Pathogenic Importance of Cysteinyl Leukotrienes in Development of Gastric Lesions Induced by Ischemia/Reperfusion in Mice

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Abbreviations:
cysteinyl leukotrienes -------------------------------------- CysLTs
leukotriene ---------------------------------------------------- LT
ischemia/reperfusion -------------------------------------- I/R
prostaglandins ----------------------------------------------- PGs
cyclooxygenase ---------------------------------------------- COX
prostaglandin E$_2$ --------------------------------------------- PGE$_2$
PGE receptor ------------------------------------------------- EP receptor
CysLT receptor type 1 ------------------------------------- CysLT,R
5-lipoxygenase --------------------------------------------- 5-LOX
Myeloperoxidase ------------------------------- MPO
Abstract

We examined the role of cysteinyl leukotrienes (CysLTs) in the gastric ulcerogenic response to ischemia/reperfusion (I/R) in mice. Experiments were performed in male C57BL/6J mice after 18 h fasting. Under urethane anesthesia, the celiac artery was clamped for 30 min, then reperfusion was achieved by removing the clamp and the stomach was examined for lesions 60 min thereafter. The severity of I/R-induced gastric damage was reduced by prior administration of pranlukast [CysLT receptor type 1 (CysLT1R) antagonist] as well as TMK688 [5-lipoxygenase (5-LOX) inhibitor]. On the contrary, these lesions were markedly worsened by pretreatment with indomethacin, and this response was abrogated by the co-administration of TMK688 or pranlukast. The gene expression of CysLT1R but not 5-LOX was up-regulated in the stomach following I/R, but both expressions were increased under I/R in the presence of indomethacin. I/R slightly increased the mucosal CysLT content of the stomach, yet this increase was markedly enhanced when the animals were pretreated with indomethacin. The increased CysLT biosynthetic response to indomethacin during I/R was attenuated by TMK688. Indomethacin alone caused a slight increase of CysLT1R expression and markedly up-regulated 5-LOX expression in the stomach. We concluded that I/R up-regulated the expression of CysLT1R in the stomach; CysLTs play a role in the pathogenesis of I/R-induced gastric damage through the activation of CysLT1R; and the aggravation by indomethacin of these lesions may be brought about by the increase of CysLT production and the up-regulation of 5-LOX expression, in addition to the decreased PG production.
Introduction

Ischemia/reperfusion (I/R) injury is caused by interruption of the blood supply to an organ tissue followed by the reintroduction of blood into the affected area. The phenomenon of I/R injury is a major clinical problem after stroke, infarction, shock, and organ transplantation (Boros et al., 1989; Stein et al., 1990). The depletion of adenosine triphosphate (ATP) and disturbance of intracellular Ca\(^{2+}\) homeostasis have been suggested as the major pathophysiological mechanism during ischemia, leading to loss of cell viability (Cheung et al., 1986; Farber et al., 1981). Reperfusion of ischemic tissues paradoxically exacerbates the injury process and leads to the release of reactive oxygen species and proinflammatory mediators, and the attraction of inflammatory cells infiltrating the tissues (Chamoun et al., 2000; Piper et al., 2003). Indeed, many studies showed that a xanthine oxidase inhibitor as well as radical scavengers protect the stomach against injury during I/R, confirming the importance of free radicals in the pathogenesis of I/R-induced gastric injury (Yasue et al., 1992; Perry et al., 1986).

Prostaglandins (PGs), cyclooxygenase (COX) products, are essential to maintain the mucosal integrity of the gastrointestinal tract, and a deficiency of endogenous PGs shows a propensity to damage the mucosa (Kunikata et al., 2001; Suzuki et al., 2001; Takeuchi et al., 2007) as well as worsens the ulcerogenic response to noxious stimuli in the gastrointestinal tract, including I/R (Takeuchi et al., 1986; Kotani et al., 2006; Tanaka et al., 2007). Leukotrienes (LTs), lipoxygenase (5-LOX) products, are also known to play a role in the inflammatory response (Steinhilber, 1999). It is thus possible that LTs are also involved in the pathogenic mechanism of
I/R-induced gastric lesions, in addition to PGs. Among the 5-LOX products, LTC₄, LTD₄, and LTE₄, referred to as cysteinyl LTs (CysLTs), are recognized as potent inflammatory mediators (Murakami et al., 1995; Gronert et al., 2001). CysLTs are generated from cell membrane phospholipid-associated arachidonic acids by 5-LOX and reported to play a pathogenic role in the development of I/R-induced damage in kidney, liver, and bladder (Sener et al., 2006 & 2007; Takamatsu et al., 2004). However, it remains unknown whether CysLTs are involved in the development of gastric lesions under I/R.

