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EFFECTS OF ACID-DEGRADED PRODUCTS OF LEMINOPRAZOLE ON ACID SECRETION, MUCUS SECRETION AND SYNTHESIS, AND INDOMETHACIN-INDUCED DAMAGE IN CELL CULTURE

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We examined the effects of four acid-degraded products of leminoprazole on [1] acid secretion by parietal cells, [2] mucus secretion and synthesis by epithelial cells and [3] indomethacin-induced damage to epithelial cells. These gastric cells were prepared from rabbit stomachs. Upon stimulation with 10 μM histamine, acid secretion by parietal cells was inhibited by leminoprazole, sulfide and 2-(isobutylmethylamino)benzylalcohol. Sulfide also inhibited dibutyryl cyclicAMP (100 μM)-stimulated secretion. However, the inhibitory effects of such compounds were observed only at high concentrations, in comparison with the antisecretory concentrations of leminoprazole. On the other hand, among acid-degraded products, only sulfide enhanced mucus secretion and synthesis by epithelial cells. The stimulatory effects of sulfide were the same as those of leminoprazole. Futhermore, the effects of sulfide as well as leminoprazole were suppressed by N^G-nitro-L-arginine methyl ester (L-NAME), and L-arginine, not D-arginine, prevented the inhibition by L-NAME. In contrast, all degraded products failed to protect epithelial cells against indomethacin-induced damage. Overall, these results suggest that only the mucus-elevating effect of administered leminoprazole may be partly due to the stimulatory effects of sulfide derived from leminoprazole on mucus secretion and synthesis by epithelial cells.

K e y   w o r d s: *leminoprazole, acid-degraded products, gastric cells, mucus*

INTRODUCTION

Leminioprazole (NC-1300-O-3, (±)-2-[[2-(isobutylmethylamino)-benzyl] sulfinyl]-1H-benzimidazole) is a unique acid pump inhibitor which has both antiulcer and mucosal protective effects (1—6). *In vitro* studies revealed that, in addition to inhibition of acid secretion from parietal cells (7), leminoprazole also stimulates mucus secretion and synthesis by epithelial cells (8, 9) and protects epithelial cells against various forms of cell damage (10, 11). These pharmacological effects are considered to largely contribute to the therapeutic effects of leminoprazole.

It is well known that acid pump inhibitors with a benzimidazole moiety are converted to active inhibitors under acidic conditions, but are rapidly degraded into several compounds (12). Similarly, leminoprazole is easily degraded into sulfide (52.9%), 2-mercaptobenzimidazole (MBI; 5.7%), benzimidazole (BI; 11.8%) and 2-(isobutylmethylamino)-benzylalcohol (2-MBIZ; 27.5%) at an acidic pH (13) (*Fig. 1*). However, it has not been confirmed that leminoprazole
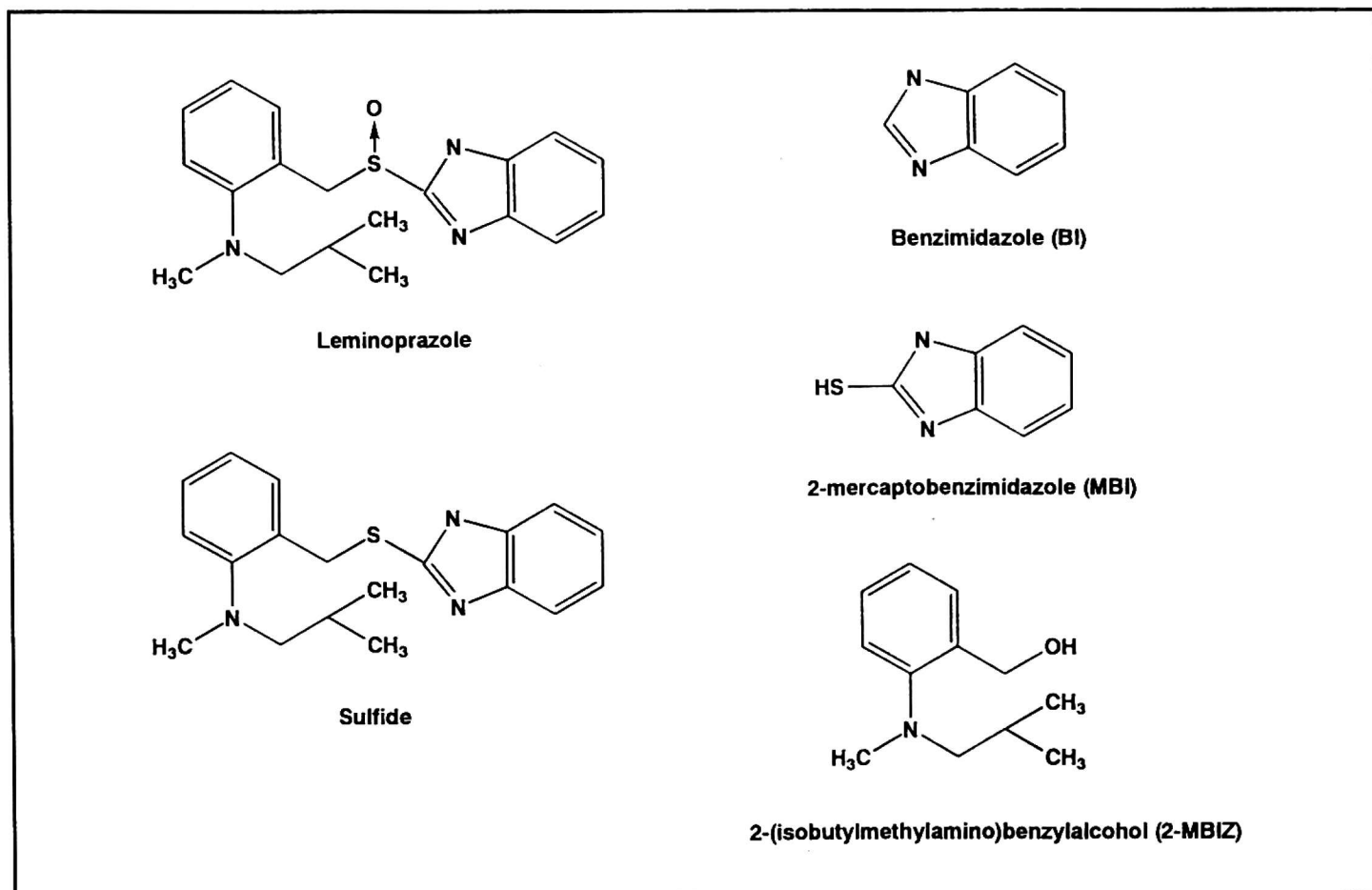

