Effects of KB-5492, a New Anti-Ulcer Agent, on Ethanol- and Acidified Aspirin-Induced Gastric Mucosal Damage In Vivo and In Vitro

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ABSTRACT—The effects of KB-5492, a new anti-ulcer agent with selective affinity for the sigma receptor, on ethanol- and acidified aspirin-induced gastric mucosal damage were studied in vivo and in vitro and compared with those of 16,16-dimethyl prostaglandin E₂ (dmPGE₂). In the in vivo study, KB-5492 (200 mg/kg, p.o.) as well as dmPGE₂ (0.01 mg/kg, p.o.) significantly prevented the acute macroscopic lesions in rat gastric mucosa induced by oral administration of either absolute ethanol or 80 mM aspirin in 150 mM HCl. The light microscopic examination revealed that KB-5492 almost completely prevented the deep mucosal lesions induced by these necrotizing agents. KB-5492 also prevented the exfoliation of surface epithelial cells, but its preventive effect was incomplete. In the in vitro study, gastric epithelial cells, isolated from the rat stomach, were cultured for 6 days until they reached confluency. Subsequently, ⁵¹Cr was incorporated into the cells. Both 10 mM aspirin (at pH 5.0) and 12.5% ethanol (at pH 7.4) induced damage to the cells and markedly increased ⁵¹Cr release from the cells. KB-5492 at 0.3 and 1 mM and dmPGE₂ at 0.3 and 1 μM significantly, but not completely, prevented both the aspirin- and ethanol-induced increases in ⁵¹Cr release from the cells. These findings indicate that KB-5492 as well as dmPGE₂ may exert a direct but limited protective effect on the surface epithelial cells in vivo.

Keywords: KB-5492, Anti-ulcer agent, Prostaglandin, Gastric epithelial cell, Gastric mucosal protection

KB-5492, 4-methoxyphenyl 4-(3,4,5-trimethoxybenzyl)-1-piperazineacetate monofumarate monohydrate, is a new anti-ulcer agent previously shown to prevent various experimental gastric mucosal lesions in rats including those induced by oral administration of absolute ethanol and acidified aspirin (1, 2). Although KB-5492, at anti-ulcer doses, did not affect either basal or histamine-stimulated gastric acid secretion in rats (1, 2), it increased gastric mucosal blood flow and prevented aspirin-induced reduction of gastric mucus (2). Therefore, KB-5492 has been considered to enhance gastric mucosal defensive factors.

Recently, sigma-receptors have been reported to exist in many organs such as the brain (3), liver (4) and spleen (5). Furthermore, sigma-receptors are abundant in the mucosal and submucosal plexus of the gastric fundus and duodenum in guinea pig and human (6, 7). Sigma receptor ligand, 1,3-di(2-tolyl) guanidine (DTG), stimulates duodenal alkaline secretion and shows protective effects on various gastric lesions in rats through a sigma receptor (8, 9). Thus, sigma receptors may play an important role in the control of mucosal function. KB-5492 was found to have a selective affinity for the sigma receptor in guinea

pig brain and porcine fundic mucosa (Y. Harada et al., unpublished data).

In the present study, the effects of KB-5492 on ethanol- and acidified aspirin-induced gastric mucosal damage were investigated in vivo and in vitro to clarify whether a direct protective effect on the gastric mucosal cells is involved in the mechanism of gastric mucosal protection by KB-5492.

MATERIALS AND METHODS

Animals

In the in vivo studies, male Sprague-Dawley rats weighing 210–240 g (Charles River Japan, Atsugi) were used. The animals were fasted but were allowed free access to water for 24 hr before the experiments. In the in vitro studies, 5- to 10-day-old Sprague-Dawley rats (Clea Japan, Osaka) were used.

In vivo studies

Effects of drugs on ethanol- and acidified aspirin-induced gastric mucosal lesions: One milliliter of absolute

ethanol (Wako, Osaka) or 80 mM aspirin (Wako) suspended in 150 mM HCl was administered to the rat orally. After 1 hr, the animals were killed. The stomach was removed, fixed by inflation with 12 ml of 1% formalin, and then incised along the greater curvature. The length (mm) of each lesion formed on the glandular portion was measured under a dissecting microscope, and the sum of the length of the lesions in each animal was calculated. For histological examination, the stomach was spread on cardboard and immersed in 10% phosphate-buffered formalin (pH 7.0) for 48 hr. A small specimen of the tissue was cut out from a non-necrotic lesion area of the fundic mucosa. The tissue was embedded in paraffin and sliced into 4- μ m sections. Subsequently, the sections were stained with hematoxylin and eosin.

