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ALTERATION IN GASTRIC MUCOSAL ACID PROTEASE ACTIVITY INDUCED BY NECROTIZING AGENTS AND PREVENTION BY PROSTAGLANDIN E_2

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Tissue levels of two gastric mucosal acid proteases, pepsinogen and cathepsin D-like acid proteinase, were determined in rat gastric mucosa damaged by various necrotizing agents and the protective effects of prostaglandins against these biological alterations were investigated. Gastric mucosal damage by each necrotizing agent used was associated with a marked decrease in tissue level of cathepsin D-like acid proteinase. Particularly, ethanol ingestion caused its significant reduction parallel to the production of gastric lesions in a time-dependent manner. On the other hand, mucosal pepsinogen level increased markedly only in ethanol-damaged gastric mucosa, indicating that this change was mediated by a different mechanism from that for cathepsin D-like enzyme. In rats pretreated with prostaglandin E_2 and prostaglandin inducers before ethanol administration, these biological alterations of two enzymes were effectively prevented as were gastric lesions. However, ethanol ingestion caused these changes to occur to the same degree in both the necrotic and non-necrotic areas of glandular mucosa. It was considered that cathepsin D-like acid proteinase was released from damaged gastric mucosa through a direct action on cellular membrane different from vasoconstrictor and platelet aggregating actions mediated by arachidonic acid metabolites.

Keywords —cathepsin D-like acid proteinase; prostaglandin E_2; pepsinogen; gastric lesion; cytoprotection; gastric mucosa; acid protease

INTRODUCTION

Robert *et al.* [1] have reported that various necrotizing agents such as ethanol, HCl, NaOH, hypertonic NaCl and boiling water produce severe gastric lesions when administered orally to rats. Such gastric damage is effectively protected by pretreatment with prostaglandins (PGs) and this effect is called cytoprotection. However, at present, this phenomenon is discussed only in the context of occurence of gastric lesions detected macroscopically as necrotic bands. Biological changes besides lesion formation in damaged gastric mucosa have not yet been well characterized in such conditions. Recently, some papers have described the biological and/or biochemical alterations which are observed in gastric mucosa damaged by various necrotizing agents. Miller *et al.* [2] reported that ethanol exposure depressed tissue levels of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein in gastric glandular mucosa. Mózsik *et al.* [3] described marked changes in tissue levels of superoxide dismutase in damaged gastric mucosa by several irritants. Whittle and Steel[4] also reported intraluminal release of cytoplasmic and lysosomal enzymes from ethanol-damaged stomach *in vitro*. However, acid proteases in damaged gastric mucosa have not been well characterized. A possibe contribution of gastric mucosal acid proteases in producing and/or augmenting gastric ulcerations has been demonstrated by some groups.[5–7] We have isolated and characterized a unique acid protease named cathepsin D-like acid proteinase (CDLAP)[8,9] from rat gastric mucosa as well as two pepsinogens[10,11] and a gastric cathepsin D.[9] This new enzyme has properties apparently distinct from other well-known acid proteases and is a type of lysosomal enzyme, judging from its characteristics including intracellular distribution and similarity to splenic enzyme. Its physiological role in rat gastric mucosa is not yet elucidated. In this study, we investigated its biological alteration in tissue level following intragastric application of various necrotizing agents and compared it with that of the major gastric acid protease, pepsinogen. The effect of PGs to prevent these alterations is described and its mechanism is also discussed.

MATERIALS AND METHODS

Materials —Male Wistar rats weighing

about 200 g were obtained from Saitama Laboratory Animals Supply (Saitama, Japan). Bovine hemoglobin and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (U.S.A.) and DEAE-Sepharose CL-6B was from Pharmacia (Sweden). Teprenone (6,10,14,18-tetramethyl-5,9,13,17-nonadecatetraen-2-one, Eisai Co., Tokyo) was prepared from its granule by solvent extraction. PGE_2 was purchased from Funakoshi (Tokyo). Other reagents used were of analytical grade.

