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Prevention by Rebamipide of Acute Reflux Esophagitis in Rats

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Proinflammatory factors, including neutrophil-derived oxygen free radicals and inflammatory cytokines, have recently been implicated in the pathogenesis of reflux esophagitis. Rebamipide has been widely used as an anti-ulcer agent. The aim of the present study was to assess the protective effect of rebamipide against acute reflux esophagitis in rats. Esophagitis was induced in rats by ligation at the limiting ridge and the lower portion of the duodenum. Vehicle or rebamipide were given as a single dose intraduodenally. Lesion index (LI), thiobarbituric acid-reactive substances (TBA-RS), myeloperoxidase (MPO) activity, mRNA and protein of tumor necrosis factor (TNF)-α and cytokine-induced neutrophil chemoattractant (CINC)-1 in the esophageal mucosa were markedly increased; pretreatment with rebamipide, however, significantly reduced both macroscopic and microscopic injuries and increases in inflammatory mediators. The results of this study indicate that rebamipide protects against the occurrence of esophagitis and has highly promising potential as a new therapeutic agent for reflux esophagitis.

KEY WORDS: reflux esophagitis; rebamipide; neutrophils; lipid peroxidation; TNF-α; CINC-1.

Recent studies have demonstrated that reactive oxygen species and cytokine-mediated infiltration of inflammatory cells are involved in the pathogenesis of gastric mucosal injuries induced by ischemia-reperfusion and nonsteroidal anti-inflammatory drugs (1–4). Certain types of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon-gamma (IFN-γ), and interleukin-8 (IL-8), which induce neutrophil accumulation in various tissues, have been implicated in the process of mucosal inflammation (5). Fitzgerald *et al.* (6) recently reported that the expression of mRNAs for proinflammatory cytokines such as IL-1α, IL-8, and IFN-γ was significantly increased in patients with reflux esophagitis. Isomoto *et al.* (7) also reported that the levels of IL-8, MCP-1, and RANTES proteins were significantly higher in the esophageal mucosa of patients with reflux esophagitis than in normal controls. Two of our recent studies indicated that IL-8 mRNA expression of esophageal mucosa in humans significantly correlated with the endoscopic severity of reflux esophagitis and with neutrophil infiltration into the epithelium and lamina propria, and that neutrophil infiltration correlated with the endoscopic severity of reflux esophagitis (8, 9). In addition, it has been shown that mucosal damage in experimental reflux esophagitis is partially mediated by oxygen-derived free radicals (10, 11). Our third study suggests that inflammatory cytokines and neutrophils play an important role in the pathogenesis of experimental reflux esophagitis induced by the reflux of gastroduodenal contents (12). These results suggest that inflammatory changes in the esophageal mucosa, such as cytokine expression, leukocyte infiltration, and

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oxygen-derived free radicals, might be involved in the pathogenesis of esophageal inflammation induced by gastric and/or duodenal content reflux.

Rebamipide {2-(4-chlorobensoylamino)-3-[2(1H)-quinolinone-4-yl] propionic acid} (CAS 11911-87-6) is an anti-ulcer and anti-gastritis agent widely used in Japan. Rebamipide has been reported to both increase endogenous prostaglandin in gastric mucosa (13) and to inhibit the production of oxygen-derived free radicals (14–16) and the adherence of neutrophils to endothelial cells (17). It has been demonstrated that rebamipide prevents various acute experimental gastric mucosal lesions and functions cytoprotectively (18–20). The effect of rebamipide on inflammatory reactions in the esophageal mucosa, however, has not fully been investigated. The present study was designed to assess the protective effect of rebamipide on the experimental model of reflux esophagitis in terms of lipid peroxidation, neutrophil infiltration, and inflammatory cytokines.

MATERIALS AND METHODS

Animals and Agents

Male Wistar rats weighing 190–210 g were obtained from Keari Co. Ltd. (Osaka, Japan), and were housed at 22°C with a 12-hr lighting time and food *ad libitum*. Food was withheld for 24 hr prior to the induction of esophagitis. Drinking water was freely available to the animals up to 2 hr before induction. All animals were kept in raised mesh-bottom cages to prevent coprophagy. Care of the animals and the experimental procedures were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine.