Fig. 1. Chemical structures of leminoprazole and its acid-degraded products.

is transformed to an active compound. Accordingly, it is possible that the acid-degraded products generated in the microenvironment of the gastric glands may contribute to the *in vivo* effects of leminoprazole. Therefore, we examined whether or not the acid-degraded products of leminoprazole exert 1.) an antisecretory effect against parietal cells, 2.) stimulatory effects on mucus secretion and synthesis by epithelial cells, and 3.) cytoprotective effect against indomethacin-induced damage to epithelial cells, in cell culture.

MATERIALS AND METHODS

Preparation of parietal cells and epithelial cells from rabbit stomachs.

These gastric cells were isolated from rabbit stomachs according to the methods of Urushidani *et al* (14) and Watanabe *et al* (15) with slight modifications. Male Japanese White rabbits (Nihon S.L.C., Shizuoka, Japan), weighing 3.0 to 4.9 kg, were anesthetized with pentobarbital (50 mg/kg, i.v.; Abbott, North Chicago, IL), and then perfused locally *via* the celiac artery with 800 ml of

phosphate-buffered saline (PBS) and 200 ml of medium A consisting of 25 mM HEPES-NaOH (pH 7.4), 132.4 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 2 mg/ml glucose and 1 mg/ml bovine serum albumin. After the stomachs had been excised, the oxyntic mucosa was scraped and minced immediately. After the tissue was washed with medium A, it was incubated in medium A containing 0.05% collagenase (Wako Chemicals, Osaka, Japan) for 40—60 min at 37°C under 95% O_2/5% CO_2, and then filtrated through a metal mesh (diameter, 300 µm). After centrifugation at 190 × g for 7 min, the pellet was resuspended in medium A containing 27% Percoll (Pharmacia Biotech, Uppsala, Sweden), followed by centrifugation at 190 × g for 15 min. For isolation of parietal cells, the cells located near the surface were obtained. The cells in low density fractions were enriched with parietal cells with a final purity greater than 75%, as determined by microscopic observation. The cells were resuspended in medium A, and were used as a suspension culture for the study of acid secretion. The remaining cell pellet was resuspended in medium A, and mucosal epithelial cells were obtained by filtration through a metal mesh (diameter, 100 µm). The viabilities of the isolated cells were greater than 80%, as determined by the trypan blue dye exclusion test (16).

Epithelial cells were inoculated into 6-well plates (1×10^6 cells/2 ml) for study of mucus secretion and synthesis or 24-well plates (1×10^5 cells/0.5 ml) for study of cytoprotection in Ham's F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco BRL), 100 units/ml penicillin, 100 units/ml streptomycin and 0.25 µg/ml amphotericin B, and then cultured at 37°C under 5% CO_2 in air, the medium being changed daily. The cells reached confluence in 2 or 3 days, and 80—90% of the cells were morphologically and functionally identical to epithelial cells, as reported previously (9). The cells grown to confluence were used for studies of mucus secretion, synthesis, and cytoprotection.

Determination of acid secretion by parietal cells.

Acid secretion was determined by the (^{14}C) aminopyrine uptake method (17). Isolated parietal cells (3×10^5 cells) were incubated in 1 ml of medium A containing each secretagogue (histamine 10 µM or dibutyryl cyclicAMP (dbcAMP) 100 µM) and/or the indicated drug in the presence of [^{14}C] aminopyrine (18.5 Bq, American Radiolabeled Chemicals, St. Louis, MO) at 37°C for 40 min. After the cells were washed with PBS, they were dissolved in 200 µl of 0.1 M KOH. Radioactivity in the cell lysate (0.1 ml) was then measured.

Determination of acid pump activity.

Membrane vesicles enriched with acid pumps were prepared from the stomachs of male Japanese White rabbits according to the method of Im et al (18). All procedures were carried out at 4°C. The oxyntic mucosa was scraped off, and then homogenized in 10 volumes of 2 mM HEPES-Tris (pH 7.4) buffer containing 0.25 M sucrose, 1 mM EGTA and 2 mM $MgCl_2$. After centrifugation at 20,000 × g for 15 min, the supernatant was centrifuged at 100,000 × g for 1 hr. The pellet was suspended in the same buffer, and purified further by density gradient centrifugation. The crude membrane sample was put on the top of a 30% sucrose cushion which had been made up in the same buffer, and centrifuged at 100,000 × g for 1 hr. The membranes sedimented above the 30% sucrose layer were collected, and used for measurement of acid pump activity.

Acid pump activity was assessed by the release of Pi from ATP. The membranes (20 µg protein) were incubated in 1 ml of 40 mM imidazole-HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, 2 mM ATP, 10 µg/ml nigercin and the indicated drug with or without 10 mM KCl at 37°C for 15 min. The reaction was terminated by an addition of 1 ml of 10% trichloroacetic acid. Pi was determined by the method of Fiske and Subbarow (19). The K^+-stimulated ATPase (acid pump) activity was taken as the difference between the Mg^{2+}-stimulated and the Mg^{2+}-K^+-stimulated ATPase activities.

Determination of mucus secretion and synthesis by epithelial cells.

The amounts of secreted and synthesized mucus were determined by the method of Keates and Hanson (20) with slight modifications. Epithelial cells were washed with Ca^{2+}, Mg^{2+}-free PBS, and then incubated at 37°C with the indicated drug in 1 ml of Dulbecco's modified Eagle medium (Gibco BRL) containing (^{3}H) glucosamine (18.5 kBq; New England Nuclear, Boston,MA). In the case of (^{14}C) leucine labeling, leucine-free Dulbecco's medium supplemented with (^{14}C) leucine (37 kBq; New England Nuclear) was used. Six hours later, 0.6 ml of the medium was recovered and mixed with 60 µl of 20% Triton X-100. The remaining cells were solubilized in 0.6 ml of 2% Triton X-100. Each Triton-containing sample was applied onto a Sepharose CL-4B column (6 ml; Pharmacia Biotech) which had been equilibrated with 2% Triton X-100, and the void fractions were collected. The radioactivity in the fractions was measured.

