Effects of Sulfhydryl-Related Compounds on Indomethacin-Induced Gastric Lesions in Rats: Role of Endogenous Sulfhydryls in the Pathogenesis

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ABSTRACT — The role of mucosal sulfhydryl (SH) in the pathogenesis of indomethacin-induced gastric lesions was investigated in rats. Indomethacin (25 mg/kg, s.c.) caused high-amplitude gastric contractions, resulting in linear hemorrhagic lesions in the corpus mucosa within 4 hr, but did not induce any changes in the mucosal SH levels. These lesions were prevented significantly by prior s.c. administration of cysteamine, glutathione (GSH) or diethylmaleate (DEM), irrespective of whether the mucosal SH was increased by the former two agents or reduced by the latter. N-Ethylmaleimide (NEM) tended to worsen such lesions without any effect on the mucosal SH contents. Gastric hypermotility caused by indomethacin was inhibited significantly by DEM, cysteamine and GSH, while acid secretion was reduced by DEM and NEM. Both cysteamine and GSH prevented the indomethacin-induced linear lesions even in the stomach perfused with 150 mM HCl, whereas in the animals treated with DEM, non-linear damage was induced exclusively in the antrum by indomethacin in the presence of acid. We conclude that the mucosal SH has no relation to the ulcerogenicity of indomethacin in the gastric corpus mucosa.

The mechanisms of gastric protection are considered to involve at least two endogenous mediators such as prostaglandins (PGs) and sulfhydryl (SH) substances in the mucosa (1). Participation of PGs has been demonstrated in various experimental ulcer models (2–4), while the role of SH has been exclusively studied in relation to gastric cytoprotection against necrotizing agents (5–7). Since endogenous SH substances, mainly present as intracellular glutathione (GSH), have a variety of actions in the body (8), they might have roles in the development of other lesion models which have different pathogenetic mechanisms. In fact, Konturek et al. (9) recently reported the involvement of mucosal SH in the pathogenesis of stress ulcers. Yet, it remains undetermined whether the mucosal SH has some relation to indomethacin-induced gastric lesions.

In the present study, we thus examined the effect of an ulcerogenic dose of indomethacin on the mucosal SH levels in the rat stomach, and investigated the relationship of endogenous SH to the ulcerogenicity of this agent by modulating the mucosal SH levels using SH-related compounds (cysteamine and GSH), an SH alkylator (N-ethylmaleimide) or an SH depletor (diethylmaleate).

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MATERIALS AND METHODS

Male Sprague Dawley rats (250–280 g, Charles River, Japan), kept in individual cages with raised mesh bottoms, were deprived of food but allowed free access to tap water for 18 hr before the experiments. All studies were carried out under unanesthetized conditions using 5–8 rats per group.

General procedures

The animals were given indomethacin s.c. in a dose of 25 mg/kg and killed 4 hr later to examine the gastric mucosa for hemorrhagic lesions. Under these conditions, gastric motility, acid secretion and SH contents in the mucosa were measured at various time periods. Dimeethylmaleate (DEM: 0.3 and 0.6 ml/kg) was used as an SH depletor; N-ethylmaleimide (NEM: 10 mg/kg) was used as an SH alkylating agent; and both cysteamine (30, 60 and 100 mg/kg) and GSH (100 and 200 mg/kg) were used as SH agents (10, 11). Each agent was given s.c. 30 min before indomethacin treatment, and changes in the above parameters were examined and correlated with those in gastric lesions induced by indomethacin.

Macroscopic evaluation of gastric lesions

The animals given indomethacin were killed 4 hr later, and the stomachs were removed, inflated by injecting 8 ml of 2% formalin, immersed in 2% formalin for 10 min to fix the gastric wall, and opened along the greater curvature. Then, the stomachs were examined for hemorrhagic lesions developed in the glandular mucosa under a dissecting microscope with a square grid (×10). The length (mm) of each lesion was measured, summed per stomach and used as a lesion score. In a separate study, the stomachs were perfused with 150 mM HCl (1 ml/hr) during a 4-hr test period according to the previously published method (12). Acid perfusion was started immediately after injection of indomethacin, the animals were killed 4 hr later, and the stomachs were treated with formalin and examined for lesions. The person measuring the lesions did not know the treatment given to the animals.

