Antithrombin reduces the ischemia/reperfusion-induced spinal cord injury in rats by attenuating inflammatory responses

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Summary
Antithrombin (AT) reveals its antiinflammatory activity by promoting endothelial release of prostacyclin (PGI₂) in vivo. Since neuroinflammation is critically involved in the development of ischemia/reperfusion (I/R)-induced spinal cord injury (SCI), it is possible that AT reduces the I/R-induced SCI by attenuating the inflammatory responses. We examined this possibility using rat model of I/R-induced SCI in the present study. AT significantly reduced the mortality and motor disturbances by inhibiting reduction of the number of motor neurons in animals subjected to SCI. Microinfarctions of the spinal cord seen after reperfusion were markedly reduced by AT. AT significantly enhanced the I/R-induced increases in spinal cord tissue levels of 6-keto-PGF₁α, a stable metabolite of PGI₂. AT significantly inhibited the I/R-induced increases in spinal cord tissue levels of TNF-α, rat interleukin-8 and myeloperoxidase. In contrast, Trp⁴⁹-modified AT did not show any protective effects. Pretreatment with indomethacin significantly reversed the protective effects of AT. An inactive derivative of factor Xa, which selectively inhibits thrombin generation, has been shown to fail to reduce SCI. Taken together, these observations strongly suggested that AT might reduce I/R-induced SCI mainly by the antiinflammatory effect through promotion of endothelial production of PGI₂. These findings also suggested that AT might be a potential neuroprotective agent.

Keywords
Antithrombin, ischemia/reperfusion, spinal cord injury, tumor necrosis factor-α, prostacyclin

Introduction
Antithrombin (AT) is an important serine protease inhibitor of the coagulation system (1). Inhibition by AT of thrombin and the other serine proteases generated in the coagulation cascade is markedly accelerated by its interaction with the endothelial surface glycosaminoglycans (GAGs) (1). Patients with congenital AT deficiency and those with the variant AT that lacks affinity for GAGs develop thrombosis, showing the importance of the interaction of AT with the endothelial cell surface GAGs for regulation of the coagulation cascade (2, 3).

AT has been shown to promote endothelial release of prostacyclin by interacting with the endothelial surface GAGs in vivo (4). Prostacyclin (PGI₂) potently inhibits platelet aggregation (5) and induces vasodilation (6), thereby maintaining proper organ microcirculation. In addition, PGI₂ inhibits the endotoxin (ET)-induced monocyte production of tumor necrosis factor-α (TNF-α) (7). TNF-α plays a role in the propagation of inflammation by activating endothelial cells and neutrophils (8). Activated neutrophils adherent to the endothelium damage the endothelial cells by releasing inflammatory mediators including neutrophil proteases and oxygen free radicals (9). The
resulting endothelial damage might increase vascular permeability, leading to tissue ischemia (10). We recently reported that AT inhibited activated neutrophil-induced increase in vascular permeability via promotion of endothelial production of PGI₂ (11). Such antiinflammatory properties of AT mediated by PGI₂ were also demonstrated in our rat model of sepsis (12, 13).

Spinal cord injury after operations on the descending thoracic and thoracoabdominal aorta is a well known and serious complication (14, 15). The cause of this complication is believed to include spinal cord ischemia during interruption of the intercostal and lumbar arteries and postoperative reperfusion injury (14, 16). Although various surgical techniques and pharmacologic interventions have been used to reduce the incidence of perioperative paraplegia, this complication remains unpredictable and unpreventable (16, 17). Clark et al. (18) reported that treatment with anti-leukocyte adhesion molecule antibodies reduced ischemic injury in the rabbit spinal cord ischemia model, suggesting that activated neutrophil-induced endothelial damage leading to spinal cord tissue ischemia might be critically involved in the development of I/R-induced spinal cord injury. We also previously reported that inhibition of TNF-α production by leukocytopenia reduced motor disturbance in the rat spinal cord ischemia model (19). These results suggest that activated neutrophils are important in the development of ischemia/reperfusion (I/R)-induced spinal cord injury.