Gastric Lesion — Rats were fasted for 24 h in individual wire bottom cages and water was removed 19 h before the experiment. Gastric lesions were produced by the method of Robert *et al.*[1] One milliliter of absolute ethanol (99.5%), 0.6 N HCl, 0.2 N NaOH, 25% HCl or saline was given orally. Animals were killed by ether anesthesia 1 h later or at various times after this treatment. Their stomachs were quickly removed, opened along the greater curvature and rinsed in ice-cold saline. After removing excess water and mucus with a soft tissue, the mucosa was laid flat on a filter paper and examined for the presence of necrotic lesions. The length of each lesion was measured in millimeters and the total length of the lesions was recorded as the ulcer index.

Enzyme Assay — The glandular portion of each rat stomach was easily separated from non-glandular forestomach by cutting along the limiting ridge. Since acid proteases characteristically distributed in the mucosal layer of the glandular area compared to the muscular layer,[8] the whole glandular tissue was used for preparation of mucosal extract. Tissue sample was homogenized in a high speed homogenizer with 5 ml of 0.05 M sodium phosphate buffer (pH 7.3). After centrifuging at 10000 × ***g*** for 10 min, the supernatant fraction was determined for pepsinogen and CDLAP activities. Both activities were assayed using 2% hemoglobin (pH 1.8) as substrate according to our methods.[8-10] Briefly, pepsinogen activity which accounts for more than 90% of total enzyme activity of gastric mucosal homogenate was determined directly using a small amount of enzyme, while CDLAP, comprising less than 10%, was assayed after 10 min-preincubation in the presence of 4 M urea to inactivate pepsinogen, pepsin and cathepsin D[9] using a larger amount of enzyme. These enzyme activities were calculated by subtracting their blank values from gross values and expressed in units (U). One unit is an amount of the enzyme which produces an absorbance of 1.0 per minute at 37 °C. Protein concentration was determined by the method of Lowry *et al.*[12] using bovine serum albumin as standard.

DEAE-Sepharose Column Chromatography — The gastric mucosal extract containing the same amount of protein determined by Folin-Lowry method was individually applied to a column (1.5 × 12 cm) of DEAE-Sepharose CL-6B which was previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.3). After washing the column with the same buffer, elution was carried out with a linear gradient of NaCl from 0 to 0.6 M. Acid protease activity was determined with 2% hemoglobin (pH 1.8) at 37 °C for 10 min.

Effect of PGE_2 and PG Inducers — PGE_2 dissolved in saline or 20% ethanol as a PG inducer[13] was given orally 15 min before the administration of absolute ethanol. Teprenone which was suspended in 0.25% carboxymethylcellulose was administered orally 30 min before ethanol ingestion. Rats were killed 1 h later and stomachs were excised. The severity of gastric lesions and the acid protease activity of the mucosal extract were determined as described above.

Enzyme Activity in Two Regions of Damaged Gastric Mucosa — Absolute ethanol (1 ml) was given orally and 1 h later rats were killed. After measurement of the ulcer index of each stomach, the glandular mucosa was divided into two regions by cutting along the necrotic bands. One was a necrotic area with dark red color and the other was a non-necrotic area with normal color seen macroscopically. After homogenizing them individually, two enzyme activities were assayed.

Statistical Analysis — Statistical differences were evaluated using the Student's *t*-test.

RESULTS

Acid Protease Activity in Gastric Mucosa Damaged by Necrotizing Agents

All necrotizing agents used in this study produced severe gastric damage characterized by dark red-colored necrosis. As shown in Table I, the two acid protease levels in damaged gastric mucosa were markedly altered after the rats were treated with the agents. CDLAP activity was significantly decreased by gastric ingestion of the agents tested, with the greatest decrease

TABLE I. *Enzyme Activity in Rat Gastric Mucosa Damaged by Various Necrotizing Agents*