Determination of mucosal sulfhydryls

The amount of SH (nonprotein SH) was measured in the gastric mucosa according to the modified method (10), originally described by Kaplowitz et al. (13). The animals were killed 0.5, 1, 2, and 4 hr after administration of indomethacin. In some cases, the animals were pretreated with DEM, NEM, cysteamine and GSH 30 min before indomethacin treatment and then were killed 1 and 4 hr later. The stomachs were removed, incised along the greater curvature and the corpus mucosa was scraped using two glass slides and kept cold on ice. The mucosal scraping was weighed, homogenized in 2 ml of phosphate buffer (0.1 N NaH2PO4 plus 0.25 N sucrose, pH 7.4) and centrifuged at 4000 r.p.m. for 15 min at 4°C. A 0.5-ml aliquot of 25% trichloroacetic acid was added to 1 ml of the supernatant of each sample and then the sample was kept for 30 min at 4°C. After centrifugation at 3000 r.p.m. for 15 min, the supernatant was used to determine GSH using DTNB [5′,5′-dithiobis (2-nitrobenzoic acid)]. Absorbance was measured at 412 nm on a Hitachi spectrophotometer (Model 200-100), and the results were expressed as micromoles per gram of wet tissue weight.

Determination of gastric motility

Gastric motility was measured using a balloon according to the previously published method (12). Briefly, under ether anesthesia, a balloon and the support catheter were placed in the glandular stomach through an incision of the forestomach. The animals then were placed in Bollman cages, and the support catheter was connected to a pressure transducer and a polygraph device (Nihon Kohden). Gastric motility was continuously monitored on a recorder (Nihon Densi Kagaku, Unicorder U-228) as intraluminal pressure recordings. The quantitative analysis was performed by measuring the amplitude of each contraction (clear spike) during a test period, determining
the mean of a rat over every 10-min period from these values, and then by calculating the mean for each time period from 5 rats per group. Approximately 30 min after basal motility had well-stabilized, the animals were given indomethacin, and gastric motility was measured for 2 hr thereafter. DEM, NEM, cysteamine and GSH were given s.c. 30 min before administration of indomethacin.

Measurement of gastric acid secretion
Since previous studies showed that gastric lesions induced by indomethacin were significantly prevented by antacids or antisecretory agents (12, 14), the effects of drugs affecting mucosal SH on acid secretion were examined. Gastric acid secretion was measured according to the previous paper (15). Briefly, under ether anesthesia, the abdomen was incised, and both the stomach and duodenum were exposed. A gastric fistula prepared by a polyethylene tube was inserted into the stomach through the pylorus from an incision made in the duodenum. The fistula was placed by a ligature around the pylorus and withdrawn through the abdominal incision, and the animals were kept in Bollman cages during a test period. The stomachs were filled with 2 ml of saline (154 mM NaCl), and the gastric solution was changed every 15 min through the fistula for a total period of 3.5 hr. The collected gastric juice was analyzed for volume and titrated with 0.1 N NaOH to pH 7.0 for titratable acidity using an autoburette (Radiometer, Copenhagen, Denmark). Acid output was calculated as the product of volume and acidity, and the results were expressed as microequivalents per 15 min. After basal acid output had stabilized, the animals were given indomethacin, and other agents were given s.c. 30 min after administration of indomethacin.

Preparation of drugs
Drugs used were indomethacin, diethylmaleate, N-ethylmaleimide (Sigma Chemicals), glutathione, DTNB (Wako Chemicals) and cysteamine 2HCl (Nacalai Tesque). Indomethacin was suspended in saline with a drop of Tween 80 (Wako), while the other agents were dissolved in saline. Each agent was prepared immediately before use and given s.c. in a volume of 0.5 ml per 100 g body weight.

Statistics
Data are presented as the mean ± S.E. of 5–8 rats per group. Statistical analyses were performed using a two-tailed Dunnett’s multiple comparison test (16) for unpaired samples, and values of P < 0.05 were regarded as significant.

RESULTS
Development of gastric lesions
Subcutaneously administered indomethacin (25 mg/kg) caused multiple hemorrhagic lesions in the corpus mucosa, along the long axis of the stomach, the lesion score being 31.1 ± 3.4 mm at 4 hr after indomethacin treatment. These lesions were dose-dependently prevented by pretreatment of the animals with SH agents such as cysteamine (30–100 mg/kg) and GSH (100, 200 mg/kg); a significant protection was observed at 100 mg/kg in both cases, the inhibition being 89.7% and 63.7%, respectively (Fig. 1). The SH depletor DEM at a higher dose (0.6 ml/kg) also significantly reduced the severity of gastric lesions induced by indomethacin, the inhibition being 46.9%. However, NEM, an SH alkylating agent, at 10 mg/kg rather showed a tendency to aggravate the lesions.