Recently, two subtypes of CysLT receptors, CysLT₁R and CysLT₂R, have been characterized, both of which are classic G protein-coupled receptors with seven transmembrane domains (Brink et al., 2003). It has been reported that CysLT₁R is highly expressed in spleen, small intestine, colon, heart, pancreas, kidney, liver and placenta (Lynch et al., 1999; Sarau et al., 1999) and detected in mast cells, mononuclear cells, and microvascular endothelia by in situ hybridization or immunohistochemistry (Lynch et al., 1999; Zhang et al., 2004). Several studies showed that the administration of a 5-LOX inhibitor or a selective CysLT₁R antagonist reduced the severity of ethanol- or nonsteroidal antiinflammatory drug (NSAID)-induced gastric injury by inhibiting neutrophil adhesion (Dengiz et al., 2007; Sala and Folco, 2001), suggesting the pathogenic importance of CysLTs/CysLT₁R in gastric lesions. Since CysLT₁R, found in pulmonary vein preparations by pharmacological assays, has now been reported to express in various tissues (Brink et al., 2003; Kanaoka and Boyce, 2004, Moos et al., 2008), it is possible that CysLT₂R may also play a role in the pathogenesis of gastric lesions as induced by I/R. However, none of selective CysLT₂R antagonists is available for in-vivo studies.
In the present study, we thus focused on CysLTs and CysLT_{1R} and examined the effects of a 5-LOX inhibitor and a selective CysLT_{1R} antagonist on I/R-induced gastric damage in mice and investigated the roles of CysLTs/CysLT_{1R} in mucosal defense during I/R. Additionally, we also examined the mechanism by which indomethacin aggravates I/R-induced lesions, in relation to CysLTs/CysLT_{1R} as well as 5-LOX in the stomach.
Methods

Animals

Male C57BL/6J mice (3 months old; SLC, Shizuoka, Japan) were used. The animals were deprived of food but allowed free access to tap water for 18 h before the experiments. All experiments were carried out using five 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 Ischemia and Reperfusion-Induced Gastric Damage

Acute gastric mucosal lesions were produced by I/R in mice, according to a modified method reported by 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 40 g; BEAR Medical Corporation, Chiba, Japan), and 30 min later reperfusion was achieved through removal of the clamp. In a preliminary study, we found that macroscopically visible lesions were consistently observed in the stomach 60 min after the onset of reperfusion following ischemia for 30 min. The effect of various treatments on the formation of lesions was assessed 60 min after the reperfusion, according to a previous study (Yoshikawa et al., 1989). 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. Pranlukast (a CysLT1R antagonist; 0.1–10 mg/kg)(Nishio et al., 2007), montelukast (a CysLT1R antagonist; 3 mg/kg) or TMK688 (a 5-LOX inhibitor; 3-30 mg/kg)(Konaka et al., 1999)
was administered p.o. 60 min before ischemia. Indomethacin (a nonselective COX inhibitor; 5 mg/kg) was given s.c. 30 min before ischemia. The area (mm$^2$) of hemorrhagic lesions developed in the stomach was measured under a dissecting microscope (Olympus, Tokyo, Japan) with a square grid (x10). 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 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 mice, according to a modified version of the method of Krawisz et al. (1984). At 60 min after I/R-treatment, the animals were sacrificed by the withdrawal of blood from the heart through perfusion with saline, and the stomach was excised. After rinsing of the tissue with cold saline, the mucosa was scraped with glass slides, weighed, and homogenized in a 50 mM phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB; pH 6.0; Sigma). The homogenized samples were subjected to freezing and thawing three times, and centrifuged at 2,000 g for 10 min at 4°C. MPO activity in the supernatant was determined by adding 50 µl of the supernatant to 50 µl of 10 mM phosphate buffer (pH 6.0) and 50 µl of 1.5 M o-dianisidine hydrochloride (Sigma) containing 0.0005% (w/v) hydrogen peroxide. The changes in absorbance at 450 nm were recorded on a microplate reader (VERSAmax; Molecular Device, OA). Sample protein content was estimated by
spectrophotometric assay (Pierce protein assay kit, Illinois), and the MPO activity was obtained from the slope of the reaction curve, based on the following equation:
Specific activity (µmol H₂O₂/min/mg protein) = (OD/min)/OD/µmol H₂O₂ x mg protein). Pranlukast (3 mg/kg) or TMK688 (30 mg/kg) was administered p.o. 60 min before ischemia, while indomethacin was given s.c. 30 min before ischemia.

Measurement of Mucosal CysLT Levels

Levels of CysLT in the gastric mucosa were measured after I/R treatment in mice. TMK688 (30 mg/kg) or pranlukast (3 mg/kg) was administered p.o. alone or 60 min before ischemia, while indomethacin was given s.c. 30 min before ischemia. 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. Then, the tissues were homogenized by Polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 12,000 g for 10 min at 4°C. After the supernatant of each sample had been evaporated with N₂ gas, the residue was resolved in assay buffer and used for the determination of CysLTs. Concentrations of CysLTs were measured using a CysLT enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI).

