Endogenous prostaglandins (PGs) are involved in adaptive gastric protection against acute injury, and cyclooxygenase (COX)-1 is responsible for the production of PGs in this phenomenon. In the present study, we examined the effect of various COX inhibitors on gastric ulcerogenic and acid secretory responses following daily exposure of the stomach to iodoacetamide (IA) and investigated the role for COX isozyme in gastric protection under subchronic mucosal irritation. Gastric mucosal irritation was induced by addition of 0.1% IA to drinking water, and the gastric mucosa was examined on the 6th day. Indomethacin (5 mg/kg) or SC-560 (selective COX-1 inhibitor, 5 mg/kg) or rofecoxib (selective COX-2 inhibitor, 5 mg/kg) was given p.o. twice 24 hr and 3 hr before the termination of IA treatment. Giving IA in drinking water for 5 days produced minimal damage in the stomach. The damage was significantly worsened by indomethacin, resulting in hemorrhagic lesions. Both SC-560 and rofecoxib also aggravated such lesions, although the effect of rofecoxib was more pronounced. Treatment with IA decreased acid secretion in pylorus-ligated stomachs, and this change was significantly reverted by indomethacin as well as SC-560 and rofecoxib. Mucosal PGE$_2$ content was increased following IA treatment, with apparent expression of COX-2 mRNA in the stomach, and the increased PGE$_2$ production was significantly suppressed by SC-560 and rofecoxib as well as indomethacin. These results suggest that endogenous PGs derived from both COX-1 and COX-2 are involved in the mucosal defense of the inflamed stomach, partly by decreasing acid secretion and contribute to maintaining the mucosal integrity under such conditions.

**Key words:** iodoacetamide, inflammation, gastric lesion, acid secretion, prostaglandin, COX isozyme, selective COX inhibitor; rat
INTRODUCTION

Prostaglandins (PGs) are involved in a variety of physiological processes in the stomach, including acid secretion, production of mucus and mucosal blood flow (1). Cyclooxygenase (COX), the key enzyme for PG production, exists as two isozymes referred to as COX-1 and COX-2. COX-1 is constitutively expressed in normal gastric mucosa and generates PGs involved in the maintenance of essential physiological functions (2 - 4), while COX-2, characterized by a rapid inducibility in response to various proinflammatory stimuli, is responsible for pathological PG production at inflammatory sites (3, 5, 6).

Application of mild irritants to the stomach damages the surface epithelium of the gastric mucosa, yet they rarely cause macroscopically visible damage because of the self-defensive mechanism including an increase of mucosal blood flow and a decrease of acid secretion (7 - 9). These functional changes subside in the presence of nonsteroidal anti-inflammatory drugs (NSAIDs), suggesting the involvement of endogenous PGs in this phenomenon (7, 8). We have previously reported, using selective COX-1 and COX-2 inhibitors, that COX-1 is the enzyme responsible for the production of PGs in such conditions (10, 11). Barnett et al (12) recently showed that even in the stomach with inflammation caused by iodoacetamide, a sulfhydryl alkylator, PGs derived from COX-1 but not COX-2 exert inhibitory effects on acid secretion. However, the role of COX-2 in the mucosal defense in the inflamed stomach remains unknown. Since COX-2 is readily up-regulated in the stomach in response to irritating stimuli (13 - 15), it is possible that PGs produced by COX-2 contribute to the mucosal defense in the inflamed stomach.

In the present study, we examined the effects of selective COX-1 and COX-2 inhibitors on gastric ulcerogenic and acid secretory responses following daily exposure of the stomach to iodoacetamide and investigated the role for COX isozyme in gastric protection under subchronic mucosal irritation caused by daily administration of iodoacetamide.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, weighing 200–230 g (Charles River, Shizuoka, Japan), were used in all experiments. Studies were carried out using 4–6 rats per group. On fasting, the animals were kept in individual cages with raised mesh bottoms and deprived of food but allowed free access to tap water for 18 hr. All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Experimental protocol

