

# Roles of Cyclooxygenase-2 and Prostacyclin/IP Receptors in Mucosal Defense against Ischemia/Reperfusion Injury in Mouse Stomach

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## ABSTRACT

We examined the roles of cyclooxygenase (COX) isozymes, prostaglandins (PGs), and their receptors in the mucosal defense against ischemia/reperfusion (I/R)-induced gastric lesions in mice. Male C57BL/6 mice, including wild-type animals and those lacking prostaglandin E<sub>2</sub> (EP)1, EP3, or prostaglandin I<sub>2</sub> (IP) receptors, were used after 18 h of fasting. Under urethane anesthesia, the celiac artery was clamped (ischemia) for 30 min, and then reperfusion was achieved for 60 min through the removal of the clamp, and the stomach was examined for lesions. I/R produced hemorrhagic gastric lesions in wild-type mice. The severity of lesions was significantly increased by pretreatment with indomethacin (a nonselective COX inhibitor) and rofecoxib (a selective COX-2 inhibitor) but not 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560; a selective COX-1 inhibitor). The expression

of COX-2 mRNA was up-regulated in the stomach following I/R but not by sham operation or ischemia alone. The ulcerogenic response was markedly aggravated in IP receptor knockout mice but not those lacking EP1 or EP3 receptors. I/R increased the levels of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> in the stomach of wild-type mice, and this response was attenuated by indomethacin and rofecoxib but not SC-560. Pretreatment of wild-type mice with iloprost, a prostacyclin (PGI<sub>2</sub>) analog, significantly prevented the I/R-induced gastric lesions in the absence and presence of indomethacin or rofecoxib. PGE<sub>2</sub> also reduced the severity of I/R-induced gastric lesions, yet the effect was much less pronounced than that of iloprost. These results suggest that endogenous PGs derived from COX-2 play a crucial role in gastric mucosal defense during I/R, and this action is mainly mediated by PGI<sub>2</sub> through the activation of IP receptors.

The damage caused by an interruption of blood supply to an organ or tissue followed by the reintroduction of blood into the affected area is called ischemia/reperfusion (I/R) injury. The phenomenon of I/R injury is a major clinical problem after stroke, infarction, shock, and organ transplantation. The depletion of adenosine triphosphate (ATP) and disturbance of intracellular calcium homeostasis have been suggested as the major pathophysiological mechanisms during ischemia, leading to loss of cell viability (Farber et al., 1981; Cheung et al., 1986). Reperfusion of ischemic tissues paradoxically exacerbates the injury process and leads to the release of reactive oxygen species and proinflammatory me-

diators and the attraction of inflammatory cells infiltrating the tissues (Chamoun et al., 2000; Piper et al., 2003). In the gastrointestinal tract, I/R injuries are known to be associated with significant morbidity and mortality during the course of hemorrhagic shock, abdominal aortic aneurysm repair, ischemic bowel disease, and necrotizing enterocolitis (Yasue et al., 1992; Riaz et al., 2002; Dimmitt et al., 2003).

Nonselective cyclooxygenase (COX) inhibitors damage the gastrointestinal mucosa in patients as an adverse reaction (Soll et al., 1991). By contrast, selective COX-2 inhibitors such as rofecoxib and celecoxib do not induce gastric lesions in rats (Vane and Botting, 1995; Laudanno et al., 2001). Hence, these COX-2 inhibitors were expected to be anti-inflammatory and chemopreventive drugs devoid of gastrointestinal toxicity, although chronic treatment with a COX-2 inhibitor delayed the healing of gastric ulcers (Mizuno et al., 1997; Miura et al., 2004). Recently, it was reported that a selective COX-2 inhibitor aggravated gastric lesions induced

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**ABBREVIATIONS:** I/R, ischemia/reperfusion; COX, cyclooxygenase; PG, prostaglandin; EP, prostaglandin E<sub>2</sub> receptor; PGI<sub>2</sub>, prostacyclin; IP, prostaglandin I<sub>2</sub> receptor; MPO, myeloperoxidase; PCR, polymerase chain reaction; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methyl sulfonyl)phenyl-2(5H)-furanone.

by I/R in rats, suggesting the involvement of endogenous prostaglandins (PGs) in the mucosal defense during I/R (Maricic et al., 1999).

PGs, especially PGE<sub>2</sub>, have been shown to exert a protective action in the stomach through the activation of EP1 receptors, although the effects on various functions are mediated by different EP receptor subtypes; cf., acid inhibition by EP3 receptors, an increase of mucus secretion by EP4 receptors, an increase of mucosal blood flow by EP2/EP4 receptors, and an inhibition of gastric motility by EP1 receptors (Takeuchi et al., 2002b). Likewise, prostacyclin (PGI<sub>2</sub>), a prostanoid mainly synthesized in the endothelium, exerts various physiological actions at the interface between blood and tissue (Konturek and Robert, 1982; Whittle et al., 1984). Since PGI<sub>2</sub> enhances the gastric mucosal microcirculation through vasodilation and inhibition of platelet aggregation, it is possible that this prostanoid contributes to the maintenance of the mucosal integrity of the stomach during I/R (Granger and Kubes, 1994; Saika et al., 1999). However, it remains unknown which type of prostanoid plays a role in mucosal defense of the stomach under I/R conditions. In the present study, we examined the effects of various COX inhibitors on I/R-induced gastric lesions in mice and further investigated which type of prostanoid receptor is involved in mucosal defense under I/R-induced conditions using animals lacking EP1, EP3, or IP receptors.

## Materials and Methods

**Animals.** Male C57BL/6 mice (3 months old; SLC, Shizuoka, Japan) were used. Mice lacking EP1, EP3, or IP receptors were generated as described previously (Sugimoto et al., 1992; Ushikubi et al., 1998). In brief, the genes encoding the EP1, EP3, and IP receptors were individually disrupted, and chimeric mice were generated. These animals were then backcrossed with C57BL/6 mice, and the resulting heterozygous littermates [EP1 (-/-), EP3 (-/-), or IP (-/-)] were bred to produce homozygous EP1 (-/-), EP3 (-/-), or IP (-/-) mice. Homozygous mice were born at the predicted Mendelian frequency, grew normally, lived longer than 1 year, and were fertile. Distribution of the EP1, EP3, and IP receptor genes was verified by Northern blot hybridization, which failed to detect messenger RNAs encoding the respective receptors in EP1 (-/-), EP3 (-/-), and IP (-/-) mice. These knockout mice were deprived of food but allowed free access to tap water for 18 h before the experiments. All experiments were carried out using four to eight mice under urethane anesthesia unless otherwise specified. The experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

