A Possible Mechanism of Protection by Polyamines against Gastric Damage Induced by Acidified Ethanol in Rats: Polyamine Protection May Depend on Its Antiperoxidative Properties

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Abstract—The protective mechanism of polyamines against acidified ethanol-induced gastric damage was studied. Their oral administration prevented the formation of gastric mucosal lesions induced by 80% ethanol in 150 mM HCl in a dose-dependent manner, with the order of the protective potency being spermine > spermidine > putrescine. The acidified ethanol-induced lesions were accompanied by a concomitant increase in gastric mucosal lipid peroxide levels, but spermine in a protective dose could prevent the increment of lipid peroxides. Polyamines, in a concentration-dependent fashion, inhibited the reduction of nitroblue tetrazolium by superoxide anion radicals generated in vitro in the xanthine-xanthine oxidase system and the lipid peroxidation in vitro induced by ferrous ion in the porcine gastric mucosal homogenate. The order of the superoxide scavenging potency and the inhibitory potency of iron-induced lipid peroxidation by polyamines corresponded to the order to the protective potency against acidified ethanol-induced gastric lesions. The present results suggest that cytoprotection by polyamines may be responsible for their antiperoxidative activities.

Naturally occurring polyamines such as putrecine, spermidine and spermine, not only seem to have an important role in the cell proliferative process (1) but also seem to protect bacterial cells (2), subcellular organella (3) and nucleic acids (4) by a stabilizing effect against deleterious environments.

Recently, polyamines such as spermine and spermidine have been shown to inhibit gastric acid secretion by affecting gastric \((\text{H}^+ + \text{K}^+)\text{-ATPase} (5)\) and the development of experimentally induced gastric lesions (6). In a previous paper, we reported that polyamines, as well as prostaglandins (PGs), protect the gastric mucosa against acidified ethanol, a severe necrotizing agent, and the protective effect of spermine may be related to endogenous nonprotein sulfhydryls (7). However, the detailed mechanisms of this so called “cytoprotection” are unknown. Our recent report showed that ethanol injury to the stomach is accompanied by an increase of the lipid peroxide level in the mucosal layer (8). As polyamines have been reported to prevent the lipid peroxidation in liver microsomes (9–11), the present study was performed to investigate the relationship between the antiperoxidative and gastroprotective activities of polyamines.

Materials and Methods

Procedure for producing gastric lesions: Male Jcl Sprague-Dawley rats (200–300 g) were fasted for 24 hr before the experiments, but allowed free access to water. One milliliter of 90% ethanol in 150 mM HCl was given orally, and the animals were killed with an overdose of ether 1 hr later. The stomachs were quickly removed, emptied of the remaining contents and inflated with 6 ml of 1% buffered formalin for 10 min. The total
length of each lesion was measured under a binocular microscope (×10) for use as the lesion index.

**Measurement of intragastric volume:** Spermine at 0.5 mmoles/kg was administered orally, and the rats were killed by an overdose of ether 30 min later. Each abdomen was opened, both the gastroesophageal junction and pylorus were closed with arterial clamps, and then the stomach was removed. The amount of fluid present in the stomach was measured.

**Measurement of gastric mucosal lipid peroxides:** Fasted animals were killed by decapitation at various times after acidified ethanol application (90% ethanol in 0.05 M HCl, 1 ml/animal, p.o.). Lipid peroxides in the glandular part of the mucosa were measured fluorometrically using the thiobarbituric acid (TBA) method according to Ohkawa et al. (12), except that the reaction time of the homogenate with TBA was modified to 120 min when the reaction reached a plateau (8). Malondialdehyde bis(dimethylacetal) was used as a standard. Protein was determined by the procedure of Lowry et al. (13).

**Measurement of gastric mucosal non-protein sulfhydryls:** Nonprotein sulfhydryls in the same tissue samples used to measure the lipid peroxides were quantitated according to the method of Ellman (14). The procedure has been described in detail previously (7).

