Role of the thrombin/protease-activated receptor 1 pathway in intestinal ischemia-reperfusion injury in rats

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Thrombin, a multifunctional serine protease, plays a critical role in hemostasis, coagulation, and thrombosis. In addition, thrombin can function as an agonist for cellular responses in a variety of cell types. Thrombin is known to have inflammatory functions via G protein-coupled receptors, including protease-activated receptor-1 (PAR1). It has been reported that PAR1 induces rat cytokine-induced neutrophil chemoattractant-1 (CINC-1), a CXC chemokine of the interleukin-8 family (7, 8), release (30) and that thrombin stimulates proinflammatory production in vivo (31) and in vitro (4, 20, 28). However, the role of the thrombin/PAR1 pathway in intestinal I-R has not yet been investigated. In this study we examined whether thrombin and PAR1 might be involved in the pathophysiology of intestinal I-R by administering antithrombin (AT), a specific inhibitor for thrombin, to rats treated with I-R. AT is a single-chain glycoprotein that binds to thrombin and a progressive inhibitor of serine proteases. It is the physiological inhibitor of the central enzyme of blood coagulation (26). It is well known that in the presence of heparin, which binds to AT, the rates of inactivation of thrombin and the other coagulation enzymes are accelerated (25). Besides the anticoagulation effect, recent experimental studies have revealed that AT had anti-inflammatory effects in models of Escherichia coli colitis (29), endotoxin-induced hypotension (13), and pulmonary vascular injury (22). However, the effects of AT on intestinal I-R injury have not yet been studied. In this study, we showed that I-R increased the thrombin activation and the PAR1 expression in the intestine was evaluated by real-time PCR. The severity of the intestinal mucosal injury was evaluated on the distal segment of the ileum by several biochemical markers and histological findings. Reperfusion significantly increased the serum levels of thrombin-antithrombin complex and enhanced rates of inactivation of thrombin and the other coagulation factors in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS

Reagents. AT was kindly provided from Benesis (Osaka, Japan). ELISA kits for rat TNF-α and CINC-1 were obtained from BioSource (Camarillo, CA) and Immuno-Biological Laboratories (Gunma, Japan), respectively. All other chemicals used were of reagent grade.

Preparation of rats for intestinal I-R. Male Wistar rats weighing 180–200 g were obtained from Keari (Osaka, Japan). The animals were housed at 22°C in a controlled environment with 12 h of artificial light per day. The animals were not fed for 24 h before the experiments but were allowed free access to water. Through a midline abdominal incision, intestinal I-R injury was induced by clamping the superior mesenteric artery for 30 min under intraperitoneal urethane anesthesia (1,000 mg/kg) in rats with and without AT treatment. After

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30 min of occlusion, reperfusion was produced by removing the clamp. At 60 min after reperfusion, all of the rats were killed by exsanguination via the abdominal aorta under intraperitoneal urethane anesthesia (1,000 mg/kg) for histological examination, biochemical studies, and RNA analysis. AT was administered to rats by intravenous physiological saline. The sham group received a sham operation. The handling of animals and all experimental procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

Measurement of thrombin-AT complex. Blood samples were collected via the inferior vena cava under intraperitoneal urethane anesthesia (10 ml) and microfuged at 1,500 rpm for 15 min at 37°C to obtain plasma samples. Briefly, 50 µl of plasma were placed into 96-well plates coated with rabbit anti-thrombin antibody and incubated for 15 min at 37°C. Then the supernatants were removed, and the wells were washed with 1% bovine serum albumin. The supernatant of mucosal homogenates were determined colorimetrically 30 min later (490-nm wavelength; MPR-A4i microplate reader; Tosoh, Tokyo, Japan) with tetramethylbenzidine as a substrate.