**Induction of Gastric Mucosal Lesion by Ischemia and Reperfusion.** Acute gastric mucosal lesions were produced by I/R (Wada et al., 1996). Briefly, under urethane anesthesia (1.25 g/kg i.p.), the celiac artery was clamped with a small clamp (disposable vascular clip, holding force of 40 g; BEAR Medical Corporation, Chiba, Japan), and 30 min later, reperfusion was achieved through removal of the clamp. After reperfusion for 60 min, the stomach was excised, inflated by injecting 0.4 ml of 2% formalin for 10 min to fix the tissue walls, and opened along the greater curvature. In wild-type mice, the effects of COX inhibitors on the I/R-induced gastric lesions were examined. Indomethacin (5 mg/kg), rofecoxib (a selective COX-2 inhibitor, 5 mg/kg), or SC-560 (a selective COX-1 inhibitor, 5 mg/kg) was administered p.o. 60 min before ischemia. The doses of these COX inhibitors were selected to show nonselective inhibition of both COX-1 and COX-2 or selective inhibition of COX-1 or COX-2, respectively (Takeeda et al., 2003). In addition, the effects of iloprost, an analog of PGI<sub>2</sub>, and PGE<sub>2</sub> on the I/R-induced gastric

lesions were examined also in both wild-type and IP receptor knockout mice. Iloprost (0.3–3 μg/kg) or PGE<sub>2</sub> (0.1–1 mg/kg) was given i.v. 5 min before reperfusion. The area (millimeters<sup>2</sup> × 10). The person measuring the lesions did not know the treatments given to the animals.

In some cases, the gastric mucosa was examined with a light microscope following I/R. The animals were killed after I/R treatment, and the stomachs were excised. The tissue samples were then immersed in 2% formalin-saline, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E).

**Determination of Myeloperoxidase Activity.** Myeloperoxidase (MPO) activity in the gastric mucosa was measured after I/R treatment in wild-type and IP receptor knockout mice, according to a modified version of the method of Krawisz et al. (1984). After 60 min after I/R treatment, the animals were sacrificed by withdrawal of blood from the heart by perfusing with saline, and the stomach was excised. After rinsing of the tissue with ice-cold saline, the mucosa was scraped with glass slides, weighed, and homogenized in a 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Sigma-Aldrich, St. Louis, MO). The homogenized samples were subjected to freezing and thawing three times and centrifuged at 2000 rpm for 10 min at 4°C. MPO activity in the supernatant was determined by adding 100 μl of the supernatant to 1.9 ml of 10 mM phosphate buffer (pH 6.0) and 1 ml of 1.5 M *o*-dianisidine hydrochloride (Sigma-Aldrich) containing 0.0005% (w/v) hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a Hitachi spectrophotometer (U-2000; Hitachi, Ibaraki, Japan). Sample protein content was estimated by spectrophotometric assay (Pierce protein assay kit; Rockford, IL), and the MPO activity was obtained from the slope of the reaction curve, based on the following equation: specific activity (μmol H<sub>2</sub>O<sub>2</sub>/min/mg of protein) = (OD/min)/OD/μmol H<sub>2</sub>O<sub>2</sub> × mg of protein.

**Measurement of Mucosal PGE<sub>2</sub> and 6-Keto-PGF<sub>1α</sub> Levels.** Levels of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>, the stable metabolite of PGI<sub>2</sub>, in the gastric mucosa were measured after I/R treatment in wild-type mice. The animals were killed under deep ether anesthesia after the 60-min reperfusion period, and the gastric mucosa was isolated, weighed, and placed in a tube containing 100% methanol plus 0.1 mM indomethacin (Futaki et al., 1994). Then the tissues were homogenized by a Polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 12,000 rpm for 10 min at 4°C. After the supernatant of each sample had been evaporated with N<sub>2</sub> gas, the residue was resolved in assay buffer and used for determination of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>. The concentrations of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> were measured using a PGE<sub>2</sub> or 6-keto-PGF<sub>1α</sub> enzyme immunoassay kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Analysis of COX-1, -2, EP1, EP3, and IP mRNA Expression by Reverse Transcription-PCR.** Wild-type animals were killed under deep ether anesthesia after I/R treatment, and the stomachs were removed, frozen in liquid nitrogen, and stored at -80°C prior to use. Tissue samples were pooled from two to three 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 (Invitrogen). The sequences of sense and antisense primers for the mouse COX-1, COX-2, EP1, EP3, and IP are shown in Table 1. An aliquot of the reverse transcription 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 56°C, and 1 min of extension at 72°C on a thermal cycler. A portion of the PCR mixture was electrophoresed in 1.5% agarose gel in Tris-EDTA-acetic acid buffer, and the gel was stained with ethidium bromide and photographed.

**Determination of Gastric Secretion.** Acid secretion was measured in wild-type mice provided with an acute gastric fistula under urethane anesthesia (1.25 g/kg i.p.). Briefly, the abdomen was incised, and both the stomach and duodenum were exposed. An acute fistula (inside diameter, 2 mm) made with a polyethylene tube was

TABLE 1  
PCR primer sequences and product size

Molecule	Primer Sequence	Product Size
		bp
GAPDH	5'-AACGACCCCTTCATTGAC-3' 5'-TCCACGACATACTCAGCAC-3'	191
COX-1	5'-CTGCATGTGGCTGTGGATGTCATC-3' 5'-GGTCTTGGTGAGGCAGACCAG-3'	389
COX-2	5'-GTCTGATGATGTATGCCACAATCTG-3' 5'-GATGCCAGTGATAGAGGGTGTGAA-3'	276
EP1	5'-AATACATCTGTGGTCTGCCAACA-3' 5'-CCACCATTCCACATCGTGTGCGT-3'	829
EP3	5'-GGTATGCCAGCCACATGAAGAC-3' 5'-CAAGATCTGGTTCAGCGAAGCC-3'	529
IP	5'-GGCAGGAGAGGATGAAAGTTTAC-3' 5'-GTCAGAGGCACAGCAGTCAATGG-3'	408

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).

inserted into the stomach from a small incision made in the fore-stomach and was held in place by a ligature. The stomach was filled with 0.4 ml of saline (154 mM NaCl) through the fistula, and the solution was changed every 20 min. The collected samples were centrifuged at 3000 rpm for 15 min and titrated to pH 7.0 against 10 mM NaOH using an autoburette (Hiranuma Comtite-8, Tokyo, Japan). Indomethacin (5 mg/kg), SC-560 (5 mg/kg), or rofecoxib (5 mg/kg) was given i.d. 1 h before instillation of saline in the stomach whereas iloprost (0.1~3 μg/kg) was given i.v. 5 min before.