Indomethacin-induced damage to epithelial cells.

Epithelial cell damage was induced by treatment with indomethacin, as described previously (10). In brief, after incubation in 0.5 ml of F12 medium containing the indicated drug at 37°C for 2 h, and washing with Ca^{2+}, Mg^{2+}-free PBS, the cells were further incubated in 0.5 ml of medium containing 50 µM indomethacin or vehicle alone at 37°C for 4 hrs.

Determination of cell viability.

Cell viability was assessed by the dye exclusion method (16). After incubation with the indicated drugs, trypan blue solution was added to the wells or the tubes. Three minutes later, the numbers of stained and non-stained cells were determined in four randomly chosen fields in each well under a microscope (Olympus CK2, Tokyo, Japan; ×100). Cell viability was calculated as follows:

Viability (%) = (Non-stained cells/(Stained cells + Non-stained cells)) × 100.

Cell viability was expressed as a percentage of the corresponding control value.

Drugs.

Leminoprazole and its acid-degraded products (provided by Nippon Chemiphar, Tokyo, Japan) were all dissolved in dimethyl sulfoxide. In all assays, the final concentration of dimethyl sulfoxide was less than 1%, which did not affect cell functions and viabilities. Other reagents and their commercial sources were as follows: histamine, Nacalai Tesque (Kyoto, Japan); dbcAMP, N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine, D-arginine and indomethacin, Sigma Chemicals (St. Louis, MO). All other chemicals were of reagent grade.

Statistical analysis.

The data are presented as means ±S.E.. Statistical differences were evaluated using Student's t-test or Dunnett's multiple comparison test, a P value of < 0.05 being regarded as significant. EC_{50} values were calculated by the Litchfield-Wilcoxon method.

RESULTS

Effects of acid-degraded products of leminoprazole on acid secretion by parietal cells.

Basal (^{14}C) aminopyrine uptake was negligible, but 10 µM histamine promoted (^{14}C) aminopyrine uptake (about 8%) by rabbit parietal cells. In the

case of acid secretion stimulated by 10 μM histamine, over 1 μM of leminoprazole exerted a potent antisecretory effect (*Fig. 2A*). Sulfide and 2-MBIZ also significantly inhibited the histamine-stimulated acid secretion in a dose-dependent manner. However, the effects of sulfide and 2-MBIZ were apparently weaker than that of leminoprazole. The EC_{50} values of sulfide and 2-MBIZ were 1.4 μM and 32.0 μM, respectively. In contrast, neither BI nor MBI had effects on acid secretion.
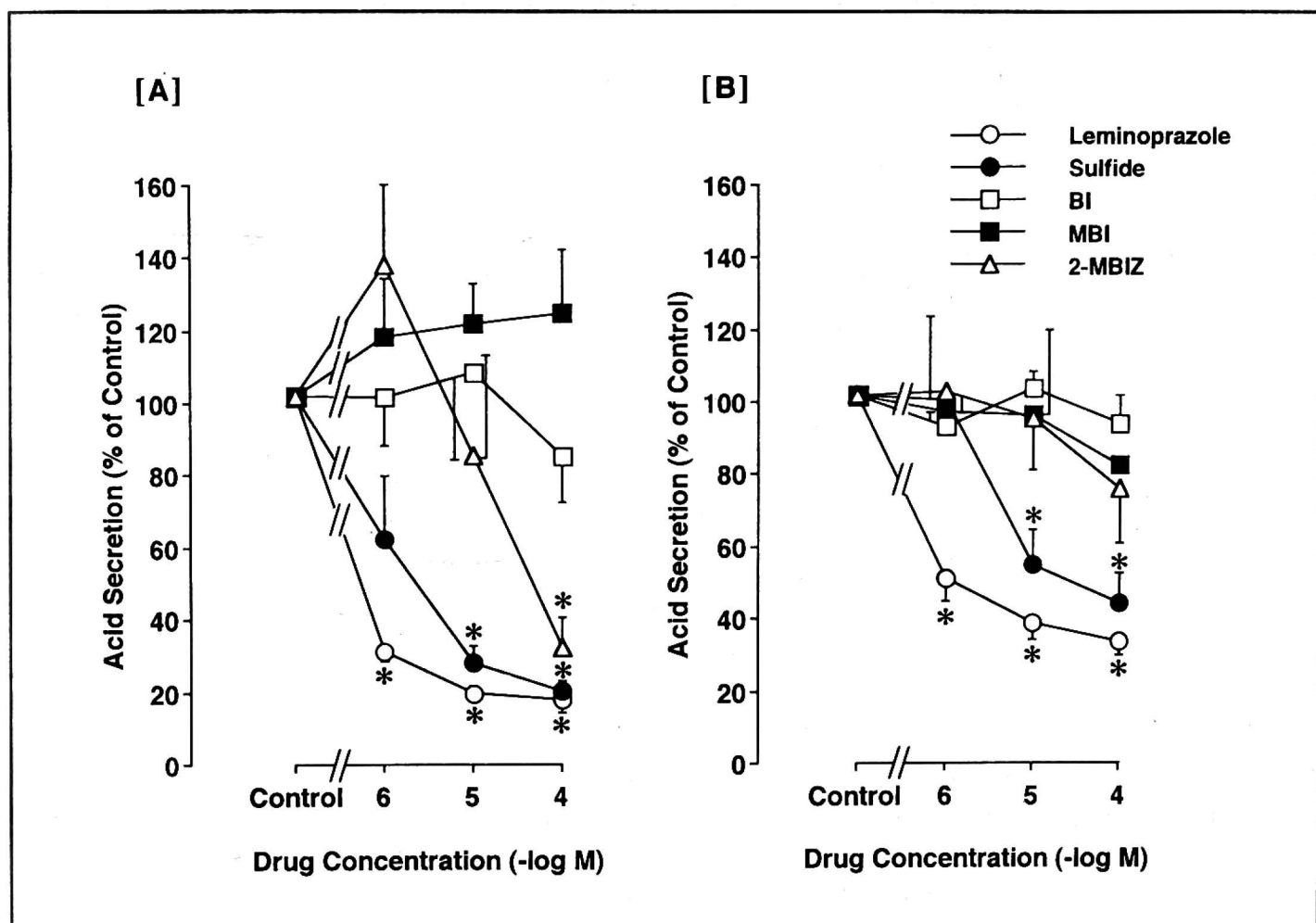

Fig. 2. Effects of leminoprazole and its acid-degraded products on acid secretion by parietal cells. Parietal cells were incubated with 10 μM histamine (A) or 100 μM dbcAMP (B) in the presence of the indicated drug. Forty minutes later, [^{14}C] aminopyrine uptake by the cells was determined. Data are presented as means ±S.E. (n = 8). *Statistically significant difference from the control (without any drugs).