Treatment	No. of rats	Ulcer index	Protein (mg)	Pepsinogen activity (U)	CDLAP activity (U)
Saline	6	–	40.55±1.50	15.70±0.21	1.44±0.01
Ethanol	7	64.4±5.3	42.06±2.32	29.32±1.70[a)]	0.64±0.05[a)]
0.6 N HCl	7	25.6±4.5	37.39±3.32	16.82±1.22	1.08±0.08[a)]
0.2 N NaOH	7	42.7±6.1	41.25±1.83	18.19±2.50	1.18±0.06[a)]
25% NaCl	6	N.D.	37.72±2.59	15.73±1.48	1.08±0.08[a)]

One ml of necrotizing agents was given to each rat and the rats were killed 1 h later. Pepsinogen and CDLAP activities were determined using hemoglobin (pH 1.8) in the absence and presence of 4 M urea, respectively, as described in Materials and Methods. Each value represents mean ± S.E. Significantly different from saline-treated group, a) $p < 0.01$. N.D., not determined.

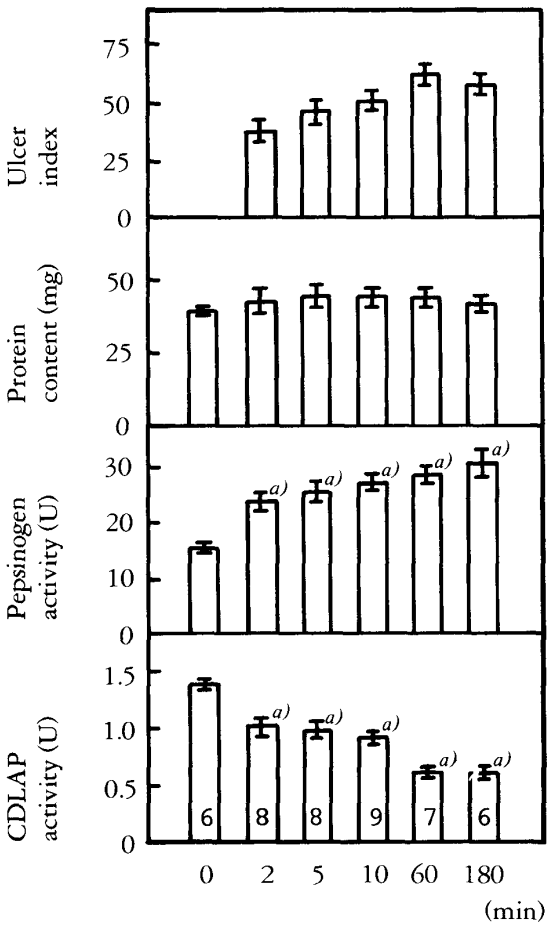

FIG. 1. *Alterations in Ulcer Index, Protein Content and Acid Protease Activities in Ethanol-Damaged Gastric Mucosa*

Rats were given orally 1 ml of ethanol and killed at the indicated time. Control group was not treated with ethanol. Each result is the mean ± S.E. of n experiments shown in the column. Significantly different from control group, a) $p < 0.01$.

obtained with ethanol. In contrast, pepsinogen activity showed a 2-fold increase in the mucosal level with ethanol treatment, whereas there was no effect from the other three treatments. Since the particle fractions obtained after centrifugation of the mucosal homogenates also showed the same patterns for both enzymes, these values reflected their mucosal levels. No significant difference in protein contents was found after these treatments.

Time-Dependent Alteration in Acid Protease Activity in Ethanol-Damaged Gastric Mucosa

Figure 1 shows the time-dependent changes in ulcer index, protein content and two acid protease activities after ethanol ingestion. Gastric lesions were produced just 2 min after oral administration of ethanol and their severity increased during the experimental period for 1 h. CDLAP level in the mucosa showed a significant and rapid decrease at 2 min after ethanol exposure and the decrease continued for 3 h. Contrary to CDLAP, a significant time-dependent increase was observed for pepsinogen, but the protein content was nearly constant during the experimental period.

