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Ebselen, a seleno-organic compound, protects against ethanol-induced murine gastric mucosal injury in both in vivo and in vitro systems

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Abstract

The inhibitory effect of the seleno-organic compound ebselen on ethanol-induced murine gastric mucosal injury was examined. In an in vivo study, absolute ethanol (50 μl/mouse, oral) produced marked gastric mucosal necrosis along with hemorrhage or edema and elevations in both lipid peroxide and peptidoleukotriene levels in the fundic mucosa. Pretreatment with ebselen (30 and 100 mg/kg, oral) significantly prevented this gastric mucosal injury and, further, remarkably decreased the elevated lipid peroxide and peptidoleukotriene levels. In an in vitro study using a murine gastric surface mucous cell line GSM06, exposure to ethanol concentration dependently elicited cell damage (7.5–17.5% ethanol) and an increase in lipid peroxides without alterations in peptidoleukotrienes (15% ethanol). Addition of ebselen (10 and 100 μM) to this system (15% ethanol) significantly inhibited the cell damage and completely prevented the increase in lipid peroxide level. These results indicate that ebselen protects against murine gastric mucosal injury both in vivo and in vitro, and that this protection may be related at least in part to its inhibitory action on lipid peroxides.

Keywords: Ebselen; Ethanol; Gastric mucosal injury, murine; Gastric surface mucous cell line GSM06; Lipid peroxide; Peptidoleukotriene

1. Introduction

2-Phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) is a seleno-organic compound which possesses glutathione peroxidase-like activity and antioxidative and 5-lipoxygenase inhibitory properties (Kuhl et al., 1985; Muller et al., 1984, 1985; Parnham and Kindt, 1984; Safayhi et al., 1985; Wendel et al., 1984). It has been indicated that this compound prevents aspirin-, diclofenac- and water-immersion restraint stress-induced gastric ulcers in rats (Leyck and Parnham, 1990; Tabuchi and Kurebayashi, 1993). Ebselen was also reported to inhibit gastric acid secretions in pylorus-ligated rats (Tabuchi and Kurebayashi, 1993) and in parietal cells by interference with sulfhydryl groups of the gastric proton pump, H^+, K^+-ATPase (Beil et al., 1990; Tabuchi et al., 1994), indicating that this antisecretory action is involved in the antiulcer effect of the compound in rats. Additionally, this compound displayed gastroprotective actions which inhibited gastric mucosal lesions induced by necrotizing agents such as concentrated hydrochloric acid and acidified ethanol in rats (Kurebayashi et al., 1989). Recently, evidence has been accumulated to indicate that oxygen free radicals (Pihan et al., 1987; Szelenyi and Brune, 1988), lipid peroxidation (Mizui and Doteuchi, 1986; Mizui et al., 1987; Parmar and Ghosh, 1981; Szabo et al., 1981) and peptidoleukotrienes (Oates and Hakkinen, 1988; Peskar et al., 1986; Tabuchi et al., 1993) in the gastric mucosa participate in the process of ulcer formation caused by necrotizing agents. The mechanisms underlying the gastroprotection provided by ebselen, however, still remain unclear. The present study was designed to investigate the protective effect of ebselen on murine gastric mucosal injury induced by ethanol in an in vivo system. This compound completely inhibited mucosal necrotic lesions and partly inhibited the damage of

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surface mucous cells. In an ensuing in vitro study using a cell line (GSM06) established from the gastric surface mucous cell in transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene (Sugiyama et al., 1993), we undertook to ascertain the direct effect of ebselen on ethanol-induced mucous cell injury. Further, in both systems, the role of lipid peroxides and peptidoleukotrienes in the prevention of the injury was investigated.

2. Materials and methods

2.1. Materials

Ebselen was obtained from A. Nattermann & Cie. GmbH, Co. (Cologne, Germany). In the in vivo studies, this compound was suspended in 0.5% carboxymethylcellulose sodium solution at a constant dosing volume of 5 ml/kg. In the in vitro studies, ebselen was dissolved in absolute ethanol (final concentration: 0.5%). Daigo's T medium (without Hepes and phenol red), insulin, transferrin, ethanolamine, sodium selenite, mouse epidermal growth factor and 6-carboxyfluorescein diacethoxymethyl ester (BCECF-AM) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum purchased from Gibco Co. (Grand Island, NY, USA) was inactivated at 56°C for 30 min before use. All other chemicals were of the best quality commercially available.