**Measurement of superoxide (O$_2^-$) scavenging activity:** The superoxide scavenging activity of polyamines was determined by the modification (15) of the assay method for superoxide dismutase using the xanthine oxidase system (16). The reaction mixture consisted of 2.4 ml of 0.5 mM sodium carbonate buffer (pH 9.2) and 0.1 ml each of 3 mM xanthine, 4 mM sodium ethylenediaminetetraacetate, 0.15% bovine serum albumin, 0.75 mM nitroblue tetrazolium (NBT) and various concentrations of polyamines. The reaction was initiated by addition of 0.1 ml of xanthine oxidase solution at room temperature, and the absorbance of formazan converted from NBT by O$_2^-$ was measured at 560 nm. Xanthine oxidase solution added to the reaction mixture was diluted by 0.05 M sodium phosphate buffer (pH 7.4) to obtain an absorbance of around 0.25 after 20 min reaction in the absence of polyamines. In a separate experiment, the rate of uric acid formation was estimated by the increase in absorbance at 293 nm in the reaction mixture from which NBT was absent.

**Ferrous ion-induced lipid peroxidation in porcine gastric mucosal homogenate:** The glandular portion of fresh porcine stomach was used. The mucosal layer was separated from the muscular layer with a sharp blade, placed in 4 volumes of ice-cold 0.05 mM phosphate-saline buffer (pH 7.4) and homogenized with a high speed homogenizer (Ultra-Turrax) for 30 sec. The homogenate was centrifuged at 1000 g for 15 min, and the supernatant fluid was preserved at −80°C until use. The stock homogenate was diluted three-fold with phosphate-saline buffer. After 0.4 ml of the diluted homogenate had been incubated at 37°C for 10 min, 20 μl of 0.4 mM FeSO$_4$ and 60 μl of various concentrations of polyamines were added to the diluted homogenate (0.4 ml), and incubation was performed at 37°C for 60 min. The reaction was stopped by adding 20 μl of 0.1% butylated hydroxytoluene in ethanol and 125 μl of 25% metaphosphoric acid. Proteins were removed by centrifugation for 2 min using a Beckman Microfuge B, and the lipid peroxides of the supernatant were quantitated by measuring the absorbances at 532 nm as TBA-reacting substances according to Ohkawa et al. (12), except that sodium dodecyl sulfate was omitted from the reaction mixture.

**Chemicals:** Spermine, spermidine and putrescine as hydrochloride salts were all purchased from Nakarai Chemicals, Ltd. Cimetidine was synthesized in our laboratories. Xanthine oxidase was obtained from Boehringer Mannheim GmbH, superoxide dismutase from Sigma Chemical Co., and malondialdehyde bis(dimethylacetal) from Merck-Schuchardt. The other chemicals used were of reagent grade.

Polyamines were orally administered at 1 ml/kg as a water solution. Cimetidine was intraperitoneally administered as a saline solution.

**Statistical analysis:** The data were analyzed by Student's $t$-test, and $P<0.05$ was regarded
as significant.

Results

Effects of polyamines on gastric lesions induced by acidified ethanol: As shown in Fig. 1, pretreatment with either spermine or spermidine given orally 30 min before administration of 90% ethanol in 150 mM HCl, dose-dependently prevented the formation of lesions which were formed predominantly in the glandular region. Spermine offered more potent protection than spermidine. Putrescine did not significantly affect the lesion formation even at the higher dose of 0.75 mmol/kg. Histological examination showed that spermine did not protect the gastric surface epithelium, but prevented severe necrosis in deeper regions, like the prostaglandins (PGs) (17). Oral administration of 0.5 mmol/kg of spermine immediately before the application of 90% ethanol in 150 mM HCl did not significantly prevent the lesion formation, but spermine given 5 min before acidified ethanol did (Fig. 2). The protection was maximal when spermine was given 30 min before acidified ethanol application, but was not observed when spermine was given 5 hr earlier.

Effects of intragastric volume on spermine protection against acidified ethanol-induced gastric lesions: As shown in Table 1A, spermine at 0.1 or 0.25 mmol/kg (in a dose which was shown to protect gastric mucosa in Fig. 1) did not significantly affect the volume of the contents, but spermine at a higher dose (0.5 mmol/kg) significantly increased it. Since the gastric fluids that accumulated in the stomach were not affected by cimetidine (20 mg/kg, i.p.) administered simultaneously with spermine, they might not have resulted from acid secretion (Table 1B). We further checked whether or not the protection by spermine was merely induced by dilution of the acidified ethanol as an irritant. The marked protection by spermine (0.5 mmol/kg) was still observed when the volume of 90% ethanol in 150 mM HCl was increased to 2 ml/animal to reduce the dilution effect. In contrast, severe gastric lesions were produced by application of 2 ml of 90% ethanol in 150 mM HCl even just after oral administration of 1 ml of water (Table 1C). This finding shows that the protective action of spermine is not due to the dilution of acidified ethanol by an increased volume of the intragastric contents.