Elution Profile of Gastric Mucosal Extract on DEAE-Sepharose Column

Each gastric mucosal extract (125 mg of protein) from rats that received orally 1 ml of saline or ethanol was chromatographed on a DEAE-Sepharose CL-6B column under the same elution conditions. As illustrated in Fig. 2, the elution profile of the extract from ethanol-treated rats was different from that of normal rats. The peak of CDLAP activity became smaller after ethanol treatment, whereas pepsinogen was increased

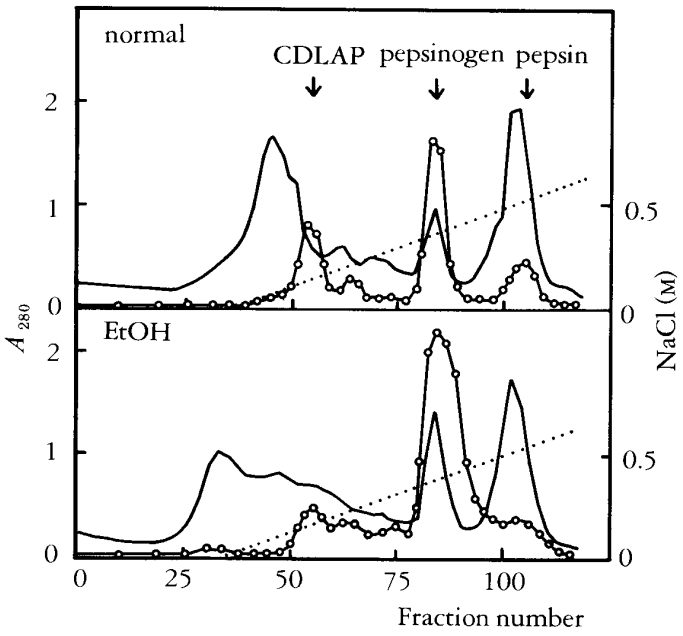

FIG. 2. *Elution Profiles of Gastric Mucosal Extracts from Normal and Ethanol-Treated Rats on DEAE-Sepharose Column*

Each gastric mucosal extract containing the same amount of protein (125 mg) was applied to a DEAE-Sepharose CL-6B column (1.6 × 12 cm) and eluted under the same elution conditions. Protein concentration (——) was monitored at 280 nm and protease activity (○—○) was determined by measuring the absorbance at 280 nm of trichroloacetic acid-soluble product after digestion of hemoglobin at pH 1.8.

quantitatively. However, the gastric mucosal pepsin content, which suggests an intramucosal activation of pepsinogen to its active form, was not affected by this treatment.

Effect of PGE_2 and PG Inducers on the Alteration in Gastric Mucosal Acid Protease Levels Induced by Ethanol

Table II demonstrates the preventive effect of PGs against gastric damage and biological changes induced by ethanol. Pretreatment with PGE_2 (200 μg/kg) or 20% ethanol (2 ml/kg) significantly prevented not only gastric lesion formation but also alterations in the two acid protease levels in gastric mucosa. Pretreatment with an antiulcer drug, teprenone, also showed a similar protective effect against gastric lesion formation and changes in mucosal enzyme levels. Teprenone strongly prevented the release of CDLAP after ethanol treatment.

Acid Protease Activity in Two Regions of Ethanol-Damaged Mucosa

Ethanol-damaged stomachs with an ulcer index of more than 50 were used in this experiment. The damaged areas of necrosis detected as thick black or red bands were separated from the remaining tissues with no macroscopic change. Figure 3 shows the enzyme activity of mucosal extract prepared from each region which was compared with that of normal rat stomach. The reduction of the specific activity of CDLAP was observed to occur to the same degree in both regions of ethanol-damaged gastric mucosa and these values were nearly one-half of the control. No significance was found between these two values. On the other hand, pepsinogen activity increased significantly in both areas but there was no significant difference between them.

