Proinflammatory role of protease-activated receptor-2 in intestinal ischemia/reperfusion injury in rats

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Abstract. Protease-activated receptors (PARs) are widely recognized for their modulatory properties during inflammation. The aim of the present study was to examine the role of PAR-2 on ischemia/reperfusion-induced small intestinal injury in rats. Intestinal damage was induced in male Wistar rats by clamping both the superior mesenteric artery and the celiac trunk for 30 min, followed by reperfusion for 60 min. Expression of PAR-2 in the intestinal mucosa was estimated by Western blot analysis and real-time PCR. Anti-rat PAR-2 cleavage site (PCS) antibody was intraperitoneally administered to the rats 1 h before the vascular clamping. The intestinal mucosal injury and inflammation were evaluated by biochemical markers and histological findings. Thiobarbituric acid (TBA) reactive substances and tissue-associated myeloperoxidase (MPO) activity were measured in the intestinal mucosa as indices of lipid peroxidation and neutrophil infiltration, respectively. Expression of cytokine-induced neutrophil chemoattractant-1 (CINC-1) in intestinal mucosa was measured by enzyme-linked immunosorbent assay. Expression of PAR-2 mRNA and protein in the intestinal mucosa was increased after reperfusion following ischemia. Reperfusion after ischemia resulted in an increase in luminal protein concentrations, hemoglobin concentrations, TBA reactive substances, MPO activity and CINC-1 protein. Pre-treatment with anti-rat PCS antibody significantly inhibited the increases in these parameters. These results suggest that PAR-2 plays an important role in the pathogenesis of ischemia/reperfusion-induced intestinal injury.

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Introduction

Ischemia itself causes tissue damage, but further injury occurs when the blood flow to ischemic tissue is restored in an attempt to maintain cell function and viability (1-3). Such ischemia/reperfusion (I/R) injury is a serious condition resulting from acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, severe burns and hemorrhagic, traumatic or septic shock. According to previous studies (1,4-6), neutrophils that accumulate in the intestinal mucosa following neutrophil-endothelial cell adhesive interactions have been attributed to intestinal I/R injury via the production of reactive oxygen species and proteases (7-9). Leukocyte accumulation is a complex phenomenon that also involves endothelium-based adhesion molecules, as well as leukocyte-chemotaxis factors. Intercellular adhesion molecules, the most important endothelial adhesion molecules, are normally expressed at a low basal level, but their expression is enhanced by various inflammatory cytokines released from post-ischemic tissues, such as interleukin-1 (IL-1), IL-8 and tumor necrosis factor-α (TNF-α).

Protease-activated receptors (PARs) are a family of seven transmembrane trimer G-protein-coupled receptors activated by specific proteases, consisting of four family members (PAR-1, PAR-2, PAR-3 and PAR-4). While PAR-1, -3 and -4 are mainly activated by thrombin, PAR-2 is activated by trypsin, mast cell tryptase and coagulation factors (VIIa and Xa) (10,11). In particular, PAR-2 is expressed in various organs, including the gastrointestinal tract, and its activation results in proinflammatory effects, including cytokine production (12-15), vasodilatation (16), edema (17) and leukocyte-endothelial interactions (17,18). Maeda *et al* reported that the administration of anti-rat PAR-2 cleavage site (PCS) antibody inhibits cytokine production and the resultant development of distant organ injury during rat acute pancreatitis (19). The anti-rat PCS antibody has been shown to bind to antigenic determinants around the trypsin cleavage site and to competitively neutralize the effects of trypsin on PAR-2.

On the other hand, a recent study demonstrated that intraduodenal injection of the PAR-2-activating peptide SLIGRL-NH(2) may inhibit the intestinal damage and improve the delayed gastrointestinal transit induced by intestinal I/R (20). However, it remains unclear whether the

functional blocking of PAR-2 exacerbates intestinal I/R injury. In addition, PAR-2 expression in intestinal I/R injury has not been well investigated. In the present study, we estimated the PAR-2 expression in intestinal mucosa exposed to I/R and the effect of the neutralizing antibody to rat-PCS to determine the role of PAR-2 in I/R-induced intestinal injury in rats.

