Characteristics of Attenuating Effects of Rebamipide, an Anti-ulcer Agent, on Oxidative Burst of Human Neutrophils

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Abstract. The aim of this study was to characterize the effects of rebamipide on the oxidative burst of human neutrophils. The neutrophil oxidative burst was measured in the presence of rebamipide and cimetidine using lucigenin- or luminol-dependent chemiluminescence (LgCL or LmCL). Rebamipide inhibited the LmCL response stimulated with opsonized zymosan, 12-myristate 13-acetate phorbol, and calcium ionophore in a dose-dependent manner, but the LgCL response was inhibited when neutrophils were stimulated with opsonized zymosan. LmCL response was also dose-dependently attenuated by rebamipide even in the presence of cimetidine. Thus, addition of rebamipide to H2-receptor antagonists can be considered for the treatment of gastric mucosal injury associated with oxidative stress.

Keywords: rebamipide, neutrophil, reactive oxygen species

Reactive oxygen species (ROS) are produced by inflammatory cells, particularly activated neutrophils, to attack invading pathogens. ROS can also impair proteins, nucleic acids, and wide range of molecular species. Excess ROS production has been recognized to play a significant role in tissue injury. ROS produced by neutrophils have been involved in the pathogenesis of gastritis and gastric ulcers induced by several pathogens such as non-steroidal anti-inflammatory drugs (NSAIDs) or Helicobacter pylori. Because there can be persisting infection of H. pylori for decades, there may be considerable neutrophil infiltration in gastric mucosa. Increased oxidative DNA damage is observed in gastric mucosa with H. pylori infection. High amount of DNA damage can lead to cell death, and this mechanism would play an important role in the development of atrophic gastritis and gastric carcinogenesis. The role of oxidative stress has been also implicated in the NSAIDs-induced gastric mucosal lesions. Therefore, for clinically available anti-ulcer agents, anti-oxidative functions could be desirable.

Rebamipide, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinone-4yl], has been shown to have protective effects against gastric mucosal injury induced by H. pylori and chemicals. Rebamipide induces production of prostaglandins in the gastric mucosa and increases gastric mucus. Antioxidative properties of rebamipide are also implicated in the protection of gastric mucosal injury. A previous study demonstrated the radical scavenging properties of rebamipide, and a recent study showed the competitive inhibitory action of rebamipide on formyl-methionyl-leucyl-phenylalanine (fMLP) receptor. However, the mechanisms of the inhibitory effects of rebamipide on neutrophil ROS production have not been fully addressed. In order to characterize the inhibitory effects of rebamipide on the oxidative burst of human neutrophils, we stimulated neutrophils by three agents and measured ROS production using two chemiluminescent probes in the presence of various concentrations of rebamipide. Since rebamipide is often used with H2-receptor antagonists, we also compared the effects of rebamipide with those of cimetidine.

Neutrophils were isolated from peripheral blood collected from 6 healthy males, aged 25 to 36 (mean 29), by Histopaque density gradient separation (Sigma, St. Louis, MO, USA). Neutrophil suspension was ad-
justed to 2.0 × 10^6 cells/μl by dilution with Hanks’ balanced salt solution (HBSS). The proportion of neutrophils in the cell preparations was >97%, and the cell viability determined by trypan blue exclusion was >99%. The stimulants used were opsonized zymosan (OZ), 12-myristate 13-acetate phorbol (PMA), and calcium ionophore (A23187, Sigma). OZ was prepared by opsonizing Zymosan A (Sigma) using pooled sera. OZ was suspended in HBSS and was used at the final concentrations of 1.0 mg/g.

