Differential Effect of Benexate Hydrochloride Betadex on Prostaglandin Levels in Stomach and Inflammatory Site in Rats

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Received January 29, 1996 Accepted July 29, 1996

ABSTRACT—We compared the effects of an anti-ulcer agent, benexate hydrochloride betadex (BHB), on prostaglandin (PG) levels in gastric tissue and inflammatory exudate in untreated and indomethacin-treated rats. BHB (100, 300 and 1000 mg/kg, p.o.) showed dose-dependent inhibition of gastric mucosal lesions induced by indomethacin (30 mg/kg, p.o.). Sustained decrease of PGs (PGE_2 and 6-keto-$PGF_{1\alpha}$) in the gastric wall was observed from 0.5 to 6 hr after indomethacin treatment. BHB (300 and 1000 mg/kg) dose-dependently led to recovery of the indomethacin-induced decrease of gastric PGs at 1 and 6 hr after dosing. It did not antagonize the indomethacin-induced decrease of PG levels in the pleural exudate of carrageenin pleurisy nor did it affect the anti-inflammatory effects of indomethacin. BHB (100 to 1000 mg/kg) alone increased gastric PGE_2 by 61% to 113%, while it decreased PGE_2 levels in the pleural exudate by 9% to 71% at 6 hr after dosing. These results suggest that sustained increase of gastric PGE_2 by BHB could be responsible for protection against indomethacin-induced gastric mucosal lesions and that BHB is a suitable anti-ulcer agent for NSAIDs without compromising their anti-inflammatory effects.

Keywords: Benexate hydrochloride betadex, Gastric mucosa, Indomethacin, Prostaglandin, Pleurisy

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their efficacy in the treatment of pain, inflammation and fever, but are well-known to cause damage to the upper gastrointestinal tract. However, withdrawal from such therapy is not always practicable in patients who need analgesics and antipyretics (1). The basis of NSAIDs-induced gastric damage is thought to be multifactorial (2, 3): i) suppression of mucosal prostaglandin (PG) synthesis via inhibition of cyclooxygenase, ii) decrease in the transmucosal potential difference, iii) decrease in mucosal blood flow, iv) effects on neutrophil function, v) inhibition of gastric HCO_3^- secretion, and vi) enhancement of gastric motility. Among these, inhibition of PG synthesis probably plays a key role because PGs, particularly PGE_2 and PGI_2, are known to function in gastric defense by inhibiting acid secretion and by increasing mucus secretion and blood flow (4).

To prevent acute gastritis and peptic ulcers induced by NSAIDs, benexate hydrochloride betadex (BHB, β-cyclodextrin clathrate of benexate) is used clinically as a defensive-type anti-ulcer agent. Its defensive effects on gastric mucosa have been demonstrated in the promotion of PG synthesis (5), protein secretion (6) and blood flow (7), and also in the inhibition of acid secretion as well as gastric motility (8) and other activities (9–11). However, although BHB inhibits indomethacin-induced gastric lesions (8) and protects against the indomethacin-induced decrease of gastric mucosal PGs in animals (5), it is not certain whether BHB affects PG levels at the inflammatory site or whether it compromises the anti-inflammatory effects of NSAIDs such as indomethacin.

In the current study, to validate the suitability of BHB as an anti-ulcer agent for NSAIDs-related gastric injury, we compared the effect of BHB on PG levels in gastric tissue and inflammatory exudates and examined its effect on inflammatory responses to carrageenin in rats treated with or without indomethacin. Since gastric PGs are easily formed by chopping and scraping of gastric mucosa (12), and the already reported increase of gastric PGs by BHB might include artifact effects (5), we collected the gastric tissue by *in situ* freezing to accurately measure the levels of endogenous gastric PGs.

MATERIALS AND METHODS

Animals

Seven-week-old male Lewis rats (Charles River Japan, Tokyo), weighing 170–220 g, were used. They were

housed in groups of 4 to 5 per cage. All animals were starved, but allowed free access to water, for 16 hr before the experiments.