The experiments were performed in four groups of rats; each group was pretreated with saline, indomethacin (a non-selective COX-1 and COX-2 inhibitor), SC-560 (a selective COX-1 inhibitor)(16) and rofecoxib (a selective COX-2 inhibitor) (17), respectively. In these groups of rats
gastric inflammation was induced by the addition of 0.1% iodoacetamide to the drinking water, based on the model described by Szabo et al (18). Controls were given drinking tap water. All experiments were performed on the 6th day after the rats were given the modified drinking water. Various COX inhibitors were given twice 24 hr and 3 hr before sacrifice. In some rats, the expression of mRNA for COX-1 and COX-2 was examined using reverse transcription-polymerase chain reaction (RT-PCR) in the stomach after the treatment with iodoacetamide. In these rats, the effects of various COX inhibitors on gastric mucosal PGE₂ content and acid secretion were also examined.

**Induction of gastric injury**

The animals were given the modified drinking water containing 0.1% iodoacetamide for 5 days (12,18). The amount of water intake and body weight were measured every days during the treatment. The animals were killed on various days, and the stomachs were removed, inflated by injecting 10 ml of 2% formalin, immersed in 2% formalin for 10 min to fix the gastric tissue wall, and opened along the greater curvature. Then, the area (mm²) of hemorrhagic lesions was measured under a dissecting microscope with a square grid (x10), summed per stomach, and used as a lesion score. In another study, the animals were treated with 0.1% iodoacetamide for 5 days, and the stomachs examined on the 6th day. Indomethacin (5 mg/kg), SC-560 (5 mg/kg) or rofecoxib (5 mg/kg) p.o. twice 24 hr and 3 hr before sacrifice. In some rats, SC-560 was given together with rofecoxib at the same dosing schedule. A person measuring the lesion did not know the treatment given to the animals.

In some cases, the stomachs treated with 0.1% iodoacetamide for 5 days were examined under a light microscope. The tissue samples were immersed in 10% formalin, processed for routine light microscopy, sectioned at 5 µm, and stained with hematoxylin & eosin.

**Determination of acid secretion**

Acid secretion was measured using a pylorus-ligation technique in the rats given 0.1% iodoacetamide in drinking water for 5 days, with or without indomethacin, SC-560 or rofecoxib. Rats were fasted for 18 hr before surgery, with free access to drinking water. Under ether anesthesia, the abdomen was opened and the pylorus was ligated. Four hours later, the animals were killed by deep ether anesthesia, the stomachs were removed, and then the gastric contents were collected. After centrifugation for 10 min at 3,000 rpm, each sample was measured for volume and titrated with 100 mM NaOH to pH 7.0 using an automatic titrator (Hiranuma, Committe-8, Ibaraki, Japan). Indomethacin (5 mg/kg), SC-560 (5 mg/kg) or rofecoxib (5 mg/kg) was given p.o. twice 24 hr and 3 hr before pylorus ligation.

**Determination of prostaglandin E₂ content**

_Gastric mucosa._ Levels of PGE₂ were measured in the stomach, with or without iodoacetamide treatment. Animals were given indomethacin, SC-560 or rofecoxib p.o., and then killed 3 hr later under deep ether anesthesia. In the latter, the animals were given the modified drinking water containing 0.1% iodoacetamide for 5 days, and indomethacin, SC-560 or rofecoxib was given p.o. twice 24 hr and 3 hr before sacrifice. In some rats, SC-560 was given together with rofecoxib at the same dosing schedule. In both studies, the corpus mucosa was isolated, weighed, and placed in a tube containing 100% ethanol plus 0.1 M indomethacin (19). The samples were then minced with scissors, homogenized and centrifuged at 12000 r.p.m. for 10 min at 4°C. The supernatant of each sample was used for determination of PGE₂ by EIA using a PGE₂- kit (Cayman Chemical Co., Ann Arbor, MI).