Rebamipide was provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), and for the purpose of this study suspended in 0.5% carboxymethylcellulose (CMC; Sigma Chemical, St. Louis, Missouri).

Preparation of Reflux Esophagitis in Rats

Reflux esophagitis was elicited by the modified method of Nakamura *et al.* (21). In brief, the rats were laparotomized under light ether anesthesia to ligate the duodenum with silk thread at the anal side of the papilla duodeni major. In addition, the limiting ridge (transitional region between the forestomach and corpus of stomach) was ligated in the esophagitis groups. Consequently, the total capacity of the stomach to reduce capacity of stomach to store gastric juice was greatly diminished, resulting in the reflux of gastric juice and duodenal bile juice into the esophagus. The sham group of rats underwent a sham operation with no ligation. The abdomen was closed with suture and the animals were placed in their cages without food and water.

The rats (total $n = 28$) were randomly divided into four groups: the sham-operated group with only 0.5% CMC (the sham group $n = 5$), the esophagitis group with CMC (the control group $n = 9$), the esophagitis group with rebamipide administered at 30 mg/kg (the control + rebamipide group $n = 9$), and the sham-operated group with rebamipide administered at 30 mg/kg (the sham + rebamipide group $n = 5$). Rebamipide or only CMC as a single dose was administered to the distal duodenum beyond papilla using syringe with 27G needle before the close of the abdomen.

Effect of Rebamipide on Reflux Esophagitis Model

Twelve hours after the induction of esophagitis, the animals were euthanized by exsanguination via the abdominal aorta under intraperitoneal urethane anesthesia (Sigma Chemical, St. Louis, Missouri, 1000 mg/kg). The 3 cm of distal (gastric side) esophagus was removed and opened by a longitudinal incision, and intraesophageal mucus was washed out with normal saline. The severity of esophagitis was evaluated by an independent observer who was not informed of the treatment method. The wet weight of the esophagus (3 cm in length) was measured. The total area (mm^2) of lesions that had developed in the esophagus was determined under a dissecting microscope (10×) with a square grid, and graded with the lesion index (LI), as follows (22): 0) no visible lesions; 1) a few erosions; 2) total area of lesions <30 mm^2; 3) total area of lesions 31 mm^2; 4) perforation. Afterward, a portion of each sample was immediately fixed in 10% formalin and the fixed tissue embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H–E) for microscopic study.

Subsequently, the esophageal mucosa was scraped into a tube using two glass slides and was homogenized with 1.5 mL of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl to assess thiobarbituric acid-reactive substances (TBA-RS), tissue-associated myeloperoxidase (MPO) activity, and the levels of two kinds of inflammatory cytokines. TBA-RS, an index of lipid peroxidation, and MPO activity, an index of neutrophil accumulation, were measured by the method of Ohkawa *et al.* (23) and by the method of Krawisz *et al.* (24), respectively.