Analyses of Expression of 5-LOX and CysLT₁R mRNAs by RT-PCR

Animals were killed under deep ether anesthesia 60 min after I/R-treatment in the absence or presence of indomethacin (5 mg/kg) given s.c. 30 min before the ischemia, and the stomachs were removed, frozen in liquid nitrogen, and stored at -80°C prior to use. In some case, the animals were given TMK688 (30 mg/kg) or
indomethacin (5 mg/kg) alone without I/R treatment, and killed 60 min later. Tissue samples were pooled from two to three mice for extraction of total RNA, which was prepared by a single-step acid phenol-chloroform extraction procedure by use of Sepasol RNA-I (Nacalai tesque, Kyoto, Japan). Total RNA primed by random hexadeoxy ribonucleotide was reverse-transcribed with the SuperScript pre-amplification system (Invitrogen, Carlsbad, CA). The sequences of sense and antisense primers for mouse GAPDH, 5-LOX, and CysLT₁R are listed 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 95°C and 1 min of annealing at 68°C by Advantage-2 on a thermal cycler. A portion of the PCR mixture was electrophoresed in a 1.5% agarose gel in Tris-EDTA-acetic acid buffer, and the gel was stained with ethidium bromide and photographed. Images were analyzed with the Image J (version 1.39), and the semiquantitative measurement of mRNA expression was presented as a ratio compared with GAPDH.

**Determination of Gastric Acid Secretion**

Acid secretion was measured in pylorus-ligated mice. Animals were deprived of food for 18 h and water for 2 h before the operation. Under light ether anesthesia, the abdomen was opened and the pylorus was ligated, and the animals were then allowed to recover from the anesthesia. TMK688 or pranlukast was given i.d. immediately after pylorus ligation. Two hours after pylorus ligation, the animals were sacrificed with an overdose of ether, the gastric contents were collected and analyzed for volume and acidity. Acidity was measured by automatic titration of the gastric contents against 0.01 M/L NaOH to pH 7.0 with a titrator (Hiranuma; Comtite

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COM-550, Tokyo, Japan). Gastric acid output (volume x acidity) was expressed as microequivalents per 1 h. Control animals were administered vehicle alone.

**Preparation of Drugs**

The drugs used were urethane (Tokyo Kasei, Tokyo, Japan), TMK688 (1-[[5′-(3″-methoxy-4″-ethoxycarbonyl-oxyphenyl)-2′,4′-pentadienoyl]aminoethyl]4-diphenylmethoxypiperidine; Banyu Pharm. Co. Ltd., Tokyo, Japan), pranlukast (4-oxo-8-[4-(4-phenylbutoxy)benzoyl amino]-2)-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate: Ono Pharm. Co. Ltd., Osaka, Japan), montelukast (2-[1-[(1R)-1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfanylmethyl]cyclopropyl]acetic acid: Cayman Chemical, Ann Arbor, MI, USA) and indomethacin (Sigma Chemicals, St. Louis, MO). Indomethacin, TMK688, pranlukast and montelukast were suspended in a hydroxypropylcellulose (HPC) solution (Wako, Osaka, Japan). Each agent was prepared immediately before use and administered p.o., i.d., or s.c. in a volume of 0.1 ml/20 g body weight. Control animals received HPC as the vehicle.

**Statistical Analysis**

Data are presented as the mean±SE for 5 to 8 rats per group. Statistical analyses were performed using a one-way analysis of variance (ANOVA) and Student’s t-test or Dunnett’s multiple comparison test where appropriate, and values of P<0.05 were considered significant.
Results

Effects of TMK688 and Pranlukast on I/R-Induced Gastric Damage in Mice

Laparotomy without clamping of the gastric artery (sham operation) did not produce any damage in the gastric mucosa. In the animals subjected to I/R treatment (30 min of ischemia followed by reperfusion for 60 min), however, multiple hemorrhagic lesions were observed in the gastric mucosa, the lesion score being $11.2\pm1.4 \text{ mm}^2$ (Figure 1A). Ischemia for 30 min did not induce any macroscopically visible damage in the mucosa (not shown). Pretreatment of the animals with TMK688 (3-30 mg/kg, p.o.), a 5-LOX inhibitor, dose-dependently prevented the I/R-induced development of gastric lesions, the inhibition at 30 mg/kg being 49.1%, which is significant as compared to control.