Indomethacin-induced gastric mucosal lesion

Indomethacin (3, 10 and 30 mg/kg), suspended in 0.6% arabic gum, was given orally. The animals were sacrificed 6 hr after indomethacin treatment under pentobarbital (40 mg/kg, i.p.) anesthesia. The stomach was removed, inflated by injecting 6 ml of 1% formalin and kept immersed in 1% formalin for 15 min to fix the mucosa. After opening the stomach along the greater curvature, the length (mm) of visible lesions in the glandular portion was measured and summed per stomach for use as the lesion index. BHB (100, 300 and 1000 mg/kg), dissolved in distilled water, was administered orally 15 min before indomethacin treatment.

Assay of gastric PGs

At given intervals after indomethacin treatment, the animals were anesthetized with pentobarbital and the abdominal wall was incised. The omentum was gently separated without touching the stomach, and the glandular portion of stomach along the great curvature was pressed *in situ* between a pair of wide metal blocks ($5 \times 4 \times 1$ cm) fixed at the tips of tongs, which had all been refrigerated in liquid nitrogen (12). The stomach was immediately cut off and kept in liquid nitrogen for at least 10 min. About 200- to 300-mg samples of the glandular portion were pulverized by hitting the cold piston with a hammer 5 times. This pulverized tissue was immediately transferred into 15-ml polypropyrene tube that had been kept cool with dry ice. The sample was stored at -80℃ until assay. PGs were extracted by a procedure based on the method of Suzuki et al. (12). The pulverized samples were mixed with 5 ml of 80% cold ethanol (HPLC grade; Wako Pure Chemicals, Tokyo) and homogenized for 30 sec using a Polytron, and left standing in an ice bath for 60 min. The samples were then centrifuged at $2000 \times g$ for 20 min at 4℃. Supernatants were diluted to a 10% ethanol solution by adding distilled water, acidified to pH 3.0 and immediately applied to an Amprep C18 column (Amersham, Little Chalfont, UK). After washing successively with 20 ml of 10% ethanol and 20 ml of petroleum ether (Wako Pure Chemicals), the PGs were finally extracted with 8 ml of ethyl acetate. The PGE_2 and 6-keto-$PGF_{1\alpha}$ in the eluate were quantitated by radioimmunoassay using ^{125}I-labeled kits (NEK-020A-10 and NEK-025A-10; New England Nuclear, Boston, MA, USA). The recovery of ^{3}H-labeled PGE_2 (8000 dpm, NET-428; New England Nuclear), added to the samples immediately after homogenization was approximately 80%. Threshold amounts, which can be detected by these assay systems, were 0.5 pg/tube for PGE_2 and 5 pg/tube for 6-keto-$PGF_{1\alpha}$.

Carrageenin-induced pleurisy and assay of PGs

Experiments were performed according to the methods of Harada et al. (13). Under ether anesthesia, 0.2 ml of 1% λ-carrageenin (Zushi Chemical, Kanagawa) in sterile saline solution was injected into the right pleural cavity through a blunt-edged 25-gauge needle. Five hours after the induction of pleurisy, the animals were sacrificed by exsanguination under ether anesthesia. For analysis of PG levels in the pleural exudate, 4 ml of saline containing 10 μg/ml of indomethacin (Sigma, St. Louis, MO, USA) was injected immediately after exsanguination to inhibit the formation of PGs during exudate collection. After 15 min, the volume of exudate was measured and collected in a polypropyrene tube, and aliquots (0.1 ml) were taken to count the number of infiltrated cells. The remaining sample was centrifuged at $2000 \times g$ for 15 min at 4℃, and the supernatant was stored at -80℃ until assay. The sample was acidified with 1 N HCl and loaded on to an Amprep C18 column (Amersham). The subsequent procedures for extraction and assay of PGs were the same as those described above for gastric PGs. Indomethacin was administered orally 1 hr before the induction of pleurisy. BHB was administered orally 15 min before indomethacin treatment.

Preparation of drugs

BHB was obtained from Teikoku Chemical Industry Co., Ltd. (Osaka); it was dissolved in distilled water just before use and administered orally at 5 ml/kg. Indomethacin (Sigma) was suspended in 0.6% arabic gum solution and administered orally at 5 ml/kg.

