Protective Effect of Rebamipide against Hydrogen Peroxide-Induced Hemorrhagic Mucosal Lesions in Rat Stomach

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ABSTRACT—Intragastric administration of 6% H₂O₂ induces gastric mucosal hemorrhagic lesions in rats. Intraperitoneal administration of rebamipide at 10 to 100 mg/kg dose-dependently prevented the H₂O₂-induced mucosal lesions. The fact that cimetidine, ranitidine and omeprazole could not prevent the gastric mucosa from developing H₂O₂-induced lesions indicates that acid secretion might not be the main cause of these lesions. The protective effect of rebamipide was partially reduced by indomethacin and completely blocked by diethyl maleate, a glutathione depressor. Gastric mucosal glutathione level and glutathione peroxidase and superoxide dismutase (SOD) activities were significantly decreased by 6% H₂O₂ instillation. Rebamipide at 100 mg/kg significantly inhibited the decreases in gastric mucosal glutathione level and SOD activity. These results suggest that H₂O₂-induced gastric mucosal lesions might partially involve a decrease in defense activities against reactive oxygen species and that the protective effect of rebamipide might be related to its ability to improve oxidative stress in gastric mucosa.

Keywords: Rebamipide, H₂O₂, Gastric mucosal lesion, Glutathione, Reactive oxygen

Reactive oxygen species are known to be involved in the pathogenesis of mucosal lesions in the gastrointestinal tract by ischemia-reperfusion and necrotizing agents such as ethanol and aspirin (1-4). Due to their high reactivity and short life, it has still not been elucidated which free radicals, i.e., the superoxide or hydroxyl radical, play an important role in these mucosal lesions (1-5). Several investigations (6, 7) have been performed using H₂O₂ as the necrotizing agent. It is well known that H₂O₂ can be metabolized to the hydroxyl radical, which is one of the most potently damaging free radicals (8).

Rebamipide has been reported to prevent various acute experimental gastric mucosal lesions and to accelerate the healing of chronic gastric ulcers (9, 10). Ogino et al. (11) showed that the antiulcer effect of rebamipide might be related to the inhibition of the production of reactive oxygen species by activated neutrophils in a diethylthiocarbamate-induced antral ulcer model in rats. Recently, it was indicated that rebamipide was one of the most potent hydroxyl radical scavengers studied by electron spin resonance (12). However, there is still no direct evidence that rebamipide protects gastric mucosa from the damage induced by reactive oxygen species. The present study was undertaken to examine the protective effect of rebamipide against H₂O₂-induced lesions and to elucidate whether or not its effect might be connected to gastric mucosal glutathione level, glutathione peroxidase activity and/or SOD activity.

MATERIALS AND METHODS

Animals
Male Wistar/ST strain rats (SLC, Shizuoka) weighing 200-300 g were housed in wire-bottom cages to prevent coprophagy. Food and water were respectively withheld from 24 hr and 20 hr prior to the experiment.

Chemicals
Rebamipide, ranitidine and omeprazole were synthesized by Otsuka Pharmaceutical Co., Ltd. (Tokushima). Cimetidine and diethyl maleate were purchased from Nacalai Tesque (Osaka). Catalase (19,900 U/mg protein), superoxide dismutase (SOD, 4,200 U/mg protein), indomethacin and glutathione were obtained from Sigma Chemical Co. (St. Louis, MO, USA). H₂O₂ was supplied by Wako Pure Chemicals (Osaka). Rebamipide, cimetidine, ranitidine and omeprazole were prepared by suspension in 0.5% carboxymethyl cellulose (CMC). Catalase
and SOD were dissolved in saline. Solutions were freshly prepared each day.