TABLE II. *Effect of PGE_2 and PG Inducer on the Alterations in Gastric Mucosal Enzyme Activity Induced by Ethanol*

Pretreatment	No. of rats	Ulcer index	Protein (mg)	Pepsinogen activity (U)	CDLAP activity (U)
Saline	8	64.1 ± 6.9	42.64 ± 2.40	32.33 ± 1.24	0.89 ± 0.06
PGE_2	9	7.3 ± 2.0[a]	42.68 ± 2.50	23.41 ± 1.45[a]	1.31 ± 0.06[a]
20% ethanol	8	10.9 ± 3.2[a]	43.65 ± 1.79	22.62 ± 1.55[a]	1.34 ± 0.04[a]
Vehicle	9	46.4 ± 5.6	44.20 ± 3.22	35.76 ± 2.00	1.02 ± 0.06
Teprenone	8	7.0 ± 1.7[b]	44.60 ± 2.85	28.62 ± 0.82[b]	1.37 ± 0.04[b]
Normal rat	6	—	40.55 ± 1.55	15.70 ± 0.21	1.44 ± 0.01

PGE_2 (200 μg/kg), 20% ethanol and saline were given orally, 2 ml/kg, 15 min prior to ethanol ingestion. Teprenone (250 mg/kg) and vehicle (0.25% carboxymethylcellulose) were given orally, 2 ml/kg, 30 min before ethanol ingestion. Rats were killed 1 h after ethanol application. Each value represents mean ± S.E. Significantly different from saline-pretreated group, a) $p < 0.01$. Significantly different from vehicle-pretreated group, b) $p < 0.01$.

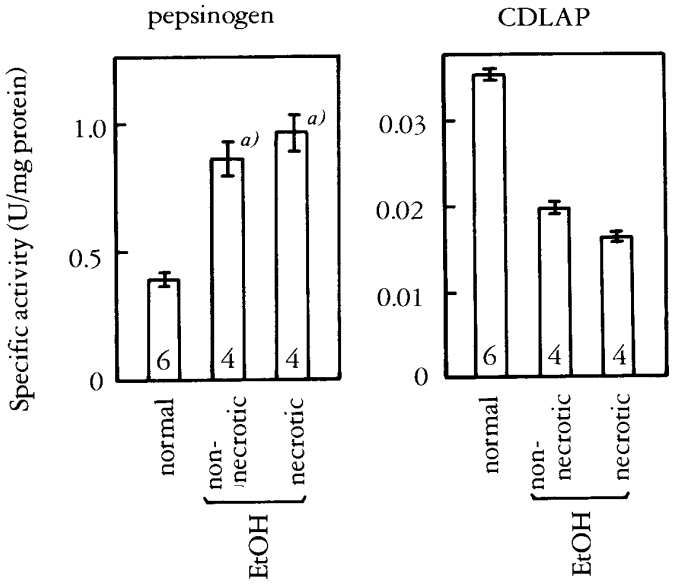

FIG. 3. *Comparison of Acid Protease Activity in Normal and Ethanol-Damaged Gastric Mucosa*
Each result is the mean ± S.E. of n experiments noted in the column. Significantly different from normal group, a) $p < 0.01$.

DISCUSSION

In this study, we found that a new type of acid protease, CDLAP, was significantly decreased in the gastric mucosal level by administration of the necrotizing agents tested. This decrease was observed just 2 min after ethanol treatment as was the production of gastric lesions, showing a very rapid biological process. It was apparently correlated with the development of gastric mucosal damage by ethanol. The tissue damage consisting of necrosis is considered to be due mainly to the potent vasoconstrictor and platelet aggregating actions mediated by endogenous substances. Whittle *et al.* [14] and Smith *et al.* [15] reported that thromboxane A_2 promoted tissue damage in the gastric mucosa and myocardial tissue, respectively, through its potent microcirculation-disordering effect. On the other hand, Peskar *et al.* [16] described recently that leukotriene C_4 was generated in ethanol-damaged gastric mucosa and was involved in the pathogenesis of gastric damage. Although these substances can induce microcirculatory disorders in several tissues, they may exert another direct action on cellular membrane. Indeed, ethanol application stimulated the *in vitro* release of intracellular enzymes from rat gastric mucosa[4] and stable thromboxane analogue reduced cathepsin D level in myocardial tissue.[15] Recently, Pendleton *et al.* [17] have described leukotriene-induced pepsin secretion from gastric glands. From these findings, we can consider that CDLAP was also released from the gastric mucosa by a direct cytolytic action mediated by such endogenous substances.