The esophageal concentration of TNF-α and cytokine-induced neutrophil chemoattractant (CINC)-1, corresponding to IL-8 in humans, were determined with enzyme-linked immunosorbent assay (ELISA) kits (Immuno-Biological Laboratories Co., Ltd, Gunma, Japan). The assay was performed according to the manufacturer's instructions. After color development, optical densities were measured at 450 nm with a microplate reader (MPR A4i, Tosoh, Tokyo). Furthermore, 6 hr after the induction of esophagitis, the expression of mRNA of TNF-α and CINC-1 was assayed with reverse transcription-polymerase chain reaction (RT-PCR) by the same methods as previously described (25, 26). In short, total RNA was isolated from esophageal mucosal tissue with the single-step guanidinium thiocyanate-phenol-chloroform method (Isogen, Nippon Gene, Toyama, Japan). The concentration of RNA was determined by the absorbance at 260 nm relative to that at 280 nm. The isolated RNA was stored at −70°C until use. One microgram of extracted RNA was reverse-transcribed into first-strand complementary DNA (cDNA) at 42°C for 40 min, using 100 U/mL of reverse-transcriptase (Takara Shuzo Co., Shiga, Japan) and 0.1 μM of oligo (dT)-adapter primer (Takara Shuzo Co.) in a 50 μL reaction mixture. An aliquot (1 μL) of the reverse transcriptase product was added to 3 mM primers for TNF-α, CINC-1, and β-actin (the internal standard). PCR was performed using the following specific primers: TNF-α: sense, 5′-ATGAGCACAGAAAGCATGATC-3′ and antisense, 5′-TACAGGCTTGTCACTCGAATT-3′; CINC-1: sense, 5′-ACAGTGGCAGGGATTCACTT-3′ and

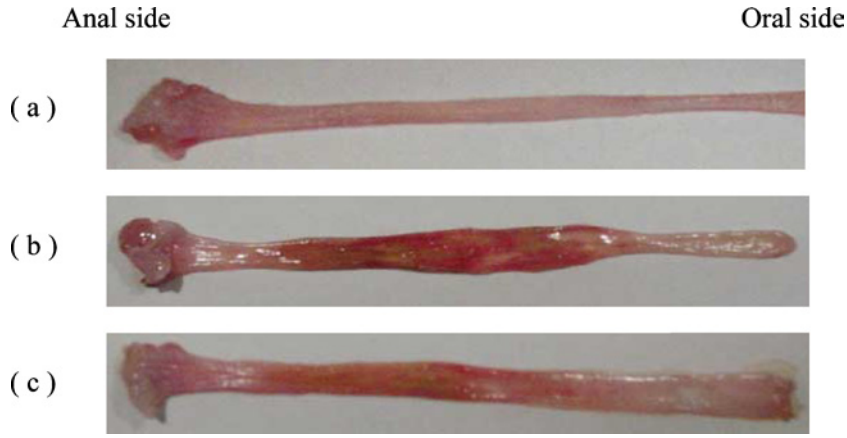


Fig 1. Macroscopic finding of the removed esophagus in (a) the sham group, (b) the control (esophagitis) group, and (c) the rebamipide group. Esophagitis was induced as described in "Materials and Methods." Rebamipide was administered at a single dose of 30 mg/kg from the distal duodenum beyond papilla. Macroscopic findings of the esophagus were estimated 12 hr after the induction of esophagitis. Representative results are shown.

antisense, 5′-CTAGCACAGTGGTTGACACT-3′; β-actin: sense, 5′-TCCTGTGGCATCCATGAAACT-3′ and antisense, 5′-GAAGCATTTGCGGTGCACGAT-3′ (25, 26). The mixture was subjected to 35 cycles (denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60s.) of amplification, followed by a final 72°C extension step for 7 min. The reaction products were separated by electrophoresis in a 2.5% agarose gel and stained with ethidium bromide.

Statistics

The esophageal LI is presented as scatter plots. Differences between groups were compared by analysis of variance (ANOVA) followed by the Mann–Whitney *U*-test, a nonparametric test. Data on wet weight, TBA-RS, MPO activity, and TNF-α and CINC-1 in esophageal tissue are expressed as the mean ± SEM, and were compared using ANOVA followed by Fisher's protected least significant difference test (Fisher's PLSD). A level of $P < 0.05$ was considered statistically significant.