DbcAMP at 100 μM was more potent than 10 μM histamine (^{14}C) aminopyrine uptake; about 15%). Leminoprazole dose-dependently inhibited the dbcAMP-stimulated acid secretion (*Fig. 2B*). Among the acid-degraded products, only sulfide at high concentrations showed an inhibitory effect. A significant effect of sulfide was observed at over 10 μM. The EC_{50} values of leminoprazole and sulfide were 1.3 μM and 93.0 μM, respectively.

After parietal cells were incubated with leminoprazole, sulfide and 2-MBIZ at 100 μM for 40 min in the presence of each secretagogue, cell viability was

determined. The viability was not affected by an drug, as compared with the vehicle-treated cells.

We examined whether or not sulfide and 2-MBIZ inhibit acid pump activity. Leminoprazole dose-dependently inhibited acid pump activity (*Fig. 3*). The EC_{50} value was 8.6 µM. In contrast, sulfide and 2-MBIZ, even at high concentrations (up to 100 µM), did not affect the activity. The other acid-degraded products also showed no effects (data not shown).
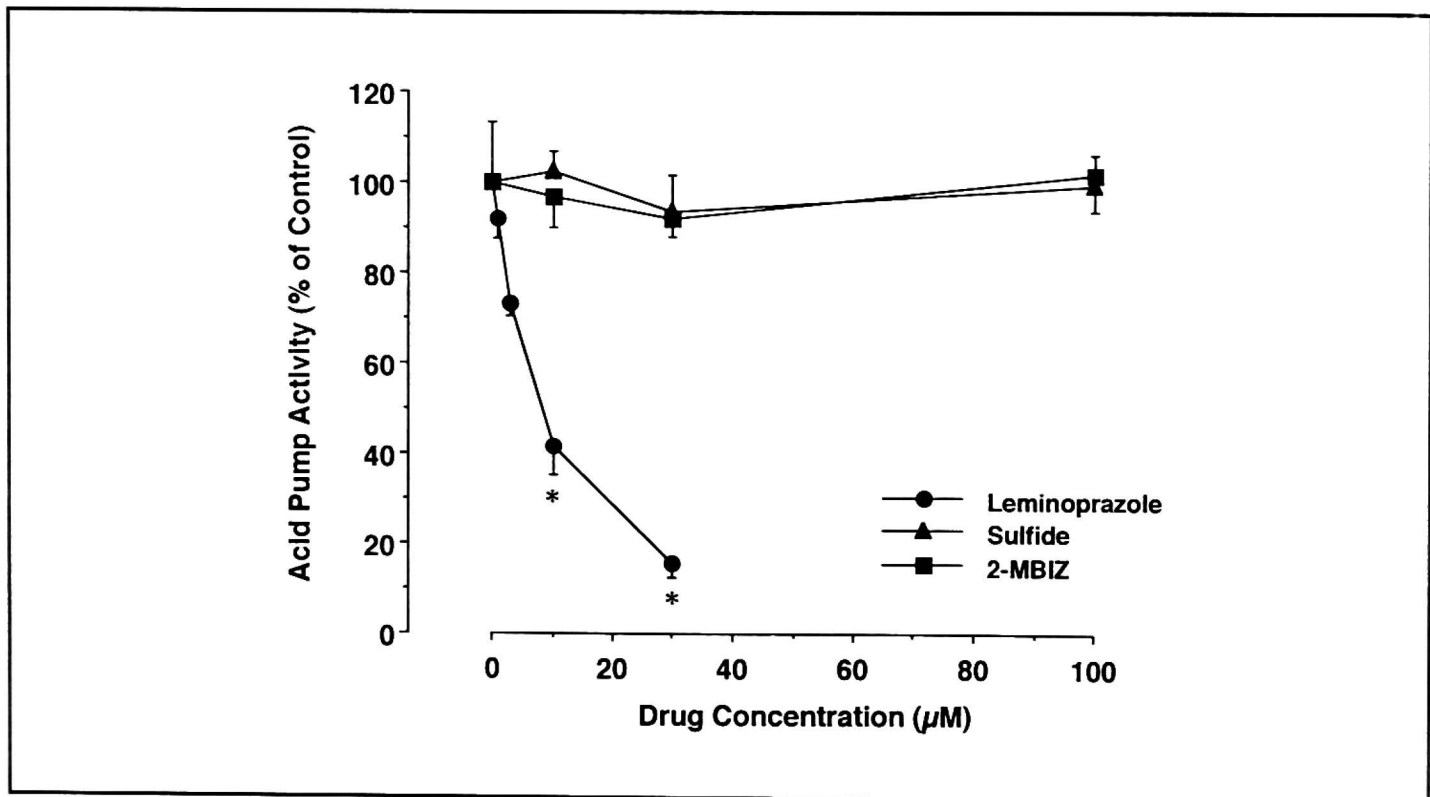

Fig. 3. Effects of leminoprazole, sulfide and 2-MBIZ on acid pump activity. Gastric membranes enriched in acid pumps were incubated with the indicated drug in the presence or absence of 10 mM KCl for 15 min. The K^+-stimulated ATPase (acid pump) activity was taken as the difference between the Mg^{2+}-stimulated and the Mg^{2+}, K^+-stimulated ATPase activities. Data are presented as means $\pm$ S.E. (n = 4). *Statistically significant difference from the control (without any drugs).

Effects of acid-degraded products of leminoprazole on mucus secretion and synthesis by epithelial cells.

