide accumulation and α-tocopherol consumption closely paralleled the development of the gastric mucosal injury (Naito, 1993). In the present study, the increase in the total area of erosions closely paralleled the accumulation of lipid peroxides in the gastric mucosa with a significant correlation. Previous studies have demonstrated that the gastric damage and the accumulation of lipid peroxides induced by ischemia-reperfusion are significantly inhibited by superoxide dismutase (Naito, 1993), as well as by rebamipide (Naito et al., 1994), a hydroxyl radical scavenger, and by allopurinol (Yoshikawa et al., 1989), a xanthine oxidase inhibitor. These results indicate that oxygen radical-mediated tissue lipid peroxidation plays a crucial role in the formation of gastric mucosal injury produced by ischemia-reperfusion, and support the possibility that the inhibition of lipid peroxidation in vivo by IT-066 is partially responsible for its anti-ulcer effect.

The exact mechanisms of the pharmacological effect of IT-066 in vivo remain unclear. However, the present EPR spin trapping method has demonstrated that this agent scavenges superoxide and hydroxyl radicals. The hydroxyl radical is known to be a most potent reactive oxygen species which reacts rapidly with biological materials, causing oxidative stress. Ching et al. (1993) have demonstrated that histamine H_2 receptor antagonists including cimetidine, ranitidine, and famotidine, are very powerful hydroxyl radical scavengers, and that their rate constants for the reaction with hydroxyl radicals range from 7.7×10^9 M^{-1} s^{-1} to 14.8×10^9 M^{-1} s^{-1}. In the present kinetic competition study, the second order rate constant for the reaction between IT-066 and hydroxyl radical at pH 7.8 was calculated to be 18.6×10^9 M^{-1} s^{-1}, which is greater than that of dimethyl sulfoxide (DMSO) (7×10^9 M^{-1} s^{-1} (Tanigawa, 1990)). DMSO, a hydroxyl radical scavenger, has been shown to inhibit the gastric mucosal injury induced by ischemia (Perry et al., 1986), and by stress and absolute ethanol (Terano et al., 1989). In addition, IT-066 inhibited AAPH-induced lipid peroxidation of the gastric mucosal homogenates in vitro. From this point of view, the beneficial effects of IT-066 on reperfusion-induced gastric mucosal injury may be attributed to its antioxidative activities.

In conclusion, the present study has demonstrated the gastroprotective action of IT-066 against ischemia-reperfusion injury. The marked effectiveness of IT-066 in this model might be attributed to its potent dual actions, the antioxidative action demonstrated here and the antisecretory action. The histamine H_2 receptor antagonist showed a higher recurrence rate after the cessation of drug administration, whereas recurrence was less with the free radical scavenging drug (Salim, 1990; Naito et al., 1993). Although the recurrence rate after treatment with IT-066 has not been examined, the dual action of IT-066 is expected to achieve not only high efficacy in treatment but also a lower recurrence rate.

References

Ching, T.L., G.R. Haenen and A. Bast, 1993, Cimetidine and other H_2 receptor antagonists as powerful hydroxyl radical scavengers, Chem.-Biol. Interact. 86, 119.

Finkelstein, E., G.M. Roser and E.J. Rauckman, 1980, Spin trapping. Kinetics of the reaction of superoxide and hydroxyl radicals with nitrones, J. Am. Chem. Soc. 102, 4994.

Fridovich, I., 1978, The biology of oxygen radicals, Science 201, 875.

Granger, D.N., G. Rutili and J.M. McCord, 1981, Superoxide radicals in feline intestinal ischemia, Gastroenterology 81, 22.

Hori, Y., T. Yoshikawa, T. Yoneta, Y. Naito, H. Morita, H. Aita, M. Seiki and E. Tagashira, 1992, J. Clin. Exp. Med. 160, 529.

Isobe, Y., M. Muramatsu, H. Aihara and S. Otomo, 1990a, H_2-receptor antagonism by a novel compound IT-066 assessed from [^{14}C]aminopyrine accumulation in rabbit parietal cells, Eur. J. Pharmacol. 178, 343.

Isobe, Y., H. Nagai, M. Muramatsu, H. Aihara and S. Otomo, 1990b, Antisecretory and antilesional effect of a new histamine H_2 receptor antagonist, IT-056, in rats, J. Pharmacol. Exp. Ther. 255, 1078.

Itoh, M. and P.H. Guth, 1985, Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesion in rats, Gastroenterology 88, 1162.

Konturek, S.J., M. Cieszkowski, J. Bilski, T. Brzozowski and A. Szlachcic, 1992, Effects of a novel H_2-receptor antagonist containing piperidinomethyl group (IT-066) on gastric secretion in dogs and on acute gastric ulcers in rats, Eur. J. Gastroenterol. Hepatol. 4, 573.

Kvietys, P.R., B. Twohig and J. Danzell, 1990, Ethanol-induced injury to the rat gastric mucosa. Role of neutrophils and xanthine oxidase-derived radicals, Gastroenterology 98, 909.

Lacy, E.R. and S. Itoh, 1982, Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin, Gastroenterology 83, 619.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

MacNaughton, W.K., G. Cirino and J.L. Wallace, 1989, Endothelium-derived relaxing factor (nitric oxide) has protective actions in the stomach, Life Sci. 45, 1869.

