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Therapeutic Effect of Rebamipide on Ammonia-induced Gastric Mucosal Hemorrhagic Lesion in Rats

Keun Huh[1], Tae-Hyub Kwon[1], Soo-Kyun Kim[1], Jin-Sook Kim[1] and Uk-Seob Shin[2]

[1]Department of Pharmacology, College of Pharmacy, Yeungnam University, Gyongsan 713-749, [2]Dongkuk Medical Center, Department of Pharmacy, Kyongju 788-714, Korea

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Rebamipide, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinone-4-yl]-propionic acid, a novel antipeptic ulcer agent, has been reported to prevent various acute experimental gastric mucosal lesions and to accelerate the healing of chronic ulcers. Therapeutic effect of rebamipide was investigated with regard to the inhibitory effect on xanthine oxidase activity and type conversion of the enzyme which play a profound role in oxygen radicals generation system. Intraperitoneal administration of rebamipide at 60 mg/kg body weight reduced the xanthine oxidase activity, lipid peroxide content in ammonia induced hemorrhagic lesion. These results suggest that the therapeutic effect of rebamipide on gastric mucosal lesion may be in part due to the inhibitory activity of xanthine oxidase and type conversion rate of the enzyme.

Key words : Rebamipide, Gastric lesion, Ammonia, Xanthine oxidase type conversion, Lipid peroxidation

INTRODUCTION

Recent studies have shown a close relationship between pathophysiological condition infected with *Helicobacter pylori* and various gastrointestinal diseases including gastritis and peptic ulcer (Goodwin *et al.*, 1986; Graham *et al.*, 1986; Hazell *et al.*, 1987; Marshall, 1983; Marshall and Langton, 1986; Mooney *et al.*, 1991; Moris and Nicholson, 1987). Pathological studies have shown that the human gastric juice of *H. pylori*-infected stomach contains high concentration of ammonia that is produced from urea by urease, a hydrolzing enzyme of *H. pylori* (Hazell and Lee, 1986; Marshall and Langton, 1986). According to the many studies on *H. pylori*-infected gastric mucosal lesion, it is widely accepted that gastric ammonia production is a potent ulcerogenic pathogenesis on the stomach and duodenum (Murakami *et al.*, 1993; Seiki *et al.*, 1989; Tsujii *et al.*, 1992).

Rebamipide(2-(4-chlorobenzoylamine)-3-[2(1H)-quinolinone-4-yl]-propionic acid) is a novel antipeptic ulcer agent recently synthesized by OTSUKA pharmaceutical Co. in Japan. However, despite studies of antiulcer effect, little is known about the precise mechanism of pharmacological action of the drug on antiulcerative effect. In the present study we, therefore, examined the pharmacological effect of rebamipide on ammonia-induced gastric mucosal lesion and the relationship between the therapeutic effect of rebamipide on gastric lesion induced by ammonia and inhibitory effect on gastric xanthine oxidase activity and lipid peroxidation.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), nicotinamide adenine dinucleotide sodium salt (NAD^+), sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA) and xanthine sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO). Rebamipide was supplied from Korea Otsuka Pharmaceutical Co. (Seoul, Korea). Other extra pure chemicals were purchased from a reagent commercial company.

Treatment of animals

Male Spraque-Dawley rats weighing 180~210 g were deprived of food but allowed free access to tap water for 24 hours before the experiments in order to reduce the variation of hepatic metabolism and easily induce gastric hemorrhagic lesions. Animals were divided into four groups of 10 rats for each *in vivo* assay. The first group was a control group and the second group was injected with rebamipide (60 mg/Kg, intraperitoneally) for 3 days. The third group was ad-

Correspondence to: Professor Keun Huh, Department of Pharmacology, College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea

ministered a single oral dose of 2.0 ml of 250 mM ammonia solution at the first day for ammonia-induced gastric mucosal damage according to the method of Tsujii *et al.* (1992). The last group was injected rebamipide for 3 days after ammonia solution treatment.

Gastrohemorrhagic lesion index

Gastrohemorrhagic lesion index was measured as follows. Three day after treatment with ammonia solution, rats were anesthetized with ether, then, their stomachs were removed with a midline laparotomy (Williams, 1956). The gastrohemorrhagic lesion index was measured by macroscopically and was expressed as the area of the antral hemorrhagic lesion (mm^2).