KB-5492 (Kanebo, Osaka) suspended in 1% gum arabic solution, or 16,16-dimethyl prostaglandin E₂ (dmPGE₂, Cayman Chemical, Ann Arbor, MI, USA) dissolved initially in absolute ethanol and then diluted with 1% gum arabic solution was administered orally 30 min before ethanol or aspirin treatment.

In vitro studies

Isolation and culture of gastric epithelial cells: The experiment was performed according to the method of Terano et al. (10). The stomach of 5- to 10-day-old rats was removed, and the corpus area was isolated and then minced. The minced tissues were suspended in Coon's modified Ham's F-12 medium (KC Biological, Lenexa, KS, USA) containing 0.1% collagenase (Wako), 0.05% hyaluronidase (Type I-S; Sigma, St. Louis, MO, USA), 100 U/ml penicillin G potassium (Meiji Seika, Tokyo), 100 μ g/ml streptomycin sulfate (Meiji Seika) and 100 μ g/ml gentamicin sulfate (Sigma), and then incubated at 37°C for 1 hr. The tissues were filtered through a nylon mesh and the filtrate was washed with Hank's balanced salt solution (HBSS; Nissui, Tokyo). The cell pellet was resuspended in Coon's modified Ham's F-12 medium containing 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD, USA), 15 mM HEPES (Dojindo Laboratories, Kumamoto), 2 μ g/ml fibronectin (Sigma) and the same antibiotics as described above. Subsequently, the cells were seeded in 96-well tissue culture plates (Corning, Corning, NY, USA) at 5×10^4 cells/well and cultured at 37°C for 6 days in a humidified atmosphere containing 5% CO₂ in air until they reached confluency.

Chromium-51 loading: The experiment was also performed according to the method of Terano et al. (11). The medium was discarded and the cells were washed with HBSS. The cells were incubated at 37°C for 2 hr in HBSS containing 370 kBq/ml ⁵¹Cr (sodium chromate, 14.8–44.4 TBq/g Cr, New England Nuclear, Boston, MA, USA) and then washed with HBSS to remove excess

isotope.

Measurement of ⁵¹Cr release: Both test drugs (i.e., KB-5492 and dmPGE₂) and damaging agents (i.e., aspirin and ethanol) were dissolved in HBSS. In the study on aspirin-induced damage, ⁵¹Cr-preloaded cells were incubated at 37°C for 2 hr in the following solutions adjusted to pH 5.0: (a) HBSS plus 10 mM aspirin, (b) test drug plus 10 mM aspirin, (c) HBSS alone and (d) test drug alone. After incubation, the radioactivity of ⁵¹Cr released into the supernatant was counted with a Gamma Counter (A-5210; Packard, Meriden, CT, USA). The radioactivity of ⁵¹Cr in the cells was also counted after the cells were solubilized with 1 N NaOH. Subsequently, ⁵¹Cr release (%) was calculated as follows:

$$^{51}\text{Cr Release (\%)} = \frac{\text{cpm in the supernatant}}{\text{cpm in the supernatant} + \text{cpm in the cells}}$$

In the study on ethanol-induced damage, test drugs, dissolved in HBSS and adjusted to pH 7.4, were preincubated with ⁵¹Cr-preloaded cells at 37°C for 20 min, and the cells were incubated for 10 min after the exposure to 12.5% ethanol. Subsequently, ⁵¹Cr release was calculated in the same manner as described above.

Statistics

Results were expressed as the mean $\pm$ S.E. Statistical significance was determined by one-way analysis of variance followed by Dunnett's or Duncan's test.

RESULTS

Effects of drugs on ethanol- and acidified aspirin-induced gastric mucosal lesions

As shown in Fig. 1, oral administration of absolute

Table 1. Effects of KB-5492 and 16,16-dimethyl prostaglandin E₂ on ethanol- and acidified aspirin-induced gastric mucosal lesions in rats

Drug	Dose (mg/kg, p.o.)	No. of rats	Lesion length (mm)	Inhibition (%)
(1) Absolute ethanol-induced lesions				
Control	—	6	159 $\pm$ 18	—
KB-5492	200	6	27 $\pm$ 4**	83
16,16-dmPGE ₂	0.01	6	13 $\pm$ 4**	92
(2) Acidified aspirin-induced lesions				
Control	—	6	143 $\pm$ 14	—
KB-5492	200	6	20 $\pm$ 8**	86
16,16-dmPGE ₂	0.01	6	14 $\pm$ 6**	90

One milliliter of absolute ethanol or 80 mM aspirin in 150 mM HCl was administered orally and the stomach was removed 1 hr later. Drugs were administered orally 30 min before ethanol or acidified aspirin treatment. Values are expressed as the mean $\pm$ S.E.