**Preparation of Drugs.** The drugs used were urethane (Tokyo Kase, Tokyo, Japan), indomethacin (Sigma-Aldrich), SC-560 (Cayman Chemical, Ann Arbor, MI), rofecoxib (synthesized in our laboratory), iloprost (Nacalai Tesque, Kyoto, Japan), and PGE<sub>2</sub> (Funakoshi, Tokyo, Japan). All COX inhibitors were suspended in a hydroxy propyl cellulose solution (Wako, Osaka, Japan). Iloprost was dissolved in saline, whereas PGE<sub>2</sub> was first dissolved in absolute ethanol and diluted with saline to the desired concentrations. Each agent was prepared immediately before use and administered p.o., i.d., i.p., or i.v. in a volume of 0.5 ml/100 g of body weight. Control animals received saline as the vehicle.

**Statistics.** Data are presented as the mean ± S.E.M. for four to eight mice per group. Statistical analyses were performed using a one-way analysis of variance and Student's *t* test or Dunnett's multiple comparison test where appropriate, and values of *P* < 0.05 were considered significant.

## Results

**I/R-Induced Gastric Lesions in Wild-Type Mice.** Laparotomy without clamping of the gastric artery (sham operation) did not produce any damage in the gastric mucosa of wild-type mice. In the animals subjected to I/R treatment (30-min ischemia followed by reperfusion for 60 min), however, multiple hemorrhagic lesions were observed in the gastric mucosa, the lesion score being 7.2 ± 4.9 mm<sup>2</sup> (*n* = 5). Ischemia for 30 min did not induce any macroscopically visible damage in the mucosa. Histologically, most of the damage induced by I/R was restricted to the surface epithelium, but some damage occurred deep in the mucosa, extending to the region of pits and glands (Fig. 1, A–D). In sham-operated animals, no damage was detected even by histological observation.

**Effect of COX Inhibitors on I/R-Induced Gastric Lesions in Wild-Type Mice.** Following I/R treatment in wild-type mice, the gastric mucosa developed multiple hemorrhagic lesions, the lesion score being 9.9 ± 3.6 mm<sup>2</sup>. Pretreatment of the animals with indomethacin (5 mg/kg p.o.) significantly aggravated these lesions, the lesion score

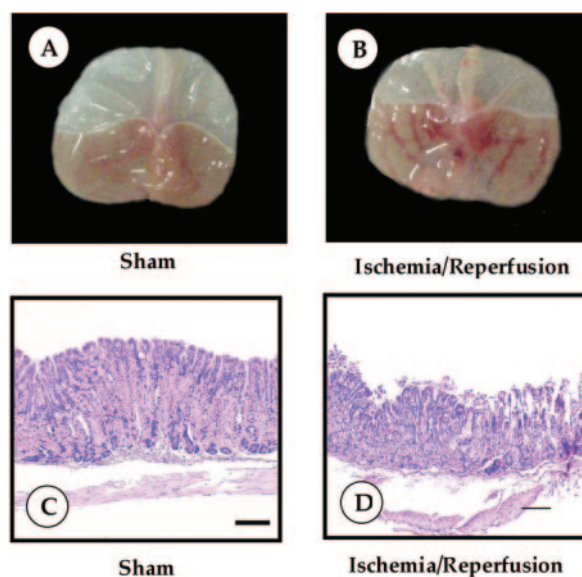


Fig. 1. Gross appearance and histological observations of the gastric mucosa in wild-type mice subjected to a sham operation or I/R treatment. A and C, sham; B and D, I/R. Bar, 100 μm

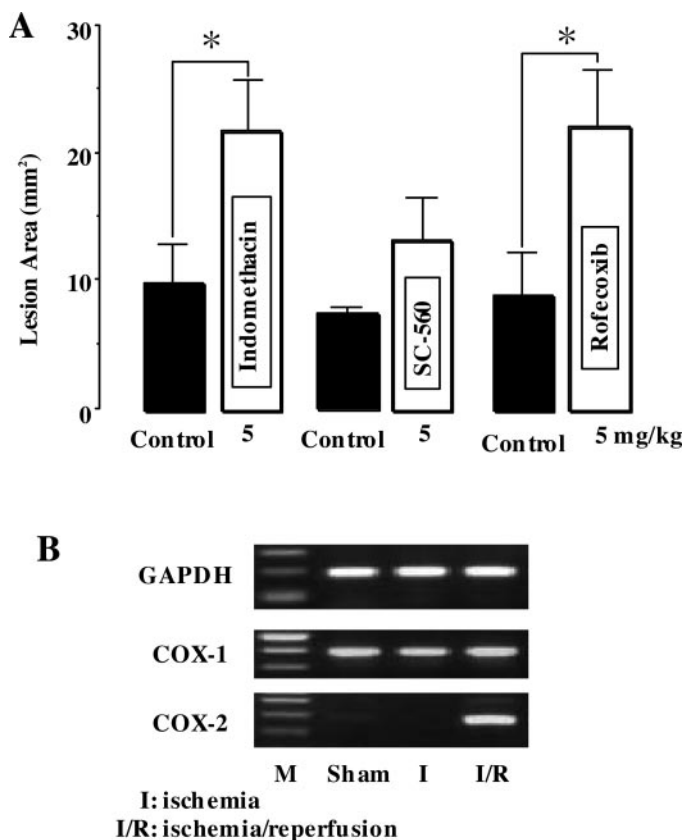


Fig. 2. A, effects of COX inhibitors on the I/R-induced gastric lesions in wild-type mice. Indomethacin (5 mg/kg), SC-560 (5 mg/kg), or rofecoxib (5 mg/kg) was given p.o. 60 min before ischemia. Data are presented as mean ± S.E.M. from five to seven mice. \*, significant difference from control at *p* < 0.05. B, COX mRNA expression in the gastric mucosa of wild-type (WT) mice subjected to a sham operation or I/R treatment. I, ischemia for 30 min.

being 22.3 ± 4.5 mm<sup>2</sup> (Fig. 2A). The severity of I/R-induced gastric lesions was also significantly increased by prior administration of the selective COX-2 inhibitor rofecoxib (5

mg/kg p.o.), and the lesion score was almost equivalent to that observed in indomethacin-pretreated animals. However, the COX-1-selective inhibitor SC-560 (5 mg/kg p.o.) had no effect on the development of gastric lesions induced by I/R.

The gene expression of glyceraldehyde-3-phosphate dehydrogenase, the housekeeping gene, as well as COX-1 was clearly detectable in the stomach of control wild-type mice and was not affected by either ischemia or I/R (Fig. 2B). Although the expression of COX-2 mRNA was negligible in the gastric mucosa of control wild-type mice, it was markedly up-regulated following I/R but not ischemia alone.