Enzyme preparation

Enzymes were prepared from rat stomach. Rats were sacrificed by ether anesthesia. Their stomachs were immediately removed and weighed. After trimming and mincing, the pieces of stomach were homogenized with 4 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) solution. The homogenate was centrifuged at 600×g for 10 min. The pellet was discarded and the supernatant was centrifuged at 10, 000×g for 20 min. The supernatant fraction was further ultracentrifuged at 105,000×g for 60 min. The resultant cytosolic fraction was used as the enzyme source for the xanthine dehydrogenase or xanthine oxidase assays.

Enzyme assay

Xanthine dehydrogenase activity was assayed by measuring, spectrophotometrically, the amount of uric acid formed from xanthine sodium with NAD^+ as a cofactor in the reaction mixture (Della and Stirpe, 1972). Xanthine oxidase activity was aerobically determined by measuring the rates of uric acid formation without NAD^+ in the reaction mixture from xanthine sodium as substrate. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 0.1 ml of enzyme source, 0.06 mM of the substrate and distilled water in a final volume of 4 ml. The reaction was carried out at 37°C for 15 min. The type conversion ratio from xanthine dehydrogenase (type D) to xanthine oxidase (type O) was represented as O/D+O.

Measurement of gastric lipid peroxide content

The content of gastric lipid peroxide was determined using the method of Ohkawa *et al.* (1979). In brief, the reaction mixture contained 0.2 ml of homogenate which was previously described as enzyme preparation above, 8.1% SDS, acetate buffer, and TBA solution were mixed well for 3 min and incubated at 95°C for 60 min. TBA reactive substance, malondialdehyde was extracted with a butanol-pyridine mixture solution. The absorbance measured at 532 nm was expressed as nanomoles of MDA.

Protein assay and statistical analysis

Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. The differences between the experimental groups were analyzed with student's t-test.

RESULTS

Therapeutic effect of rebamipide against ammonia-induced gastrohemorrhagic lesions in rats

The gastrohemorrhagic lesion assessed macroscopically was significantly increased by oral administration of single dose of 250 mM ammonium hydroxide solution. As shown in Table I, the gastrohemorrhagic lesion area is observed as being 45.7±12.6 mm^2 at 3 day after ammonium hydroxide treatment. The control group or those given only rebamipide 60 mg/kg suspended with 0.5% Carboxymethylcellulose (CMC), Intraperitoneal (IP) for 3 days did not reveal and gastric mucosal lesions. Rebamipide treatment markedly reduced the development area of gastric mucosal hemorrhagic lesions caused by ammonium hydroxide solution (20.2±5.3 mm^2).

Effect of rebamipide on MDA production in gastric mucosa in ammonia- treated rats

The effect of rebamipide on lipid peroxidation of gastric tissue in control and ammonium hydroxide solution-treated animals are shown in Fig. 1. The mean level of rebamipide alone was not significantly different from that observed in control animals. However, MDA level in the ammonia-treated animals were significantly increased as compared with that in the saline-treated animals (4.20±0.12 nmoles/mg protein).

Table. I. Therapeutic effect of rebamipide against ammonia-induced gastrohemorrhagic lesion in rats

	Number of rats	Gastrohemorrhagic lesion score (mm^2)	Inhibition ratio of injury (%)
Control	10	6.0±1.0	-
Ammonia	10	45.7±12.5	0
Rebamipide	10	5.8±1.6	-
Rebamipide+ Ammonia	10	20.2±5.3***a	35.8

Ammonia solution (250 mM, p.o.) treated rats were injected to the rebamipide (60 mg/kg, i.p.) for 3 days and sacrified 1 hr later from last treatment. The assay procedure was described in the Materials and Methods. Values are expressed as mean±S.E. for 10 animals. a, represent significantly difference compared to ammonia treated group (***: $P<0.001$).

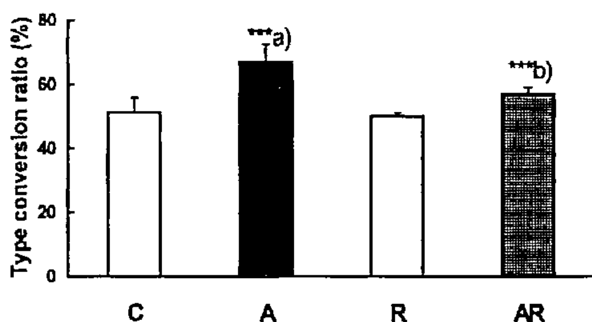

Fig. 1. Effect of rebamipide on gastric lipid peroxidation in ammonia-treated rats. C: control, A: ammonia (250 mM, p.o.), R: rebamipide (60 mg/kg, i.p.), AR: rebamipide+ammonia-treated group. a) represent significantly difference compared to the control group (***: P<0.001). b) represent significantly difference compared to ammonia treated group (***: P<0.001).