**H$_2$O$_2$-induced gastric mucosal necrosis**

Rebamipide at 10, 30 and 100 mg/kg was administered i.p. 30 min prior to intragastric administration of 6% H$_2$O$_2$. Cimetidine, ranitidine or omeprazole at 100 mg/kg was administered i.p. 1 hr prior to H$_2$O$_2$. Catalase at 500,000 U/kg and/or SOD at 30,000 U/kg were administered s.c. 30 min before H$_2$O$_2$ instillation. Control animals received 0.5% CMC or saline instead of drugs. Parenteral administration was used to avoid possible interference from the production of mucosal lesions by adaptive cytoprotection (13). In separate experiments, indomethacin at 5 mg/kg or diethyl maleate at 1 ml/kg was given s.c. 1 hr prior to the 6% H$_2$O$_2$ instillation. Following these procedures, 6% H$_2$O$_2$ in a volume of 1 ml/body was administered i.g. through an oral gastric tube. Thirty minutes after H$_2$O$_2$ administration, the rats were sacrificed by dislocation of the cervical vertebrate and the stomach was excised. After light fixation with 10 ml of 3% buffered formalin, the stomach was opened along the great curvature and washed with saline. The total glandular and hemorrhagic mucosal lesion area were measured by an image analyzer (Luzex-IID; Nikon, Tokyo).

**Determination of GSH level, GSHpx and SOD activities**

Rebamipide at 10, 30 and 100 mg/kg was administered i.p. 30 min prior to intragastric administration of 6% H$_2$O$_2$. Control animals received i.p. 0.5% CMC. The animals were sacrificed by cervical dislocation 30 min after H$_2$O$_2$ instillation. In a separate experiment, rebamipide at 10, 30 and 100 mg/kg was administered i.p. to normal rats, which were sacrificed 1 hr after administration. The stomach was immediately excised and opened along the great curvature. After rinsing with ice-cooled saline, the entire gastric mucosa was scraped with a slide glass and weighed. Ten percent of the mucosal homogenates was prepared in 50 mM potassium phosphate buffer pH 7.0 using a Polytron homogenizer (Kinematica, Lucerne, Switzerland; output 5 to 6). The homogenates were stored at $-80$°C until the determination of total glutathione (GSH) level and glutathione peroxidase (GSHpx) and superoxide dismutase (SOD) activities. GSH level was determined according to the method of Sies and Akerboom (14) and expressed as nmol/mg protein. Protein was determined by the method of Lowry et al. (15). GSHpx activity was spectrophotometrically measured by the method of Paglia and Valentine (16) and expressed as units (U)/mg protein. SOD activity was determined by the nitrite method (17) and expressed as nitrite units (NU)/mg protein.

**Statistical analyses**

The data was expressed as the mean ± S.E. The statistical difference was determined by Student's two-tailed t-test or one-way analysis of variance followed by two-tailed Dunnett's or Scheffe's test. A difference at P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of rebamipide, antisecretory agents and reactive oxygen metabolite scavenging enzymes on H$_2$O$_2$-induced gastric mucosal lesions**

Intragastric administration of 6% H$_2$O$_2$ produced hemorrhagic, necrotic mucosal lesions in the fundic area. Intraperitoneal administration of rebamipide at 10 to 100 mg/kg prevented the mucosal lesions in a dose-dependent manner (Fig. 1). Pretreatment with cimetidine, ranitidine or omeprazole was not able to prevent the gastric mucosa from developing H$_2$O$_2$-induced lesions (Fig. 2). Catalase at 500,000 U/kg, s.c. and/or SOD at 30,000 U/kg, s.c. also failed to protect the gastric mucosa from H$_2$O$_2$-induced hemorrhagic mucosal lesions (Fig. 2).

**Effect of rebamipide with indomethacin or diethyl maleate on H$_2$O$_2$-induced gastric mucosal lesions**

Hemorrhagic mucosal lesions induced by 6% H$_2$O$_2$ were not aggravated by pretreatment with indomethacin.
at 5 mg/kg, s.c. 30 min before H₂O₂ instillation. The protective effect of rebamipide was partially reversed by pretreatment with indomethacin (Fig. 3). Pretreatment with diethyl maleate at 1 ml/kg, s.c. did not change the magnitude of the H₂O₂-induced gastric mucosal lesions, but completely abolished the protective effect of rebamipide.

Effect of H₂O₂ and rebamipide on gastric mucosal total GSH level and GSHpx and SOD activities in gastric mucosa

Gastric mucosal total GSH was significantly decreased (41%) 30 min after intragastric administration of 6% H₂O₂ (Table 1). Rebamipide at 10 to 100 mg/kg, i.p. 30 min prior to H₂O₂ instillation dose-dependently inhibited the decrease in mucosal GSH, and the inhibiting effect was significant at 100 mg/kg. GSHpx and SOD activities in gastric mucosa were also significantly reduced by 6% H₂O₂ administration to 19% and 41% of those of normal rats, respectively. Rebamipide at 10 to 100 mg/kg, i.p. showed
a tendency to inhibit the reduction of GSHpx activity in a dose-dependent manner. Rebamipide at 100 mg/kg, i.p. significantly inhibited the reduction of gastric mucosal SOD activity. On the other hand, in normal rats, intraperitoneal rebamipide at 10 to 100 mg/kg did not significantly affect gastric mucosal GSH level or GSHpx and SOD activities.