_Carrageenan-airpouch model._ The effect of various COX inhibitors on PGE₂ content was also examined in the exudates of a carrageenan-airpouch model. An airpouch was induced as described in
detail previously (20, 21). In brief, 20 ml of air was injected s.c. on the back of the rat on the first day. Two days later, another 10 ml of air was injected at the same site. On the fifth day after the first injection, a further 10 ml of air was injected into the pouch. Twenty-four hours later, carrageenan (2 ml of an 1% w/v solution in saline) was injected into the airpouch. All of the injections were performed under light ether anesthesia. Six hours after the carrageenan injection, the rats were anesthetized with ether, and the pouch was carefully opened by making a small incision. Then, the exudate was collected and transferred to a tube. An aliquot of the exudates was frozen on dry ice and stored at -20°C for subsequent measurements of PGE₂ concentration as described above. Indomethacin, SC-560 or rofecoxib was given p.o. 1 hr before the last injection of carrageenan into the airpouch.

**Analyses of COX-1 and COX-2 mRNAs by reverse transcription-polymerase chain reaction (RT-PCR)**

The animals given the modified drinking water containing 0.1% iodoacetamide for 5 days were killed under deep ether anesthesia, and their stomachs were removed, frozen in liquid nitrogen, and stored at -80°C until use. The tissue samples were pooled from 2~3 rats for extraction of total RNA, which was prepared by a single-step acid phenol-chloroform extraction procedure by use of TRIzol (Invitrogen, Carlsbad, CA). Total RNA primed by random hexadeoxy ribonucleotide was reverse-transcribed with the SUPERSCRIPT preamplification system (GIBCO BRL). The sequences of sense and antisense primers for rat COX-1 and COX-2 as well as glyceraldehyde-3-phosphate dehydrogenase (G3PDH) are referred to the previous papers (5, 22, 23). An aliquot of the RT reaction product served as a template in 35 cycles of PCR with 1 min of denaturation at 94°C, 0.5 min of annealing at 58°C and 1 min of extension at 72°C on a thermal cycler. A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in TAE buffer (Tris buffer 40 mM, EDTA 2 mM and acetic acid 20 mM; pH 8.1), and the gel was stained with ethidium bromide and photographed.

**Preparation of Drugs**

Drugs used were iodoacetamide (Nacalai tesque, Kyoto, Japan), indomethacin, (Sigma Chemicals, St. Louis, Mo), SC-560 (Cayman Chemical, Ann Arbor, MI), rofecoxib (Banyu, Tokyo, Japan) and carrageenan (Nacalai tesque). All COX inhibitors were suspended in a hydroxy propyl cellulose (HPC) solution (Wako, Osaka, Japan). Other agents were dissolved in saline. Each agent was prepared immediately before use and administered i.p. or p.o. in a volume of 0.5 ml per 100 g body weight.

**Statistics**

Data are presented as the mean ± SE from 4~6 rats per group. Statistical analyses were performed using a two-tailed Dunnett’s multiple comparison test, and values of p<0.05 were regarded as significant.

**RESULTS**

**Effects of various COX inhibitors on PGE₂ production in the gastric mucosa and the carrageenan-induced airpouch**

Oral administration of indomethacin at 5 mg/kg caused a marked decrease in the mucosal PGE₂ content by about 80% at 1 hr after administration in the
stomach (Fig. 1). Likewise, SC-560 at 5 mg/kg also caused a significant decrease in PGE₂ content. However, rofecoxib at 5 mg/kg had no effect on mucosal PGE₂ content in the stomach. On the other hand, indomethacin given p.o. at 5 mg/kg significantly reduced exudate PGE₂ content in the carrageenan-induced airpouch. Rofecoxib at 5 mg/kg also decreased the exudate PGE₂ content as effectively as indomethacin, while SC-560 at 5 mg/kg did not significantly affect the production of PGE₂ in the carrageenan-induced airpouch model.

Since SC-560 and rofecoxib at 5 mg/kg significantly inhibited the production of PGE₂ in the gastric mucosa and the airpouch exudate, respectively, these drugs at the dose were used in the subsequent studies as a selective COX-1 or COX-2 inhibitor.

Changes in body weight and gastric mucosa during iodoacetamide treatment

Treatment of animals with 0.1% iodoacetamide in drinking water decreased the body weight gradually, depending on the duration of the treatment, and a significant effect was observed after 5 days treatment, the decrease being 10.1% (Fig. 2). Oral intake of iodoacetamide for 2 days provoked minimal damage in the gastric mucosa, and the lesion score progressively increased to 3.8±0.7 mm² after 5 days treatment. On the other hand, the amount of water intake was not significantly altered by replacing tap water with 0.1% iodoacetamide, the average value being 17.6 ml (15±4~23±4 ml) during a 5-days test period.