Changes in mucosal sulfhydryls
The levels of SH in the gastric mucosa were 1.5–2.1 μmol/g tissue, and these values were not significantly altered at any time (0.5, 1, 2 and 4 hr) after indomethacin treatment (25 mg/kg) (Fig. 2). In normal rats, the amount of the mucosal SH was significantly reduced by DEM (0.6 ml/kg), increased by both cysteamine (100 mg/kg) and GSH (200 mg/kg), but showed no change after treatment with NEM (10 mg/kg) (not shown). These agents had similar effects on the mucosal SH levels in the presence of indomethacin. Pretreatment of
the animals with DEM caused a significant reduction (82.0%) in the mucosal SH contents when determined 1 hr after administration of indomethacin (Fig. 3). In contrast, the mucosal SH contents in the indomethacin-treated animals were significantly increased by either cysteamine (42.4%) or GSH (87.1%) at the same time period (1 hr). The mucosal SH contents remained lowered for the 4-hr experimental period in the animals treated with DEM, while the SH increasing effects of both cysteamine and GSH were not observed when examined at 4 hr after indomethacin treatment (not shown).

Gastric motility changes

The stomachs in control rats contracted at a frequency of 14.3 ± 3.2/10 min with an amplitude of 18.4 ± 2.4 cmH₂O. Gastric motility was markedly enhanced in response to s.c. administration of indomethacin (25 mg/kg), and this persisted for the 4 hr-test period (Fig. 4). Such motility changes caused by indomethacin was significantly inhibited by both cysteamine (30, 100 mg/kg) and GSH (100, 200 mg/kg) in a dose-dependent manner. In the animals given cysteamine at 100 mg/kg, the motility was suppressed below the basal values and remained lowered for at least 2 hr, resulting in fluctuations of the contractility at baseline levels (Fig. 5). DEM at a lower dose (0.3 ml/kg) did not significantly affect the in-
crease of gastric motility induced by indomethacin, but a higher dose (0.6 ml/kg) potently inhibited the motility response to a degree similar to that observed with 200 mg/kg of GSH.

**Acid secretory changes**

The stomachs in control rats secreted acid in an amount of 16–30 μEq/15 min under the present conditions, and this activity was not significantly affected by indomethacin (25 mg/kg, s.c.). Acid secretion remained unchanged in the animals treated with GSH (200 mg/kg). Although cysteamine (100 mg/kg) tended to increase acid secretion, this effect was not significant when compared to the control (Fig. 6). In contrast, both DEM (0.6 ml/kg) and NEM (10 mg/kg) significantly inhibited acid secretion in the indomethacin-treated rats. In the former, acid output was reduced for a relatively short period (1 hr), followed by a complete return to control values 2 hr later, while NEM produced a potent (>80%) and persistent inhibition of acid secretion during the entire experimental period.
**Gastric lesions in acid-perfused stomach**

It is well-known that gastric lesions induced by indomethacin are inhibited by an antacid or antisecretory agent (12). Since DEM at the protective dose also showed a significant antisecretory effect, we examined the effects of SH-related agents on gastric lesions induced by indomethacin in the presence of exogenous acid (150 mM HCl). Indomethacin (25 mg/kg, s.c.) caused linear hemorrhagic lesions in the stomach perfused with 150 mM HCl, the severity and location of damage being similar to those observed in the stomach without acid perfusion (Fig. 7). SH-related compounds such as cysteamine (100 mg/kg) or GSH (200 mg/kg) showed a significant protection against such damages in the acid-perfused stomach, the inhibition being 56.6% or 62.7%, respectively. However, DEM at 0.6 ml/kg, which significantly reduced the mucosal SH levels, did not protect the acid-perfused stomach against indomethacin; the lesion score was 23.1 ± 5.1 mm, which is not significantly different from that (22.8 ± 4.1 mm) in the control rats. In the animals pretreated with DEM, small or large non-linear damage was induced...
by indomethacin exclusively in the antrum with an incidence of 100%, but linear lesions in the corpus mucosa were similarly prevented like those in the stomach without acid perfusion.

DISCUSSION

The present study showed that indomethacin-induced gastric lesions were prevented, irrespective of whether the mucosal SH levels were increased by cysteamine and GSH or decreased by DEM, indicating no relation of endogenous SH to development of such lesions in response to this agent. This is supported by the finding that indomethacin by itself did not significantly alter SH contents in the gastric mucosa during a 4 hr-test period, although hemorrhagic damage was already observed in the stomach 2 hr after administration.