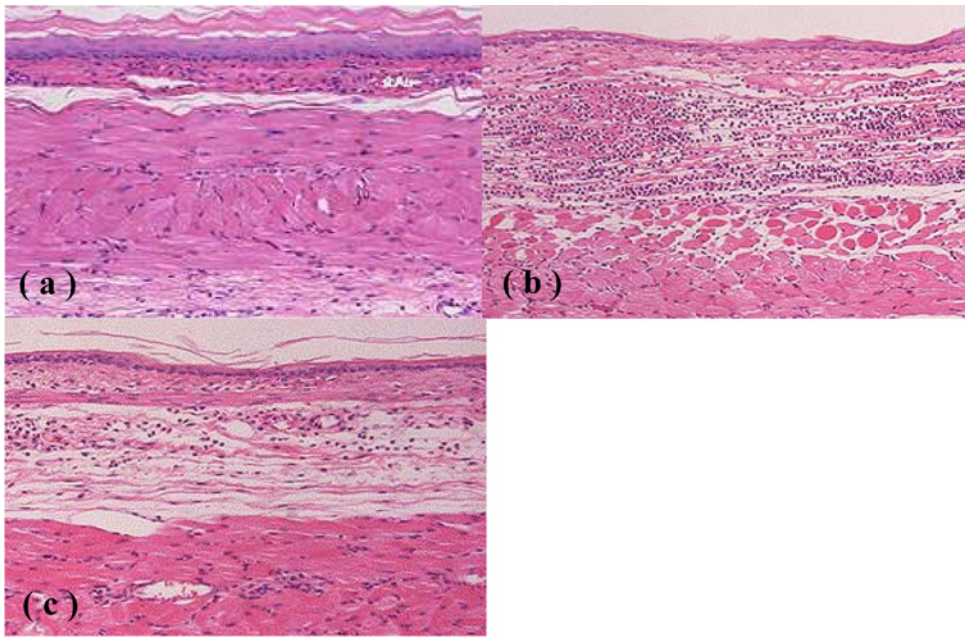


Fig 2. Microscopic finding of the esophagus in (a) the sham group, (b) the control (esophagitis) group, and (c) the rebamipide group. Removed esophagus was immediately fixed in 10% formalin and stained with hematoxylin and eosin (100× magnification). Representative results are shown.

RESULTS

The Assessment of Rebamipide

Gross morphology of the esophagus revealed edema and longitudinal redness of the mucosa and thickening of the wall in the control esophagus; the changes in the control esophagus with rebamipide, however, were less than those in the control esophagus (Figure 1). The histological study with H–E stain showed that, 12 hr after the induction of esophagitis, the control esophagus showed hyperemia of epithelial layers and edema in mucosa and submucosa with the infiltration of numerous inflammatory cells, and especially prominent neutrophils in clusters; these changes were reduced by the administration of rebamipide (Figure 2).

The esophageal LI and wet weight in the control esophagus were significantly higher than in the normal esophagus. Rebamipide significantly inhibited increases in the LI and wet weight (Figure 3a and b). Significant increases in TBA-RS (Figure 4a) and in MPO activity (Figure 4b) were observed in the control esophagus. These increases were significantly suppressed by the administration of rebamipide. Rebamipide itself did not affect the LI, wet weight, TBA-RS and MPO activity.

In the cytokine analysis, the protein levels of TNF-α (Figure 4c) and CINC-1 (Figure 4d) in the esophageal mucosa increased in the control esophagus; again, these increases were significantly inhibited by the administration of rebamipide. The mRNA expression of TNF-α and CINC-1 was strongly detected in the esophageal mucosa of the control esophagus compared with the normal esophagus. In the control esophagus with rebamipide, mRNA expression of these genes was also lower than those in the control esophagus (Figure 5). The protein and mRNA expression of these cytokines were not affected by rebamipide itself.