To further investigate the involvement of CysLTs in the pathogenesis of I/R-induced gastric lesions, we examined the effect of pranlukast, a CysLT1R antagonist, on the gastric ulcerogenic response to I/R. As shown in Figure 1B, the severity of the gastric lesions was reduced by the prior administration of pranlukast (0.1-10 mg/kg, p.o.), in a dose-dependent manner, and a significant effect was observed at 1 mg/kg or greater, the inhibition at 1, 3 and 10 mg/kg being 44.4%, 72.2% and 74.0%, respectively. A significant protection was similarly obtained by montelukast (3 mg/kg), another CysLT1R antagonist, the inhibition being 73.6% ($3.9\pm0.7 \text{ mm}^2$ vs $14.8\pm1.8 \text{ mm}^2$).

In a sham-operated animal without I/R treatment, no damage was detected even by histological observation (Figure 2A). By contrast, severe damage was
observed histologically in the stomach after I/R treatment; most of the damage was restricted to the surface epithelium, but some damage occurred deep in the mucosa, extending to the region of pits and glands (Figure 2B). When the animal was pretreated with TMK688 (30 mg/kg, p.o.) or pranlukast (3 mg/kg, p.o.), the severity of the histological damage was markedly reduced, and only slight damage was observed in the surface epithelium (Figure 2 and 2C).

**Effects of TMK688 and Pranlukast on I/R-Induced Gastric Damage in Mice Pretreated with Indomethacin**

It has been shown that COX inhibition stimulates the production of LTs by inducing a shift in balance from protective PGs to proulcerogenic CysLTs (Peskar, 1991) and that I/R-induced gastric injury was markedly aggravated by prior administration of indomethacin, a nonselective COX inhibitor (Kotani et al., 2006). We therefore examined the effects of 5-LOX inhibitor and CysLT₁R antagonist on I/R-induced gastric ulceration in the mice pretreated with indomethacin.

When the stomach was subjected to I/R in the presence of indomethacin (5 mg/kg, s.c.), the severity of gastric lesions was significantly increased, the lesion score being 20.3±2.3 mm², about 2.5 times greater than that (8.3±1.5 mm²) for I/R alone (Figure 3). This effect of indomethacin was significantly abrogated by co-treatment with either TMK688 (30 mg/kg, p.o.) or pranlukast (3 mg/kg, p.o.), the inhibition being 40.4% and 70.0%, respectively.

**Effects of TMK688 and Pranlukast on MPO Activity in Mouse Stomachs Following I/R**
It is known that I/R-induced gastric injury is accompanied by an increase in neutrophil recruitment in the mucosa (Kotani et al., 2006; Kobata et al., 2007). In the present study, the severity of the I/R-induced gastric lesions was decreased by prior administration of TMK688 and pranlukast. To confirm that the mucosal inflammatory response during I/R is also affected by these treatments, we examined the effects of TMK688 and pranlukast on the changes in MPO activity in the gastric mucosa following I/R.

Gastric mucosal MPO activity in sham-operated mice was less than 0.02 µmole H$_2$O$_2$/min/mg protein. The MPO activity was markedly increased after I/R, the values being 0.042±0.007 µmole H$_2$O$_2$/min/mg protein (Figure 4). This response was significantly abrogated by the prior administration of pranlukast (3 mg/kg, p.o.), the inhibition being 59.5%. TMK688 (30 mg/kg, p.o.) also suppressed the increase of MPO activity induced by I/R, though the effect was not statistically significant.

We further examined the effects of TMK688 and pranlukast on the changes in MPO activity in the gastric mucosa following I/R in the presence of indomethacin. The mucosal MPO activity in control mice was 0.044±0.006 µmole H$_2$O$_2$/min/mg protein when determined 60 min after I/R-treatment (Figure 5). The I/R-induced increase of MPO activity was markedly enhanced in the animals pretreated with indomethacin (5 mg/kg, s.c.), the values being 0.11±0.013 µmole H$_2$O$_2$/min/mg protein. This response was significantly suppressed by the prior administration of TMK688 (30 mg/kg, p.o.) or pranlukast (3 mg/kg, p.o.), the inhibition being 56.4% or 58.2%, respectively.
Changes in Mucosal CysLT Content of Mouse Stomach Following I/R with or without Indomethacin

To further investigate the role of CysLTs in the I/R-induced development of gastric lesions, we measured the mucosal CysLT levels in the mouse stomach in the absence or presence of indomethacin.

The levels of CysLTs in the gastric mucosa were not changed following I/R treatment compared with the sham operation, the values being 3.3±0.2 ng/mg tissue in the sham-operated group and 2.4±0.3 ng/mg tissue in the I/R-treated group. Pretreatment with TMK688 (30 mg/kg, p.o.) inhibited the production of CysLTs in the gastric mucosa subjected to I/R treatment and significantly decreased the levels to approximately half of control values (Figure 6). Pretreatment of the animals with indomethacin (5 mg/kg, s.c.), however, significantly increased the mucosal levels of CysLTs to 7.0±1.3 ng/mg tissue under I/R, and this response was almost totally suppressed by co-treatment with TMK688 (30 mg/kg), the inhibition being 90.6%. Pranlukast (3 mg/kg, p.o.) had no effect on the mucosal CysLT levels in the stomach following I/R treatment, in the absence or presence of indomethacin.