Determination of the mucosal content of TNF-α and CINC-1. The concentrations of the inflammatory cytokines TNF-α and CINC-1 in the supernatant of mucosal homogenates were determined by rat TNF-α- and CINC-1-specific ELISA kits according to the manufacturer’s instructions. Briefly, 100 µl of cell supernatants were placed into 96-well plates coated with rabbit anti-rat GRO/CINC-1 IgG or TNF-α IgG and incubated for 1 h at room temperature. Following this, the supernatants were removed and the wells were washed with 1% bovine serum albumin and 0.05% Tween 20 in PBS. Subsequently, horseradish peroxidase-conjugated rabbit anti-rat GRO/CINC-1 or TNF-α antibody (Fab’ fragments) was added, and the amount of CINC-1 or TNF-α was determined colorimetrically 30 min later (490-nm wavelength; MPR-A4i microplate reader; Tosoh, Tokyo, Japan) by using tetramethylbenzidine as a substrate.

RNA analysis. The mRNA expression of intestinal PAR1 was determined by real-time PCR, and those of intestinal TNF-α and CINC-1 were determined by RT-PCR at 60 min after reperfusion. For both methods, total RNA was isolated from intestinal mucosal tissue by the acid guanidinium phenol chloroform method with an ISOGEN kit (Nippon Gene, Tokyo, Japan) and the concentration of RNA was determined by the absorbance at 260 nm in relation to that at 280 nm. The isolated RNA was stored at −70°C until it was used for the real-time PCR and RT-PCR. One microgram of extracted RNA 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 µM of oligo(dT)-adapter primer (Takara Biomedicals) in a 50-µl reaction mixture. Real-time PCR for PAR1 was carried out with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using the DNA-binding dye SYBR green I for the detection of PCR products. The concentration of the inflammatory cytokines. Tissue-associated MPO activity, an index of neutrophil infiltration, was determined by a modification of the method described by Grisham et al. (6). The MPO activity was assessed by measuring the H2O2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at 655 nm and 25°C.

Assessment of intestinal mucosal injury induced by I-R. The animals were killed after reperfusion, and the intestine was removed and submitted for examination. A distal segment of the ileum (30 cm) was excised from a region 5 cm proximal to the terminal ileum and submitted for examination. A distal segment of the ileum (30 cm) was excised from a region 5 cm proximal to the terminal ileum and submitted for histological examination and measurement of biochemical parameters. For histological evaluation, formalin-fixed tissues were stained with hematoxylin and eosin and evaluated by light microscopy by a pathologist who was not informed of the experimental conditions for any given specimen. Subsequently, the intestinal mucosa was scraped off by using two glass slides and homogenized in a homogenizer with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl to measure tissue-associated myeloperoxidase (MPO) activity and the levels of the inflammatory cytokines. Tissue-associated MPO activity, an index of neutrophil infiltration, was determined by a modification of the method described by Grisham et al. (6). The MPO activity was assessed by measuring the H2O2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance of 1.0/min at 655 nm and 25°C.

Fig. 1. Changes in serum levels of thrombin-antithrombin (AT) complex (TAT) in rats subjected to ischemia-reperfusion (I-R). ◆ Sham group; ● group receiving I-R + vehicle. Data represent means ± SE of 5–6 rats. *P < 0.01 vs. sham.

Fig. 2. Protease-activated receptor 1 (PAR1) expression in the small intestine mucosal injury induced by I-R. Data represent means ± SE of 5–6 rats. #P < 0.01 vs. sham.
followed by 40 cycles of amplification for 3 s at 95°C and 31 s at 60°C, with subsequent melting curve analysis increasing the temperature from 60 to 95°C. The primers had the following sequences: for PAR-1, sense 5'-AGGCCCTCCCTGAACATCCT-3' and antisense 5'-ACGGCCGGCTTCTTGAC-3'; and for β-actin, sense 5'-GCCGTTGAGTACTTGAGTGACT-3'. Relative quantification of gene expression with real-time PCR data was calculated relative to β-actin.

In RT-PCR for TNF-α, CINC-1, and β-actin, an aliquot (1 μl) of the reverse transcriptase product was added to 3 mM primers for TNF-α, CINC-1, and β-actin (the internal standard). The reaction was performed as follows: 25 cycles of amplification (denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s), followed by a final extension step of 7 min at 72°C. The primers had the following sequences: for TNF-α, sense 5'-ATGAGCACAGAAGCATGATC-3' and antisense 5'-TACAGGCTTGTCACTCGAATT-3'; for CINC-1, sense 5'-CTGTGCTGGCCACCAGCCGC-3' and antisense 5'-ACAGTCCTTGGAACTTCTCTG-3'; and for β-actin, sense 5'-ATCGTGGGCCGCCCTAGGCA-3' and antisense 5'-TGGCCTTAGGGTTCAGAGGGG-3'. The PCR products were separated electrophoretically in a 25 g/l agarose gel and stained by ethidium bromide.