The role of endogenous SH in mucosal protection has been demonstrated in ethanol-induced gastric injury; the development of damage was accompanied by a lowering of mucosal SH, while exogenous SH compounds prevented the damage and restored the reduced SH levels (2–4). Similar results were obtained in the animals subjected to stress (9). However, a few studies supported the role of SH in the mucosal protection, by showing that DEM, a SH depletor, exhibited cytoprotection against ethanol-induced gastric lesions, despite causing a significant reduction in the mucosal SH levels (10, 17). In the present study, we found that indomethacin caused hemorrhagic damage in the stomach without any effect on the mucosal SH levels. Yet, these lesions were significantly inhibited by prior administration of SH compounds such as cysteamine and GSH. These agents increased the mucosal SH contents in the absence or presence of indomethacin, suggesting that the mucosal protection by SH compounds may be due to elevation of the mucosal SH levels. However, indomethacin-induced gastric lesions were also prevented by the SH depletor DEM, despite causing a significant reduction in the mucosal SH levels. Accordingly, the present results may suggest no relation of mucosal SH to the ulcerogenicity of indomethacin as well as the protective mechanism of SH compounds. Certainly, we cannot exclude a possible relation between protein SH and mucosal protection, because in this study, the mucosal SH was determined without biochemical restriction posed by the colorimetric GSH/nonprotein SH assay. However, Liu et al. (18) recently reported that indomethacin at an ulcerogenic dose did not alter both protein and nonprotein SHs in the gastric mucosa of rats.

We previously reported that gastric hypermotility may be a major factor in the development of gastric lesions in response to indomethacin (12, 14). Either GSH, cysteamine or DEM significantly inhibited the enhanced gastric motility after indomethacin treatment, suggesting that the antigastric motility may be involved in the protective mechanism against these lesions. In agreement with the previous finding (19), NEM rather worsened the gastric lesions induced by indomethacin. This may be explained by enhancement of the vascular permeability which is characteristically observed in the early stage of lesion formation in response to indomethacin (19, 20). These results confirmed the importance of gastric motility in the pathogenetic mechanism of indomethacin-induced lesions and further suggested that there is no relation of mucosal SH to gastric motility changes. It is evident that the anti-lesion activity of SH compounds such as cysteamine and GSH was not accounted for by the antisecretory activity, because these agents had no effect on acid secretion. This is supported by the finding that such agents also prevented indomethacin-induced lesions even in the stomach perfused with exogenous acid. On the other hand, the anti-lesion activity of DEM disappeared in the stomach in the presence of acid. Since DEM inhibited acid secretion as well as motility, it is possible that the protective mechanism of DEM may be attributable to acid inhibition. Even in the stomachs perfused with acid, DEM prevented the fold-related linear lesions in the corpus mucosa in
response to indomethacin. However, under these conditions, indomethacin produced a different type of damage (non-linear lesions) exclusively in the antrum, resulting in no difference in the total lesion score between DEM-treated and control groups. Thus, it may be assumed that the prevention of linear lesions in the corpus mucosa is related to inhibition of the gastric motility response to indomethacin, yet different mechanisms might operate in the development of the antral lesions in the stomach perfused with exogenous acid in the presence of DEM.

It has been reported that oxygen-derived free radicals are involved in the pathogenesis of indomethacin-induced gastric lesions (20, 21). The increased lipid peroxidation was observed in the gastric mucosa after indomethacin treatment, while oxyradical scavengers (SOD or DMSO) suppressed the lipid peroxidation and reduced the severity of gastric lesions induced by indomethacin (20). Since GSH plays a role in reducing the reactive oxygen-mediated cytotoxicity (22, 23), this action may relate in part to the protection afforded by SH compounds. Along this line, DEM reduces the mucosal GSH contents and impairs the antioxidative process, leading to accumulation of oxyradicals and cellular damage (24). Induction of antral damage by DEM in the presence of acid may be associated with the mucosal SH deficiency caused by this agent. Further studies would be needed to examine the role of mucosal SH in the pathogenesis of such antral lesions.

In conclusion, the present study suggests that the mucosal SH does not play a major role in the ulcerogenicity of indomethacin in the stomach. The antagonism by GSH, cysteamine and DEM may be attributable to inhibition of the gastric motility response to indomethacin but not due to an increase or decrease in the mucosal SH levels.

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