Taken together, these observations strongly suggest that AT might reduce I/R-induced spinal cord injury by inhibiting neutrophil activation through the promotion of endothelial production of PGI₂. In the present study, we examined these possibilities using the rat model of I/R-induced spinal cord injury.

**Materials and methods**

**Reagents**

AT was the generous gift of Mitsubishi-Welpharma (Osaka, Japan). AT was purified from heat-treated pooled human plasma by adsorption on fixed heparin according to a modified version of technique of Miller-Anderson et al. (20). The AT concentrate used in the experiments revealed a single band on sodium dodecyl sulfatepolyacrylamide gel electrophoresis. Further characterization of the AT concentrate demonstrated that the heparin concentration was less than 0.01 U/ml and that it was free of pathogen. Dimethyl-(2-hydroxy-5-nitrobenzyl) sulfonium bromide (DHNBSB), indomethacin (IM), and myeloperoxidase (MPO) were purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

**Preparation of Trp⁴⁹-modified AT**

The Trp⁴⁹ residue of AT was chemically modified by a version of the method of Karp et al (21). In brief, DHNBSB was mixed with a continuously stirred solution containing 40 µM AT, 0.1 M Tris-HCl (pH8.0) and 0.15 M NaCl. The final concentration of DHNBSB was calculated as 8 mM. After stirring for 15 minutes at 22°C, the insoluble hydroxynitrobenzyl alcohol which formed as a hydrolysis product, was removed by centrifugation. The resulting solution was next subjected to chromatography on a column (2.6 x 60 cm) of Sephacryl S-200HR that had been equilibrated with 0.1 M Tris-HCl (pH8.0) and 0.15 M NaCl and then subjected to chromatography on a column (3 x 6 cm) of heparin-Sepharose CL6B, as previously described (3). The extent of derivatization of AT was determined spectrophotometrically in 2 M NaOH at a wave length of 410 nm (molar extinction coefficient, 1.85 x 10⁴ M⁻¹). (22).

**Animal model of transient spinal cord ischemia**

Adult pathogen-free male Wistar rats (Nihon SLC, Hamamatsu, Japan), weighing 300-380 g were used. The study protocol was approved by the Kumamoto University Animal Care and Use Committee, and care and handling of the animals were in accord-ance with the National Institutes of Health guidelines. Spinal cord ischemia was induced using a balloon catheter as previously described (19, 23). Under pentobarbital anesthesia (45 mg/kg, i.p., Abbott Laboratory, North Chicago, IL), a 2F Fogarty catheter (Baxter, Irvine, CA) was inserted into a left femoral artery. To induce spinal cord ischemia, the balloon placed at the end of the aortic arch was inflated with 0.05 ml of distilled water for 20 minutes. The efficiency of the occlusion was evidenced by sustained loss of any detectable pulse measured by doppler sonography on the right femoral artery. At the end of the ischemic period, the catheter was deflated and removed. During surgical preparation, body temperature was maintained between 35.5 and 37.5 °C with a thermal pad and a heat lamp. This technique creates spinal cord injury in the lumbar-sacral region that causes paraplegia in a reproducible manner (19, 23).

In sham operation animals the balloon catheter was placed in aorta but not inflated. AT (250 U /kg) or Trp⁴⁹-modified AT (250 U /kg) were administered intravenously to rats 30 minutes prior to ischemia. IM (20 mg /kg) were injected subcutaneous-ly 60 minutes prior to ischemia. Control animals received saline instead of drugs.

After the indicated period of reperfusion rats were killed for histologic and biochemical analysis. The lumbar enlargements of the spinal cords were removed after formalin perfusion fixation for histopathologic analysis, or taken, immediately plunged into liquid nitrogen for rapid freezing and stored -80°C for later biochemical analysis.