Statistical analyses

Data are presented as the mean$\pm$S.E.M. Statistical significance was determined by Student's *t*-test (unpaired, two-tailed) or Dunnett's test. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Effect of BHB on indomethacin-induced gastric mucosal lesions

Oral administration of indomethacin dose-dependently caused gastric mucosal lesions 6 hr after dosing (Table 1). As severe mucosal lesions were consistently observed with 30 mg/kg, we chose this dose of indomethacin for the following experiments. BHB offered protection against indomethacin-induced lesions dose-dependently, with 19%, 27% and 85% inhibition at 100, 300 and 1000 mg/kg, respectively.

Table 1. Effect of benexate hydrochloride betadex (BHB) on indomethacin-induced gastric mucosal lesion in rats

BHB (mg/kg, p.o.)	Indomethacin (mg/kg, p.o.)	Gastric mucosal lesion incidence	Gastric mucosal lesion lesion index (mm)
Experiment 1			
	3	4/6	1.9±1.1
	10	6/6	14.1±5.7
	30	6/6	57.3±9.7
Experiment 2			
0	30	8/8	82.3±3.8
100	30	8/8	66.9±4.3
300	30	8/8	60.3±10.0*
1000	30	8/8	12.2±3.5**

Gastric mucosal lesion was measured 6 hr after indomethacin treatment. BHB was administered 15 min before indomethacin treatment. Data represent the mean±S.E. *P<0.05, **P<0.01 vs indomethacin alone group in Experiment 2 (Dunnett's test).

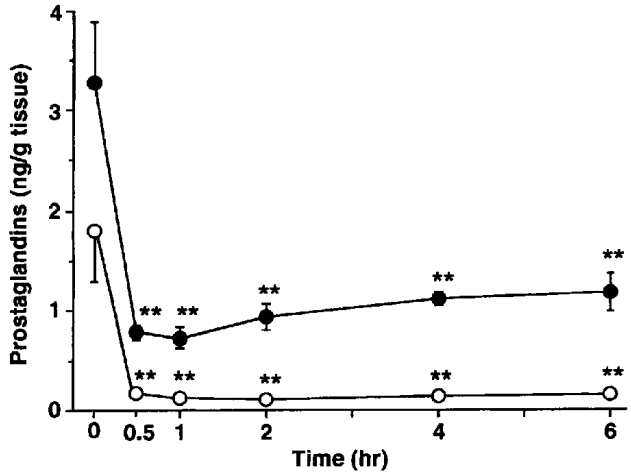

Fig. 1. Time course changes of endogenous gastric prostaglandin (PG) levels following indomethacin treatment (30 mg/kg, p.o.) in rats. Each point represents the mean±S.E. of 5 animals. **P<0.01 vs normal control (0 hr, Student's *t*-test). ○ PGE_2, ● 6-keto-$PGF_{1\alpha}$.

Effect of BHB on indomethacin-induced decrease of gastric PGs

PGE_2 and 6-keto-$PGF_{1\alpha}$ levels in normal gastric walls were 1.80±0.56 and 3.28±0.61 ng/g tissue (n=5), respectively. A lesion-inducing dose (30 mg/kg) of indomethacin caused a profound and sustained decrease of PGs from 0.5 to 6 hr with a peak effect at around 1 hr after the treatment (Fig. 1). The decrease of PGE_2 was steeper than that of 6-keto-$PGF_{1\alpha}$: the remaining PGE_2 was less than 10% of the normal gastric wall from 0.5 to 6 hr, while the level of 6-keto-$PGF_{1\alpha}$ tended to recover from 22% of the control level at 1 hr to 36% at 6 hr.

Preteatment with BHB dose-dependently prevented the indomethacin (30 mg/kg)-induced decrease of PGs not only 1 hr but also 6 hr after indomethacin treatment, when recovery of PGs by BHB (1000 mg/kg) was more pronounced at 6 hr than at 1 hr (Fig. 2). PGE_2 recovered from 22% to 31% of the normal control at 1 hr and from 29% to 70% at 6 hr (Fig. 2; A and B). 6-Keto-$PGF_{1\alpha}$ also recovered from 36% to 50% of normal control at 1 hr and from 66% to 99% at 6 hr (Fig. 2: A and B).