Expression of CysLT₁R and 5-LOX mRNAs in Mouse Stomach Following I/R

GAPDH mRNA, the housekeeping gene, was clearly detectable in the stomach of control mice, and its expression remained unaffected following I/R. Although CysLT₁R was expressed in the gastric mucosa subjected to the sham-operation, the expression was markedly up-regulated following I/R treatment when examined 1 h after reperfusion (Figures 7A and 7B). Ischemia alone had no effect on
the CysLT₁R expression (not shown). Indomethacin (5 mg/kg, s.c.) alone slightly up-regulated the expression of CysLT₁R in the gastric mucosa but did not further enhance the increased expression following I/R.

5-LOX mRNA was also observed in the normal stomach following the sham operation and remained unchanged after I/R treatment when examined 1 h after reperfusion. Ischemia alone did not affect the 5-LOX expression in the gastric mucosa (data not shown). However, the mucosal expression of 5-LOX under I/R was significantly increased by pretreatment of the animals with indomethacin (5 mg/kg) when compared to the control mouse stomach subjected to I/R alone (Figures 8A and 8B). It was also found that indomethacin by itself caused a marked up-regulation of 5-LOX expression in the stomach, and the increased expression by indomethacin was not further enhanced by additional I/R-treatment. TMK688 (30 mg/kg, p.o.) did not affect the expression of 5-LOX as well as CysLT₁R in the gastric mucosa with or without I/R treatment (not shown).

**Effects of TMK688 and Pranlukast on Gastric Acid Secretion**

The acid output in normal mouse stomachs was 1.00±0.12 µEq/h when determined by a 2-h pylorus-ligation (Table 2). Neither TMK688 (30 mg/kg, i.d.) nor pranlukast (3 mg/kg, i.d.) had any effect on the basal acid output in mice.
Discussion

The supply of blood to ischemic tissues paradoxically exacerbates the injury process and leads to the production of free radicals and pro-inflammatory mediators, and the attraction of inflammatory cells infiltrating the tissues (Farber et al., 1981; Piper et al., 2003). While there is a substantial body of experimental data characterizing the factors that reduce or enhance the severity of I/R-induced gastric lesions, the roles of CysLTs in the pathogenesis remain unknown. The present study demonstrated that I/R markedly up-regulated CysLT,R expression in the gastric mucosa with a slight increase of CysLT production and that both a 5-LOX inhibitor and a CysLT,R antagonist suppressed the development of gastric lesions in response to I/R, strongly suggesting the involvement of CysLTs as a pathogenic factor in I/R-induced gastric damage. In addition, we also observed a marked aggravation of these lesions on pretreatment with indomethacin, consistent with our previous observations (Takeuchi et al., 1986; Kotani et al., 2006), and further showed the underlying mechanism of this response to be associated with the up-regulation of 5-LOX expression and CysLT production in the stomach.

The arachidonic acids released by phospholipase A₂ serve as substrates for the production of a group lipid mediators known as LTs, which induce pro-inflammatory signaling through the activation of CysLTs and LTB₄ receptors. 5-LOX is the key enzyme in the biosynthesis of LTs from arachidonic acids. LTs, potent mediators of inflammatory and allergic reactions, are locally released from leukocytes and other 5-LOX-expressing cells and exert their effects by binding to specific membrane receptors (Maekawa et al., 2002). CysLT receptors exist in two sybtypes,
CysLT₁R and CysLT₂R, and they are expressed in various tissues and inflammatory cells (Brink et al., 2003; Lynch et al., 1999; Sarau et al., 1999). Sala and Folco (2001) reported that a selective CysLT₁R antagonist reduced the severity of ethanol- or NSAID-induced gastric lesions by inhibiting neutrophil adhesion, suggesting the pathogenic importance for CysLT₁R. Certainly, CysLT₂R may also play a role in the pathogenesis of gastric lesions. However, none of selective CysLT₂R antagonist is available for in-vivo studies. In the present study we thus focused on CysLTs/CysLT₁R and investigated the effects of a 5-LOX inhibitor and a selective CysLT₁R antagonist on I/R-induced gastric damage.