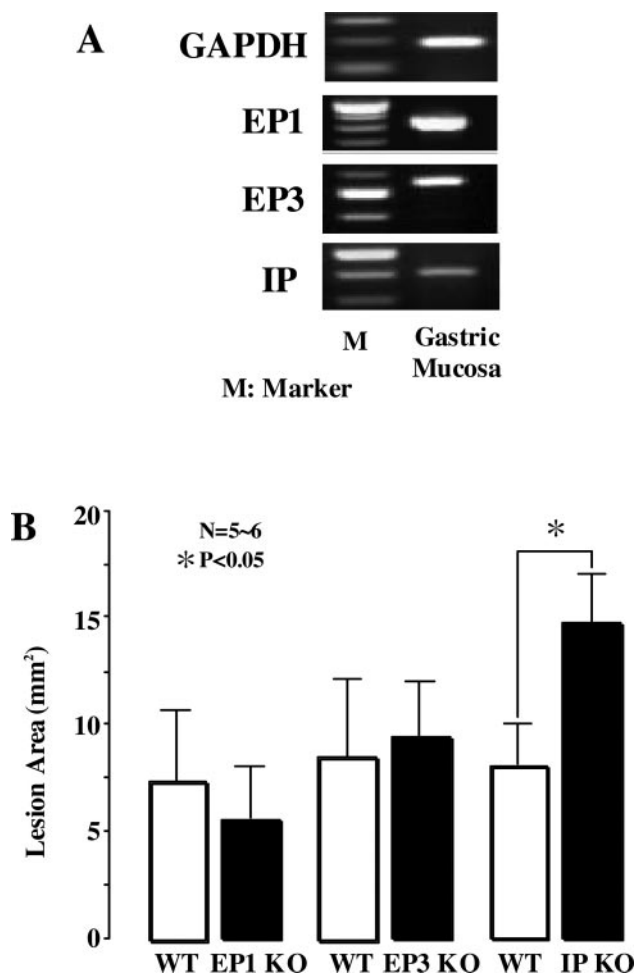
**I/R-Induced Gastric Lesions in EP1, EP3, and IP Receptor Knockout Mice.** To further investigate which prostanoid receptor is involved in the mucosal defense against I/R-induced gastric lesions, we compared the gastric ulcerogenic response to I/R in wild-type mice and the animals lacking EP1, EP3, or IP receptors. As shown in Fig. 3A, the expression of EP1, EP3, and IP receptor mRNAs was clearly detectable in the stomach of wild-type mice.

Following I/R treatment, wild-type mice in each group developed hemorrhagic lesions in the gastric mucosa, the

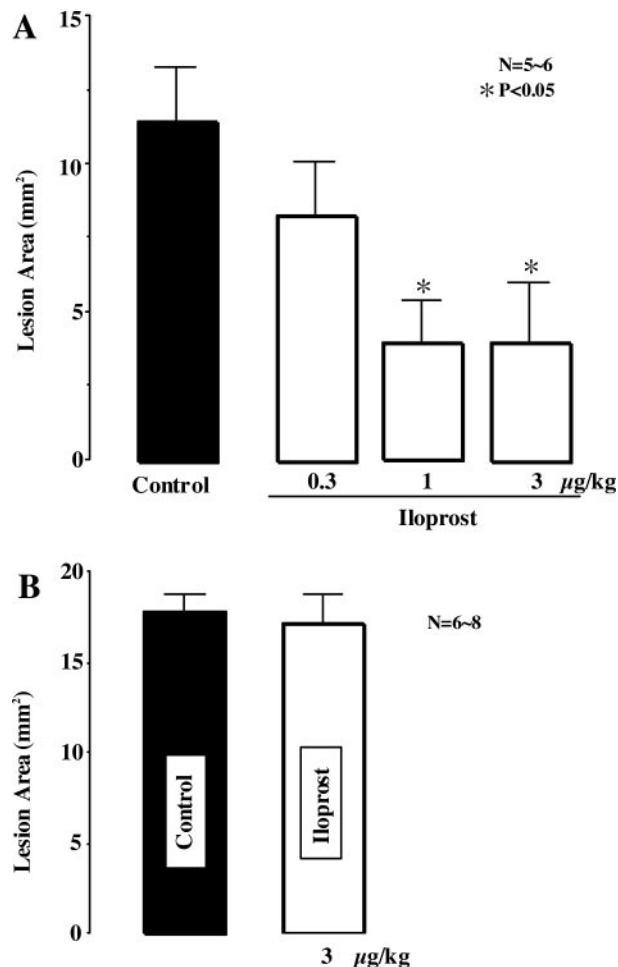
lesion score being  $7.6 \pm 2.8$  to  $8.0 \pm 3.2$  mm<sup>2</sup>. Development of gastric lesions was observed in the animals lacking EP1, EP3, or IP receptors, although the severity of the lesions differed in these groups of mice. As shown in Fig. 3B, the gastric ulcerogenic response to I/R was significantly increased in IP receptor knockout mice, the lesion score reaching roughly 2 times that in wild-type mice. However, the severity of these lesions in EP1 or EP3 receptor knockout animals was not significantly different compared with wild-type mice.

**Effect of Iloprost on I/R-Induced Gastric Lesions in Wild-Type Mice.** Since the severity of the I/R-induced gastric lesions was found to increase in IP receptor knockout animals, we examined the effect of a stable PGI<sub>2</sub> analog, iloprost, on the ulcerogenic response to I/R in wild-type mice, in the absence or presence of COX inhibitors.

Pretreatment of the animals with iloprost (0.3~3 μg/kg i.v.) dose-dependently prevented the development of I/R-induced gastric lesions in wild-type mice, the degree of protection being 72.4% (Fig. 4A). However, this agent, even at 3 μg/kg, had no effect on these lesions in the animals lacking IP receptors (Fig. 4B). On the other hand, the I/R-induced gas-



**Fig. 3.** A, expression of mRNA for prostanoid receptors (EP1, EP3, and IP) in the mouse stomach. M, marker. B, gastric lesions induced by I/R in wild-type mice and those lacking EP1, EP3, or IP receptors. Under urethane anesthesia, the celiac artery was clamped, and then reperfusion was achieved 30 min later by removal of the clamp. After a 60-min reperfusion period, hemorrhagic lesions were induced in the gastric mucosa. Data are presented as mean  $\pm$  S.E.M. from five to seven mice. \*, significant difference from each group of wild-type mice at  $p < 0.05$ .