Rabbit gastric epithelial cells steadily incorporated both (^{3}H) glucosamine and (^{14}C) leucine into high molecular weight materials, and secreted the labeled ones, which indicated that the cells both synthesized and secreted mucus. Leminoprazole at 1 µM significantly enhanced both mucus secretion and synthesis (*Fig. 4*). The increases in the secretion and synthesis caused by leminoprazole were determined by both (^{3}H) glucosamine and (^{14}C) leucine labeling to be around 40%, compared with the corresponding controls. Of interest was that sulfide exerted stimulatory effects on mucus secretion and synthesis. However, the other acid-degraded products were not effective, although MBI tended to slightly stimulate the secretion and synthesis of mucus.
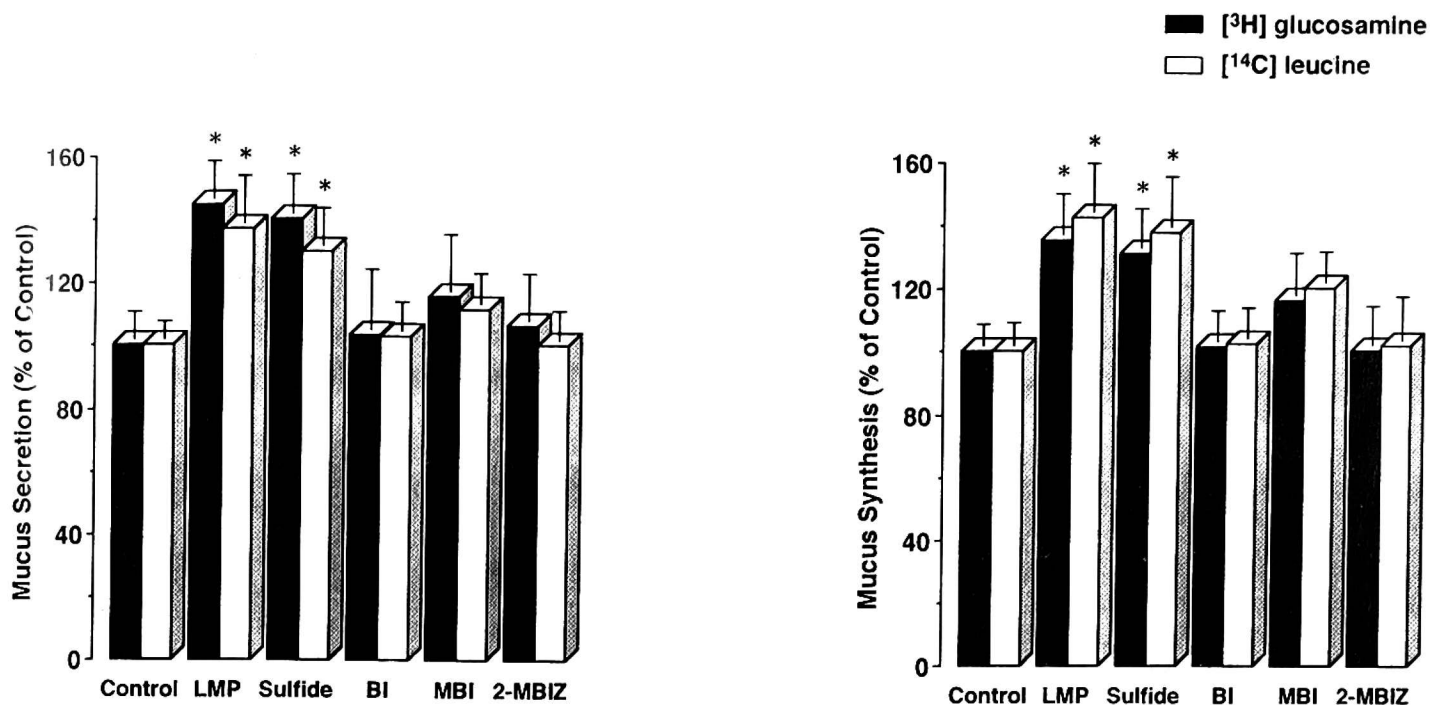
Fig. 4. Effects of leminoprazole (LMP) and its acid-degraded products on mucus secretion and synthesis by epithelial cells. Epithelial cells were incubated with the indicated drug in the presence of [³H] glucosamine or [¹⁴C] leucine for 6 h. The radioactivities in the high molecular weight materials were determined. Data are presented as means ±S.E. (n = 8). *Statistically significant difference from the control (without any drugs).

The dose-dependent effects of leminoprazole and sulfide on mucus secretion and synthesis are shown in *Fig. 5*. Both drugs stimulated the secretion and synthesis at 0.3 μM, and significant effects were found at 1 μM. Thus, the effects of sulfide were the same as those of leminoprazole.
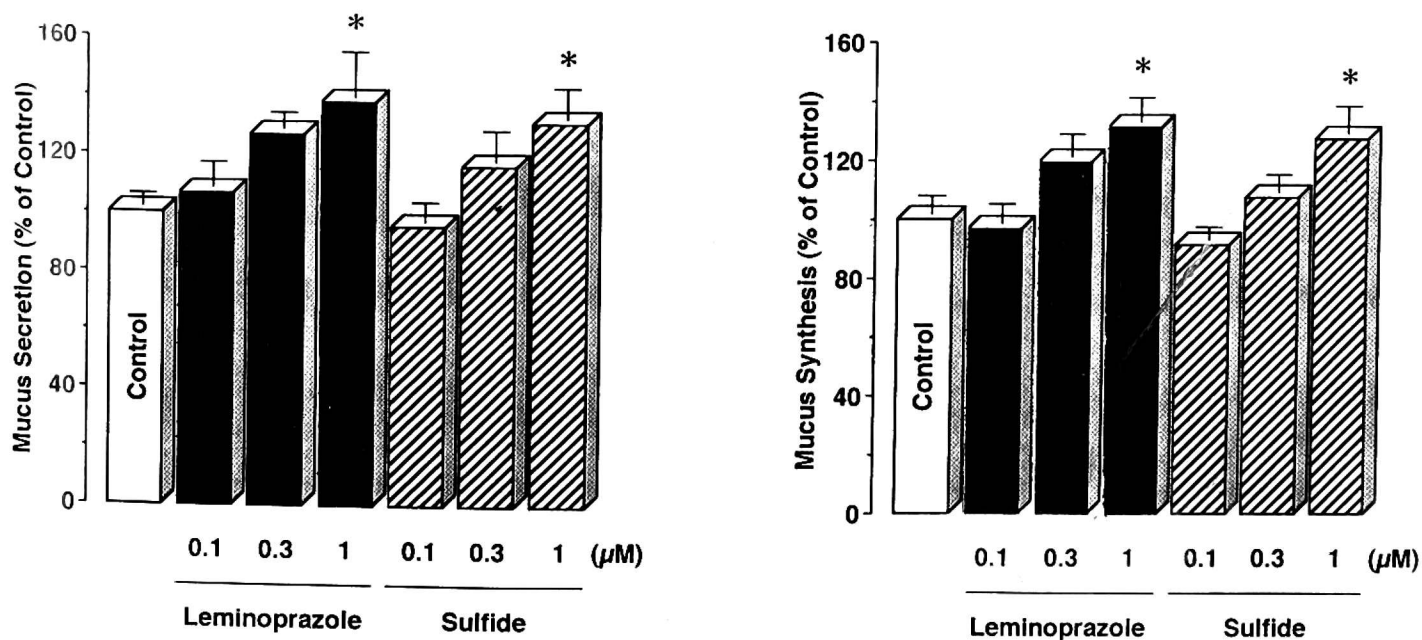