Materials and methods

Chemicals. All chemicals were prepared immediately before use. Thiobarbituric acid (TBA) and 3,3',5,5'-tetramethylbenzidine were obtained from Wako Pure Chemicals (Osaka, Japan). 1,1,3,3-Tetramethoxypropane was obtained from Tokyo Kasei (Tokyo, Japan). The cytokine-induced neutrophil chemoattractant-1 (CINC-1) enzyme-linked immunosorbent assay (ELISA) kit was obtained from Immuno-Biological Laboratories Co. (Gunma, Japan). All other chemicals used were of reagent grade.

Surgical procedure and evaluation of intestinal injury. Male Wistar rats weighing 180-200 g were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The animals were housed at 22˚C in a controlled environment with 12 h of artificial light per day, and were allowed access to rat chow and water *ad libitum*. Intestinal I/R injury was induced according to the method described in previous studies (21,22). Briefly, the rats were anesthetized with urethane (1 mg/kg, intraperitoneally). After midline laparotomy, the celiac and superior mesenteric arteries were isolated near their aortic origins. Intestinal ischemia was induced by clamping both the superior mesenteric artery and the celiac trunk, resulting in a total occlusion of these arteries. After 30 min of occlusion, the clamps were removed. Anti-rat PCS antibody (19) was intraperitoneally administrated 1 h before ischemia. The animals were randomized into groups receiving anti-rat PCS or rabbit non-specific IgG (1 mg/kg). Animals were maintained and experimental procedures were carried out in accordance with the NIH guidelines for the use of experimental animals. All experimental protocols were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

The animals were sacrificed after reperfusion, and the intestines were removed and examined. First, a 30-cm proximal intestinal loop was formed, and then saline (10 ml) was injected into the loop. The contents were centrifuged and divided into aliquots. Intestinal injury after reperfusion was evaluated by measuring the luminal levels of hemoglobin and protein and by histological examination. Intestinal bleeding was quantified indirectly as the hemoglobin concentration in luminal lavage fluid using a kit according to the manufacturer's protocol (Wako Pure Chemicals). Luminal protein levels were also determined using a Bio-Rad Protein Assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, K.K., Tokyo, Japan). For histological evaluation, formalin-fixed tissues were stained with H&E and evaluated by light microscopy.

Expression of PAR-2 mRNA and protein in intestinal I/R injury. To examine the time course of PAR-2 mRNA expression in the intestine during I/R, the intestinal mucosa was scraped off using two glass slides before ischemia (N), at 30 min of ischemia (I30/R0) and at 30 min (I30/R30) and 60 min (I30/R60) of reperfusion. Total RNA was isolated by the acid guanidinium phenol chloroform method with an Isogen kit (Nippon Gene, Tokyo, Japan). Extracted RNA (1 μg) was reverse transcribed into first-strand cDNA at 42˚C for 40 min using 100 U/ml of reverse transcriptase (Takara Biomedicals, Shiga, Japan) and 0.1 mM of oligo(dT)-adapter primer (Takara Biomedicals) in a 50-μl reaction mixture. Real-time PCR for PAR-2 was carried out with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the DNA-binding dye SYBR Green I for the detection of PCR products. The reaction mixture (RT-PCR kit, code RRO43A; Takara Biochemicals) contained 12.5 ml Premix Ex *Taq*, 2.5 ml SYBR Green I, custom-synthesized primers, ROX reference dye and cDNA (equivalent to 20 ng total RNA) for a final reaction volume of 25 ml. The PCR settings were as follows: an initial denaturation for 15 sec at 95˚C was followed by 40 cycles of amplification for 3 sec at 95˚C and 31 sec at 60˚C, with subsequent melting curve analysis at increasing temperatures from 60 to 95˚C. The primers had the following sequences: for PAR-2, sense 5'-AAAATCGAA-3'; antisense 5'-CAGCCCGTGCTG-3', and for β-actin, sense 5'-TATCCACC-3'; antisense 5'-TATCCA CCTTCCAG-3'. Relative quantification of gene expression with real-time PCR data was calculated relative to β-actin.