2.2. Animals

6- to 7-week-old male C57BL/6 mice (Charles River Japan, Kanagawa, Japan) weighing 15–25 g were used for the investigations. The animals were housed in cages in ventilated animal rooms with controlled temperature (23 ± 2°C) and relative humidity (55 ± 15%) and with 12 h of light (8:00 to 20:00). They were maintained on standard laboratory chow (F-2, Funabashi Farm, Chiba, Japan) and tap water ad libitum. The mice were fasted overnight in cages with wire mesh floors to prevent coprophagy, but allowed free access to water up to the commencement of the experiments.

2.3. Effect of ebselen on gastric mucosal lesions induced by ethanol in vivo

Ebselen (10, 30 or 100 mg/kg) was administered by gavage 30 min before a single oral treatment with absolute ethanol (50 μl/mouse). The ebselen dosage was chosen on the basis of the result of a previous study (Kurebayashi et al., 1989). 30 min after absolute ethanol treatment, the stomach was resected under light ether anesthesia and inflated by injecting 0.5 ml of 2% buffered formaldehyde into the lumen side. The stomach was then incised along the greater curvature, and the length (mm) of gastric mucosal lesions was measured. The sum of lesion lengths per stomach was used as a lesion index. Additional animals given 0.5% carboxymethylcellulose sodium solution in the same manner served as vehicle controls.

For microscopic examination of the gastric mucosa, the tissue specimens were fixed in 10% buffered formaldehyde and routinely processed with hematoxylin and eosin stain.

2.4. Measurement of ebselen levels in serum

In order to identify the levels of ebselen appropriate for the in vitro study, serum levels in animals receiving ebselen were measured with an atomic absorption spectrometer (Lewis et al., 1986). Accordingly, ebselen was administered orally to groups of four mice each at a dose of 10, 30 or 100 mg/kg and blood samples were withdrawn from the abdominal vena cava under ether anesthesia 30, 45, 60 or 120 min later. The serum ebselen level was expressed as a selenium concentration (μM).

2.5. Cell culture

The gastric surface mucous cell line GSM06 was established from primary culture of cells from adult transgenic C57BL/6 mice harboring the temperature-sensitive simian virus 40 large T-antigen gene (Sugiyama et al., 1993). GSM06 cells produce periodic acid-Schiff- and class I concanavalin A-horseradish peroxidase-positive glycoproteins (Katsuyama and Spicer, 1978) and show temperature-sensitive growth. At a permissive temperature (33°C), the cells grow to confluent monolayers and keep in tight contact with neighboring cells with a population doubling time of about 29 h, but fail to grow at a non-permissive temperature (39°C). GSM06 cells (1.6×10^5 cells/cm^2) were suspended in Daigo's T medium supplemented with 10% fetal bovine serum, 20 μg/l insulin, 20 μg/l transferrin, 1.22 μg/l ethanolamine, 0.0914 μg/l sodium selenite and 10 μg/l epidermal growth factor, seeded in a collagen-coated plastic culture vessel (Corning, Co., NY, USA) and incubated at 33°C for 24 h in a humidified 5% CO_2-95% air atmosphere.

2.6. Effect of ebselen on GSM06 cell injury induced by ethanol in vitro

Ethanol cytotoxicity was evaluated by a method using the fluorescent dye, 2′,7′-bis(carboxyethyl) carboxyfluorescein (BCECF), which is retained only in viable cells (Kolber et al., 1988; Suzuki et al., 1992).

Namely, BCECF-AM, which permeates through the cell membrane (non-fluorescent compound), is hydrolyzed by cytosolic enzymes to BCECF, and this non-permeable fluorescent compound is accumulated in the cytosolic space. GSM06 cells were cultured in a collagen-coated 96-well culture plate, washed once with Daigo's T medium, and incubated with Daigo's T medium containing BCECF-AM (10 μM) at 37°C for 40 min. Thereafter, BCECF-labeled cells were washed 3 times with the Daigo's T medium and cultured in Daigo's T medium with 0.5% (final concentration) ethanol (vehicle) or ebselen at 37°C for 10 min. After being washed once with Daigo's T medium, the cells were incubated in Daigo's T medium containing 7.5–17.5% ethanol at 37°C. 15 min later, the supernatants were collected and the cells in each well were lysed by 0.5% polyoxyethylene octylphenyl ether (Triton X-100) in Daigo's T medium. The samples were diluted 4-fold with 50 mM bicarbonate buffer (pH 9.0), and the fluorescence intensity of BCECF was measured using a spectrofluorometer (Titertek Fluoroskan II, Flow Laboratories, McLean, VA, USA) (excitation wave length: 485 nm; emission wave length: 538 nm). Cytotoxicity (%) was calculated from the following equation:

$$\text{Cytotoxicity}(\%) = \frac{F_{\text{super}} - F_{\text{spon}}}{F_{\text{super}} + F_{\text{cell}} - F_{\text{spon}}} \times 100$$

where F_{super} is the supernatant fluorescence intensity, F_{spon} the spontaneous fluorescence intensity (treated with Daigo's T medium alone), and F_{cell} the cell fluorescence intensity.

2.7. Measurement of lipid peroxides and peptidoleukotrienes

In the in vivo study, after gastric mucosal injury was induced with ethanol by the same procedure as described above, the mucosal layer in the glandular part of the stomach was separated from the muscle layer by tweezers, and washed twice with ice-cold physiological saline. The specimen obtained was used for the following assays. In the in vitro study, GSM06 cells were cultured in a 25 cm^2-collagen-coated culture bottle and incubated with Daigo's T medium containing 15% ethanol at 37°C for 15 min, then washed twice with ice-cold physiological saline, and prepared for further assays. Lipid peroxides in homogenated samples were determined fluorometrically as thiobarbituric acid-reactive substances according to the method of Ohkawa et al. (1979). For measurement of peptidoleukotrienes, the specimens exposed to ethanol in both systems were incubated in Daigo's T medium with gentle shaking at 37°C for 15 min. After that, the supernatant of the culture media was collected by centrifugation (1400 × g, 10 min) and assayed for peptidoleukotriene using a commercial peptidoleukotriene enzyme immunoassay kit (Amersham Japan Co., Tokyo, Japan) which cross-reacts with leukotriene C_4 (100%), leukotriene D_4 (100%) and leukotriene E_4 (70%). Protein concentration was measured by using the method of Lowry et al. (1951).

2.8. Statistical analyses

The data are presented as the means ± S.E.M. The statistical analysis was carried out using Dunnett's multiple comparison test. A P value less than 0.05 was regarded as significant.

3. Results

3.1. Effect of ebselen on gastric mucosal lesions induced by ethanol in mice

As shown in Figs. 1 and 2, oral administration of absolute ethanol induced visually severe hemorrhage in the mucosa and, microscopically, desquamation and disruption of surface mucous cells, profound edema in the glandular stomach and deep necrotic lesions. A single oral pretreatment with ebselen at 10–100 mg/kg in this model significantly inhibited the ulcer index from 30 mg/kg, and the inhibition was 83 and 92% at 30 and 100 mg/kg, respectively. Microscopically, this compound at 30 mg/kg tended to improve the above lesions, such as desquamation of surface mucous cells and edema, to a greater extent. At 100 mg/kg, ebselen completely reversed almost all lesions except for disruption of surface mucous cells.

3.2. Serum ebselen level

The results are summarized in Fig. 3. A single oral administration of ebselen at 10–100 mg/kg increased serum levels dose dependently over the observation period. Serum ebselen levels 30 min after dosing, which were used to determine the test compound concentration to be employed in the in vitro study, were 3.8, 9.0 and 20.1 μM at 10, 30 and 100 mg/kg, respectively. Therefore, 0.1–100 μM of ebselen was selected as concentrations for the subsequent in vitro studies.

3.3. Effect of ebselen on GSM06 cell injury induced by ethanol

As shown in Figs. 4 and 5, ethanol induced cytotoxicity in a concentration-dependent manner (7.5–17.5%). Considering the detection of the protective action of ebselen on the basis of these data, 15% ethanol was chosen as a cytotoxic concentration (75% cytotoxicity)

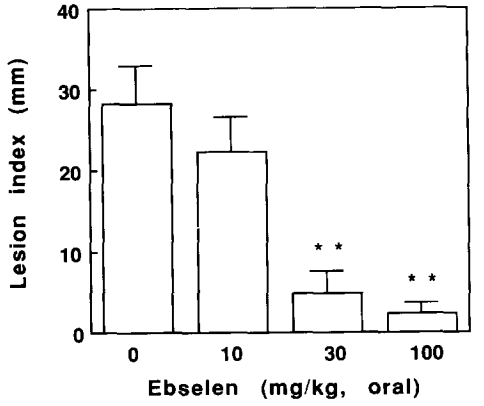

Fig. 1. Effect of ebselen on gastric mucosal lesions induced by ethanol in mice. Ebselen was administered by gavage 30 min before an oral treatment with absolute ethanol (50 μl/mouse). 30 min later, gastric lesions were visually examined. Each column and vertical bar represents the mean ± S.E.M. for six animals. $^{**}P < 0.01$ vs. zero-dose group (vehicle plus ethanol) (Dunnett's multiple comparison test).