**P < 0.01, significantly different from the control (Dunnett's test).

Fig. 1. Macroscopic appearance of rat gastric mucosa 1 hr after the oral administration of absolute ethanol and acidified aspirin. One milliliter of absolute ethanol or 80 mM aspirin in 150 mM HCl was administered orally and the stomach was removed 1 hr later. Drug or vehicle (1% gum arabic) was administered orally 30 min before ethanol or acidified aspirin treatment. A: vehicle + absolute ethanol, B: KB-5492 (200 mg/kg) + absolute ethanol, C: dmPGE₂ (0.01 mg/kg) + absolute ethanol, D: vehicle + acidified aspirin, E: KB-5492 (200 mg/kg) + acidified aspirin, F: dmPGE₂ (0.01 mg/kg) + acidified aspirin.

ethanol and that of 80 mM aspirin in 150 mM HCl both induced macroscopic lesions in the gastric mucosa 1 hr later. KB-5492 and dmPGE₂, administered orally at 200 and 0.01 mg/kg, respectively, prevented these lesions. As shown in Table 1, both drugs significantly reduced the lesion length as compared with the control.

Light microscopic observations

Figures 2 and 3 show the typical microscopic appearances of the gastric mucosa obtained 1 hr after the oral administration of absolute ethanol and acidified aspirin, respectively. Not only the exfoliation of surface epithelial cells, but also lesions reaching the deeper layers were ob-

served in the gastric mucosa exposed to absolute ethanol and acidified aspirin. KB-5492 and dmPGE₂, administered orally at 200 and 0.01 mg/kg, respectively, almost completely prevented the deep mucosal lesions. Both drugs also prevented the exfoliation of surface epithelial cells, but their preventive effects were incomplete.

Effects of drugs on ethanol- and aspirin-induced damage to cultured gastric epithelial cells

More than 90% of the cultured cells were histochemically identified as mucus-producing epithelial cells by periodic acid-Schiff staining, which was consistent with the result obtained by Terano et al. (10–12).

Fig. 2. Light microscopy of rat gastric mucosa 1 hr after the oral administration of absolute ethanol. The exfoliation of surface epithelial cells is indicated by arrows. A: normal, B: vehicle + absolute ethanol, C: KB-5492 (200 mg/kg) + absolute ethanol, D: dmPGE₂ (0.01 mg/kg) + absolute ethanol. Hematoxylin and eosin staining ($\times 300$).

As shown in Fig. 4, 12.5% ethanol markedly increased the ^{51}Cr release from cultured gastric epithelial cells. KB-5492 at 0.3 and 1 mM significantly and concentration-dependently prevented the ethanol-induced increase in ^{51}Cr release from the cells. DmPGE₂ slightly but significantly prevented the increase in ^{51}Cr release at 0.1, 0.3 and 1 μM .

As shown in Fig. 5, 10 mM aspirin also markedly increased the ^{51}Cr release from the cells. KB-5492 at 0.3 and 1 mM and dmPGE₂ at 0.3 and 1 μM slightly but sig-

nificantly and concentration-dependently prevented the aspirin-induced increase in ^{51}Cr release.

Neither 1 mM KB-5492 nor 1 μM dmPGE₂ alone affected the ^{51}Cr release from the cells.

DISCUSSION

In the present study, KB-5492 at an anti-ulcer dose of 200 mg/kg (1, 2), as well as dmPGE₂ at 0.01 mg/kg (13, 14), prevented the acute macroscopic lesions in rat gastric

Fig. 3. Light microscopy of rat gastric mucosa 1 hr after the oral administration of 80 mM aspirin in 150 mM HCl. The exfoliation of surface epithelial cells is indicated by arrows. A: normal, B: vehicle + acidified aspirin, C: KB-5492 (200 mg/kg) + acidified aspirin, D: dmPGE₂ (0.01 mg/kg) + acidified aspirin. Hematoxylin and eosin staining ($\times 300$).

mucosa induced by oral administration of either absolute ethanol or 80 mM aspirin in 150 mM HCl. However, in the light microscopic examination, the preventive effects of KB-5492 and dmPGE₂ on the exfoliation of surface epithelial cells induced by these necrotizing agents were incomplete. Our result that dmPGE₂ did not completely prevent the ethanol-induced damage to surface epithelial cells is consistent with those obtained by Lacy and Ito (15), Tarnawski et al. (16) and Schmidt et al. (17). However, our result that dmPGE₂ did not completely pre-

vent aspirin-induced damage to surface epithelial cells is inconsistent with that obtained by Ohno et al. (18). This discrepancy may be due to the difference in experimental conditions between our study and theirs; the gastric mucosa was exposed to 30 or 100 mg/kg of aspirin suspended in 1% carboxymethylcellulose for 10 min in the study by Ohno et al. (18), whereas the mucosa was exposed to 80 mM (72 mg/kg) aspirin suspended in 150 mM HCl for 60 min in our study.