Furthermore, to determine the expression of PAR-2 protein, proteins were also obtained from the intestine. The total proteins were mixed with sodium dodecyl sulfate (SDS) sample buffer. The samples were then subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and were blotted onto a PVDF membrane (ATTO Corporation, Tokyo, Japan). The membrane was blocked with 2% BSA in TBS-T (TBS and 0.1% Tween-20) at room temperature for 30 min. Western blot analysis was carried out using rabbit polyclonal anti-PAR-2 (1:1,000; Santa-Cruz) at room temperature for 1 h. After three washes with TBS-T, the membrane was incubated with anti-rabbit IgG-HRP (1:3,000; GE Healthcare Ltd., Amersham Place, UK) at room temperature for 45 min. The signals were visualized using an ECL kit (GE Healthcare Ltd.) according to the manufacturer's instructions. The band intensities were determined using CS Analyzer ver. 2.0 (ATTO Corporation).

Measurements of TBA reactive substances and myeloperoxidase (MPO) activity. As an index of lipid peroxidation, the total concentration of TBA reactive substances was measured in the intestinal mucosa (23). The intestinal mucosa was scraped off using two glass slides and homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl in a Teflon Potter-Elvehjem homogenizer. The level of TBA reactive substances in the mucosal homogenates was expressed as nanomoles of malondialdehyde per milligram of protein, using 1,1,3,3-tetramethoxypropane as the standard. The total protein in the tissue homogenates was measured with a Bio-Rad Protein Assay kit according to the manufacturer's protocol.

Tissue-associated MPO activity in the intestinal mucosa was determined by a modification of the method of Grisham *et al* (7) as an index of neutrophil accumulation. The mucosal homogenates were centrifuged at 20,000 x g for

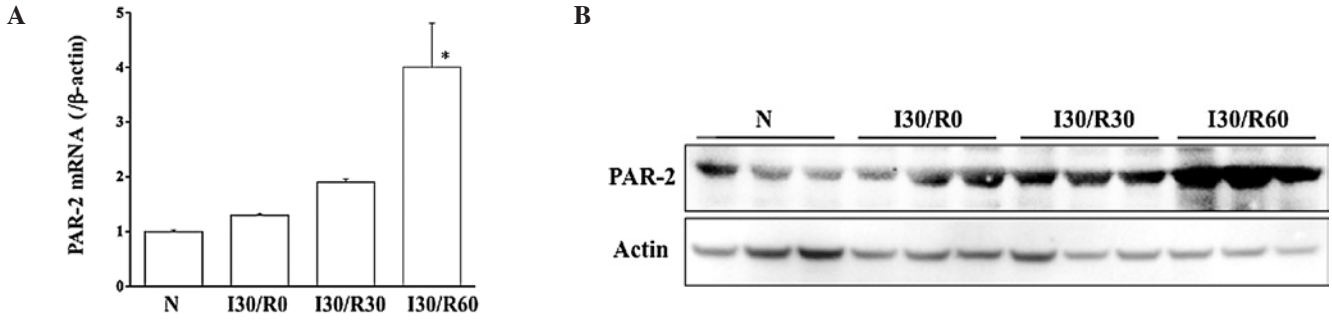

Figure 1. Expression of PAR-2 in intestinal mucosa exposed to ischemia followed by reperfusion. (A) PAR-2 mRNA expression was assessed using real-time PCR. Intestinal damage was induced in male Wistar rats by clamping both the superior mesenteric artery and the celiac trunk for 30 min followed by reperfusion for 60 min. PAR-2 mRNA was evaluated before ischemia (N), at 30 min of ischemia (I30/R0) and at 30 min (I30/R30) and 60 min (I30/R60) of reperfusion. Data represent the mean ± SE of 6 rats. *p<0.01 compared to N. (B) PAR-2 protein expression was assessed by immunoblotting with a polyclonal antibody against PAR-2. Actin antibody was used as an internal control. Representative data from three observations are shown.

15 min at 4˚C to pelletize the insoluble cellular debris. The pellet was then re-homogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20,000 x g for 15 min at 4˚C, and the supernatants were reserved. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO required to cause a change in absorbance of 1.0/min at 645 nm and 25˚C.