First, we found that both pranlukast, a CysLT₁R antagonist, and TMK688, a 5-LOX inhibitor, significantly reduced the severity of I/R-induced gastric lesions. CysLTs are reportedly known to increase vascular permeability, neutrophil invasion and inflammatory cytokine production (Maekawa et al., 2002; Sala and Folco, 2001; Profita et al., 2008). Because I/R-induced damage is a neutrophil-dependent response (Zimmerman et al., 1990), it is assumed that the protective effect of pranlukast is brought about, at least partly, by the suppression of these inflammatory responses such as neutrophil infiltration, through the blockade of CysLT₁R. Anderson et al. (2009) reported that montelukast, a CysLT₁R antagonist, inhibits the proinflammatory action of neutrophils in vitro. In the present study, we confirmed the neutrophil infiltration in the stomach during I/R-treatment, as represented by a marked increase of MPO activity, and further observed that pranlukast as well as TMK688 attenuated the increase of MPO activity following I/R treatment. These all results strongly suggest the involvement of CysLTs/CysLT₁R in the pathogenesis of I/R-induced gastric injury. We also found that the expression of CysLT₁R mRNA in the gastric
mucosa was significantly increased under I/R conditions, although the mucosal CysLT content remained unchanged before and after I/R treatment. In other organs such as brain and kidney, the expression of CysLT1R mRNA was reportedly up-regulated by ischemia alone or I/R treatment (Fang et al., 2007; Matsuyama et al., 2008). It is assumed that the increased sensitivity to CysLT in the mouse stomach under I/R conditions is, at least partly, accounted for by the up-regulation of CysLT1R expression, although I/R did not increase CysLT production in the stomach. By contrast, indomethacin alone slightly up-regulated the CysLT1R expression but did not further enhance the increased expression caused by I/R, excluding a possibility of the up-regulation of CysLT1R responsible for the aggravating mechanism by indomethacin of the I/R-induced gastric injury.

We previously reported that I/R caused 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, suggesting the involvement of COX-2/PGs in mucosal defense during I/R (Kotani et al., 2006). As 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). Similar results were also reported by Maricic et al. (1999) who showed that I/R-induced gastric damage was aggravated by the administration of selective COX-2 inhibitors such as NS-398 and DFU. Since several studies showed that CysLT production in the stomach or the small intestine was increased by pretreatment with indomethacin (Wallace et al., 1990; Nishio et al., 2007), it is possible that the metabolism of
arachidonic acid leads to the production of LTs via 5-LOX when another pathway to PGs is blocked by COX inhibition. In the present study, we observed that both CysLT production and 5-LOX mRNA expression in the stomach were significantly increased in the presence of indomethacin under I/R conditions. It should also be noted that indomethacin alone, but not I/R, markedly up-regulated the expression of 5-LOX in the gastric mucosa. Moreover, the co-administration of TMK688, a 5-LOX inhibitor, markedly suppressed the increase of CysLT production and the aggravation of I/R-induced gastric lesions under indomethacin pretreatment, without any effect on the expression of CysLT,R. These results together with previous findings (Kotani et al, 2006) suggest that the aggravation by indomethacin of I/R-induced gastric damage is brought about by not only a decrease of PG production but also an increase of 5-LOX expression/CysLT production as well. At present, however, the mechanism how indomethacin up-regulates 5-LOX expression in the stomach remains unknown.

Although a selective COX-2 inhibitor, rofecoxib, increased the severity of I/R-induced gastric injury, similar to indomethacin (Kotani et al., 2006), the mechanisms for aggravation seem to differ in these cases. The worsening effect of these two agents was significantly prevented by the co-administration of prostacyclin (PGI2)(Kotani et al., 2006). We have recently found that the aggravation by rofecoxib, but not indomethacin, of I/R-induced gastric injury was significantly abrogated by ozagrel the inhibitor of thromboxane A2 (TXA2) synthesis as well as seratrodast the TXA2 receptor antagonist (Nakamori et al., 2009). These results suggest that the worsening effect of rofecoxib may be partly accounted for by the imbalance of TXA2/PGI2 ratio in the stomach and is apparently different from that of indomethacin which may be related to the increase of CysLT production and 5-LOX expression, in
addition to PG deficiency.

It has been reported that gastric acid secretion was substantially decreased after ischemia and remained decreased for several hours even after reperfusion (Takeuchi et al., 1986; Nakamoto et al., 1998). However, Kitano et al. (1997) reported that cimetidine, an histamine H₂ receptor antagonist, had a protective effect against I/R-induced gastric damage 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 damage in mice, suggesting the partial involvement of gastric acid in the pathogenesis of these lesions. In the present study, however, since basal gastric acid secretion in mice was significantly affected by neither pranlukast nor TMK688, the protective effects of these agents against I/R-induced gastric ulceration were accounted for by actions other than the inhibition of acid secretion. These results support an idea that the prophylactic effects of pranlukast or TMK688 may be brought about by the suppression of the inflammatory responses such as neutrophil infiltration and cytokine production during I/R, through the inhibition of CysLT production.