Fig. 5. Concentration-dependent effects of leminoprazole and sulfide on mucus secretion and synthesis by epithelial cells. Epithelial cells were incubated with the indicated concentrations of leminoprazole and sulfide in the presence of (³H) glucosamine for 6 h. The radioactivities in the high molecular weight materials were determined. Data are presented as means ±S.E. (n = 8). *Statistically significant difference from the control (without any drugs).

We reported that nitric oxide might play a key role in the stimulatory effects of leminoprazole on mucus secretion and synthesis by rabbit epithelial cells (9). To evaluate whether or not the mechanism underlying the effects of sulfide is identical to those of leminoprazole, we examined the effect of L-NAME (a nitric oxide synthase inhibitor) on the sulfide-induced increases in mucus secretion and synthesis (*Fig. 6*). L-NAME dose-dependently inhibited both the leminoprazole- and the sulfide-stimulated mucus secretion and synthesis. Treatment with 1 mM L-NAME abolished the stimulatory effects of 1 μM
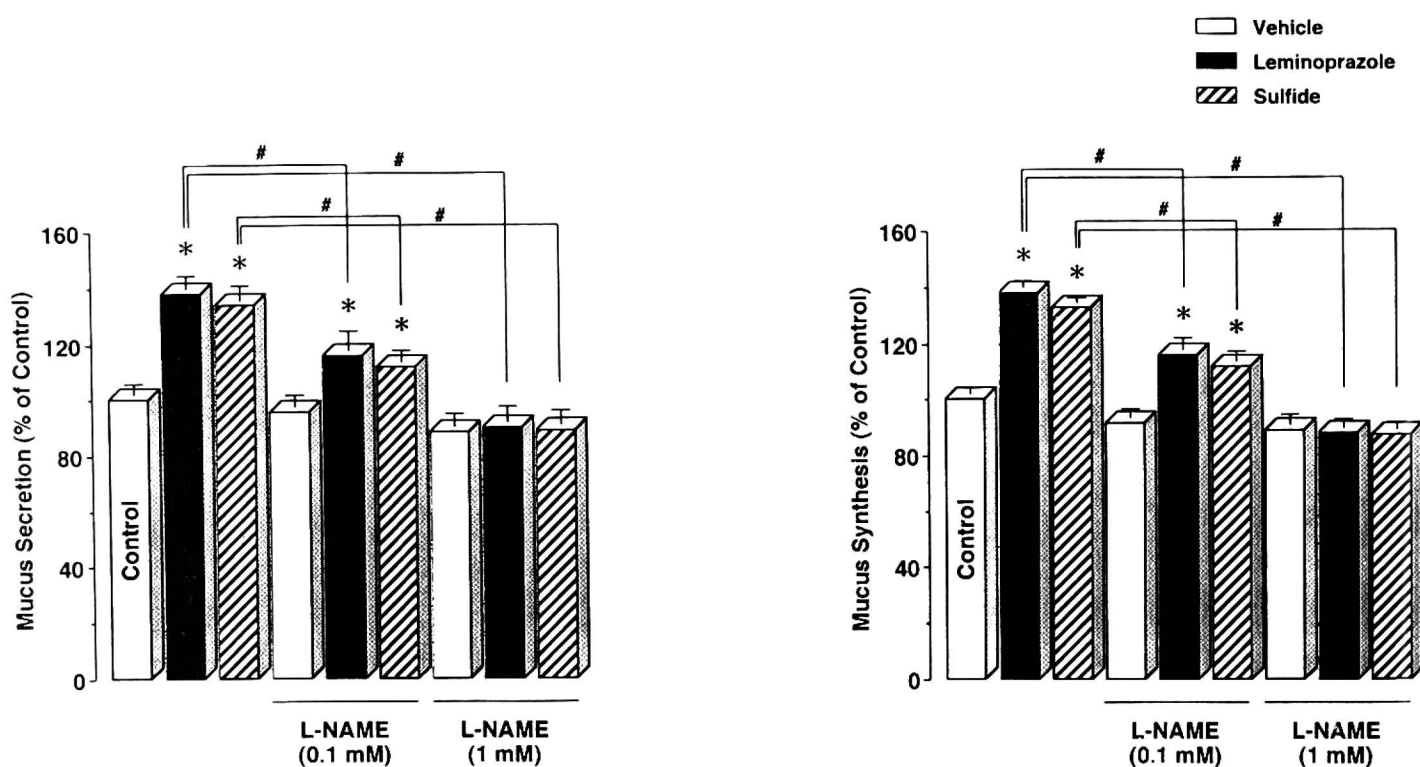

Fig. 6. Effect of L-NAME on the leminoprazole- and sulfide-stimulated secretion and synthesis of mucus by epithelial cells. Epithelial cells were incubated with 1 μM leminoprazole or 1 μM sulfide, with or without L-NAME, in the presence of (^{3}H) glucosamine for 6 h. The radioactivities in the high molecular weight materials were determined. Data are presented as means ± S.E. (n = 8). *, # Statistically significant difference from the control (without any drugs) and the drug-induced increases in the secretion and synthesis, respectively.

leminoprazole and 1 μM sulfide. Furthermore, 5 mM L-arginine restored the inhibition by L-NAME of the effects of leminoprazole and sulfide (*Fig. 7*). The restored effects of leminoprazole and sulfide were nearly the same to the effects of them without L-NAME, the increases in mucus secretion and synthesis being about 40% and 50%, respectively. However, D-arginine had no effect.