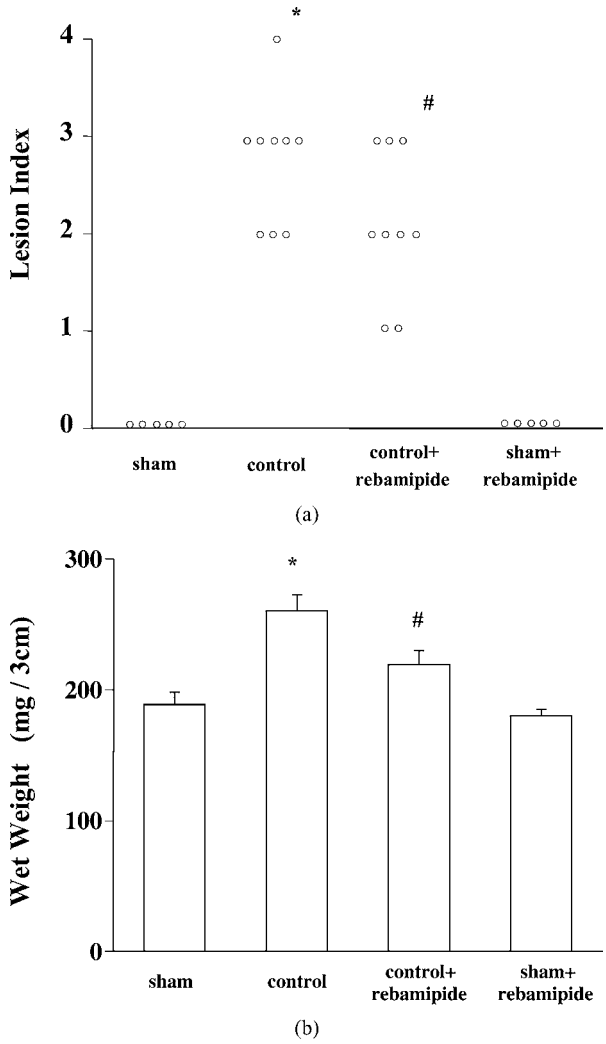


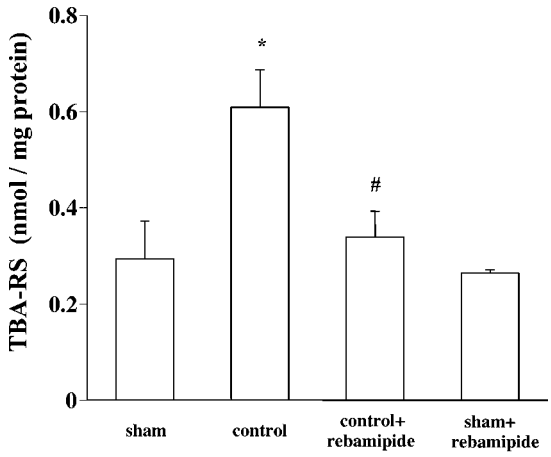
Fig 3. The change of (a) esophageal LI and (b) esophageal wet weight in esophageal mucosa. Esophageal LI was assessed by a 0–4 point scale according to histological change determined under a dissecting microscope (10× magnification). Statistical comparisons between groups were made using Mann–Whitney *U*-test. Esophageal wet weight was measured by the longitudinal length of 3 cm. Data represent the mean ± SEM of 5–9 rats. $^{*}P < 0.01$ when compared to the sham group, $^{\#}P < 0.05$ when compared to the control group.

DISCUSSION

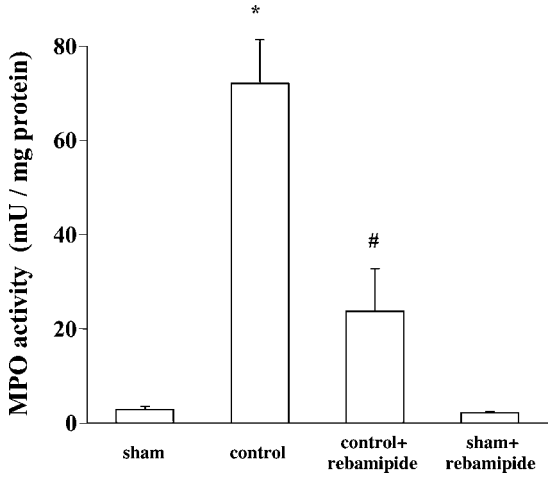
The results of the present study indicate that rebamipide inhibited the increase in MPO activity, TBA-RS, and inflammatory cytokines in the esophageal mucosa, suggesting that rebamipide contributed to the prevention of esophagitis induced by reflux of gastro-duodenal contents.