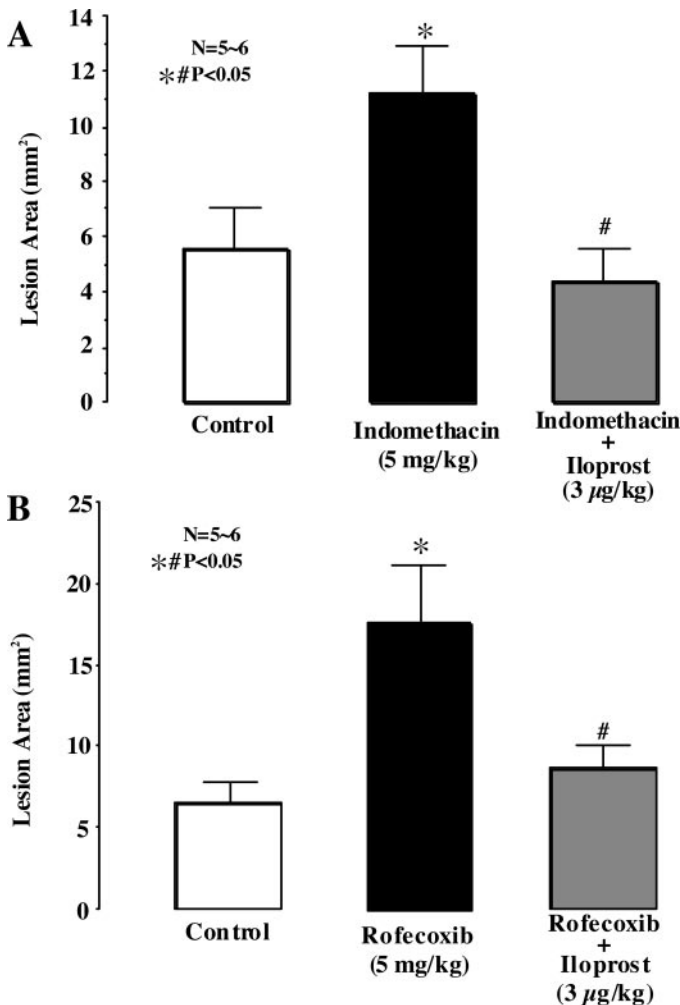


**Fig. 4.** A, effect of iloprost, the IP agonist, on I/R-induced gastric lesions in wild-type mice. Iloprost (0.3~3 μg/kg) was given i.v. 5 min before reperfusion. Data are presented as mean  $\pm$  S.E.M. from six to eight mice. \*, significant difference from control at  $p < 0.05$ . B, effect of iloprost on I/R-induced gastric lesions in IP receptor knockout mice. Iloprost (3 μg/kg) was given i.v. 5 min before reperfusion. Data are presented as mean  $\pm$  S.E.M. from six to eight mice.

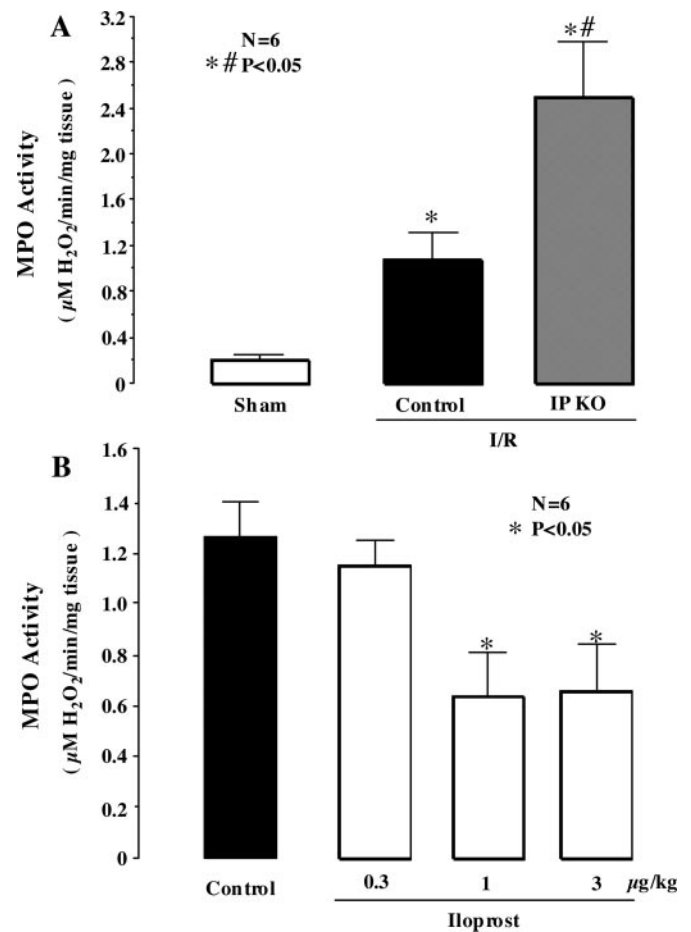
tric lesions were markedly worsened by prior administration of either indomethacin (5 mg/kg p.o.) or rofecoxib (5 mg/kg p.o.), the degree of aggravation being 52.2 or 67.3%, respectively (Fig. 5A). The aggravating effect of these COX inhibitors was significantly abrogated by pretreatment with iloprost (1 μg/kg i.v.; Fig. 5B).

**Mucosal MPO Activity during I/R in Wild-Type and IP Receptor Knockout Mice.** The severity of I/R-induced gastric lesions was increased in IP receptor knockout mice and reduced by supplementation of PGI<sub>2</sub>, respectively. To confirm that the mucosal inflammatory response during I/R is also affected by these treatments, we measured MPO activity in the gastric mucosa of both wild-type and IP receptor knockout mice after I/R in the presence or absence of iloprost.

Tissue-associated MPO activity in the gastric mucosa of sham-operated mice was less than 0.3 μmol H<sub>2</sub>O<sub>2</sub>/min/mg of tissue. Gastric MPO activity in wild-type mice was markedly increased after I/R, reaching about 4 times over the control levels, the values being 1.11 ± 0.2 μmol H<sub>2</sub>O<sub>2</sub>/min/mg of tissue (Fig. 6A). The MPO activity was further increased in IP receptor knockout animals in response to I/R, the values



**Fig. 5.** Effect of iloprost (1 μg/kg) on the I/R-induced gastric lesions in wild-type mice pretreated with COX inhibitors. Indomethacin (5 mg/kg) or rofecoxib (5 mg/kg) was given p.o. 60 min before ischemia. Iloprost (1 μg/kg) was given i.v. 5 min before reperfusion. Data are presented as the mean ± S.E.M. from five to six mice. Significant differences at *p* < 0.05: \*, from the corresponding control; #, from treatment with (A) indomethacin or (B) rofecoxib.