Effects of acid-degraded products of leminoprazole on indomethacin-induced damage to epithelial cells.

Exposure of rabbit epithelial cells to 50 μM indomethacin caused a significant reduction in viability, as determined by the dye exclusion method (*Fig. 8*). The loss of viability induced by indomethacin was 43.8 ± 3.1%.
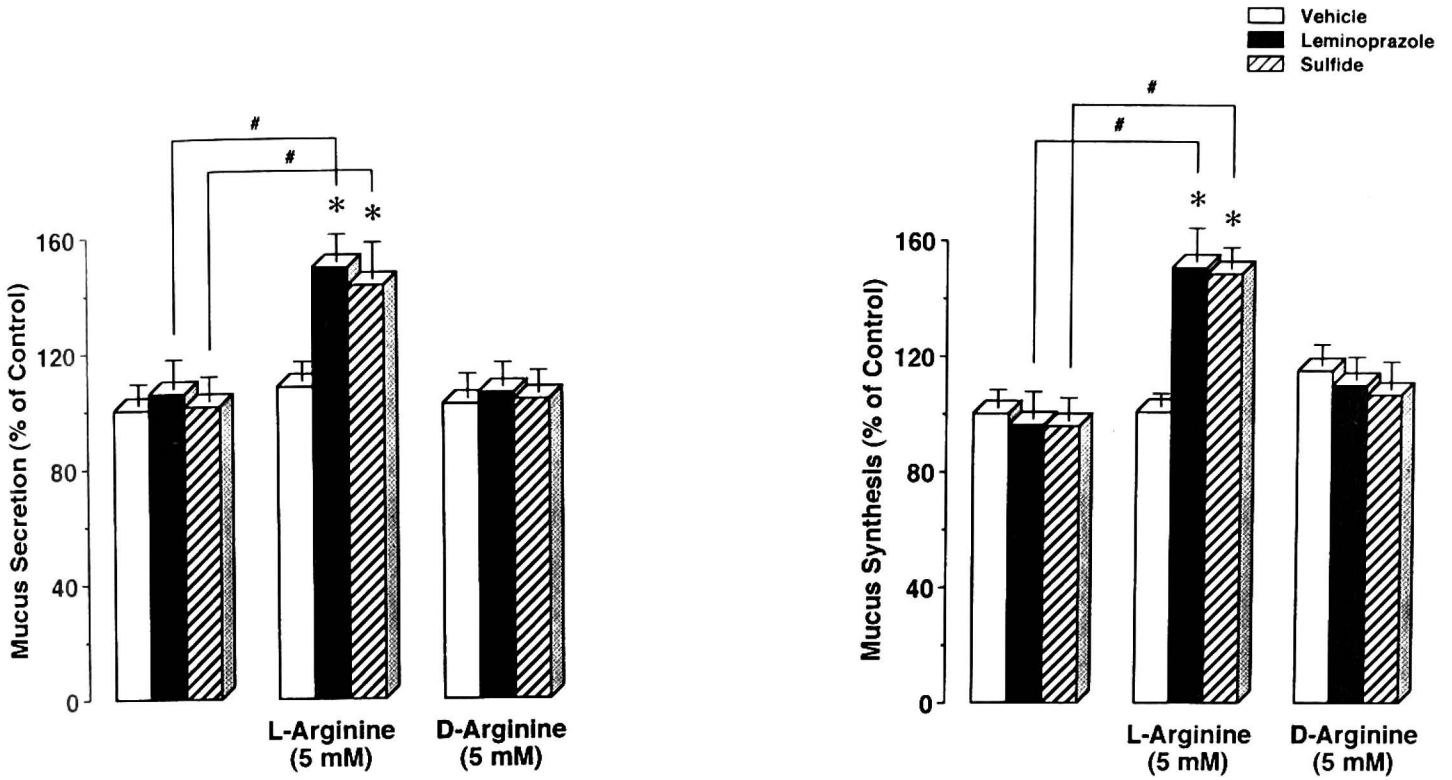
Fig. 7. Effects of L-arginine and D-arginine on the L-NAME-induced decreases in leminoprazole- and sulfide-stimulated secretion and synthesis of mucus by epithelial cells. Epithelial cells were incubated with 1 µM leminoprazole or 1 µM sulfide, with 5 mM L-arginine or D-arginine, in the presence of 1 mM L-NAME and (^{3}H) glucosamine for 6 h. The radioactivities in the high molecular weight materials were determined. Data are presented as means ± S.E. (n = 8.*, #Statistically significant difference from the corresponding vehicle and the corresponding group without arginines, respectively.
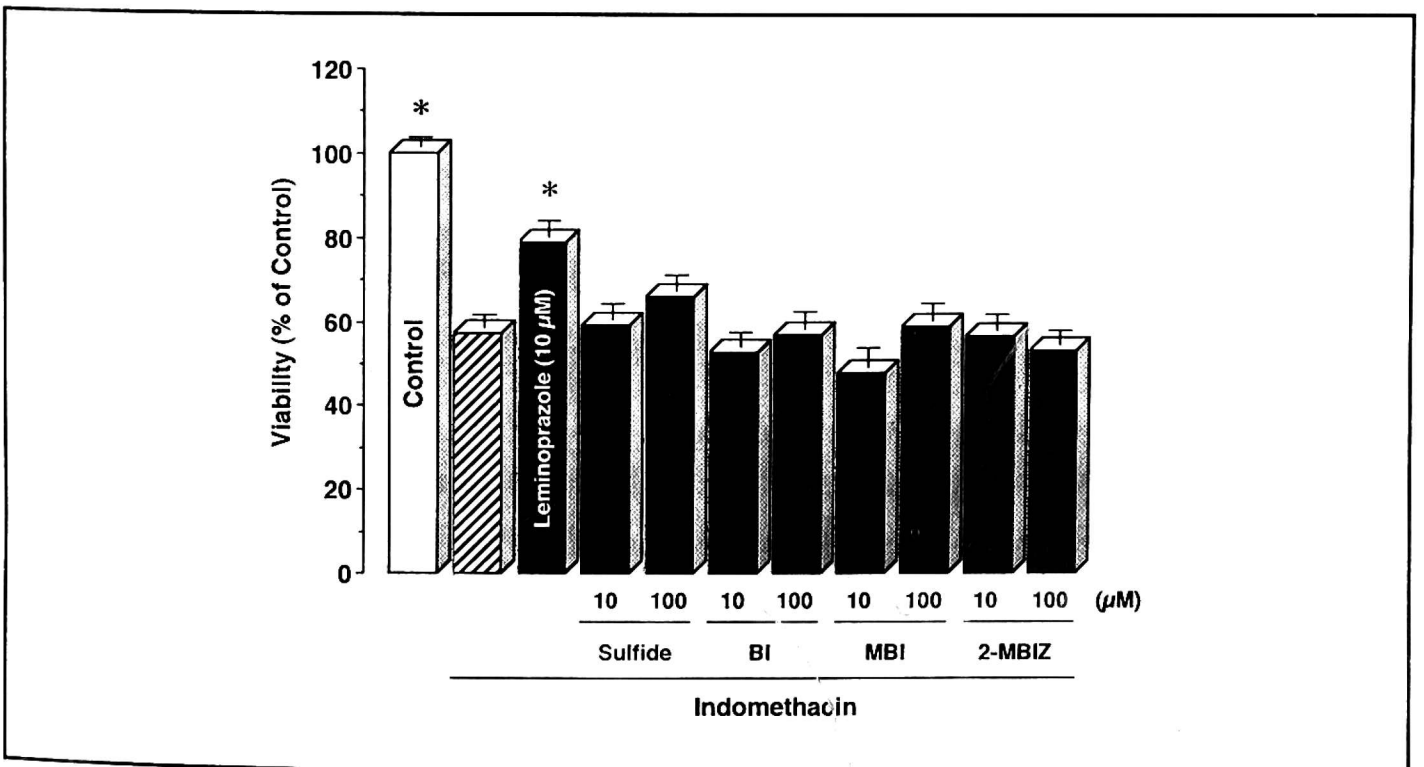