Statistical analysis. All results were expressed as means ± SE. The data were compared by two-way ANOVA, and differences were considered to be significant if the P value was <0.05 based on Scheffe’s multiple-comparison test. All analyses were performed using the StatView 5.0-J program (Abacus Concepts, Berkeley, CA).

RESULTS

Reperfusion increased thrombin activity and PAR1 expression. Thrombin activation was assessed by measuring the TAT complexes. The serum level of TAT complexes was significantly increased after 30 min of ischemia, compared with that in the sham group, and this increase in the level of TAT complexes was further enhanced at 60 min after reperfusion (Fig. 1). Reperfusion also significantly enhanced the expression of PAR1 in the intestinal mucosa (Fig. 2).

AT inhibited intestinal I-R injury. Reperfusion after 30 min of ischemia resulted in an increase of the luminal protein and hemoglobin concentrations at 60 min after reperfusion (Fig. 3). In contrast, treatment with AT at 5 min before ischemia inhibited the increase of luminal protein and hemoglobin at a dose of 30 U/kg, and there was no significant difference between sham and AT group (Fig. 3). Neither vehicle alone nor AT alone affected the luminal protein and hemoglobin concentrations (data not shown). Therefore, in the following experiments, we used AT at a dose of 30 U/kg and examined its pharmacological action. In the control group (I-R group), multiple erosions and bleeding were observed macroscopically after I-R injury, whereas these changes were inhibited by treatment with AT. The protective effect of AT was further
confirmed microscopically. Representative hematoxylin and eosin-stained sections of the ileum from sham, I-R, and I-R plus AT-treated animals are depicted in Fig. 4. We found the characteristic histological findings after I-R injury, shortening of the villi, loss of the villous epithelium (erosion), and prominent mucosal neutrophil infiltration, whereas the ileum of sham animals exhibited normal mucosal architecture with intact villi. In contrast, administration of AT markedly inhibited these changes caused by I-R. AT alone did not produce any macroscopic or microscopic lesions in the rat intestine (data not shown).

Effect of AT on mucosal MPO activity. In the intestinal mucosa exposed to I-R injury, MPO activity was significantly increased compared with that in the sham group. This increase of MPO activity after reperfusion was significantly inhibited by treatment with AT (Fig. 5).

Effect of AT on mucosal cytokine levels. The CINC-1 and TNF-α levels in mucosal homogenate were increased significantly in the I-R group compared with those in the sham group. AT markedly reduced both the CINC-1 and TNF-α concentrations compared with those in the I-R group (Figs. 6 and 7).

Effect of AT on the expression of CINC-1 and TNF-α mRNA. The expressions of CINC-1 and TNF-α mRNA in the intestine were upregulated by I-R injury. These increases in the expressions of CINC-1 and TNF-α mRNA were also inhibited by AT (Fig. 8).

DISCUSSION

In the present study, we demonstrated that the serum level of TAT complexes, an index of thrombin activation, and the intestinal mRNA level of PAR1, a specific receptor of thrombin, were both increased after intestinal I-R and that the treatment with AT, a synthetic thrombin inhibitor, markedly ameliorated the intestinal injury and inflammation induced by I-R in rats. These data suggest that enhanced activation of the thrombin/PAR1 pathway plays a key role in the pathogenesis of I-R-induced intestinal injury.

To examine the role of the thrombin/PAR1 pathway in I-R-induced intestinal injury, we first measured the serum level of TAT complexes, an index of thrombin activation, and the intestinal mRNA level of PAR1, a specific receptor of thrombin, which were both increased after intestinal I-R and that the treatment with AT, a synthetic thrombin inhibitor, markedly ameliorated the intestinal injury and inflammation induced by I-R in rats. These data suggest that the signaling pathway mediated by the interaction between thrombin and PAR1 plays a crucial role in the pathogenesis of I-R-induced intestinal injury.