Given the findings of the present study, we concluded that I/R treatment up-regulated the expression of CysLT₁R in the stomach and that CysLTs play a crucial role in the pathogenesis of I/R-induced gastric lesions through the activation of CysLT₁R. In addition, the aggravation by indomethacin of I/R-induced gastric injury may be brought about by the increase of CysLT production as well as the up-regulation of 5-LOX expression, in addition to the decreased PG production. The present study cannot exclude a possibility that CysLT₂R may play a role in the gastric ulcerogenic response to I/R, in addition to CysLT₁R. Further study using a selective
CysLT₂R antagonist is needed to clarify this point.
References


Krawisz JE, Sharon P and Stenson WF (1984) Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity: assessment of inflammation in


Footnotes

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Legends for Figures

Figure 1. Effects of TMK688 (A) and pranlukast (B) on I/R-induced gastric lesions in mice. Under urethane anesthesia, the celiac artery was clamped (ischemia), and reperfusion was achieved 30 min later by removal of the clamp. After a 60-min reperfusion period, gastric lesions were evaluated. TMK688 (3~30 mg/kg) or pranlukast (0.1~10 mg/kg) was given p.o. 60 min before ischemia. Data are presented as the mean±SE for five to six mice. *Significant difference from control at P<0.05.

Figure 2. Microscopical observation of the gastric mucosa subjected to sham- and I/R-treatment. Under urethane anesthesia, the celiac artery was clamped (ischemia), and reperfusion was achieved 30 min later by removal of the clamp. After a 60-min reperfusion period, the stomachs were excised. TMK688 (30 mg/kg) or pranlukast (1~10 mg/kg) was given p.o. 60 min before ischemia. Figures show: (A) sham-treatment; (B) I/R-treatment; (C) I/R plus TMK688 (30 mg/kg, p.o.); (D) I/R plus pranlukast (3 mg/kg, p.o.). Bar=100 µm.

Figure 3. Effects of TMK688 and pranlukast on I/R-induced gastric lesions in mice pretreated with indomethacin. Under urethane anesthesia, the celiac artery was clamped (ischemia), and reperfusion was achieved 30 min later by removal of the clamp. After a 60-min reperfusion period, gastric lesions were evaluated. Indomethacin (5 mg/kg) was given s.c. 30 min before ischemia, while TMK688 (30 mg/kg) or pranlukast (3 mg/kg) was given p.o. 60 min before. Data are presented as the mean±SE for five to seven mice. Significant difference at P<0.05: *from
control; # from vehicle.

Figure 4. Effects of TMK688 and pranlukast on changes in gastric mucosal MPO activity induced by I/R in 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. TMK688 (30 mg/kg) or pranlukast (3 mg/kg) was given p.o. 60 min before ischemia. Data are presented as the mean±SE for five to six mice. Significant difference at P<0.05: * from sham; # from control (I/R alone).

Figure 5. Effects of TMK688 and pranlukast on changes in gastric mucosal MPO activity induced by I/R in mice pretreated with indomethacin. 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. Indomethacin (5 mg/kg, s.c.) was given 30 min before ischemia, while TMK688 (30 mg/kg) or pranlukast (3 mg/kg) was given p.o. 60 min before. Data are presented as the mean±SE for five to six mice. Significant difference at P<0.05: * from control (I/R alone); # from vehicle (I/R plus indomethacin).

Figure 6. Changes in mucosal CysLT levels in the mouse stomach subjected to sham- and I/R-treatment. 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, the mucosal CysLT levels were measured.
Indomethacin (5 mg/kg) was given s.c. 30 min before ischemia, while pranlukast (3 mg/kg) or TMK688 (30 mg/kg) was given p.o. alone or 60 min before. Data are presented as the mean±SE for six to eight mice. Significant difference at P<0.05: $ from sham; * from control; # from vehicle.

Figure 7. (A) Mucosal expression of CysLT,R mRNA in the mouse stomach given indomethacin alone or subjected to I/R-treatment in the absence or presence of indomethacin. Indomethacin (IM: 5 mg/kg) was given s.c. 30 min before ischemia. The expression of CysLT,R mRNA was analyzed by RT-PCR 2 h after indomethacin alone or 1 h after reperfusion. (B) Densitometric quantitation was performed with image J software, and the results are expressed as the ratio of CysLT,R to GAPDH. Data are presented as the mean±SE for five mice. Significant difference at P<0.05; * from sham; # from indomethacin alone.

Figure 8. (A) Mucosal expression of 5-LOX mRNA in the mouse stomach given indomethacin alone or subjected to I/R-treatment in the absence or presence of indomethacin. Indomethacin (IM: 5 mg/kg) was given s.c. 30 min before ischemia. The expression of 5-LOX mRNA was analyzed by RT-PCR 2 h after indomethacin alone or 1 h after reperfusion. (B) Densitometric quantitation was performed with Image J software, and the results are expressed as the ratio of 5-LOX to GAPDH. Data are presented as the mean±SE for five mice. Significant difference at P<0.05: * from sham; # from control.
Table 1.

