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

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Tsuboi H, Naito Y, Katada K, Takagi T, Handa O, Kokura S, Ichikawa H, Yoshida N, Tsukada M, Yoshikawa T. Role of the thrombin/protease-activated receptor 1 pathway in intestinal ischemia-reperfusion injury in rats. Am J Physiol Gastrointest Liver Physiol 292: G678–G683, 2007. First published October 5, 2006; doi:10.1152/ajpgi.00361.2006.—CXC chemokines, including human interleukin-8 and rat cytokine-induced neutrophil chemoattractant-1, play a crucial role in the pathogenesis of intestinal inflammation induced by ischemia-reperfusion (I-R). Thrombin and its specific receptor, protease-activated receptor 1 (PAR1), act as important players in inflammation. However, the association between thrombin activation and chemokine production during I-R has not been well studied. We investigated whether thrombin and PAR1 might be involved in the pathophysiology of intestinal I-R, using an in vivo model. Intestinal damage was induced by clamping the superior mesenteric artery for 30 min followed by reperfusion in male Wistar rats. Thrombin-antithrombin complex was measured as an indicator of thrombin activation, and PAR1 expression in the intestinal mucosa 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 the production of cytokine-induced neutrophil chemoattractant-1 (CINC-1), a CXC chemokine of the interleukin-8 family. These results suggest that the thrombin/PAR1 pathway is involved in the pathophysiology of intestinal I-R injury. It is well known that activated neutrophils can cause tissue damage by releasing cytotoxic free radicals (6, 11, 21).

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) (3, 15). 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 and that AT dramatically attenuated the I-R-induced intestinal injury and inflammation.

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
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.

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 infusion at 5 min before clamping the superior mesenteric artery, whereas control rats (I-R group) received an equivalent volume of 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 at four given time points (before and 30 min after ischemia, and 30 min and 60 min after reperfusion) and centrifuged at 1,500 rpm for 30 min and 60 min after ischemia, and 30 min and 60 min after reperfusion) and centrifuged at 1,500 rpm for 30 min later (490-nm wavelength; MPR-A4i microplate reader; Tosoh, Tokyo, Japan) with tetramethylbenzidine as a substrate.

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 to measurement of luminal protein and hemoglobin. Saline (10 ml) was injected into the intestinal loop from oral side to collect the hemoglobin levels in the intestinal lumen were measured for evaluation of intestinal injury. The hemoglobin level, which reflects intestinal bleeding, was measured with a kit (Wako Pure Chemical Industries, Osaka, Japan), and the protein concentration was also measured by the Lowry method using a commercially available kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. A distal segment of the ileum (10 cm) was excised from a region 5 cm proximal to the terminal ileum and submitted to 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.

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) with 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 reaction mixture (RT-PCR kit, Code RRO43A, Takara Biochemicals) contained 12.5 μl Premix Ex Taq, 2.5 μl SYBR green I, custom-synthesized primers, ROX reference dye, and cDNA (equivalent to 20 ng total RNA) to give a final reaction volume of 25 μl. The PCR settings were as follows: the initial denaturation for 15 s at 95°C was followed by 39 cycles of 5 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The primer sequences are shown in Table 1. The PCR products were analyzed by agarose gel electrophoresis and confirmed to be specific. The mRNA levels of PAR1 were normalized to the endogenous control 18S rRNA using the 2ΔCt method. The mRNA expression of intestinal PAR1 was determined by real-time PCR system (Applied Biosystems, Foster City, CA) 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 μl Premix Ex Taq, 2.5 μl SYBR green I, custom-synthesized primers, ROX reference dye, and cDNA (equivalent to 20 ng total RNA) to give a final reaction volume of 25 μl. The PCR settings were as follows: the initial denaturation for 15 s at 95°C was followed by 39 cycles of 5 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The primer sequences are shown in Table 1. The mRNA levels of PAR1 were normalized to the endogenous control 18S rRNA using the 2ΔCt method.
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'-AGCCITCCCTGCAACTCT-3' and antisense 5'-AGCCGCTTCTTGCAC-3'; and for β-actin, sense 5'-GAG-CAACATCCTCCTAATGCTT-3' and antisense 5'-GCCGTG-GATACCTTGGAGTGACT-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'-ATGAGCACAGA-CATGATC-3' and antisense 5'-TACAGGCTTGTCACTCGATT-3'; for CINC-1, sense 5'-CTGTGCTGGCCACCAGCGC-3' and antisense 5'-ACAGTCTTGGAACTTCTCTG-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, 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.

To clarify the role of thrombin in the present study, we investigated the effect of AT, a synthetic thrombin inhibitor, on I-R-induced intestinal injury and inflammation. In this study, intestinal injury was assessed by a variety of methods, including the measurement of luminal concentrations of protein and hemoglobin, and histological findings. The result of each assessment showed that AT administration significantly inhibited these parameters. In addition, we demonstrated that AT significantly inhibited the expression of proinflammatory cytokines (CINC-1 and TNF-α) and neutrophil accumulation determined by MPO activity in the intestine after I-R. These results indicate that AT has a protective role against I-R-induced intestinal mucosal injury as well as an anti-inflammatory role against I-R-induced inflammation. The protection against I-R injury by AT has been supported by several studies, in which AT treatment ameliorated the renal and hepatic injury during I-R, indicating the generation and activation of thrombin was induced by I-R. These data are consistent with the report by Schoots et al. (27), who used a similar model of intestinal I-R and have demonstrated that the I-R resulted in local intestinal thrombin generation, as reflected by increases in TAT complex levels. More interestingly, the intestinal mRNA expression of PAR1 was increased after I-R in the present study. These data suggest that enhanced activation of the thrombin/PAR1 pathway plays a key role in the pathogenesis of I-R-induced intestinal injury. In our study, the concentration of TAT was decreased at 60 min and significantly increased at 90 min, which might indicate that TAT complexes were activated again during 60 min after ischemia.
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 overexpress 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