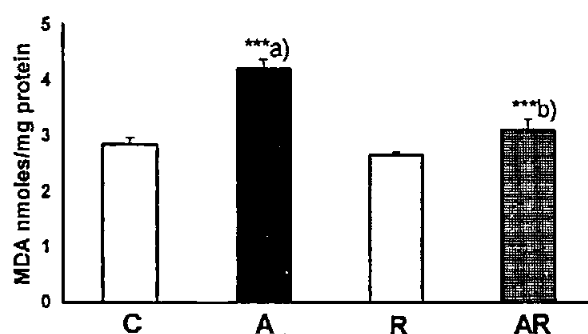

Fig. 2. Effect of rebamipide on type conversion of gastric xanthine oxidase in ammonia-treated rats. C: control, A: ammonia (250 mM, p.o.), R: rebamipide (60 mg/kg, i.p.), AR: rebamipide+ammonia-treated group. a) represent significantly difference compared to the control group (***: P<0.001). b) represent significantly difference compared to ammonia treated group (***: P<0.001).

The MDA level in the rebamipide treated animals (2.41±0.30 nmoles/mg protein) was markedly lower than the only ammonium hydroxide solution treated group.

Inhibitory effect of rebamipide on the gastric xanthine oxidase activity in ammonia-treated rats

Inhibitory effect of rebamipide on the gastric xanthine oxidase and dehydrogenase activity in ammonia-treated rats are summarized in Table II. The xanthine oxidase activities in stomach tissue shows 0.209± 0.012 uric acid nmoles/mg protein/min in control animals. The enzyme activity was slightly decreased by the administration of rebamipide, which was not significantly different from that observed in vehicle group. When ammonium hydroxide solution was treated to the animal, the xanthine oxidase (type O) activity was significantly increased to 0.281±0.049 uric acid nmoles/mg protein/min. Whereas, in rebamipide treated animals the ammonium hydroxide-induced increase of enzyme activity was significantly reduced to control levels. On the other hand, in each group, such as the rebamipide-treated, ammonium hydroxide-treated, or rebamipide and ammonium hydroxide-treated group, xanthine dehydrogenase (type D) activity was not significantly affected. These results suggested that rebamipide inhibits selectively the xanthine oxidase activity induced by ammonia.

Table II. Effect of rebamipide on gastric xanthine oxidase and dehydrogenase activity in ammonia-treated rats

	Specific	Activity[a]
	Xanthine oxidase (O)	Xanthine dehydrogenase (D)
Control	0.209±0.012	0.199±0.015
Ammonia	$0.281\pm0.049^{**b)}$	0.190±0.007
Rebamipide	0.196±0.012	0.196±0.021
Rebamipide+ Ammonia	$0.230\pm0.024^{*c)}$	0.184±0.024

a) Uric acid n moles/mg protein/min, b) represent significantly difference compared to the control group (**:P<0.01), c) represent significantly difference compared to ammonia treated group (*:P<0.05).

Effect of rebamipide on the type conversion of gastric xanthine oxidase in ammonia-treated rats

Natural xanthine oxidizing enzymes exist mainly xanthine dehydrogenase (type D) in biological conditions. It is postulated that endogenous xanthine dehydrogenase is converted to oxygen radical producing xanthine oxidase (type O) during the oxidative stress. As shown in Fig. 2, no significant differences were observed in the type conversion ratio between in saline-treated group and only rebamipide treated group (51% and 50%). On the other hand, the type conversion ratio of xanthine oxidase in ammonia-treated animals showed about 67% which is 1.31 fold higher than that of control animals. Treatment with rebamipide after ammonia administration significantly decreased the type conversion ratio as compared to that of ammonium hydroxide treated animals. These results suggested that the type conversion of xanthine oxidase may be related with gastrohemorrhagic lesions by ammonium hydroxide.

DISCUSSION

It has been well known that *H. pylori* infection, a prevalent human-specific pathogen, is a causative agent in chronic active gastritis (Goodwin *et al.*, 1986; Graham *et al.*, 1986), gastric and duodenal ulcer (Hazell *et al.*, 1987; Moris and Nicholson, 1987). Recent studies have shown a close relationship between gastroduodenal diseases and the level of ammonia

produced by *H. Pylori* (Seiki *et al.*, 1989; Murakami *et al.*, 1986; Murakami *et al.*, 1987).