Effect of BHB on indomethacin-induced decrease of PGs in carrageenin pleurisy

Since BHB protected against the undesirable effects of indomethacin, such as gastric lesions and decrease of stomach PGs, we next tested whether BHB affected the anti-inflammatory effect of indomethacin. In the carrageenin pleurisy model in rats, 30 mg/kg indomethacin caused large decreases of PGs (less than 10% of vehicle control) as well as significant inhibition of plasma exudation and leukocyte infiltration (Table 2). Pretreatment

Table 2. Effect of benexate hydrochloride betadex, (BHB) on anti-inflammatory activity of indomethacin in carrageenin-induced pleurisy in rats

BHB (mg/kg, p.o.)	Indomethacin (mg/kg, p.o.)	No. of rats	PGE_2 (pg/rat)	6-Keto-$PGF_{1\alpha}$ (pg/rat)	Exudate volume (ml)	Leukocyte No. (×10^6/rat)
Normal						
—	—	6	31±3	415±48	0±0	7±0
Carrageenin pleurisy						
0	0	6	166±28	3727±181	1.23±0.08	112±5
0	30	6	15±1**	179±14**	0.37±0.02**	69±5**
1000	30	6	17±0** NS	194±14** NS	0.38±0.02** NS	72±4** NS

Pleural exudates were collected 5 hr after the induction of carrageenin pleurisy. Indomethacin was administered 1 hr before the induction of pleurisy, and BHB was administered 15 min before indomethacin treatment. In the normal animal group, the pleural cavity was washed with 4 ml of saline containing 10 μg/ml of indomethacin. Data represent the mean±S.E. **P<0.01 vs vehicle alone control (dose=0/0, Student's *t*-test). NS: not significant.