Osaka) and was diluted with HBSS and used at the final concentrations of 1.0 mg/g. 1.0 mg/g. Os was suspended in HBSS and was used at the final concentrations of 1.0 mg/g.

the solution was adjusted to an isotonic state of 10 mM Luminol was thoroughly dissolved with 1 N NaOH and the solution was adjusted to an isotonic state of 10 mM at pH 7.4 and used at the final concentration of 0.1 mM. Rebamipide and cimetidine were initially diluted by red microliters of rebamipide (Otsuka Pharmacy Co., Tokushima) or cimetidine (Sigma) solution (0 to 1.0 mM) of cimetidine in the neutrophils. We used 96-well microplates. Each well was added with 50 μl of neutrophil suspension and 50 μl of luminogen or luminol. Immediately after the addition of 50 μl of neutrophil stimulating agents, measurement of chemiluminescence was started using a Lumi Box H-1000 (Microtec-Nichion, Funabashi). Effect of rebamipide on luminol-dependent chemiluminescence (LmCL) response was also measured in the presence of 0.1 or 1.0 mM of cimetidine. The highest value in the CL response was expressed as the peak height (PH) and used for the statistical analysis. PH observed in neutrophils without rebamipide and cimetidine served as the control (100%). Pair comparison based on the Bonferroni’s standard P value less than 5% were considered significant.

Figure 2 shows the effects of rebamipide and cimetidine on lucigenin-dependent chemiluminescence (LgCL) response. LgCL was attenuated by rebamipide dose-dependently only when neutrophils were stimulated with OZ (r = −0.486, P < 0.01). In PMA-stimulated neutrophils, rebamipide attenuated LgCL response significantly when the concentration of rebamipide was larger than 1.0 mM (P < 0.05). However, A23187-stimulated LgCL was not attenuated by rebamipide in this study. Cimetidine did not affect LgCL response significantly at the concentration of 0 – 1.5 mM. Effects of rebamipide on LmCL responses stimulated by three stimulants incubated with cimetidine are shown in Table 1. Rebamipide had dose-dependent attenuating effects on LmCL response in the presence of 0.1 or 1.0 mM of cimetidine. In the presence of 0.1 mM of cimetidine, the calculated correlation (r) was −0.594 (P < 0.01) for OZ stimulated LmCL, −0.401 (P = 0.06) for PMA stimulated LmCL, and −0.635 (P < 0.01) for A23187 stimulated LmCL. The calculated correlation was also significant in the presence of 1.0 mM of cimetidine: r = −0.784 (P < 0.001), −0.850 (P < 0.001), and −0.785 (P < 0.001) for OZ, PMA, and A23187 stimulated LmCL, respectively.

In the present study, we used two chemiluminescent probes and three stimulants. LgCL and LmCL have different luminescence mechanisms, and they have been used in differential measurements for ROS. OZ is a phagocytic particle that binds to receptors existing on the neutrophil cell surface, while PMA directly binds to protein kinase C (PKC) without functional modification at the receptor level. Calcium-ionophore increases calcium concentration in the neutrophils. We used 96-well microplates to measure LgCL and LmCL stimulated with three stimulants at the same time. LgCL mainly detects superoxide anion (O_2^-) produced as a starting substance of ROS metabolism and considered to reflect NADPH-oxidase activity (10). In this study, rebamipide attenuated LgCL stimulated by OZ in a dose-dependent manner. OZ activates membrane-binding phospholipase C (PLC) through G protein by binding to receptors expressed on the neutrophil surface. Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate
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Fig. 1. Effects of rebamipide and cimetidine on LmCL response induced by OZ (A), PMA (B), and calcium ionophore (C). Results obtained in the absence of rebamipide and cimetidine served as the control (100%). Each column represents the mean ± S.D. of the six tests.

Fig. 2. Effects of rebamipide and cimetidine on LgCL response induced by OZ (A), PMA (B), and calcium ionophore (C). Results obtained in the absence of rebamipide and cimetidine served as the control (100%). Each column represents the mean ± S.D. of the six tests.