It is widely accepted that lipid peroxidation by oxygen radical may also play an important role in various type of tissue damages (Joseph *et al.*, 1990; Kusterer *et al.*, 1987). Xanthine oxidase acts as a major factor of oxygen free radicals generation and induces oxidative stress by increasing the lipid peroxidation in gastrointestinal tract (Granger *et al.*, 1981; Janasch *et al.*, 1981; Parks and Granger, 1983). Xanthine oxidase catalyses the reaction in which hypoxanthine is converted to xanthine and then to uric acid. Naturally, under normal conditions the xanthine-utilizing enzyme exists predominantly as xanthine dehydrogenase (type D), which utilizes NAD^+ as an electron acceptor and produces NADH (Royd and Mccord, 1983). It is demonstrated that the enzymes would be converted from dehydrogenase to oxidase form (type D→type O) during either ischemia or reperfusion (Granger *et al.*, 1981; Mccord and Roy, 1982; Roy and Mccord, 1982), which rather utilizes O_2 as an electron accepter generating superoxide anion radicals. Recent studies indicate that an important series of events occur at the onset of gastrointestinal ischemia which, in the stomach, may make xanthine oxidase an important source of oxygen radicals which in turn might contribute to ischemia induced injury (Parks and Granger, 1983).

The results of present experiment suggest that the phenomena of increasing xanthine oxidase activity resulted from type conversion of the enzyme may play in pathogenesis of ammonia-induced gastrohemorragic lesions. Rebamipide significantly reduced the gastrohemorrhagic lesions as well as the lipid peroxide content and decreased the xanthine oxidase activity in gastric tissue of rats in ammonia-induced hemorrhagic lesions. These results suggest that rebamipide has a therapeutic effect on gastrohemorrhagic lesions by inhibiting lipid peroxidation reaction of polyunsaturated fatty acid in cell membrane ascribed to reducing xanthine oxidase activity. In the previous study from this laboratory, it was observed that the rebamipide has a preventive effect against ethanol-induced gastric mucosal hemorrhagic lesion by inhibit the xanthine oxidase activity in rats. (Huh *et al.*, 1996). Taken together the previous and present results, we conclude that the inhibitory effect on the enzyme activity and type conversion of xanthine oxidase may partially result in the therapeutic effect of rebamipide on ammonia-induced gastric hemorrhagic lesion. We have observed that the increment of type conversion from xanthine dehydrogenase to oxidase by ammonia was significantly inhibited by rebamipide injection. Recently, Kuppusamy and Zweier (1989) reported that direct production of hydroxyl radical by xanthine oxidase in a cell and tissue induced the oxidative cellular damage, which is not prevented by superoxide dismutase. Based upon this, the antiulcer effect of rebamipide may be connected to inhibition of the xanthine oxidase activity and type conversion the enzyme which relating to the generation of reactive oxygen species in ammonia-induced gastric mucosal hemorrhagic lesions.

Based on all the results, it is concluded that rebamipide may exert a therapeutic effect on ammonia-induced gastric mucosal lesions through following two ways that rebamipide decreases the gastric lipid peroxide level, inhibits the type conversion of xanthine oxidase and the enzyme activity in gastric stomach tissue. However, further studies are required to establish the effectiveness of rebamipide in artificially induced antral ulcer and to define the mechanism by which the production of active oxygen species leads to morphological and functional injury to the gastric mucosa.

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REFERENCES CITED

Della Corte, E. and Stirpe, F., The regulation of rat liver xanthine oxidase, Involvement of thio groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme. *Biochem. J.*, 126, 739-749 (1972).

Goodwin, C. S., Amstrong, J. A. and Marshall, B. J., *Campylobacter pyloridis*, gastritis and peptic ulceration. *J. Clin. Pathol.*, 39, 353-365 (1986).

Graham, D. Y., Klein, P. D., Evans, D. J., Alpert, L. C., Opekun, A. R. and Boutton, T. W., *Campylobactor pylori* detected noninvasively by the ^{13}C-urea breath teat. *Lancet*, 1, 1174-1177 (1987).

Granger, D. N., Rutili, G. and Mccord, J. M., Superoxide radicals and feline intestinal ischemia. *Gastroenterol.*, 81(1), 22-29 (1981).