Rebamipide is an anti-ulcer and anti-gastritis agent with several anti-inflammatory actions: scavenging oxygen-derived free radicals (14–16) and inhibiting the production of oxygen radicals by activated neutrophils (17, 27). In addition, it has been reported that rebamipide reduces the adherence of neutrophils to endothelial cells as well as the CD18 expression on neutrophils (17).

The esophagitis model used in this study has been attributed to activated neutrophils, oxygen-derived free radicals, and inflammatory cytokines (12). In the present study, it has been deemed probable that rebamipide circulating in the blood protects against experimental esophagitis because rebamipide could not directly run back to the esophagus after administration from the distal duodenum beyond papilla. We reported that approximately 300 ng/mL rebamipide could inhibit the neutrophil adherence to endothelial cells via inhibiting CD11/CD18 expression on neutrophils and the production of oxygen-derived free



(a)



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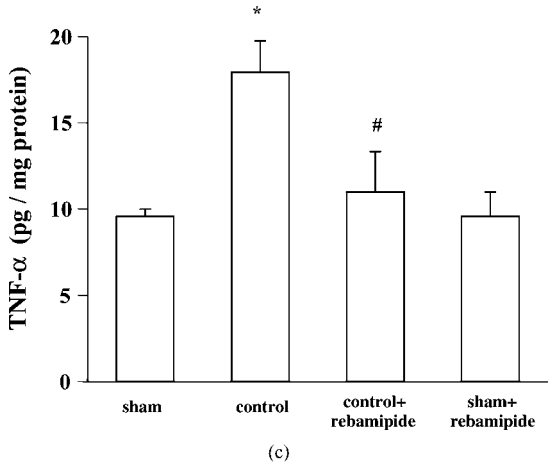
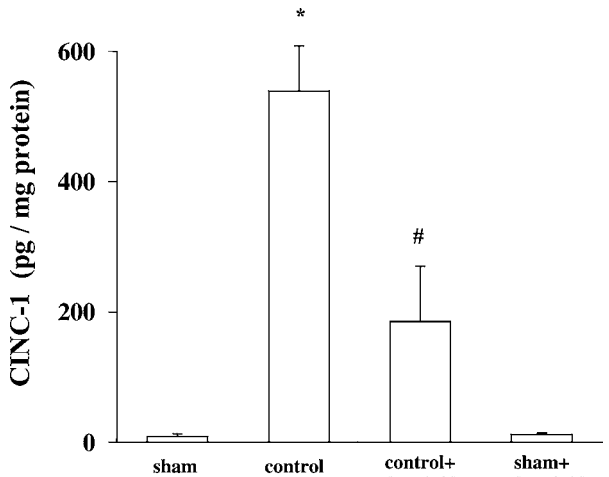


(c)

Fig 4. The change of (a) TBA-RS, (b) MPO activity, (c) TNF-α protein and d) CINC-1 protein in esophageal mucosa. The scratched esophageal mucosa was homogenized, and the samples were used to measure TBA-RS, MPO activity, TNF-α, and CINC-1. Data represent the mean $\pm$ SEM of 5–9 rats. $^{*}P < 0.01$ when compared to the sham group, $^{\#}P < 0.05$ when compared to the control group.



(d)

Fig 4. Continued

radicals from activated neutrophils (17). In the present study, although the concentrations of rebamipide in the esophagus and serum were not measured, it is probable that intraduodenal administration of 30 mg/kg of rebamipide was sufficient to modulate neutrophil functions. In fact, MPO activity, an index of neutrophil infiltration, and the infiltration of numerous inflammatory cells in the esophageal mucosa were decreased by the administration of rebamipide, suggesting that rebamipide was able to inhibit activation of neutrophils.