Fig. 8. Effects of leminoprazole and its acid-degraded products on indomethacin-induced damage to epithelial cells. Epithelial cells were pretreated with the indicated drug for 2 h, and then exposed to 50 µM indomethacin for 4 h. Cell viability was determined by the dye exclusion method. Data are presented as means ± S.E. (n = 8). *Statistically significant difference from the control (without any drugs).

Pretreatment of cells with 10 μM leminoprazole significantly prevented the loss in viability. The inhibition caused by leminoprazole was 49.5%. In contrast, acid-degraded products had no effect on indomethacin-induced damage to epithelial cells.

DISCUSSION

The present results indicate that, among the acid-degraded products of leminoprazole, sulfide and 2-MBIZ have an inhibitory effect on acid secretion by parietal cells. The possibility of the functional inhibition of parietal cells by sulfide and 2-MBIZ being a result of their cytotoxity was ruled out, since these drugs did not cause a reduction in cell viability. Fryklund and Wallmark (21) reported that sulfide derivatives of omeprazole at high concentrations mitigate the secretory function of parietal cells in isolated rabbit gastric glands despite its failure in inhibiting acid pump activity. It was suggested that sulfides dissipate the proton gradient, probably by serving as a permeable buffer. Similarly, in the cases of sulfide and 2-MBIZ of leminoprazole, high concentrations were required for inhibition of acid secretion. Furthermore, we confirmed that leminoprazole significantly inhibits acid pump activity, but none of its acid-degraded products have an inhibitory effect. All above points being considered, it is speculated that the inhibition of acid secretion by sulfide and 2-MBIZ may result from their buffering effects. However, it is apparent that the antisecretory effects of sulfide and 2-MBIZ were much weaker than that of leminoprazole. It is highly unlikely that the acid-degraded products contribute to the *in vivo* antisecretory effects of leminoprazole.

We found that sulfide enhances the secretion and synthesis of mucus by epithelial cells. We previously reported that leminoprazole stimulates mucus secretion and synthesis, probably through nitric oxide produced by constitutive nitric oxide synthase (9). Similar to the case of leminoprazole, the increases in mucus secretion and synthesis induced by sulfide were also inhibited by L-NAME. In addition, L-arginine restored the inhibition by L-NAME of the effects of leminoprazole and sulfide, but D-arginine had no effect. These results indicate that nitric oxide might be involved in the effects of sulfide. These results also suggest that leminoprazole and sulfide may act on the same target site(s) in epithelial cells. The concentration of sulfide required for acceleration of mucus secretion and synthesis (0.3—1 μM) was lower than that required for the inhibition of acid secretion (10—100 μM). The buffering effect sulfide is unlikely to be related to the stimulatory effects on mucus secretion and synthesis. It should be noted that the stimulatory effects of sulfide were the same as those of leminoprazole. Since a considerable amount of sulfide can be produced by exposure of leminoprazole to acid (13), sulfide may contribute to

the mucus-elevating effect of administered leminoprazole. In addition, these results indicate that the sulfoxide structure is not necessarily essential for stimulation of mucus secretion and synthesis.

Recently, we found that leminoprazole directly protects epithelial cells against mild damage caused by ethanol, taurocholate and indomethacin, and that its cytoprotective effect might be mediated through *de novo* synthesized specific proteins (10, 11). All acid-degraded products failed to prevent cell damage caused by indomethacin, suggesting that the defensive responses of epithelial cells were elicited by leminoprazole itself. In fact, none of acid-degraded products induced the synthesis of leminoprazole-specific proteins (data not shown). In addition, we previously reported that neither nitric oxide nor elevated mucus are involved in the cytoprotection by leminoprazole against indomethacin-induced cell damage (10). Taken together, we reconfirmed that the specific proteins induced by leminoprazole itself might play an important role in the cytoprotective effect.

Overall, these results suggest that only the mucus-elevating effect of administered leminoprazole may be partly due to the stimulatory effects of sulfide derived from leminoprazole on mucus secretion and synthesis by epithelial cells.

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