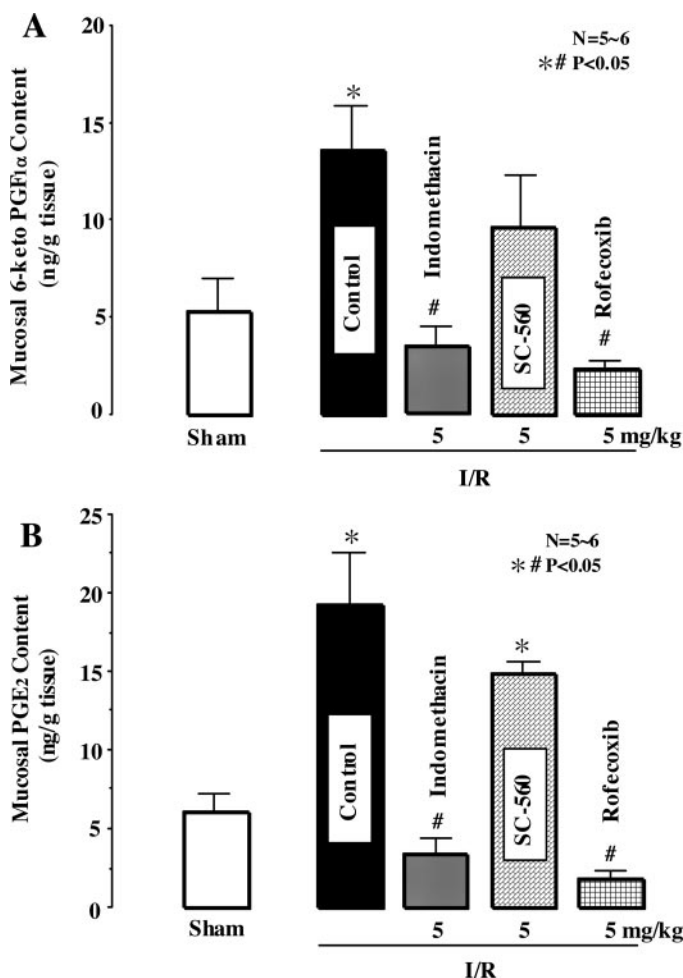


**Fig. 6.** A, I/R-induced changes in gastric MPO activity in wild-type and IP receptor knockout mice. Under urethane anesthesia, the celiac artery was clamped (ischemia), and then reperfusion was achieved 30 min later by removal of the clamp. After a 60-min reperfusion period, gastric MPO activity was measured. Data are presented as the mean ± S.E.M. from six mice. Significant difference at *p* < 0.05: \*, from sham; #, from control. B, the effect of iloprost on I/R-induced gastric MPO activity in wild-type mice. Iloprost (0.3~3 μg/kg) was given i.v. 5 min before reperfusion. Data are presented as mean ± S.E.M. from six mice. \*, significant difference from control at *p* < 0.05.

being 2.56 ± 0.41 μmol H<sub>2</sub>O<sub>2</sub>/min/mg of tissue, which is significantly greater than that observed in wild-type mice. On the other hand, the increase in gastric MPO activity following I/R in wild-type mice was dose-dependently suppressed by prior administration of iloprost (0.3~3 μg/kg), and a significant effect was observed at 1 and 3 μg/kg, the inhibition being 44.1 and 44.0%, respectively (Fig. 6B).

**Gastric Mucosal 6-Keto-PGF<sub>1α</sub> and PGE<sub>2</sub> Contents in Wild-Type Mice.** Levels of 6-keto-PGF<sub>1α</sub>, the stable metabolite of PGI<sub>2</sub>, in the gastric mucosa were significantly increased in wild-type mice following I/R treatment compared with a sham operation (Fig. 7A). This increase was significantly prevented by prior administration of indomethacin (5 mg/kg p.o.) and rofecoxib (5 mg/kg p.o.) but not SC-560 (5 mg/kg p.o.). Likewise, the mucosal PGE<sub>2</sub> content was also significantly increased by I/R treatment, and the response was significantly attenuated by both indomethacin and rofecoxib but not SC-560 (Fig. 7B).

**Effect of Various COX Inhibitors and Iloprost on Gastric Acid Secretion.** The stomach of wild-type mice secreted acid at a rate of about 3.01 to 3.42 μEq for 2 h.



**Fig. 7.** Effect of COX inhibitors on mucosal levels of 6-keto-PGF<sub>1α</sub>, the stable metabolite of PGI<sub>2</sub> (A), and PGE<sub>2</sub> (B) in the stomach of wild-type mice subjected to I/R treatment. Indomethacin (5 mg/kg), SC-560 (5 mg/kg), or rofecoxib (5 mg/kg) was given p.o. 60 min before ischemia. Data are presented as the mean ± S.E.M. from five mice. Significant difference at  $p < 0.05$ : \*, from sham; #, from control.

Neither indomethacin, SC-560, nor rofecoxib had any effect on basal acid secretion in wild-type mice, the values being equivalent in all groups (Table 2). Likewise, iloprost (0.3~3 μg/kg) given i.v. did not significantly affect acid output at any dose, although a slight decrease (12.6%) was observed at the highest dose, 3 μg/kg. In addition, no difference in basal acid

**TABLE 2**

Effect of various COX inhibitors and iloprost on gastric secretion in wild-type mice

Acid secretion was measured in wild-type mice provided with an acute gastric fistula under urethane anesthesia. The stomach was filled with 0.4 ml of saline through the fistula, the solution was changed after 20 min, and the collected samples were used to measure acid output. Indomethacin (5 mg/kg), SC-560 (5 mg/kg), or rofecoxib (5 mg/kg) was given i.d. 1 h before the instillation of saline in the stomach, whereas iloprost (0.1~3 μg/kg) was given i.v. 5 min before. Data are presented as the mean ± S.E.M. from four to five mice.

Group	Dose	No. of Mice	Total Acid Output μEq/2 h
Saline		4	3.02 ± 0.36
Indomethacin	5 mg/kg	5	2.67 ± 0.39
SC-560	5 mg/kg	5	2.86 ± 0.29
Rofecoxib	5 mg/kg	5	3.21 ± 0.17
Iloprost	0.3 μg/kg	5	3.01 ± 0.40
	1 μg/kg	5	3.18 ± 0.10
	3 μg/kg	5	2.64 ± 0.55

secretion was observed between wild-type and IP receptor knockout mice, the values being  $3.01 \pm 0.40$  μEq/2 h and  $3.51 \pm 0.43$  μEq/2 h, respectively (Table 3).

**Effect of PGE<sub>2</sub> on I/R-Induced Gastric Lesions in Wild-Type Mice.** The severity of I/R-induced gastric lesions remained unaltered in EP1 or EP3 receptor knockout mice. However, because the levels of PGE<sub>2</sub> content in the gastric mucosa were found to increase following I/R with the up-regulation of COX-2 expression, there is a possibility that PGE<sub>2</sub> plays some role in mucosal defense of the stomach during I/R through a different EP receptor subtype. We therefore examined the effect of PGE<sub>2</sub> on I/R-induced gastric lesions in both wild-type and IP receptor knockout mice.