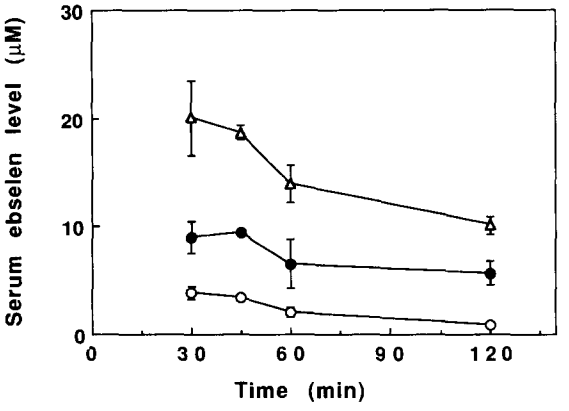

Fig. 3. Serum ebselen levels after a single oral administration. Blood samples were withdrawn from the abdominal vena cava under ether anesthesia 30, 45, 60 and 120 min after oral treatment with ebselen at a dose of 10 (○), 30 (●) or 100 (△) mg/kg ($n = 4$).

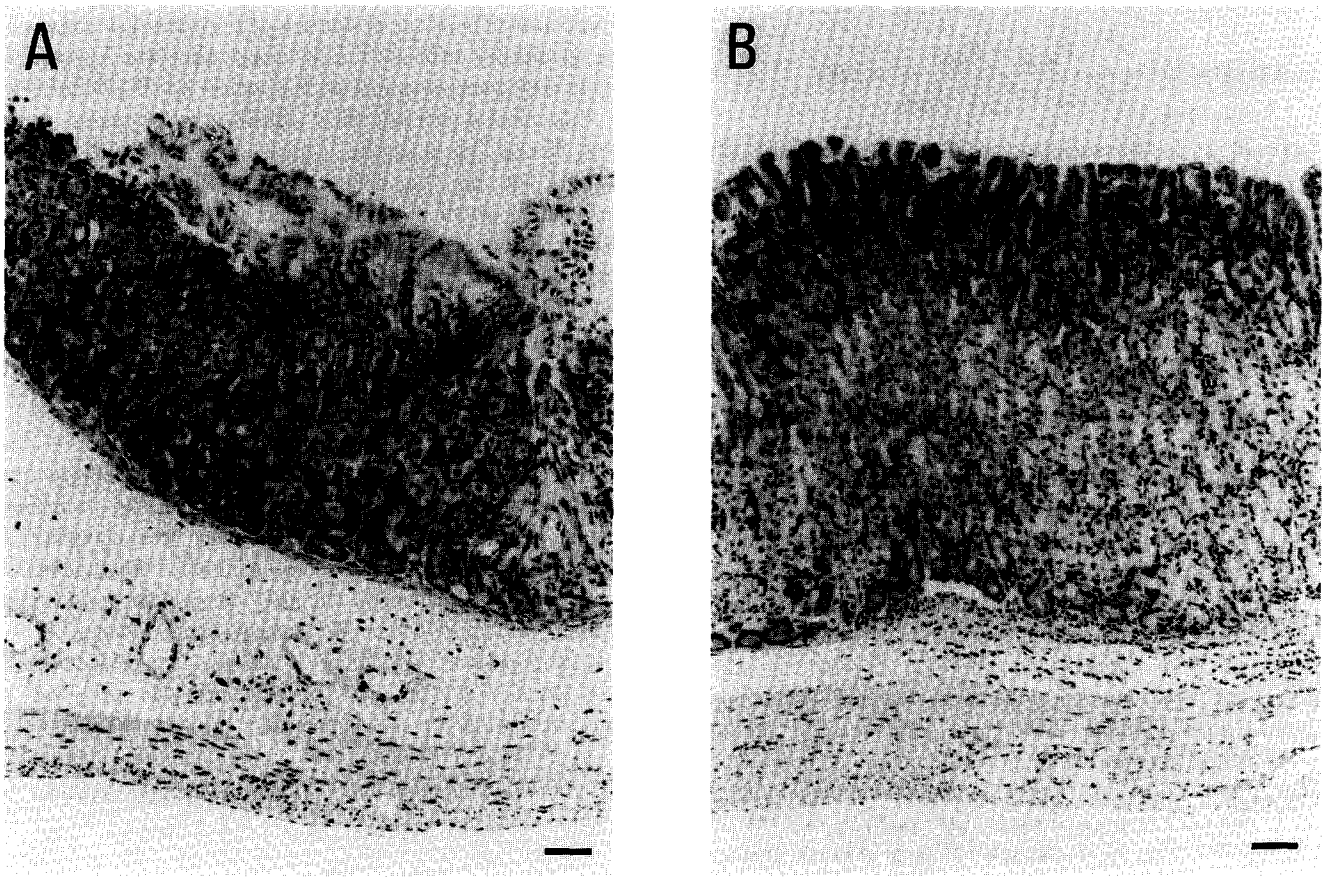

Fig. 2. Microscopic findings of gastroprotection by ebselen against ethanol-induced gastric injury. Ebselen (100 mg/kg) was administered orally 30 min before a single oral treatment with absolute ethanol (50 μl/mouse). 30 min later, gastric mucosa was fixed with 10% buffered formaldehyde. (A) Ethanol alone-treated gastric mucosa: note desquamation and disruption of surface mucous cells, edema in the gastric fundic mucosa and deep necrotic lesions. (B) Ebselen plus ethanol-treated gastric mucosa: note protection against changes induced by ethanol. Bar = 50 μm.

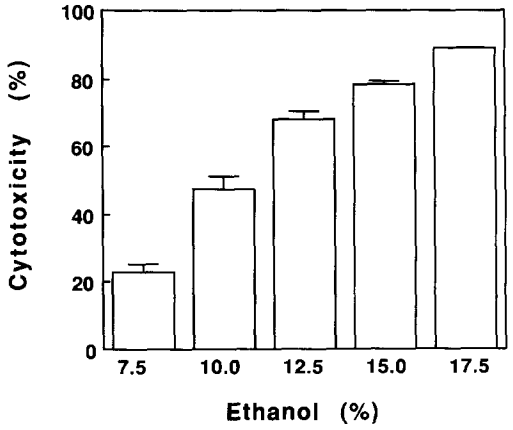

Fig. 4. GSM06 cell injuries induced by various concentrations of ethanol. The cells (1.6×10^5 cells/cm^2) were cultured in Daigo's-T medium containing 10 μM of 