Fig 1. A: Effects of various COX inhibitors on gastric mucosal PGE2 content in rats. The animals were given indomethacin (5 mg/kg), SC-560 (5 mg/kg) or rofecoxib (5 mg/kg) p.o. and killed 3 hr later. B: Effects of these agents on PGE₂ content in the carrageenan-induced airpouch in rats. Indomethacin (5 mg/kg), SC-560 (5 mg/kg) or rofecoxib (5 mg/kg) was given p.o. 1 hr before the last injection of carrageenan in the pouch. Data are presented as the mean±SE from 5~6 rats. *Significant difference from control, at P<0.05.
Effects of various COX inhibitors on gastric lesions induced by iodoacetamide treatment

Oral intake of 0.1% iodoacetamide in drinking water for 5 days irritated the stomach, resulting in hemorrhagic damage in the mucosa; the lesion score was 2.8±0.3 mm². Indomethacin, when administered p.o. twice at 24 hr and 3 hr before sacrifice, caused a marked aggravation of the lesions in response to iodoacetamide treatment, the lesion score being 19.0±2.7 mm² (Fig. 3). Histologically, iodoacetamide treatment alone produced apparent damage in the stomach, mostly in the surface epithelial cells, but by additional treatment with indomethacin the damage was deep in the mucosa (Fig. 4). The severity of these lesions was also significantly increased by either SC-560 or rofecoxib, the lesion score was 6.8±0.8 or 11.7±2.3 mm², respectively. When the animals were treated together with SC-560 plus rofecoxib, gastric lesions induced by iodoacetamide extended to the level observed in indomethacin-treated rats, the lesion score being 18.6±2.1 mm².

Effects of iodoacetamide and various COX inhibitors on mucosal PGE₂ content

The mucosal PGE₂ content in the stomach significantly increased from 39.2±4.6 to 66.1±6.9 ng/g tissue by treatment with the modified drinking water containing 0.1% iodoacetamide for 5 days (Fig. 5). The increase in mucosal PGE₂ was markedly suppressed by indomethacin that was given p.o. twice 24 hr and 3 hr before sacrifice; the values were less than 5 ng/g tissue. Likewise, SC-560 significantly reduced the mucosal PGE₂ content, the values decreasing even below normal levels. The increase in PGE₂ content was also significantly suppressed by treatment with rofecoxib, although the values were not significantly lower as compared to normal rats. The combined administration of
SC-560 plus rofecoxib markedly decreased the mucosal PGE$_2$ content to the same degree as observed in indomethacin-treated rats.

Expression of COX-1- and COX-2-mRNAs in the stomach following iodoacetamide treatment

In the animals given tap water, COX-1 gene expression was observed in the gastric mucosa, while the expression of COX-2 was hardly detected (Fig. 6). On the other hand, the gene expression of both COX-1 and COX-2 was clearly observed in the stomach of animals given the modified drinking water containing 0.1% iodoacetamide for 5 days.
Changes in acid secretion following iodoacetamide treatment

Normal animals fed on chow and tap water secreted acid at a rate of about 7 ml for 4 hr in pylorus-ligated stomachs, and total acid output was 177.2±18.7 µEq/hr. Treatment with 0.1% iodoacetamide in drinking water for 5 days markedly reduced the acid output to 44.6±7.2 µEq/hr, the inhibition being 74.8% (Fig. 7). The reduced acid output in the animals treated with iodoacetamide was significantly reverted by either indomethacin, SC-560 or rofecoxib, each given p.o. twice at 24 hr and 3 hr before pylorus ligation, the acid output being 142.8±20.6, 139.9±16.9 or 103.0±19.6 µEq/hr, respectively. The effect of rofecoxib was less pronounced as compared to other two agents, and the acid output was still significantly lower than that in normal rats.