We observed the relationship between the severity of gastric lesions produced and the degree of the reduction of CDLAP level in the gastric mucosa. The reduction of CDLAP was, however, observed in both the necrotic and non-necrotic areas. Consequently, this evidence indicated that this enzyme could not be one of the major endogenous necrotizing substances in rat gastric mucosa. A similar result for lysosomal cathepsin D which was reduced in both ischemic and non-ischemic areas in cats with coronary occlusion was described by Smith *et al.* [15] The release of such enzymes may be one of the accompanying biological changes occurring in damaged gastric mucosa. However, Watanabe *et al.* [5] reported that gastric cathepsin D in part contributed to gastric ulcerations in rats. The CDLAP level in rat gastric mucosa was considerably higher than that of cathepsin D and CDLAP had a higher proteolytic activity than cathepsin D.[8,9] The possibility that CDLAP thus released may act as a tissue-destructive hydrolase to augment the damage in the gastric tissue remains to be investigated.

On the other hand, ethanol ingestion caused an increased pepsinogen level in the gastric mucosa. This increase did not result in the accumulation of pepsin in the mucosa, as judged by chromatographic profiles. Since rat pepsin was shown to be highly resistant to alkali inactivation,[10] the pepsin peak in chromatography reflects its intramucosal level. Although intramucosal pepsin derived from its zymogen is reported to be an important factor in the production of some gastric ulcerations,[6,7] we can conclude that intracellular pepsin is not responsible for the development of gastric damage in this case. The increase in mucosal pepsinogen level may also be an accompanying biological change following ethanol exposure, because this phenomenon was detected only in ethanol-damaged mucosa. However, the finding that it was substantially prevented by the pretreatment of PGE_2 should be explained in the future, together with the elucidation of the mechanism of pep-

sinogen induction by ethanol in Wistar rats.

PGE_2 is reported to effectively prevent both gastric lesion formation and several biological alterations induced by various necrotizing agents.[2,4] In our study, PGE_2 could reduce the release of CDLAP from the gastric mucosa. This effect was further confirmed by pretreatment with two kinds of PG inducers. The pretreatment with 20% ethanol, which is known to be a mild irritant to induce endogenous PGs,[13] showed the same effect as PGE_2 (200 μg/kg). Teprenone, a stimulator of PGE_2 biosynthesis,[18] exerted a protective effect against gastric damage and also reduced the biological alterations induced by ethanol. In addition to PGE_2, prostacyclin is also reported to reduce tissue damage and the release of lysosomal enzymes in myocardial infarction,[19] endotoxin shock[20] and liver injury.[21] Most of these effects of PGE_2 and prostacyclin apparently reflect their potent vasodilator and anti-aggregating activities, reversing the vasoconstriction and platelet aggregating actions of thromboxane A_2, leukotriene C_4 and other endogenous substances. However, the prevention of lysosomal enzyme release by PGs is considered to result from their direct protecting effect on the cellular membrane. Our preliminary result shows that PGE_2 can maintain the viability of rat gastric mucosal cells in ethanol-containing medium. Pendleton and Stavorski[22] suggested the presence of functionally different leukotriene receptor subtypes in the gastric mucosa by using a selective leukotriene antagonist. The stimulation of pepsin secretion by leukotrienes seems to be mediated through one of these receptor systems. These results strongly support the belief that these arachidonic acid metabolites also act directly on the cellular membrane and then induce or suppress certain biological alterations such as enzyme release.

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