Determination of the mucosal content of CINC-1. The concentration of rat CINC-1, which is a potent member of the IL-8 family (24), in the mucosal homogenates was determined with ELISA kits according to the manufacturer's instructions.

Statistical analysis. The results are presented as the mean ± SEM. Overall differences between groups were determined by one-way analysis of variance (ANOVA). In cases where the one-way ANOVA was significant, differences between individual groups were analyzed using Bonferroni's multiple comparisons test. A level of $P<0.05$ was considered significant. All analyses were performed using the GraphPad Prism 4 program (GraphPad Software Inc., San Diego, CA, USA) for the Macintosh computer.

Results

Expression of PAR-2 mRNA and protein in the intestinal mucosa exposed to I/R. Expression of PAR-2 mRNA was evaluated using real time-PCR. PAR-2 mRNA expression was increased in a time-dependent manner after I/R treatment and significantly increased at I30/R60 (Fig. 1A). According to the increases in PAR-2 mRNA expression after I/R, the expression of PAR-2 protein also increased in a time-dependent manner after I/R treatment (Fig. 1B).

Effect of anti-rat PCS antibody on I/R-induced intestinal injury. Fig. 2A shows typical histological features in the anti-rat PCS antibody-treated group and the control group. The ischemia-reperfusion resulted in large areas of epithelial crypt loss, a predominant neutrophil infiltration throughout the mucosa, ulceration and mucosal bleeding (Fig. 2A-a). By contrast, the treatment with anti-rat PCS antibody showed smaller erosions with few neutrophils (Fig. 2A-b). Lavage protein concentrations were significantly elevated by approximately 5-fold after ischemia-reperfusion. Intraperitoneal pre-treatment with anti-rat PCS antibody performed 1 h before the ischemia inhibited the increases in luminal protein concentrations 60 min after reperfusion (Fig. 2B). Non-specific IgG or anti-rat PCS antibody alone did not enhance the luminal protein concentrations. Furthermore, anti-rat PCS antibody dramatically reduced the increase in luminal hemoglobin concentration, as well as that in luminal protein (Fig. 2C).

Effect of anti-rat PCS antibody on TBA reactive substances and MPO activity. The extent of lipid peroxidation was determined by measuring the TBA reactive substances in the small intestine. In the sham-operated group, there were no differences in the levels of intestinal TBA reactive substances between the non-specific IgG- and anti-rat PCS antibody-treated rats. Reperfusion after ischemia caused a significant increase in TBA reactive substances compared to levels in the sham-operated animals. The increase in TBA reactive substances in the intestinal mucosa was significantly inhibited by anti-rat PCS antibody (Fig. 3A).

Neutrophil accumulation was also evaluated by the measurement of tissue-associated MPO activity in the intestinal mucosal homogenates. In the sham-operated groups, there were no differences in the MPO activities between the non-specific IgG- and anti-rat PCS antibody-treated rats. MPO activity in the intestinal mucosa markedly increased after I/R, compared to that in the sham-operated group. The increase in MPO activity in the intestinal mucosa after reperfusion was significantly inhibited by treatment with the anti-rat PCS antibody (Fig. 3B).

Effect of anti-rat PCS antibody on CINC-1 protein contents in the intestinal mucosa. To further analyze the effects of the anti-rat PCS antibody on reperfusion-induced intestinal neutrophil accumulation, the intestinal mucosal protein contents of CINC-1 was assessed using ELISA. The content of CINC-1 protein significantly increased after I/R. The increase in CINC-1 in the intestinal mucosa was significantly inhibited by the anti-rat PCS antibody (Fig. 3C).