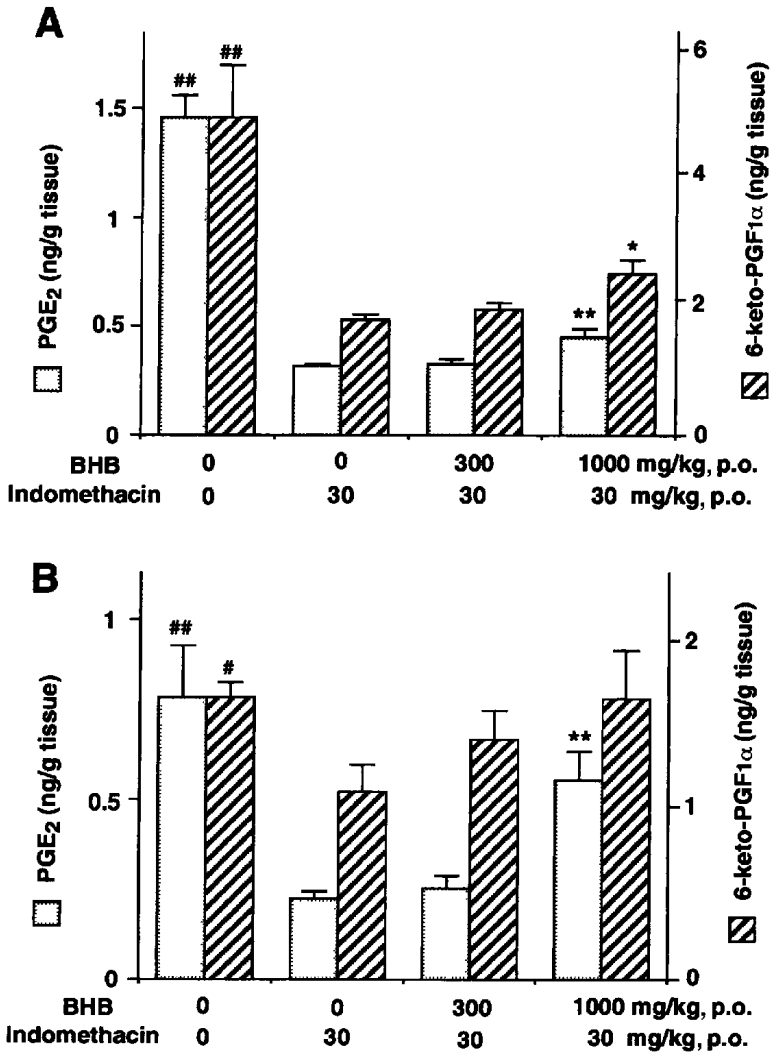

Fig. 2. Effect of betadex hydrochloride betadex (BHB) on indomethacin-induced decrease of gastric prostaglandin (PG) levels in rats. Gastric tissues were collected 1 hr (A) or 6 hr (B) after indomethacin treatment. BHB was administered 15 min before indomethacin treatment. Experiments shown in panels A and B were conducted on different days. Values are the mean ± S.E. of 6 to 8 animals. $^{\#}P<0.05$, $^{\#\#}P<0.01$ (Student's *t*-test), $^{*}P<0.05$, $^{**}P<0.01$ (Dunnett's test) vs indomethacin alone group.

with BHB (1000 mg/kg) did not affect these effects, indicating that BHB did not weaken the anti-inflammatory effects of indomethacin at inflammatory sites.

Effect of BHB on PG levels of stomach and pleural exudate

Next we studied the effect of BHB alone on PG levels in the stomach and pleural exudate to confirm the differential effect of BHB in the stomach versus inflammatory site. Significant and marked increases of both PGE_2 and 6-keto-$PGF_{1\alpha}$ (by 170% and 233%, respectively) were observed 1 hr after BHB (1000 mg/kg) treatment, with the increase of 6-keto-$PGF_{1\alpha}$ being significant at lower doses of BHB (Fig. 3A). Significant increases of gastric PGs were still noted at 6 hr;% increases with 100, 300 and 1000 mg/kg BHB for PGE_2 were 61%, 79% and 113%, respectively, and for 6-keto-$PGF_{1\alpha}$, were 116%, 90% and 161%, respectively (Fig. 3B).

By contrast, BHB decreased PGE_2 levels in the pleural exudate of carrageenin pleurisy in a dose-dependent manner (9%, 40% and 71% inhibition with 100, 300 and 1000 mg/kg, respectively), while the suppression of 6-keto-$PGF_{1\alpha}$ remained around 50% (Table 3). Leukocyte infiltration but not plasma exudation was also inhibited by the higher doses of BHB, suggesting that BHB itself may possess mild anti-inflammatory activity at doses effective against indomethacin-induced gastric mucosal lesion.