Fig. 5. Effect of various COX inhibitors on gastric mucosal PGE₂ content in rats treated with iodoacetamide. Animals were killed after treatment with 0.1% iodoacetamide for 5 days, and gastric mucosal PGE₂ was determined by EIA. Indomethacin (5 mg/kg), SC-560 (5 mg/kg), rofecoxib (5 mg/kg) or SC-560 plus rofecoxib was given p.o. twice 24 hr and 3 hr before sacrifice. Data are presented as the mean±SE from 4–6 rats. Significant difference at P<0.05; *from normal; # from control.

Fig. 6. Gene expression of COX-1, COX-2 and G3PDH in the rat gastric mucosa following the treatment of iodoacetamide. The animals were treated with iodoacetamide in drinking water for 5 days. M: marker.
DISCUSSION

The stomach has inert defensive ability against noxious stimuli and hardly ulcerates even in the presence of noxious stimuli. Indeed, the application of mucosal irritating agents damages the surface epithelium of the stomach, yet they rarely cause macroscopically visible damage because of the self-defensive mechanism including an increase of mucosal blood flow and a decrease of acid secretion (7 - 9). These functional responses in the stomach after damage are considered to play a role in the repair process of the mucosa such as the restitution of the surface epithelium (7). We previously reported that a decrease in acid secretion after exposure of the stomach to bile acids (taurocholate Na) is mediated by endogenous PGs produced by COX-1 (10). However, the damage produced by bile acids is acute in nature and recovers very quickly, so that the functional alteration also subsides within a few hours. In the present study, we used a gastiris model induced by daily administration of 0.1% iodoacetamide (12, 18) and showed that endogenous PGs derived from both COX-1 and COX-2 contribute to the mucosal defense of the inflamed stomach, partly by decreasing acid secretion and maintaining the mucosal integrity under such conditions.

First, we tested the activity of SC-560 or rofecoxib as a selective COX-1 or COX-2 inhibitor. Mucosal PGE₂ content in the normal stomach was significantly decreased by SC-560 but not rofecoxib, whereas rofecoxib but not SC-560 suppressed PGE₂ production in a carrageenan-induced airpouch model. Since intrapleural injection of carrageenan produces an increase of PGE₂ production and induction of de novo synthesis of COX-2 in pleural exudates cells (24, 25), there is no doubt that this rofecoxib action is due to suppression of COX-2

![Fig. 7. Effect of various COX inhibitors on gastric acid secretion in rats treated with iodoacetamide. The animals were treated with 0.1% iodoacetamide in drinking water for 5 days. Acid secretion was measured in pylorus-ligated rats after 18 hr fasting. Indomethacin (5 mg/kg), SC-560 (5 mg/kg) or rofecoxib (5 mg/kg) was given p.o. twice 24 hr and 3 hr before pylorus ligation. Data are presented as the mean±SE from 5–6 rats. Significant difference at P<0.05; †from normal; # from control.](Image)
activity. On the other hand, since COX-1 is constitutively expressed in normal gastric mucosa (2, 26), it is considered that the inhibitory action of SC-560 on PGE₂ production in the normal stomach is due to suppression of COX-1 activity. Thus, the present results confirmed that SC-560 and rofecoxib at the dose used, i.e., 5 mg/kg, selectively inhibits COX-1 and COX-2 activity, respectively.