PGE<sub>2</sub>, given i.v. at 0.1 to 1 mg/kg 5 min before reperfusion, dose-dependently reduced the severity of I/R-induced gastric lesions in wild-type mice, and a significant effect was obtained only at 1 mg/kg, the degree of protection being 39.1%, which is less pronounced compared with that of iloprost at 3 μg/kg (67.3%; Fig. 8A). The protective effect of PGE<sub>2</sub> was observed even in IP receptor knockout mice, although the degree of protection was 25.8%, slightly less than that in wild-type mice (Fig. 8B).

## Discussion

Ischemia followed by reperfusion leads to tissue injury (Farber et al., 1981; Cheung et al., 1986; Piper et al., 2003). Whereas there is a substantial body of experimental data characterizing the factors that promote gastric lesions under I/R-induced conditions, tissue defense reactions that counterbalance the noxious effects of I/R remain less understood. The present study clearly demonstrated that endogenous PGs derived from COX-2 play a crucial role in the mucosal defense of the stomach under I/R-induced conditions, and this action is mainly mediated by PGI<sub>2</sub> through activation of IP receptors.

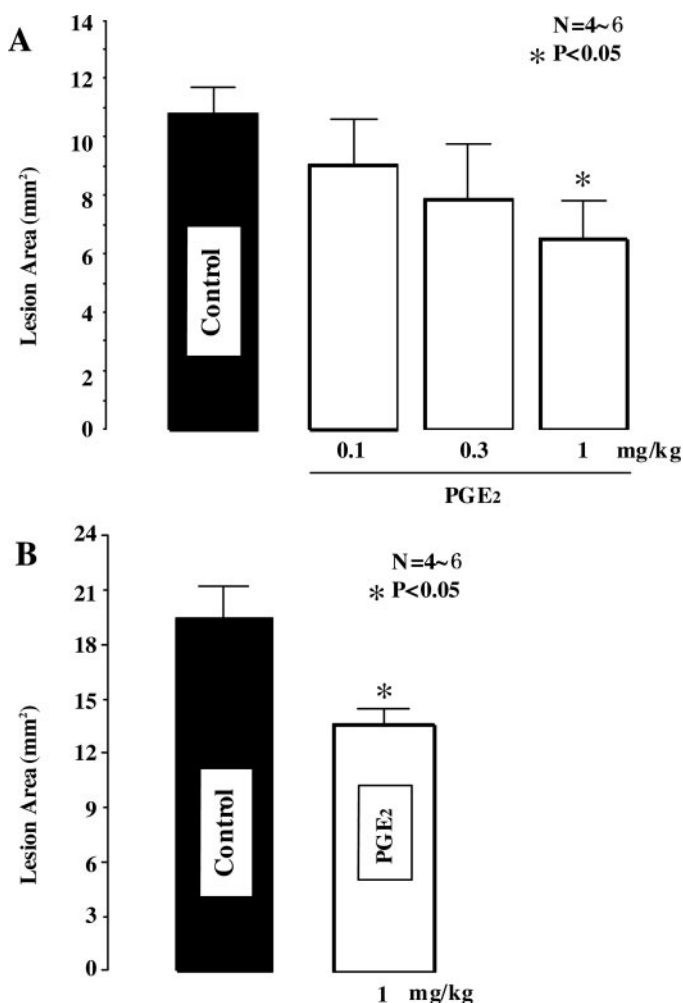
It has been thought that COX-1 functions as a housekeeping enzyme, catalyzing the formation of PGs that contribute to the maintenance of the mucosal integrity of the stomach through the modulation of various functions (Soll et al., 1991; Vane and Botting, 1995), whereas COX-2, the inducible enzyme up-regulated by proinflammatory cytokines and growth factors, mediates pathological reactions such as inflammation and tumor growth (Davies et al., 1997; Koga et al., 2004). Recent studies, however, showed that COX-2 is also involved in mucosal defense under certain conditions (Muscara et al., 2000; Tanaka et al., 2002) and plays an important role in the healing of gastric ulcers (Mizuno et al., 1997). In the present study, we found that the selective COX-2 inhibitor rofecoxib significantly aggravated the development of gastric lesions in response to I/R, similar to indomethacin, confirming the in-

**TABLE 3**

Basal acid secretion in wild-type and IP receptor knockout mice

Acid secretion was measured in wild-type and IP receptor knockout mice provided with an acute gastric fistula under urethane anesthesia. The stomach was filled with 0.4 ml of saline through the fistula, the solution was changed after 20 min, and the collected samples were used to measure acid output. Data are presented as the mean ± S.E.M. from four mice.

Group	No. of Mice	Total Acid Output μEq/2 h
Wild-type mice	4	3.01 ± 0.40
IP knockout mice	4	3.51 ± 0.43



**Fig. 8.** A, effect of PGE<sub>2</sub> on I/R-induced gastric lesions in wild-type mice. PGE<sub>2</sub> (0.1~1 mg/kg) was given i.v. 5 min before reperfusion. Data are presented as the mean ± S.E.M. from five to six mice. \*, significant difference from control at *p* < 0.05. B, effect of PGE<sub>2</sub> on I/R-induced gastric lesions in IP receptor knockout mice. PGE<sub>2</sub> (1 mg/kg) was given i.v. 5 min before reperfusion. Data are presented as mean ± S.E.M. from four to six mice.

involvement of COX-2/PGs in mucosal defense during I/R. These results are consistent with the findings of Maricic et al. (1999), who showed that I/R-induced gastric damage was aggravated by administration of selective COX-2 inhibitors such as NS-398 and DFU as assessed both macroscopically and histologically. They also showed that I/R increased COX-2 mRNA levels in the gastric mucosa and that dexamethasone aggravated the I/R-induced gastric lesions at a dose that inhibited COX-2 expression following I/R (Maricic et al., 1999). We also found in the present study the up-regulation of COX-2 expression in the stomach following I/R. These findings suggest that the gastric mucosa has the capacity to rapidly up-regulate COX-2 expression in response to various stimuli. It should be noted that ischemia alone neither caused any macroscopic damage nor up-regulated COX-2 expression in the gastric mucosa. Schmedtje et al. (1997) reported that hypoxia increased the expression of the COX-2 gene in human vascular endothelial cells in vitro. It is possible that the expression is up-regulated by ischemia alone when examined at a later period. Notwithstanding, it is assumed that COX-2 expression is up-regulated as one of the

protective mechanisms when the stomach is exposed to ulcerogenic stimuli.