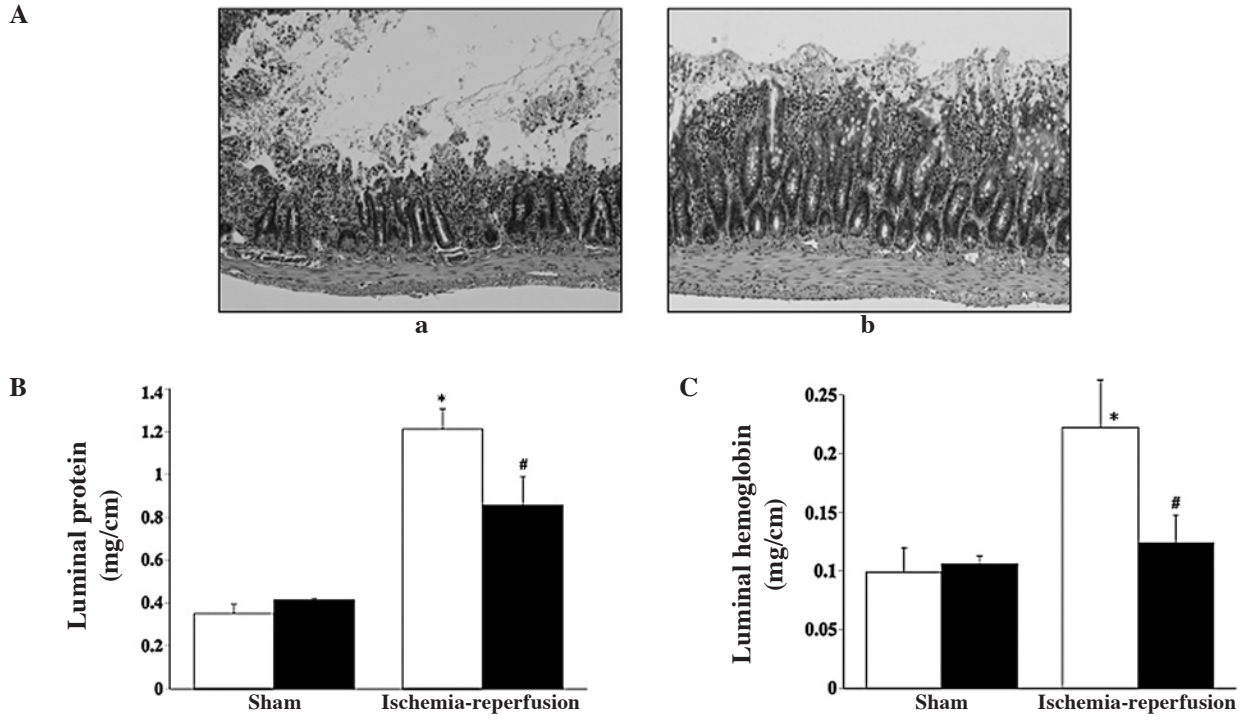

Figure 2. Effect of anti-rat PAR-2 cleavage site (PCS) antibody on histology (A), protein levels (B) and intraluminal hemoglobin (C) in rats subjected to I/R. (A) Histological appearance of the small intestine in rats subjected to I/R (a) and I/R with pre-treatment of the anti-rat PCS antibody (b). Histological examination revealed a loss and shortening of the crypt, mucosal erosions and infiltration of inflammatory cells into the ileum of the rats subjected to I/R. By contrast, treatment with anti-rat PCS antibody produced smaller erosions and a much lower level of inflammatory cell infiltration. H&E staining (x40). (B and C) Intestinal damage was evaluated by intraluminal protein and hemoglobin. After making a 30-cm proximal intestinal loop, saline (10 ml) was injected into the loop. The contents were centrifuged, and subsequently the protein and the hemoglobin concentrations in the supernatants were measured. Data represent the mean ± SEM of 7 rats. $^{*}p<0.01$ compared to sham-operated rats. $^{\#}p<0.05$ compared to the rats subjected to I/R receiving nonspecific IgG. Open bars, non-specific IgG; closed bars, anti-rat PCS antibody.