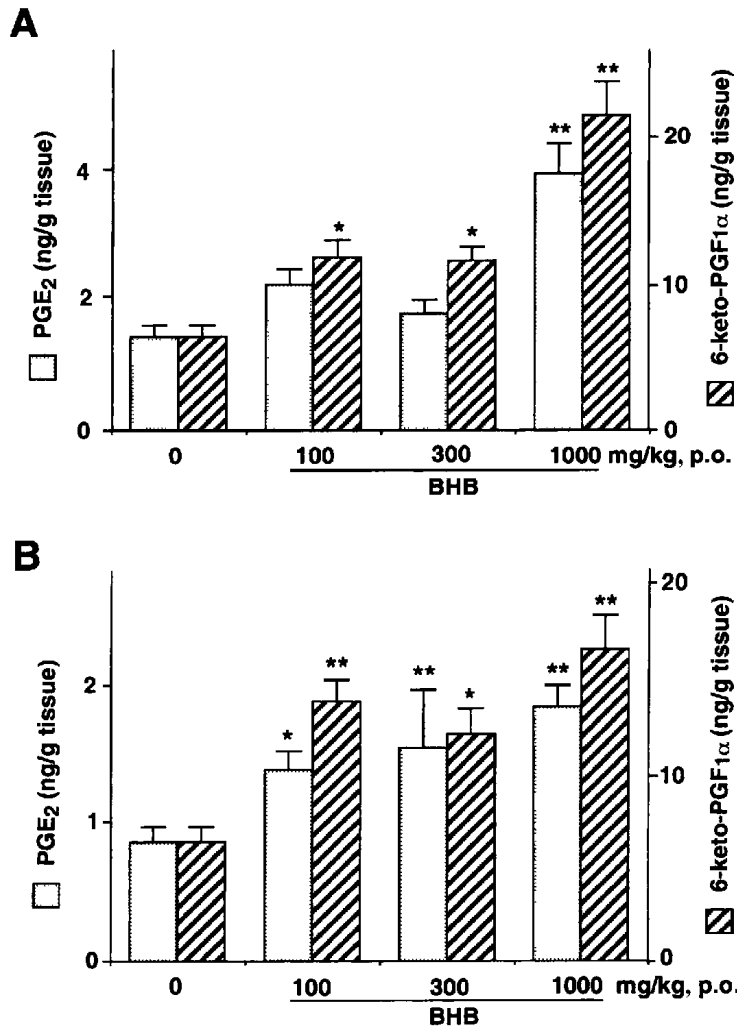

Fig. 3. Effect of benexate hydrochloride betadex (BHB) on gastric prostaglandin (PG) levels in rats. Gastric tissues were collected 1 hr (A) or 6 hr (B) after BHB treatment. Experiments shown in panels A and B were conducted on different days. Values are the mean±S.E. of 7 to 8 animals. *$P<0.05$, **$P<0.01$ vs vehicle control (dose=0, Dunnett's test).

Table 3. Effect of benexate hydrochloride betadex (BHB) on carrageenin-induced pleurisy in rats

BHB (mg/kg, p.o.)	No. of rats	PGE_2 (pg/rat)	6-Keto-$PGF_{1\alpha}$ (pg/rat)	Exudate volume (ml)	Leukocyte No. ($\times 10^6$/rat)
0	6	131±13	2522±139	1.08±0.03	113±4
100	6	119±19	1276±113**	1.07±0.02	103±3
300	5	79±7*	1410±99**	1.14±0.04	94±5*
1000	6	38±5**	1127±160**	1.02±0.02	93±5**

Pleural exudates were collected 5 hr after the induction of pleurisy. BHB was administered 1 hr before the induction of pleurisy. Data represent the mean±S.E. *$P<0.05$, **$P<0.01$ vs vehicle control (dose=0, Dunnett's test).

DISCUSSION

The present findings clearly show that BHB could offer protection against indomethacin-induced gastric mucosal lesion and decrease of endogenous gastric PGs without antagonizing the anti-inflammatory activity and

suppression of PG levels caused by indomethacin at the inflammatory site. More interestingly, BHB alone differentially affected PG levels in gastric tissue and inflammatory sites: gastric PGs were increased for at least 6 hr, while those in inflammatory exudates were decreased by BHB.

In the present study, the normal endogenous levels of PGs (PGE_2 was around 1 ng/g tissue and 6-keto-$PGF_{1\alpha}$, around 3 ng/g tissue) were similar to those reported by Suzuki et al. (12), but much lower than those (50–100 ng/g tissue) in other studies (14, 15), the latter probably reflecting the artifact generation of PGs due to the chopping and scraping of gastric mucosa. The strong and sustained decrease of gastric PGs, particularly PGE_2 by the lesion-inducing dose of indomethacin, is consistent with already documented results (16, 17), and we also confirmed significant recovery by BHB of the indomethacin-induced decrease of gastric PGs with doses capable of inhibiting indomethacin-induced gastric mucosal lesion (5). These findings suggest that sustained recovery of gastric PGs, particularly PGE_2, by BHB could be one of the mechanisms responsible for its inhibitory activity against indomethacin-induced gastric lesion.

The differential effect of BHB on PG levels in gastric and inflammatory tissue can be explained by the unique absorption, distribution and metabolism of BHB. After oral dosing of BHB in rats, only BHB is distributed in gastric tissue without metabolism in the stomach. After gastric emptying, BHB is rapidly metabolized in the small intestine to benzylsalicylate, salicylate, benzyl alcohol and guanidinomethylcyclohexanecarbonic acid (GMCHA). The main metabolite in plasma is salicylate, which can be found at a concentration above 200 μM from 30 min to 10 hr after 340.7 mg/kg, p.o. of BHB, with the levels of benzyl alcohol and GMCHA being 1/10 that of salicylate (18). Also, in human subjects, salicylate is the main metabolite of BHB in plasma with similar pharmakokinetics observed in rats (19). Salicylate is known to inhibit PG synthesis at the inflammatory site but not in gastric mucosa, which contrasts with typical NSAIDs like aspirin and indomethacin that inhibit PG production in both inflammatory and gastric tissues (20). Furthermore, definite inhibition of PGE_2 production in inflammatory exudates was reported in relation to the pharmacokinetics of salicylate in plasma and inflammatory exudates after oral dosing of salicylate (21). Thus, it is highly probable that the increase of gastric PG levels are due to BHB, while their decrease in pleural exudates are due to salicylate.