6-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) at 37°C for 40 min. Thereafter, 2',7'-bis(carboxyethyl) carboxyfluorescein (BCECF)-labeled cells were incubated in Daigo's-T medium containing 7.5–17.5% ethanol at 37°C for 15 min, and the fluorescence intensity of BCECF was measured with a spectrofluorometer (EX: 485 nm, EM: 538 nm). Cytotoxicity (%) was calculated as described in Materials and methods. Each column and vertical bar represents the mean ±S.E.M. for 10 wells.

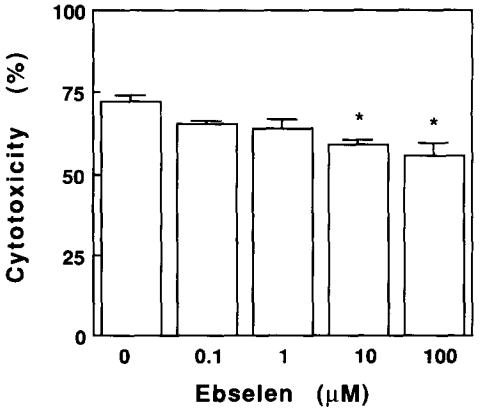

Fig. 5. Effect of ebselen on the cytotoxicity of GSM06 cells induced by 15% ethanol. The experiment was done by the same procedure as described in Fig. 4. Ebselen was added to 2',7'-bis(carboxyethyl) carboxyfluorescein (BCECF)-labeled cells 10 min before ethanol (15%) exposure. Each column and vertical bar represents the mean ±S.E.M. for 10 wells. $^*P<0.05$ vs. zero-dose group (vehicle plus ethanol) (Dunnett's multiple comparison test).

for the further study. Pretreatment with ebselen at 10 and 100 μM significantly prevented the cytotoxicity induced by 15% ethanol and the inhibition percentages were 18 and 23%, respectively.