**DISCUSSION**

It has been reported that several necrotizing agents, i.e., ethanol and aspirin, induce gastric mucosal hemorrhagic lesions by promoting the generation of reactive oxygen metabolites (3, 4). Furthermore, it has been indicated that compounds and enzymes that are capable of scavenging or inhibiting the generation of reactive oxygen species are able to protect the gastric mucosa from such hemorrhagic lesions (18, 19). In this study, intragastric administration of 6% H₂O₂ induced gastric mucosal hemorrhagic lesions in rats. H₂O₂ seems to be a suitable agent for investigating reactive oxygen metabolite-induced gastric mucosal lesions because H₂O₂ itself is a reactive oxygen metabolite.

Rebamipide is a protective antiulcer agent with activities to increase prostaglandin level in gastric mucosa and stimulate mucus secretion (9, 20, 21). In this study, rebamipide at 30 and 100 mg/kg, i.p. significantly reduced gastric mucosal hemorrhagic lesions induced by intragastric H₂O₂. It has also been reported that rebamipide can prevent gastric mucosa developing from acute gastric mucosal lesions induced by ethanol and aspirin (9, 10), which are thought to generate reactive oxygen metabolites (3, 4). Recently, Yoshikawa et al. (12) indicated that rebamipide has a potent activity to scavenge the hydroxyl radical, which is thought to be one of the most toxic free radicals. These results suggest that hydroxyl radical generated from H₂O₂ in gastric mucosa might be related to these lesions and that rebamipide’s scavenging of the hydroxyl radical may play some roles in its protective effect.

Pretreatment with indomethacin partially diminished the protective effect of rebamipide against 6% H₂O₂-induced lesions. Kleine et al. recently reported that intraperitoneal administration of rebamipide stimulated prostaglandin synthesis in the rat stomach (22). Furthermore, 16,16-dimethyl PGE₂ was previously reported to protect gastric mucosa from 6% H₂O₂-induced injury (7). This data suggests that the elevation of gastric prostaglandins might, in part, be due to the protective effect of rebamipide. Pretreatment with diethyl maleate, which is a depressor of GSH, completely blocked the protective effect of rebamipide. These findings suggest that the protective effect of rebamipide on gastric mucosal glutathione level may play a more important role in the detoxication of H₂O₂ and H₂O₂-derived oxygen metabolites than the stimulation of prostaglandin synthesis in gastric mucosa.

On the other hand, neither cimetidine, ranitidine nor omeprazole was able to protect the gastric mucosa from 6% H₂O₂-induced lesions. Aggravation rather than no effect by antisecretory agents was reported by Laudanno et al. (6). The same phenomena were indicated in ethanol-induced mucosal lesions (23). These findings suggest that the gastric mucosa could not be protected by the inhibition of acid secretion from 6% H₂O₂-induced lesions.

Although there have been reports of the free radical scavenging enzymes catalase and/or SOD being able to protect the gastric mucosa from injuries caused by reactive oxygen metabolites (3), they failed to protect against

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**Table 1.** Effects of intragastric administration of 6% H₂O₂ and pretreatment with rebamipide on total glutathione level (GSH) and glutathione peroxidase (GSHpx) and superoxide dismutase (SOD) activities in the gastric mucosa