Recently, several studies on the expression of cytokines in esophagitis have reported that the expression levels of IL-8 mRNA were significantly higher in patients with reflux esophagitis (6), and that the expression of IL-8 mRNA in the esophageal mucosa of patients with gastroesophageal reflux disease was correlated with the endoscopic grade of esophagitis or with inflammatory cell infiltration (9). Interestingly, it has been reported that the expression of cytokines such as IL-1β, TNF-α, MCP-1, MIP-1α, MIP-2, and CINC-2$\alpha$was increased in the early phase of rat chronic esophagitis (28). This acute esophagitis model is considered to be similar to the early phase of chronic esophagitis. In the present experimental esophagitis model the expression of TNF-α and CINC-1 mRNA is detectable in the early phase (3 and 6 hr after the induction of esophagitis), prior to significant increase in LI, wet weight and TBA-RS (12). These data suggest that an early increase in cytokine expression may be implicated in the pathogenesis of reflux esophagitis. In this study, rebamipide reduced the expression of TNF-α and CINC-1 mRNA in the early phase, 6 hr after induction. It is controversial whether the main source of IL-8 in human

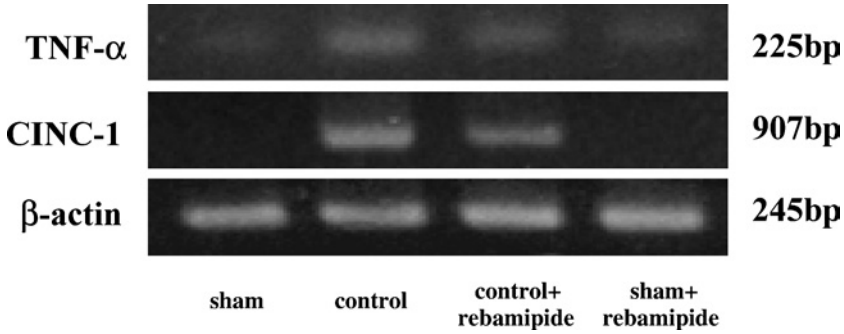


Fig 5. The expressions of TNF-α and CINC-1 mRNA in the esophageal tissues 6 hr after induction of esophagitis. Isolated total RNA from esophageal mucosa was reverse-transcribed into cDNA, which was used in TNF-α, CINC-1 and β-actin PCR. Final products were separated by agarose gel electrophoresis. Representative results are shown.

esophagus comes from neutrophils, endothelial cells, or epithelial cells (9, 29–31). It has been also reported that rebamipide can directly inhibit IL-8 production from cultured gastric epithelial cells. In this experimental model, further examinations need to be made to determine the source of inflammatory cytokines and inhibitory mechanisms of these cytokines by rebamipide.

Oxygen-derived free radicals are known to cause peroxidation of polyunsaturated fatty acids in cell membranes. In this study, TBA-RS (an index of lipid peroxidation that is a sensitive marker of membrane damage caused by free radicals) in the esophageal mucosa increased significantly in the control group. Previous reports indicated that reflux esophagitis was partially mediated by oxygen-derived free radicals (10, 11). A recent study conducted by the authors has demonstrated that lipid peroxidation in the esophageal mucosa is inhibited by anti-neutrophil serum treatment (12). These findings suggest that enhanced lipid peroxidation in the esophageal mucosa is partially associated with neutrophil-derived oxygen free radicals. In addition, it has been shown that administration of free radical scavengers such as superoxide dismutase inhibited the incidence of esophagitis (32–34). Recently it has been reported that antioxidant treatment should be considered as supplementary therapy in the prevention or treatment of reflux esophagitis with acid suppression (35). Our present study also suggests that rebamipide inhibited mucosal lipid peroxidation via the scavenging and inhibition of oxygen-derived free radical production.

In conclusion, the present study indicates that the early increase of cytokine expression, activated neutrophils, and lipid peroxidation derived from neutrophils are involved in the pathogenesis of acute reflux esophagitis. The study also suggests that rebamipide might be used for the prevention and treatment of reflux esophagitis through its anti-oxidant, anti-neutrophil and anti-cytokine effects.

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