Hazell, S. L., Hennessy, W. B., Borody, T. J., Carrick, J., Ralston, M., Brady, L. and Lee, A., *Campylobacter pyloridis* gastritis II: Distribution of bacteria and associated inflammation in the gastroduodenal environment. *Am. J. Gastroenterol.*, 82, 297-301 (1987).

Huh, K., Shin, U. S. and Lee, S. H., The effect of rebamipide on gastric xanthine oxidase activity and type conversion in ethanol-treated rats. *Free. Radic. Biol. Med.*, 20, 967-971 (1996).

Janasch, E. D., Graund, C., Bruder, G., Heid, H. W., Keenan, T. W. and Franke W. W., Localization of xanthine oxidase in mammary gland epithelium

and capillary endothelium. *Cell*, 25, 67-82 (1981).
Joseph, L., Skibba, T. H., Stadnlcka, A., Powers, R. H. and Kalbfleisch, J.H., Lipid peroxidation caused by hyperthemic perfusion of rat liver. *Biochem. Pharmacol.*, 40, 1411-1414 (1990).
Kusterer, K., Pihan, G. and Szabo, S., Role of lipid peroxidation in gastric mucosal lesions induced by HCl, NaOH or ischemia. *Am. J. Physiol.*, 252, G 811-816 (1987).
Kuppusamy, P and Zweier, J. L., Characterization of free radical generation by xanthine oxidase. *J. Biol. Chem.*, 264, 9880-9884 (1989).
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).
Marshall, B. J., Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet*, 1, 1273-1275 (1983).
Marshall, B. J. and Langton, S. R., Urea hydrolysis in patients with *Campylobacter pyloridis* infection. *Lancet*, 1, 965-966 (1986).
Mccord, J. M and Roy, R. S., The pathophysiology of superoxide, roles in inflammation and ischemia. *Can. J. Physiol. Pharmacol.*, 60, 1346-1352 (1982).
Mooney, C., Keenan, J., Munster, D., Wilson, I., Allardyce, R. and Bagshaw, P., Neutrophil activation by *Helicobactor pylori. Gut.*, 32, 853-857 (1991).
Moris, A. and Nicholson, G., Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. *Am. J. Gastroenterol.*, 82, 192-199 (1987).
Murakami, M., Mizuno, M., Ashida, Y., Saita, H., Hozu, S., Inoue, R., Inada, M. and Miyke, T., Effect of prostaglandin on NH_3-induced gastric mucosal lesions (abstr). *Gastroenterol.*, 90, 1560 (1986).
Murakami, M., Yoo, J. K., Mizuno, M., Saita, H., Inada, M. and Miyke, T., Effects of ammonia, urea and urease on the rat gastric mucosa (abstr). *Gastroenterol.*, 97, 1544 (1987).
Murakami, M., Saita, H., Teramura, S., Dekigai, H., Asagoe, K., Kusaka, S. and Kita, T., Gastric ammonia has a potent ulcerogenic action on the rat stomach. *Gastroenterol.*, 105, 1710-1715 (1993).
Ohkawa, H., Ohishi, H. and Yagi, K., Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.*, 95, 351-358 (1979).
Parks, D. A. and Granger, D. N., Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. *Am. J. Physiol.*, 245, G285-289 (1983).
Roy, R. S. and Mccord, J. M., Conversion of xanthine dehydrogenase to xanthine oxidase in oxy radicals and their scavenger system, in Greenwald, R. and Cohn, G. (eds.). *Cellular and Molecular Aspects*, Elsevier science, New York, p.145 (1983).
Roy, R. and Mccord, J. M., Ischemia-induced conversion of xanthine dehydrogenase to xanthine oxidase (abstr). *Fed. Proc.*, 41, 767 (1982).
Seiki, M., Murakami, M., Yoo, T. K., Teranuma, S., Saita, H. and Inada, M., Effects of ammonia on the gastric mucosal barrier in rats and dogs. *Jpn. J. Pharmacol.*, 51, 143-145 (1989).
Tsujii, M., Kawano, S., Tsuji, S., Fusamoto, H., Kamada, T. and Sato, N., Mechanism of gastric mucosal damage induced by ammonia. *Gastroenterol.*, 102, 1881-1888 (1992).
Williams, A.W., Effect of alcohol on gastric mucosa. *Br. Med. J.*, 1, 256-259 (1956).