Salicylate reduces gastrointestinal lesions induced by NSAIDs and absolute ethanol (22). Salicylate also has been shown to interact with aspirin and indomethacin, decreasing the inhibitory effect of aspirin and indomethacin on gastric mucosal cyclooxygenase (COX) activity ex vivo regardless of oral or parenteral administration (23). Therefore, it is plausible that salicylate generated in plasma after oral BHB treatment could contribute to the protection against indomethacin-induced gastric lesion and decrease of gastric PGs levels, besides the direct effect of BHB on gastric mucosa.

Recently, a selective increase of COX-2 over COX-1 was reported in the stomachs bearing chronic gastric ulcer induced by subserosal injection of acetic acid in mice (24), suggesting that the increases in COX-2 activity may participate in ulcer healing through production of PGE_2, an agiogenic prostanoid known to induce the expression of a strong angiogenic factor such as vascular endothelial growth factor (25). Therefore, it might be argued that the presence of salicylate in plasma after oral administration of BHB could delay ulcer healing because salicylate acts like COX-2 selective NSAIDs that usually inhibit production of PGs in inflammatory sites but not in the normal stomach (26). This is probably not the case because the long-lasting increase of gastric PGs with BHB despite the presence of salicylate in plasma could compensate for the inhibition of gastric PG synthesis caused by COX-2 inhibitors as long as BHB is administered consecutively during chronic gastric ulcer, although the effect of BHB on both the healing of preexisting ulcers and PG levels during chronic ulcers should be determined (27).

Since gastric mucosa comprises different types of cells such as epithelial, mucous, parietal and chief cells, the cellular origin of PGs is difficult to determine. However, recent immunohistochemical studies on the localization of PGs in human and rat gastric mucosa revealed that gastric PGE_2 is produced mainly from parietal cells, and PGI_2 is mainly from vascular endothelial cells and partly from parietal cells and surface epithelial cells (28, 29). Because PGE_2 shows cytoprotection against gastric mucosal irritants and inhibits acid secretion from parietal cells (4), simultaneous release of PGE_2 with acid from parietal cells is thought to play a crucial role in the protection against the gastric mucosal injury provoked by excessive acid secretion (30). In fact, BHB can induce of PGE_2 release from rat cultured gastric parietal-like cells within 30 min with a clear peak at 100 μM in vitro (Y. Hori, unpublished data).

The sustained increase of gastric PGs with BHB could be explained by the fact that about 50% and 10% of BHB remained in the stomach after 1 and 6 hr, respectively (18). Although the precise mechanism by which BHB afforded the long-lasting increase of gastric PGs in the present experiment is unknown, some possibilities are: induction and/or activation of phospholipase A_2 that liberates arachidonic acid from the lipid bilayer of gastric mucosal cells; induction and/or activation of cyclooxy-

genases that accelerate PG synthesis from arachidonic acid; inhibition of PG metabolizing enzyme, 15-hydroxy-PG-dehydrogenase, which has been claimed to be the action mechanism of other anti-ulcer agents (31). Further elucidation must await experiments using cultured gastric mucosal cells.

In conclusion, BHB caused recovery from the indomethacin-induced decrease of gastric PGs without affecting the suppression of PG levels at the inflammatory site. Furthermore, BHB alone increased PG levels in gastric tissue, while it moderately reduced them in inflammatory exudates. These results clearly indicate that BHB does not compromise the anti-inflammatory effects of NSAIDs, and therefore is a suitable anti-ulcer agent for use with NSAIDs.

Acknowledgment

We wish to thank Ms. M. Kobayashi for her excellent technical assistance.

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