Table 1. Attenuation of LmCL by rebamipide in the presence of cimetidine

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Cimetidine</th>
<th>Rebamipide (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>0.1 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OZ</td>
<td>100</td>
<td>96.5 ± 16.0</td>
</tr>
<tr>
<td>PMA</td>
<td>100</td>
<td>102.1 ± 11.6</td>
</tr>
<tr>
<td>A23187</td>
<td>100</td>
<td>92.4 ± 18.9</td>
</tr>
<tr>
<td>1.0 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OZ</td>
<td>100</td>
<td>85.1 ± 16.0</td>
</tr>
<tr>
<td>PMA</td>
<td>100</td>
<td>90.4 ± 8.6</td>
</tr>
<tr>
<td>A23187</td>
<td>100</td>
<td>96.3 ± 20.9</td>
</tr>
</tbody>
</table>

Data are expressed as means ± S.D. (%) in the six tests.
(IP3) and 1,2-diacylglycerol (DG). IP3 increases calcium concentration within the cell and DG activates PKC. These two pathways are responsible in the mediation of NADPH-oxidase activation (11). PMA directly binds to PKC and activates NADPH-oxidase, while calcium-ionophore directly increases calcium concentration within the cell. In the present results, rebamipide also had attenuating effects on PMA-stimulated LgCL, but did not affect LgCL stimulated by A23187. These results may indicate that rebamipide modulates the PIP2-PKC pathway without scavenging O2^-·. On the other hand, no effects were observed on LgCL in the presence of cimetidine. Thus, cimetidine does not have scavenging effects on O2^-· and inhibitory effects on production of superoxide.

LmCL mainly reflects hypochlorite (HOCl/OCl^-), hydroxyl radicals (·OH), and O2^-·, and it is considered to reflect myeloperoxidase (MPO) activity caused by degranulation (12). In the present study, attenuating effects of cimetidine on LmCL responses stimulated by the three stimulants were similar (nearly 30%). Cimetidine has been shown to be capable of scavenging hydroxy radical and hypochlorous acid (13). Furthermore, cimetidine does not affect MPO activity (13). Since cimetidine did not affect LgCL, attenuating effects on LmCL might be largely due to scavenging ROS but not by modulating production of ROS. Although attenuating effects of rebamipide were similar in LmCL stimulated by three different stimulants, the attenuating rate was larger than that of cimetidine. Rebamipide has been shown to have scavenging effect on hydroxyl radical (14) and our results might suggest that scavenging effects of rebamipide were larger than those of the same concentration of cimetidine. Another possibility is the inhibitory effect of rebamipide on MPO activity. In previous studies, rebamipide inhibited MPO activity in the gastric mucosa but cimetidine did not (8, 15). Another study also demonstrated that several H2-receptor antagonists did not affect MPO activity (13). Therefore, it was possible that the inhibition of MPO plays a role in the higher attenuating effect of rebamipide on LmCL.

Rebamipide has been shown to suppress fMLP-induced elevation of intracellular ionized calcium concentration (15). However, in the present study, rebamipide did not affect LgCL induced by A23187 and effects on LmCL stimulated by A23187 was similar to that stimulated by OZ or PMA. Therefore, rebamipide does not seem to have particular effects on intracellular ionized calcium concentration. A recent study showed rebamipide suppressed fMLP-stimulated ROS production by inhibiting fMLP receptor competitively (9). This mechanism could explain the suppression of fMLP-induced elevation of intracellular ionized calcium concentration in an earlier study (15) and our observations.

In conclusions, rebamipide, an anti-ulcer agent, has suppressing effects on ROS production of human neutrophils. Rebamipide would have properties other than radical scavenging effects, which was possessed by cimetidine. Although the concentration of rebamipide was high and the attenuating effects were not strong, the results of the present study might explain, at least in part, effects of rebamipide on gastric mucosal oxidative damage. Taking into consideration its mucosal protective properties, addition of rebamipide to H2-receptor antagonist would be useful for the treatment of gastric mucosal lesions, in which oxidative stress seems to play a major role.

References

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