**PCR Primer Sequences and Product Size**

<table>
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<tr>
<th>Molecule</th>
<th>Primer Sequence</th>
<th>Product Size (bp)</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>Forward; 5’-AACGACCCC TTCATTGAC-3’  &lt;br&gt;Reverse; 5’-TCCACGACATACTCAGCAC-3’</td>
<td>191</td>
</tr>
<tr>
<td>CysLT₁R</td>
<td>Forward; 5’-CAACGAACTATCCACCTTCACC-3’  &lt;br&gt;Reverse; 5’-AGCCTTCTCCTAAAGTTTCCAC-3’</td>
<td>163</td>
</tr>
<tr>
<td>5-LOX</td>
<td>Forward; 5’-CCCCCGAAGCTCCCAGTGACC-3’  &lt;br&gt;Reverse; 5’-TCCCGGGCCTTAGTGTTGATA-3’</td>
<td>482</td>
</tr>
</tbody>
</table>

GAPDH: glyceraldehyde-3-phosphate dehydrogenase  
CysLT₁R: cysteinyl leukotriene receptor type 1  
5-LOX: 5-lipoxygenase  
bp: base pair (s)
Table 2.

Effects of TMK688 or Pranlukast on Gastric Acid Secretion in Normal Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>No. of Mice</th>
<th>Gastric Acid Output (µEq/h)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>5</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>Pranlukast</td>
<td>3 mg/kg</td>
<td>5</td>
<td>0.88 ± 0.30</td>
</tr>
<tr>
<td>TMK688</td>
<td>30 mg/kg</td>
<td>5</td>
<td>1.01 ± 0.50</td>
</tr>
</tbody>
</table>

Acid secretion was measured in pylorus-ligated mice for 2 h under normal conditions. TMK688 (30 mg/kg) or pranlukast (3 mg/kg) was given i.d. immediately after the pylorus ligation. Data are presented as the mean±SE for five mice.
Figure 1

(A) Gastric Lesions (mm²) for TMK688

- Control
- 3 mg/kg
- 10 mg/kg
- 30 mg/kg

N=6
* P<0.05

(B) Gastric Lesions (mm²) for Pranlukast

- Control
- 0.3 mg/kg
- 1 mg/kg
- 3 mg/kg
- 10 mg/kg

N=5=8
* P<0.05

Ischemia/Reperfusion
Figure 2

A. Sham-Treatment
B. I/R-Treatment
C. I/R plus TMK688
D. I/R plus Pranlukast
Figure 3

Gastric Lesions (mm²)

Control  Vehicle  30 TMK688  3 mg/kg Pranlukast

Indomethacin (5 mg/kg)

Ischemia/Reperfusion

N=5-7
* P<0.05 vs. Control
# P<0.05 vs. Vehicle
Figure 4

- **Sham**
- **Control**
- **30**
- **3 mg/kg**
- **TMK688**
- **Pranlukast**

**Ischemia/Reperfusion**

*M=5-6
* P<0.05 vs. Sham
# P<0.05 vs. Control (I/R)
Figure 5

MPO Activity
(μmol H₂O₂/min/mg protein)

Control

Vehicle

Ischemia/Reperfusion

Indomethacin (5 mg/kg)

TMK688 (30 mg/kg)

Pranlukast (3 mg/kg)

* N=5-6
# P<0.05 vs. Control
# P<0.05 vs. Vehicle
Figure 6

Mucosal CysLT Levels (ng/mg tissue)

N=6-8
$ P<0.05$ vs. Sham
* $ P<0.05$ vs. Control
# $ P<0.05$ vs. Vehicle

Saline
Indomethacin (5 mg/kg)

Ischemia/Reperfusion

Sham
Control
Pranlukast
Vehicle
Pranlukast
30 mg/kg

TMK688
3
30
Figure 7

A

GAPDH

CysLT₁R

Marker  Sham  IM  I/R  IM+I/R

IM: Indomethacin
I/R: Ischemia/Reperfusion

B

Ratio of mRNA Expression (%)
(CysLT₁R/GAPDH)

N=5
*
P<0.05 vs. Sham
#
P<0.05 vs. IM alone

Sham  IM (5 mg/kg)  Control  IM

##
Figure 8

A) GAPDH and 5-LOX mRNA expression levels in different groups:
- Marker
- Sham
- IM
- I/R
- IM+I/R

B) Ratio of mRNA Expression (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>Value</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM (5 mg/kg)</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td>* #</td>
<td></td>
</tr>
</tbody>
</table>

N=5
* P<0.05 vs. Sham
# P<0.05 vs. Control

IM: Indomethacin
I/R: Ischemia/Reperfusion