Daily administration of 0.1% iodoacetamide in drinking water for 3 days caused erosive lesions in the gastric mucosa, and the severity of damage remained unaltered even after administration for another 2 days. Szabo et al. (18) demonstrated that the treatment with iodoacetamide caused significant increases in the number of neutrophils in the gastric mucosa. This finding was confirmed by Barnett et al. (12), who showed that the same treatment caused 3 fold increase in gastric myeloperoxidase activity. We also observed histologically the infiltration of neutrophils in the mucosa and submucosa after administration of iodoacetamide for 5 days. By the way, several factors are involved in the mucosal defensive mechanism of the stomach, including PGs, nitric oxide (NO) and capsaicin-sensitive afferent neurons (27 - 29), yet PGs are considered as the most important substance to this end. Indeed, the propensity to ulceration under noxious stimuli such as bile acids or stress is generally increased by depletion of endogenous PGs caused by NSAIDs (10, 30, 31). Consistent with these observations, the severity of gastric lesions induced by iodoacetamide treatment was markedly aggravated, approximately 6 fold the control values, by inducing PG deficiency by indomethacin for the last 24 hr before sacrifice. Both SC-560 and rofecoxib also significantly exacerbated these lesions, though the degree being less pronounced than that induced by indomethacin. In addition, we observed significant increases in the mucosal PGE₂ contents in the stomach after a 5 days-treatment with iodoacetamide and found that this response was significantly inhibited by SC-560 and rofecoxib as well as indomethacin. These results suggest that endogenous PGs are involved in the mucosal defensive mechanism of the stomach against subchronic irritation with iodoacetamide and that both COX-1 and COX-2 are responsible for production of PGs under such conditions. This idea was supported by the finding that the expression of COX-2 mRNA was observed in the stomach treated with iodoacetamide. Previous studies showed that the up-regulation of COX-2 expression was not observed in the stomach within 1 hr after acute exposure of the mucosa to bile acids (10), and the ulcerogenic response in such stomachs was worsened by SC-560 but not rofecoxib (11). Other studies demonstrated that COX-2 was readily up-regulated in the stomach in response to acidified bile acids or 0.2 N HCl (14, 15). It is possible that the expression of COX-2 depends on the severity or duration of irritation. Anyway, the present study indicates the distinct mechanisms underlying the mucosal defense in the stomach exposed to acute or subchronic irritation and that PGs produced by COX-2 contribute to the mucosal defense in the inflamed stomach.
Functional alterations such as a decrease in acid secretion or an increase of mucosal blood flow play a role in the mechanism of gastric mucosal defense under adverse conditions. We previously reported, using a selective COX-2 inhibitor, that endogenous PGs produced by COX-1 are involved in maintaining the gastric hyperemic response and mucosal integrity in the presence of acid following barrier disruption by bile acids (10, 11). In a preliminary study we examined changes in the mucosal blood flow during treatment with iodoacetamide, yet we could not detect apparent changes in the mucosal blood flow in the absence or presence of COX inhibitors. On the other hand, acid secretion also decrease after exposure of the stomach to mild irritation such as bile acids, the response being mediated by endogenous PGs derived from COX-1 (11). Consistent with the finding, we observed a marked decrease in acid secretion following treatment with iodoacetamide. However, the decreased acid response was significantly reverted by not only SC-560 but also rofecoxib, in addition to indomethacin, though the effect of rofecoxib was less pronounced when compared to other two COX inhibitors. Nonetheless, these results suggest that both COX-1 and COX-2 are involved in the mechanism underlying a decrease of acid secretion in the stomach following treatment with iodoacetamide. In contrast, Barnett et al (12) recently reported that even in the stomach inflamed by iodoacetamide PGs derived from COX-1 but not COX-2 exert inhibitory effects on acid secretion. In their study, an apparent increase in COX-2 expression was found in the gastric mucosa, most marked in the parietal cells as well as the infiltrating neutrophils, yet there was no discernible effect of iodoacetamide on gastric PGE\textsubscript{2} synthesis. Although the reason for the discrepancy between these two studies remains unexplained, it may be due to different experimental conditions; they examined the effect of COX inhibitors on acid secretion on the 5th day after the rats were given 0.1% iodoacetamide in drinking water, while we performed the experiment on the 6th day after the same treatment. In the present study, iodoacetamide might induce more severe inflammation in the stomach, leading to a stronger induction of COX-2, and thereby COX-2-derived PGs exerted a negative influence on acid secretion.

Given the present findings, it is concluded that endogenous PGs derived from both COX-1 and COX-2 are involved in the mucosal defense of the inflamed stomach, partly by decreasing acid secretion and contribute to maintaining the mucosal integrity under such conditions. Although COX-1 accounts for the majority of PG synthesis in the normal stomach, recent studies suggest that COX-2-derived PGs also play a role in the maintenance of gastric mucosal integrity (14, 15, 32). Thus, it is assumed that both isozymes of COX are important in the mucosal defense of stomach under normal and adverse conditions. Other mediators such as NO or sensory neurons also play important role in the regulatory mechanism of gastric function and protection (27 - 29), but their participation in the mucosal defense in the inflamed stomach remains unknown. Further study should certainly be required on this point.
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