Fig. 5. Effect of AT on MPO activity in the intestinal mucosa injury induced by I-R. Data represent means ± SE of 5–6 rats. #P < 0.01 vs. sham; *P < 0.01 vs. I-R + vehicle.

Fig. 6. Effect of AT on cytokine-induced neutrophil chemoattractant-1 (CINC-1) levels in the intestinal mucosa injury induced by I-R. Data represent means ± SE of 4 to 6 rats. #P < 0.01 vs. sham; *P < 0.05 vs. I-R + vehicle.

Fig. 7. Effect of AT on TNF-α levels in the intestinal mucosa injury induced by I-R. Data represent means ± SE of 4 to 6 rats. #P < 0.01 vs. sham; *P < 0.05 vs. I-R + vehicle.
induced by I-R (9, 18). Whereas previous reports using in vivo models have shown that AT exerts an anti-inflammatory effect at high dose such as 250 U/kg, in the present study we found that AT even at a low dose (30 U/kg) was effective to protect intestinal mucosa from I-R injury. Although the reason for discrepancy between their and our results is not clear and should be further investigated, the amount of AT used in this study is similar to that used in the clinical field (1). Taken together, these results suggest AT may exert a therapeutic effect on tissue ischemia and diseases related to I-R.

To examine the mechanism of cytoprotection by AT, we measured several biochemical parameters in the present study. The intestinal tissue levels of CINC-1, TNF-α, and MPO were significantly increased after I-R in rats, and these variables reflecting leukocyte activation were significantly reduced by administering AT, suggesting that AT might reduce the intestinal injury by inhibiting leukocyte activation. In particular, the inhibition of proinflammatory cytokines demonstrated in the present study might be considered the crucial mechanism of the AT-induced anti-inflammatory effect. Recent reports have demonstrated that thrombin activates PAR1 by proteolytic cleavage at a unique site within the amino terminus of the receptor (20) and that the intracellular signaling via PAR activation increases the mRNA expression of proinflammatory cytokines (4, 20, 28). Therefore, the inhibition of CINC-1 and TNF-α by AT observed in the present study may have been due to the inhibition of thrombin. The responsible cells that over-express CINC-1 and TNF-α under the condition of I-R are considered a various type of cells such as neutrophils, monocytes, epithelial cells, and endothelial cells. In addition to the inhibition of thrombin by AT, we should consider the direct effects of AT on each cells (endothelial cells and neutrophils).

Harada et al. (10) reported that AT exerted an anti-inflammatory effect by increasing the production of prostaglandin I₂ and E₂ through activation of cyclooxygenase in rats. However, in a study using the same rat model, we previously reported that inhibitors of cyclooxygenase 1 or 2 did not affect the severity of intestinal injury induced by I-R (19). Our preliminary experiment supports a direct action of AT in a “thrombin-independent” manner on activated endothelial cells, in which proinflammatory cytokine IL-8 production by activated human umbilical venous endothelial cells is inhibited by pretreatment with AT (unpublished data). Therefore we speculated that the anti-inflammatory effect of AT is dependent not only on its indirect effect by inhibiting thrombin pathway but also on its direct effect by modulating intracellular signaling in intestinal model of I-R. Further studies will be needed to clarify the mechanism of PAR-dependent and -independent anti-inflammatory actions of AT. In addition to the interaction between endothelial cells and AT, Minamiya et al. (17) recently showed that AT binds to glycosaminoglycans (e.g., syndecan-4) on the neutrophils, thereby reducing neutrophil accumulation in the lung and ultimately resulting in a reduction of neutrophil-dependent oxygen radical production. We have made plans to identify the target receptors or intracellular molecules associated with AT-induced cytoprotection in a future study.

In conclusion, we demonstrated the significance of the activated thrombin and PAR1 in intestinal I-R injury in rats and showed that treatment with AT, a synthetic thrombin inhibitor, markedly ameliorated the intestinal injury and inflammation induced by I-R. These results suggest that inhibition of the thrombin/PAR1 pathway may be useful for protection against the intestinal I-R injury associated with shock, surgical treatment, or organ transplantation in humans.

REFERENCES