On the other hand, we observed in this study that the selective COX-1 inhibitor SC-560 did not significantly affect the severity of I/R-induced gastric lesions, suggesting no role for COX-1 in mucosal defense during I/R. However, Hiratsuka et al. (2004) reported that SC-560 reduced the severity of I/R-induced gastric lesions in mice, probably due to down-regulation of free radical production during reperfusion by decreasing blood flow. The discrepancy in these results remains unexplained at present, yet it may be due to different experimental conditions such as the dose of SC-560; they used 40 mg/kg of this agent, 8 times higher than that (5 mg/kg) in the present study.

The most important finding of the present study is that the severity of I/R-induced gastric lesions was markedly increased in IP receptor knockout mice but not in the animals lacking EP1 or EP3 receptors. These results suggest that although I/R stimulated the generation of PGs to increase the mucosal levels of both PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub>, the stable metabolite of PGI<sub>2</sub>, in the stomach, the type of prostanoid responsible for mucosal defense during I/R is PGI<sub>2</sub> not PGE<sub>2</sub>. These results are understandable, because the expression of COX-2 in the gastric mucosa following I/R was observed mainly in the endothelial cells (Hiratsuka et al., 2004) and because PGI<sub>2</sub> is a major prostanoid produced in the endothelial cells (Konturek and Robert, 1982). We observed in this study that iloprost, a stable analog of PGI<sub>2</sub>, significantly prevented the I/R-induced gastric lesions in the absence or presence of COX inhibitors, supporting the involvement of endogenous PGI<sub>2</sub> in mucosal defense during I/R. This PGI<sub>2</sub> analog has an affinity for not only IP receptors but also EP receptors as well (Narumiya and FitzGerald, 2001). In the present study, however, iloprost had no effect on the development of I/R-induced gastric lesions in IP receptor knockout mice, excluding the involvement of EP receptors in the protective action of this agent. Harada et al. (1999, 2000) reported that iloprost prevented stress-induced gastric lesions, primarily by inhibiting leukocytes from accumulating. Since I/R injury is a neutrophil-dependent response (Zimmerman et al., 1990), it is assumed that selective COX-2 inhibitors promote the adherence of leukocyte to the vascular endothelium during I/R, thereby resulting in aggravation of the lesions in the stomach (Muscara et al., 2000). In the present study, we observed a marked increase in MPO activity in the gastric mucosa following I/R in wild-type mice, and this response was significantly reduced by iloprost and further enhanced in IP receptor knockout animals, confirming the inhibitory role for PGI<sub>2</sub>/IP receptors in the neutrophil-related process of I/R-induced gastric injury. These results all strongly suggest that endogenous PGI<sub>2</sub> produced by COX-2 plays a role in mucosal defense during I/R through the activation of IP receptors.

We previously examined, using various subtype-specific EP receptor agonists and antagonists, the relationship between EP receptor subtypes and PGE<sub>2</sub>-induced gastric cytoprotection and found that PGE<sub>2</sub> exhibits a protective action against a variety of gastric lesions mediated by the activation of EP1 receptors (Araki et al., 2000; Suzuki et al., 2001; Takeuchi et al., 2002a). The present results, however, suggest that neither EP1 nor EP3 receptors participate in mucosal defense during I/R in the stomach, although the muco-

sal PGE<sub>2</sub> content was significantly elevated following I/R. Of course, the present data do not totally exclude the involvement of PGE<sub>2</sub> in mucosal defense during I/R. It has been reported that PGE<sub>2</sub> protects against ischemia- or I/R-induced injury in brain, liver, and heart through EP2 or EP4 receptors (McCullough et al., 2004; Xiao et al., 2004; Kuzumoto et al., 2005). We also found in the present study that PGE<sub>2</sub> significantly reduced the severity of these lesions in both wild-type and IP receptor knockout mice, yet the effective dose was much higher and the effect was much less pronounced compared with iloprost. In a preliminary study, we also observed that the effect of PGE<sub>2</sub> was significantly affected by neither EP1, EP3, nor EP4 antagonists (data not shown). At present, it remains unknown whether or not this effect of PGE<sub>2</sub> is physiological action, yet there is a possibility that PGE<sub>2</sub> exhibits a protective effect against the I/R-induced gastric lesions, probably through EP2 receptors. Hoshino et al. (2003) reported that PGE<sub>2</sub> inhibited the irritant-induced apoptosis via EP2/EP4 receptors and speculated that this action is involved in the gastroprotective action of PGE<sub>2</sub> in vivo conditions. However, we previously reported that neither specific EP2 nor EP4 agonists protected the stomach against acidified ethanol or indomethacin in rats (Araki et al., 2000; Suzuki et al., 2001). Furthermore, no evidence has been reported the involvement of apoptotic changes in the I/R-induced gastric lesions. On the other hand, it is known that PGE<sub>2</sub> inhibits the neutrophil migration in the gastric mucosa via EP2/EP4 receptors (Suzuki et al., 2001). Thus, it is assumed that PGE<sub>2</sub> prevents I/R-induced gastric lesions via inhibition of the neutrophil-related process but not apoptosis, similar to PGI<sub>2</sub>.

It has been reported that gastric acid secretion is substantially decreased after ischemia and remained reduced for several hours even after reperfusion (Takeuchi et al., 1986; Nakamoto et al., 1998). However, Kitano et al. (1997) showed that cimetidine, the histamine H<sub>2</sub> receptor antagonist, had a protective effect on I/R-induced gastric lesions through the suppression of acid secretion. In a preliminary study, we observed that lansoprazole, a proton pump inhibitor, also significantly reduced the severity of I/R-induced gastric lesions in wild-type mice (data not shown), suggesting the participation of gastric acid in the pathogenesis of these lesions. Since iloprost at the highest dose (3 μg/kg) caused a slight decrease in acid secretion, consistent with the finding by Seidler et al. (1989), it is possible that the protective effect of this prostanoid, especially at high doses, on I/R-induced gastric lesions is partly accounted for by its antisecretory action.

Based on all the results of the present study, we confirmed that I/R induced gastric lesions with the up-regulation of COX-2 expression and that the damage was significantly aggravated by indomethacin as well as the selective COX-2 inhibitor rofecoxib but not the selective COX-1 inhibitor SC-560. We further showed for the first time that the I/R-induced gastric lesions were significantly worsened in IP receptor knockout mice but not in the animals lacking EP1 or EP3 receptors. Thus, it is assumed that endogenous PGs derived from COX-2 play a crucial role in gastric mucosal defense during I/R, and this action is mainly mediated by PGI<sub>2</sub> through the activation of IP receptors.

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