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total GSH (nmol/mg prot.)</th>
<th>GSHpx (U/mg prot.)</th>
<th>SOD (NU/mg prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (H₂O₂, p.o.)</td>
<td>7</td>
<td>54.8 ± 1.5</td>
<td>0.455 ± 0.014</td>
<td>29.6 ± 1.0</td>
</tr>
<tr>
<td>Control (6% H₂O₂, p.o.)</td>
<td>6</td>
<td>32.3 ± 1.3</td>
<td>0.367 ± 0.027</td>
<td>17.6 ± 0.9</td>
</tr>
<tr>
<td>Rebamipide 10 mg/kg, i.p.</td>
<td>6</td>
<td>33.9 ± 1.7</td>
<td>0.381 ± 0.012</td>
<td>16.2 ± 1.0</td>
</tr>
<tr>
<td>Rebamipide 30 mg/kg, i.p.</td>
<td>7</td>
<td>36.9 ± 2.8</td>
<td>0.393 ± 0.010</td>
<td>20.3 ± 1.2</td>
</tr>
<tr>
<td>Rebamipide 100 mg/kg, i.p.</td>
<td>6</td>
<td>42.8 ± 3.4*</td>
<td>0.426 ± 0.025</td>
<td>22.3 ± 1.0*</td>
</tr>
<tr>
<td>Control (0.5% CMC, i.p.)</td>
<td>7</td>
<td>41.1 ± 1.1</td>
<td>0.455 ± 0.023</td>
<td>27.4 ± 1.0</td>
</tr>
<tr>
<td>Rebamipide 10 mg/kg, i.p.</td>
<td>7</td>
<td>43.1 ± 1.4</td>
<td>0.432 ± 0.022</td>
<td>26.2 ± 0.8</td>
</tr>
<tr>
<td>Rebamipide 30 mg/kg, i.p.</td>
<td>7</td>
<td>44.1 ± 1.3</td>
<td>0.463 ± 0.023</td>
<td>25.6 ± 0.6</td>
</tr>
<tr>
<td>Rebamipide 100 mg/kg, i.p.</td>
<td>7</td>
<td>41.4 ± 0.8</td>
<td>0.439 ± 0.023</td>
<td>25.8 ± 0.4</td>
</tr>
</tbody>
</table>

Values indicate the mean ± S.E. *P < 0.05, **P < 0.01, vs. the normal group (Student’s t-test). *P < 0.05, vs. the control group (Dunnett’s test).
6% H$_2$O$_2$ lesions in this study. It seems reasonable to assume that SOD scavenges superoxide and generates H$_2$O$_2$, but the ineffectiveness of catalase can not be explained in this context. The fact that the intravenous administration of catalase at 100,000 to 1,000,000 U/kg also failed to protect the gastric mucosa (data not shown) might suggest that exogenous catalase was not able to reach the site to fully exhibit its scavenging activity against intragastric H$_2$O$_2$ because of its large molecular size (about MW 200,000).

In this study, gastric mucosal GSH level and GSHpx and SOD activities were significantly decreased by instillation of 6% H$_2$O$_2$. It has been reported that sulfhydryl compounds mediate gastric cytoprotection (24). Oxygenation of proteins by H$_2$O$_2$ and H$_2$O$_2$-metabolites, which is partly caused by GSH depression, might be related to the decrease of GSHpx and SOD activities (18). Rebamipide at 10 to 100 mg/kg dose-dependently inhibited the decrease of gastric mucosal GSH, and this inhibition was significant at 100 mg/kg. These findings are in accord with the result that pretreatment with diethyl maleate abolished the protection provided by rebamipide. It was thought that the mechanism of the inhibition of H$_2$O$_2$-induced GSH depletion by rebamipide in gastric mucosa was due to (1) the stimulation of GSH synthesis, (2) the result of the inhibition of gastric mucosal lesion, which was not related to GSH, or (3) the inhibition of GSH consumption in gastric mucosa. In normal rats, rebamipide did not significantly affect gastric mucosal total GSH level or GSHpx and SOD activities, suggesting that the inhibition of GSH-depletion by rebamipide might be related to its inhibitory effect on the consumption of GSH during H$_2$O$_2$ attack. The H$_2$O$_2$-induced decreases in both GSHpx and SOD activities were also inhibited by pretreatment with rebamipide. Because Cu,Zn-SOD activity has been reported to be inhibited by the hydroxyl radical (25), the scavenging effect of rebamipide on the hydroxyl radical may be related to the protection of SOD activity.

In conclusion, our results suggest that rebamipide protects gastric mucosa from H$_2$O$_2$-induced mucosal lesions and that its protective effect might be involved in improving oxidative stress in gastric mucosa through inhibition of the decrease in gastric mucosal GSH level and GSHpx and SOD activities.

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