3.4. Effect of ebselen on lipid peroxides and peptidoleukotrienes both in mice and in GSM06 cells treated with ethanol

In the in vivo studies, as seen in Table 1, oral administration of absolute ethanol significantly increased lipid peroxide (50.0 ± 3.4 nmol/100 mg protein) and peptidoleukotriene (248 ± 19 pg/mg protein per 15 min) levels in the gastric mucosa, and their values were about 1.5- and 1.8-fold higher, respectively, than those of the vehicle-treated group. Pretreatment with ebselen dose dependently or markedly prevented these elevated levels from 10 mg/kg for lipid peroxides and from 30 mg/kg for peptidoleukotrienes.

In the in vitro studies, as shown in Table 2, exposure of GSM06 cells to 15% ethanol elicited an elevation in lipid peroxide level to 122 ± 1.1 nmol/100 mg protein, about 1.7-fold higher as compared to the vehicle control group, while the peptidoleukotriene level remained below the detection limit (< 5 pg/mg protein

Table 1
Effect of ebselen on lipid peroxides and peptidoleukotrienes in the gastric mucose of mice treated with absolute ethanol

Treatment	Dose (mg/kg)	Lipid peroxides (nmol/100 mg protein)	Peptidoleukotrienes (pg/mg protein per 15 min)
Vehicle alone		33.7±2.0 [b]	137± 9.2 [b]
Ethanol alone		50.0±3.4	248±19
+ebselen	10	39.4±2.0 [a]	205± 9.7
+ebselen	30	37.5±2.1 [b]	158± 6.8 [b]
+ebselen	100	29.6±1.3 [b]	154±15 [b]

Ebselen was administered by gavage 30 min before a single treatment with absolute ethanol (50 μl/mouse). Lipid peroxides in the homogenated gastric mucosa were determined fluorometrically as thiobarbituric acid-reactive substances, and peptidoleukotrienes released into the medium were measured by peptidoleukotriene enzyme immunoassay. The data represent the means±S.E.M. for six animals. Vehicle alone: 0.5% carboxymethylcellulose sodium solution (CSS) plus purified water (oral), ethanol alone: CSS plus absolute ethanol. [a] $P<0.05$, [b] $P<0.01$ vs. ethanol alone group (Dunnett's multiple comparison test).

Table 2
Effect of ebselen on lipid peroxides and peptidoleukotrienes in GSM06 cells treated with 15% ethanol

Treatment	Concentration (μM)	Lipid peroxides (nmol/100 mg protein)	Peptidoleukotrienes (pg/mg protein per 15 min)
Vehicle alone		70.3±0.6 [a]	< 5.0
Ethanol alone		122 ±1.1	< 5.0
+ebselen	1	105 ±4.6 [a]	< 5.0
+ebselen	10	95.4±3.5 [a]	< 5.0
+ebselen	100	66.2±2.9 [a]	< 5.0

Ebselen was added to the culture medium of GSM06 cells (1.6×10^5 cells/cm^2) 10 min before ethanol exposure, and then incubated for 15 min at 37°C. Lipid peroxides in homogenated cells were determined fluorometrically as thiobarbituric acid-reactive substances, and peptidoleukotrienes released into the medium were measured by peptidoleukotriene enzyme immunoassay. The data represent the means±S.E.M. for six bottles. Vehicle alone: 0.5% ethanol+Daigo's T medium; Ethanol alone: 0.5% ethanol+15% ethanol in Daigo's T medium. [a] $P<0.01$ vs. ethanol alone group (Dunnett's multiple comparison test).

per 15 min). Pretreatment with ebselen markedly and dose dependently prevented the elevation of lipid peroxide levels at 1 μM and higher.

4. Discussion

In the present study, orally administered ebselen effectively prevented the gastric mucosal necrotic lesions induced by ethanol in mice. This finding was consistent with our previous observations that ebselen inhibited the injury induced by concentrated hydrochloric acid and acidified ethanol in rats (Kurebayashi et al., 1989). We confirmed that pretreatment with indomethacin did not affect the protective effect of ebselen against necrotizing agent-induced gastric injury (Kurebayashi et al., 1989). In our preliminary experiments, indomethacin (5 mg/kg, s.c.) hardly affected the inhibitory action of ebselen in ethanol-treated mice (Tabuchi et al., unpublished data). It appears appropriate therefore to consider that the gastroprotective action of ebselen on ethanol-induced injury is not due to a stimulation of endogenous prostaglandin synthesis by itself (Robert et al., 1983).