Effects of acidified ethanol on lipid peroxide levels and nonprotein sulfhydryl levels: The
lipid peroxides in the gastric mucosa remarkably increased after the application of 90% ethanol in 150 mM HCl. The lipid peroxide level reached the maximum at 5 min after administration of acidified ethanol and was maintained for at least 1 hr (Fig. 3). On the other hand, nonprotein sulfhydryls significantly decreased upon administration of 90% ethanol in 150 mM HCl. As shown in Table 2, spermine (0.5 mmoles/kg) completely prevented the increase of the lipid peroxide level at 5 min after application of 90% ethanol in 150 mM HCl.

**Superoxide scavenging activity of polyamines:** Figure 4A shows that spermine inhibited the reduction of NBT mediated by O$_2^-$ (the increase of absorbance at 560 nm) in a concentration-dependent manner over the range of 3.3–33.3 mM. The inhibitory action of spermine was not attributed to direct inhibition of xanthine oxidase since the rate of uric acid formation (the increase of absorbance at 293 nm) was hardly modified (Fig. 4B). This finding indicates that spermine acts as an O$_2^-$ scavenger. Spermidine and putrescine as well as spermine could scavenge O$_2^-$, with the order of scavenging potency being spermine > spermidine > putrescine (Fig. 5).

**Effects of polyamines on Fe$^{2+}$-dependent lipid peroxidation in porcine gastric mucosal homogenate:** The lipid peroxides produced were 1.52±0.05 nmoles/mg protein (the mean±S.D. of 3 observations) by incubation with 0.4 mM Fe$^{2+}$ alone for 60 min. The polyamines added simultaneously with 0.4

Table 2. Effect of spermine on lipid peroxidation induced by acidified ethanol in the gastric mucosa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lipid peroxide level (nmoles/100 mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>74.8±2.5</td>
</tr>
<tr>
<td>90% EtOH-150 mM HCl</td>
<td>116.8±12.0*</td>
</tr>
<tr>
<td>Spermine, 0.5 mmoles/kg, p.o. + EtOH-HCl</td>
<td>73.4±2.8†</td>
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Each value represents the mean±S.E. of 6 animals. Statistical significance of difference from the control: *P<0.01. Statistical significance of difference from the group given acidified ethanol alone: †P<0.01.
Fig. 3. Changes of the lipid peroxide level and nonprotein sulfhydryl level in the gastric mucosa after acidified ethanol administration. Each point represents the mean ± S.E. of 5 animals. Statistical significance of difference from the nontreated group: *P<0.05, ***P<0.01.

Fig. 4. Effect of spermine on superoxide generation (absorbance at 560 nm) and uric acid production (absorbance at 293 nm) in xanthine-xanthine oxidase system. Each point represents the mean of 3 experiments. ●, none; ▲, 3.3 mM; ■, 16.7 mM; ○, 33.3 mM.

Fig. 5. Scavenging action of polyamines against superoxide generated by xanthine-xanthine oxidase system. Each point represents the mean of 3 experiments. ●, spermine; ▲, spermidine; ■, putrescine.

Fig. 6. Effect of polyamines on Fe²⁺-induced lipid peroxidation in the porcine gastric mucosal homogenate. Each point represents the mean of 4 experiments. ●, spermine; ▲, spermidine; ■, putrescine.
mM Fe²⁺ partially inhibited lipid peroxide formation in a concentration-dependent fashion. The order of the inhibitory potency of these polyamines was spermine > spermidine > putrescine (Fig. 6). Superoxide dismutase (625 units/ml) had no effect on 0.4 mM Fe²⁺-induced lipid peroxidation.