Miyagawa, H., T. Yoshikawa, T. Tanigawa, N. Yoshida, S. Sugino and M. Kondo, 1988, Measurement of serum superoxide dismutase activity by electron spin resonance, J. Clin. Biochem. Nutr. 5, 1.

Mizui, T. and M. Doteuchi, 1986, Lipid peroxidation: a possible role in gastric damage induced by ethanol in rats, Life Sci. 38, 2163.

Muramatsu, M., Y. Isobe, I. Arai, K.H. Hirose, I.C. Usuki, H. Nagai, H. Aihara and S. Otomo, 1990, Effects of the new H_2-receptor antagonist 3-amino-4-[4-[4-(1-piperidinomethyl)-2-pyridyloxy]-*cis*-2-butenylamino]-3-cyclobutene-1,2-dione hydrochloride on gastric acid secretion and ulceration, Arzneim.-Forsch. 40, 49.

Muramatsu, M., K.H. Hirose, H. Aihara and S. Otomo, 1991, Time-dependent interaction of a new H_2-receptor antagonist, IT-066, with the receptor in the atria of guinea pig, Jpn. J. Pharmacol. 57, 13.

Naito, Y., 1993, Role of superoxide anion radical in acute gastric mucosal and vascular injury induced by ischemia-reperfusion in rats, J. Kyoto Pref. Univ. Med. 102, 87.

Naito, Y., T. Yoshikawa, K. Matsuyama, S. Nishimura, N. Yagi and M. Kondo, 1993, Effects of free radical scavengers on ulcer healing process acetic acid-induced ulcer in rats, Dig. Dis. Sci. 38, A-7.

Naito, Y., T. Yoshikawa, K. Matsuyama, N. Yagi, Y. Nakamura, S. Nishimura and M. Kondo, 1994, Effect of rebamipide, a novel anti-ulcer agent, on acute gastric mucosal injury induced by ischemia-reperfusion in rats, Pathophysiology 1, 161.

Niki, E., 1987, Antioxidants in relation to lipid peroxidation, Chem. Phys. Lipids 44, 227.

Oates, P.J. and J.P. Hakkinen, 1988, Studies on the mechanism of ethanol-induced gastric damage in rats, Gastroenterology 94, 10.

Ohkawa, H., N. Ohnishi and K. Yagi, 1986, Assay for lipid peroxides for animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95, 351.

Perry, M.A., S. Wadhwa, D.A. Parks, W. Pickard and D.N. Granger, 1986, Role of oxygen radicals in ischemia-induced lesions in the cat stomach, Gastroenterology 90, 362.

Robert, A., J.E. Nezamis, C. Lancaster and A.J. Hanchar, 1979, Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury, Gastroenterology 77, 433.

Salim, A.S., 1990, Oxygen-derived free radicals and the prevention of duodenal ulcer relapse: a new approach, Am. J. Med. Sci. 300, 1.

Tanigawa, T., 1990, Determination of hydroxyl radical scavenging activity by electron spin resonance, J. Kyoto Pref. Univ. Med. 99, 133.

Terano, A., H. Hiraishi, S. Ota, J. Shiga and T. Sugimoto, 1989, Role of superoxide and hydroxyl radicals in rat gastric mucosal injury induced by ethanol, Gastroenterol. Jpn. 24, 488.

Trier, J.S., S. Szabo and C.H. Allan, 1987, Ethanol-induced damage to mucosal cappilaries or rat stomach. Ultrastructural features and effects of prostaglandin F and cysteamine, Gastroenterology 74, 13.

Whittle, B.J., B.J. Lopez and S. Moncada, 1990, Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat, Br. J. Pharmacol. 99, 607.

Yagi, K., 1976, A simple fluorometric assay for lipid peroxides in blood plasma, Biochem. Med. 15, 212.

Yoshikawa, T., S. Ueda, Y. Naito, S. Takahashi, H. Oyamada, Y. Morita, T. Yoneta and M. Kondo, 1989, Role of oxygen-derived free radicals in gastric mucosal injury induced by ischemia or ischemia-reperfusion in rats, Free Radic. Res. Commun. 7, 285.

Yoshikawa, T., Y. Naito, T. Tanigawa, T. Yoneta and M. Kondo, 1991a, The antioxidant properties of a novel zinc-carnosine chelate compound, *N*-(3-aminopropionyl)-L-histidinato zinc, Biochim. Biophys. Acta 1115, 15.

Yoshikawa, T., Y. Naito, T. Tanigawa, T. Yoneta, M. Yasuda, S. Ueda, H. Oyamada and M. Kondo, 1991b, Effect of zinc-carnosine chelate compound (Z-103), a novel antioxidant, on acute gastric mucosal injury induced by ischemia-reperfusion in rats, Free Radic. Res. Commun. 14, 289.

Yoshikawa, T., Y. Naito, T. Tanigawa and M. Kondo, 1993a, Free radical scavenging activity of the novel anti-ulcer agent rebamipide studied by electron spin resonance, Arzneim.-Forsch./Drug Res. 43, 363.

Yoshikawa, T., S. Nakamura, S. Takahashi, Y. Naito and M. Kondo, 1993b, Effect of sofalcone on gastric mucosal injury induced by ischemia-reperfusion and its antioxidant properties, J. Clin. Gastroenterol. 17, S111.