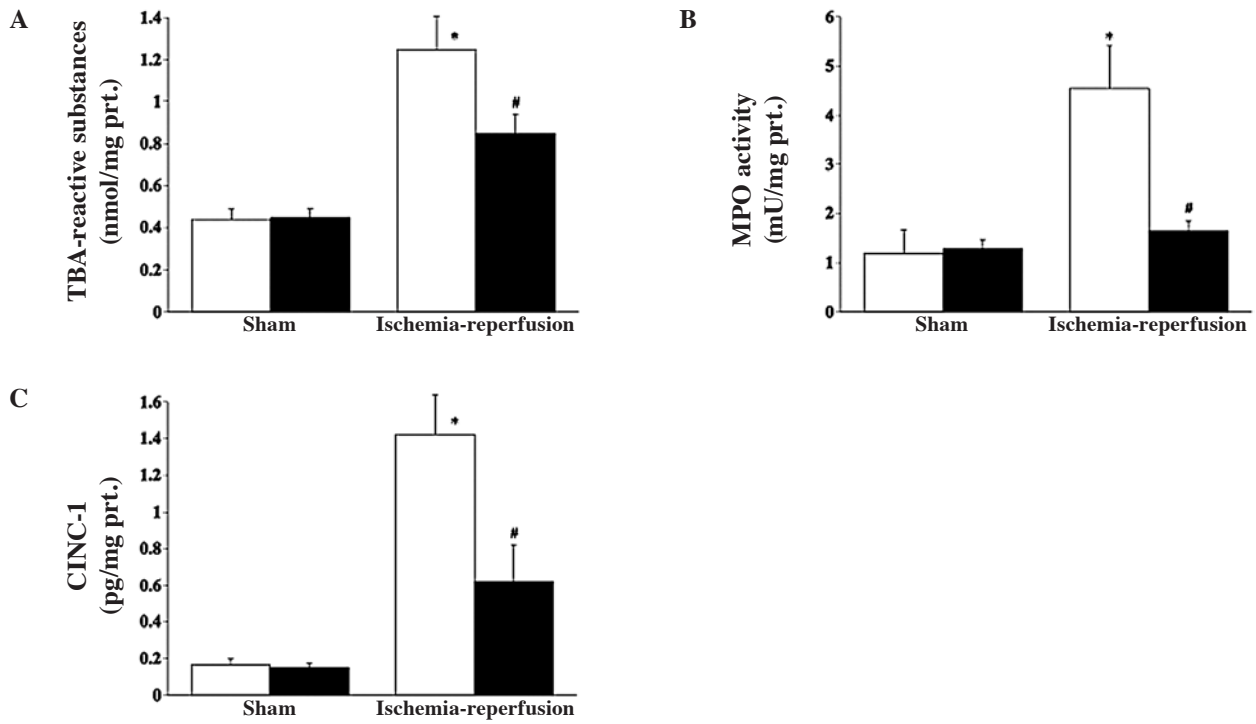

Figure 3. Effect of anti-rat PCS antibody on the increase in lipid peroxide concentration (A), neutrophil accumulation (B) and cytokine-induced neutrophil chemoattractant-1 (CINC-1) contents (C) in the intestinal mucosa of rats subjected to I/R. The intestinal mucosa was scraped off and then homogenized. Lipid peroxide levels in the intestinal homogenates were determined by measuring the levels of thiobarbituric acid (TBA) reactive substances and expressed as nanomoles of malondialdehyde per milligram of protein (A). Tissue-associated myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation in the intestinal mucosa (B). The concentration of CINC-1 in the supernatant of mucosal homogenates was determined using the ELISA kit, specifically for rat CINC-1 (C). Data represent the mean ± SEM of 7 rats. $^{\#}p<0.01$ compared to sham-operated rats. $^{*}p<0.05$ compared to rats subjected to I/R receiving nonspecific IgG. Open bars, non-specific IgG; closed bars, anti-rat PCS antibody.

Discussion

In the present study, we demonstrated that the expression level of PAR-2 mRNA and protein in the intestinal mucosa was increased after intestinal I/R. It has been reported that PAR-2 is expressed in a number of cell types, including platelets, leukocytes, endothelial and vascular smooth muscle cells, and epithelial cells such as those in the respiratory tract, uterus, ovary and the gastrointestinal tract (12-15). In particular, PAR-2 in the intestine is localized in endothelial cells, myocytes, enterocytes, enteric neurons, terminals of mesenteric afferent nerves and immune cells (26-28). It was reported that PAR is increased by inflammatory stimulants, such as TNF-α, IL-1α or LPS (29-31). In the present study, although PAR-2 localization after I/R was not determined, it is possible that some types of inflammatory mediators promoted PAR-2 expression in the small intestine. Further examination of the mechanisms involved in the increased expression of PAR-2 induced by I/R is required.