**Discussion**

The present results confirmed our previous finding that polyamines such as spermine and spermidine prevent the gastric lesions produced by acidified ethanol (7) and showed that protection by spermine is not due to dilution of the ethanol. The onset of the protective action of spermine was exhibited as early as 5 min. Therefore, spermine protection is not likely to be related to the synthesis of macromolecules such as DNA, RNA and protein. Furthermore, polyamines can inhibit phospholipase A₂, which catalyzes the rate-limiting step in the PG biosynthesis (18), and spermine protection is not reversed by pretreatment with indomethacin (7). These observations rule out the mediation of PGs in polyamine protection.

One of the recent topics in the pathophysiology of gastrointestinal injuries is the role of free radicals. Active oxygen species such as O₂⁻ and its interconversion product, hydroxyl radical (OH⁻), attack unsaturated membrane lipids and induce lipid peroxidation that causes various types of tissue damage (19). Recent studies indicate that oxygen-derived free radicals participate in gastrointestinal lesion formation induced by ischemia (20–22). Certain free radical scavengers, e.g., sulphydryl agents (23), butylated hydroxyltoluene (24), and (+)-cyanidanol (25), have been reported to offer protection against several experimental gastric lesions. These reports suggest that toxic free radicals which result in lipid peroxidation may play an important role in the pathogenesis of gastric injuries. We have observed that butylated hydroxyltoluene and allopurinol, a xanthine oxidase inhibitor, can prevent the lesion formation induced by ethanol. A detailed report will appear elsewhere. Our recent report has shown that the lipid peroxide level in the gastric mucosa increases soon after absolute ethanol challenge, and cytoprotective PGs prevent the gastric mucosal lipid peroxidation (8). In this study, we found that spermine in a protective dose, as well as PGs, inhibits the increase of lipid peroxides immediately after acidified ethanol application. The degree of lipid peroxidation and the lowering of nonprotein sulfhydryls in the gastric mucosa after acidified ethanol challenge were more pronounced than those after absolute ethanol application (8). This is likely to be related to the fact that acidification of ethanol aggravates the lesion formation (7).

Recently, Itoh and Guth (21) and Perry et al. (22) suggested the involvement of O₂⁻ in ischemia-induced gastric injuries. Though the mechanisms by which lipid peroxidation is induced in the gastric mucosa after oral administration of acidified ethanol are unknown, O₂⁻ may play a role in them. The present study revealed that naturally occurring polyamines such as spermine and spermidine possess the ability to scavenge O₂⁻ in high concentrations. On the other hand, lipid peroxidation induced by acidified ethanol may be initiated by active species other than oxygen-derived free radicals such as O₂⁻ and OH⁻. Fe²⁺ has been found to induce lipid peroxidation of liver microsomes (26) or chromaffine granules (27), and superoxide dismutase or catalase cannot prevent Fe²⁺-induced lipid peroxidation. Szabo et al. (28) have reported that oral ethanol challenge causes early vascular injury and increases vascular permeability in the gastric mucosa. Ferrous ion from extravasated plasma and erythrocytes may induce lipid peroxidation in the gastric mucosa. Recently, polyamines have been shown to inhibit lipid peroxidation in rat liver microsomes by Kitada et al. (9, 10). In agreement with these reports, we found that polyamines partially inhibited the Fe²⁺-induced lipid peroxidation in porcine gastric mucosal homogenate. Antiperoxidative actions of polyamines may be caused by their binding to the anionic site in membrane phospholipids (10, 11). Interestingly, the order of O₂⁻-scavenging potency and inhibitory potency of Fe²⁺-induced lipid peroxidation of polyamines was spermine > spermidine > putrescine, which corresponded to the order
of their protective potency against gastric damage induced by acidified ethanol. We previously observed that spermine can prevent the decrease of gastric mucosal nonprotein sulfhydryls, mainly reduced glutathione (GSH), after acidified ethanol application (7). GSH is well-known as an antioxidant which protects tissues against free radicals and decreases when the free radical production is extremely enhanced (29). The maintenance of GSH levels in the gastric mucosa made possible by spermine (7) could be explained in terms of the present observations that spermine as well as GSH possesses antiperoxidative ability.

These results suggest that the protective effect of polyamines may arise from their antiperoxidative activities which suppress lipid peroxidation induced in the gastric mucosa after acidified ethanol application, although there still remains the possibility that polyamines can indirectly inhibit lipid peroxidation via other beneficial effects.

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