Microscopic examination revealed that the pretreatment of mice with ebselen (30–100 mg/kg) undoubtedly reduced necrotic lesions in the stomach produced by absolute ethanol, but failed to completely inhibit the disruption of surface mucous cells. This mimics the results of a study using exogenous 16,16-dimethyl-prostaglandin E_2 in rats (Lacy and Ito, 1982). In this study of ebselen on ethanol-induced cytotoxicity using GSM06 cells, exposure to ethanol alone induced cell injury in a concentration-dependent manner (7.5–17.5%). These findings are almost identical to previously reported observations for primary cultured monolayers from rat gastric mucosal cells (Mutoh et al., 1990a,b). Addition of ebselen (10 and 100 μM) to this in vitro test system significantly prevented the cytotoxicity, although the inhibition percentage was only 20% at 100 μM. The difference in protective actions noted between the in vivo and in vitro systems may reflect the different experimental conditions and the degree of original gastric lesions induced by ethanol.

Gastric lesions caused by necrotizing agents have been attributed to the production of free radicals resulting from lipid peroxidation (Pihan et al., 1987; Szelenyi and Brune, 1988). In the present experiments, the gastric mucosal lipid peroxide level, as assessed by measuring the amounts of thiobarbituric acid-reactive substances, significantly increased after absolute ethanol treatment. This finding is in agreement with previous rat studies (Mizui and Doteuchi, 1986; Mizui et al., 1987). The 5-lipoxygenase products including peptidoleukotrienes are believed to be formed in gastric mucosal lesions induced by necrotizing agents. For example, studies have indicated that intragastric instillation of absolute ethanol increases gastric mucosal release of leukotriene C_4 in parallel to the incidence of gastric lesions in rats (Peskar et al., 1986; Peskar, 1991). In mice given absolute ethanol by gavage, alcohol caused a marked elevation of peptidoleukotriene level in the fundic mucosa similar to that of previous observations in rats (Peskar et al., 1986; Peskar, 1991). This suggests that lipid peroxides and peptidoleukotrienes partly participate in the pathogenesis of absolute ethanol-induced gastric mucosal lesions. In the in vivo study, the lowest dose of ebselen (10 mg/kg) scarcely inhibited gastric mucosal lesions caused by ethanol. However, this compound significantly prevented increases in both lipid peroxide and peptidoleukotriene levels at 10–100 mg/kg and 30–100 mg/kg, respectively. The variation in effective dosage level noted for these two variables was considered to reflect differences in antioxidative properties and inhibitory effects on 5-lipoxygenase, as well as in glutathione peroxidase-like activities. Some or all of these actions may be responsible for the gastroprotective properties of ebselen.

In the in vitro study, 15% ethanol produced both severe cytotoxicity (70–80%) and an increase in lipid peroxide level. Mutoh et al. (1990a) have reported that cultured rat gastric mucosal cells exposed to ethanol generate oxygen radicals and that the production of oxygen radicals is closely linked with ethanol-induced damage to the cells. The elevation of lipid peroxides in GSM06 cells upon exposure to ethanol was, therefore, thought to be brought about by the generation of oxygen free radicals. Inhibition of lipid peroxides by ebselen was noted at a low concentration of 1 μM and was complete at 100 μM. Ebselen prevented ethanol-induced cytotoxicity by only 20% even at a concentration of 100 μM. Although the difference between these levels of inhibition is not easily explained, certain factors, as described before, together with lipid peroxides may be involved in the maintenance of cell survival under in vitro conditions. However, the protection provided by ebselen is considered to be related at least in part to the inhibitory action of this compound on lipid peroxides. The peptidoleukotriene level was below the detection limit (5 pg/mg protein per 15 min). This fact indicates that gastric surface mucous cells may be a minor source of peptidoleukotrienes in the stomach. Huber et al. (1993) stated that chromogranin A-positive neuroendocrine cells but not mucous cells may represent a major store of leukotriene B_4 or leukotriene C_4 in the rat gastric mucosa.

In conclusion, ebselen protects against murine gastric mucosal injury both in vivo and in vitro. This protection may be related at least in part to the inhibitory action of ebselen on lipid peroxides.

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