PAR, particularly PAR-2, has been reported to play important roles in the pathogenesis of inflammatory and immunological disorders in various organs (12-15,19,25,32). It was also found that colonic administration of PAR-2 agonists up-regulates PAR-2 expression and induces an inflammatory reaction characterized by granulocyte infiltration, increased wall thickness, tissue damage and elevated T-helper cell type 1 cytokines (33,34). Thus, the inhibition of PAR-2 may yield an anti-inflammatory effect in intestinal inflammation. Indeed, Maeda *et al* (19) reported an inhibitory effect of anti-rat PCS antibody, a neutralizing antibody to PAR-2, on cytokine production and tissue damage during acute pancreatitis. Herein, we investigated the effect of anti-rat PCS antibody in an intestinal I/R rat model in order to determine the relationship between PAR-2 and I/R-induced intestinal injury.

In the present study, we showed that anti-rat PCS antibody significantly inhibited the expression of proinflammatory cytokines (CINC-1) and neutrophil accumulation determined by MPO activity in the intestinal mucosa after I/R. It was previously reported that CINC-1 levels increased during small intestinal I/R injury and that CINC-1 was related to the extent of mucosal damage with neutrophil accumulation (21,22,35). In addition, a previous study demonstrated that pre-treatment with an anti-CINC-1 monoclonal antibody attenuated reperfusion injury in the small intestine, in association with a reduction in TNF-α and MPO levels, thereby prolonging survival (36). Together with our present results, these data indicate that CINC-1 plays an important role in the onset of I/R injury in the small intestines of rats. Since CINC-1 is principally involved in neutrophil-specific chemotactic activity, the inhibition of neutrophil accumulation by anti-rat PCS antibody may be partially dependent on a reduction in CINC-1 production. Our results are consistent with reports that the activation of PAR-2 induces proinflammatory cytokines and chemokines from vascular endothelial cells, monocytes, the respiratory tract, the gastrointestinal tract, pancreas, neurons and skin (12-15,19,25,32).

In the present study, intestinal injury was assessed using a variety of methods, including the measurement of luminal protein and luminal hemoglobin, and histology. The result of each assessment showed that anti-rat PCS antibody administration significantly inhibited these parameters. In addition, we demonstrated that the administration of anti-rat PCS antibody significantly inhibited lipid peroxidation of the intestinal mucosa after I/R. Lipid peroxidation induced by oxygen radicals is believed to be an important cause of cell membrane damage. It has been shown that intestinal I/R injury is caused by oxygen-derived free radicals that are produced by xanthine oxidase in the small intestinal tissues and by NADPH oxidase in activated neutrophils (3,37,38). In this study, the increase in TBA reactive substances, an index of lipid peroxidation, in the intestinal mucosa after I/R was significantly inhibited by treatment with anti-rat PCS antibody. However, it has not yet been reported that PAR-2 directly regulates the production of free radicals. Therefore, the inhibition of lipid peroxidation by the anti-rat PCS antibody may be based, at least in part, on the inhibition of infiltrative neutrophils, which is an important source of free radical production.

On the other hand, it has also been reported that PAR-2 plays an important role in the anti-inflammatory reaction and cytoprotective effects, including the inhibition of experimental colitis (39) by administration of a PAR-2 agonist. Several studies have reported that PAR-2 mediates the protective effect against myocardiac or intestinal I/R injury (20,40-42). These reports indicate that PAR-2 activation with a PAR-2-activating peptide administered to animal models reduces I/R-induced tissue damage. Our present study is the first to demonstrate that PAR-2 blockage with a neutralizing antibody to PAR-2 has an anti-inflammatory effect in intestinal I/R injury. Further investigation is required to clarify the function of PAR-2 in the development of intestinal I/R injury.

In conclusion, the present study indicates that treatment with an anti-rat PCS antibody protects against the intestinal injury induced by I/R, which is associated with a marked decrease in CINC-1 overexpression and neutrophil infiltration into the intestinal mucosa. These results suggest that PAR-2 may play a crucial role in the pathogenesis of intestinal inflammation elicited by I/R. In the future, regulation of PAR-2 activity may have great potential as a new therapeutic target for ischemia-reperfusion injury.

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