Tarnawski et al. (16) have suggested that the integrity

Fig. 4. Effects of KB-5492 (KB) and 16,16-dimethyl prostaglandin E_2 (PG) on ethanol-induced increase in ^{51}Cr release from cultured rat gastric epithelial cells. Test drugs, dissolved in Hank's balanced salt solution (HBSS, pH 7.4), were added to the cultured cells, and the cells were incubated at 37°C for 20 min. Subsequently, ethanol (12.5% finally) was added to the cells, and the cells were incubated at 37°C for 10 min. Each column represents the mean $\pm$ S.E. of 9 cultures. * $P < 0.05$ and ** $P < 0.01$ represent significant differences (Duncan's test).

Fig. 5. Effects of KB-5492 (KB) and 16,16-dimethyl prostaglandin E_2 (PG) on aspirin-induced increase in ^{51}Cr release from cultured rat gastric epithelial cells. Test drugs plus aspirin (10 mM finally), both dissolved in Hank's balanced salt solution (HBSS, pH 5.0), were added to the cultured cells. Subsequently, the cells were incubated at 37°C for 2 hr. Each column represents the mean $\pm$ S.E. of 9 cultures. * $P < 0.05$ and ** $P < 0.01$ represent significant differences (Duncan's test).

of sub-epithelial proliferative zone and rapid restoration of the surface epithelium by the migration from this zone is important to the protective effect of dmPGE_2 against ethanol-induced gastric mucosal damage. In the present study, KB-5492 as well as dmPGE_2 almost completely prevented both ethanol- and acidified aspirin-induced lesions in the deeper layers corresponding to the proliferative zone (16), which may partly contribute to the mechanism of gastric mucosal protection by KB-5492.

Terano et al. (11) have reported that aspirin-induced damage to cultured gastric epithelial cells evaluated by ^{51}Cr release from ^{51}Cr -preloaded cells is highly correlated with that evaluated by the cell viability determined by the

trypan blue exclusion test. In addition, using both methods, they demonstrated the protective effect of dmPGE_2 against aspirin-induced damage to cultured gastric epithelial cells (11). In the present study, dmPGE_2 slightly prevented the aspirin-induced increase in ^{51}Cr release from cultured gastric epithelial cells, which is consistent with the result obtained by Terano et al. (11). In addition, dmPGE_2 slightly prevented the ethanol-induced increase in ^{51}Cr release from the cells at the same concentrations as in aspirin-induced damage. These findings confirm that dmPGE_2 directly protects the gastric epithelial cells in vitro regardless of damaging substances. In a similar manner to dmPGE_2 , KB-5492 slightly prevented both the aspirin- and ethanol-induced increases in ^{51}Cr re-

lease from the cells. Therefore, KB-5492 is also considered to exert a direct protective effect against the damage to the gastric epithelial cells induced by these necrotizing agents *in vitro*. However, KB-5492 and dmPGE₂ incompletely prevented both the aspirin- and ethanol-induced increases in ⁵¹Cr release from the cells. This may explain the incomplete protection by KB-5492 and dmPGE₂ against the microscopic damage to surface epithelial cells *in vivo* induced by both absolute ethanol and acidified aspirin. Taking these considerations collectively, KB-5492 as well as dmPGE₂ may exert a direct but limited protective effect on the surface epithelial cells *in vivo*.

Recently, Lippe and Szabo (19) demonstrated that dmPGE₂ delays the absorption of ethanol and aspirin from the gastric mucosa, and they suggested that this is involved in the mechanism by which dmPGE₂ protects the gastric mucosa against these damaging agents. Therefore, the effect of KB-5492 on the absorption of these damaging agents from the gastric mucosa also must be studied in the future.

In conclusion, KB-5492 as well as dmPGE₂ protects the gastric mucosa *in vivo* against the damage induced by both ethanol and acidified aspirin. However, KB-5492 does not seem to play a key role in the mechanism of gastric mucosal protection by a direct protective effect on the surface epithelial cells.

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