**Evaluation of survival and neurologic outcome**

To evaluate the effect of AT on survival, animals were allowed to survive for 21 days. The hind-limb neurologic function was assessed in a blind manner using Tarlov’s scale (24) and inclined-plane test (25). Tarlov’s motor scale is as follows: 0, no
voluntary movement (complete paraplegia); 1, perceptible movement at the joint; 2, good joint mobility but an inability to stand; 3, ability to stand and walk; and 4, complete recovery. In inclined-plane test, we used a smooth surface inclined plane, and the maximum inclination of the plane, at which rats could maintain themselves for 5 seconds without falling from it, was recorded.

**Histopathologic studies**

The lumbar enlargements of the spinal cord were removed and postfixed in the fixative overnight. Transverse semiserial 5 μm, paraffin-embedded sections were prepared to be stained with hematoxylin and eosin (H&E). When significant injury was present, neurons in the anterior horn of the gray matter show eosinophilic, structurally cytoplasm and loss of nuclear hematoxylin staining (26). The cells were considered viable if the cells demonstrated basophilic stippling. The total number of intact neurons in the ventral gray matter region was counted using 5 serial sections of each animal.

**Staining with 2, 3, 5, -Triphenyltetrazolium Chloride**

Staining with 2, 3, 5, -Triphenyltetrazolium Chloride (TTC) has been shown to be a reliable method for the detection of infarction in the central nervous system (26, 27). The lumbar enlargements of the spinal cords were removed from rats killed 24 hours after the transient ischemia, cut into 3-mm coronal sections, and immersed in a 2% TTC solution at 37°C for 30 minutes, followed by fixation in 10% phosphate buffered formalin. The rostral surface of the TTC-stained section was photographed.

**Assay of levels of 6-keto-PGF₁α, TNF-α, and rat interleukin-8 (IL-8) in spinal cord tissue**

Levels of 6-keto-PGF₁α, a stable metabolite of PGI₂, TNF-α, and rat IL-8 in spinal cord tissue were determined before and over time after reperfusion according to the previously described methods (19, 28). Briefly, the frozen tissue sample was weighed and homogenized (1:10, weight/ volume) in 100 mM phosphate buffer (pH 7.4) containing 0.05% of sodium azide in an ice bath. The homogenate was sonicated for 30 seconds and centrifuged (2,000 x g, at 4°C, for 20 minutes). 6-keto-PGF₁α was extracted from the supernatant using columns packed with ethyl-bonded silica gel (ethyl C2; Amersham). The columns were prepared by washing them with 2 ml of water. The supernatant acidified with 1 M HCl was applied to the column, and this was washed with 5 ml of water, 5 ml of 10% ethanol, and 5 ml of hexane. The elution of 6-keto-PGF₁α was performed with 5 ml of methyl formate, after which the solvent was evaporated under a stream of nitrogen gas. The concentration of 6-keto-PGF₁α was determined with a specific enzyme immunoassay kit (Amersham, Buckinghamshire, UK) (28). The results are expressed as nanograms of 6-keto-PGF₁α per gram of tissue.

The concentration of TNF-α and rat IL-8 in the supernatant were determined using enzyme-linked immunosorbent assay kits for rat TNF-α (Biosource, Camarillo, CA) and for rat IL-8 (Amersham), respectively (19). The results are expressed as picograms of cytokine per gram of tissue.

**Measurement of MPO activity in spinal cord tissue**

The presence of MPO, an enzyme specific for neutrophils, was used as an index of neutrophil accumulation in the spinal cord (29). MPO activity was measured by a previously described method (30). The spinal cord tissue was weighed and homogenized (1:10, weight/ volume) in 100 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma) in an ice bath and sonicated for 30 seconds. After centrifugation (2,000 x g, at 4°C, for 20 minutes), supernatant was assayed for MPO activity in 0.6 ml of 100 mM phosphate buffer (pH 6.0) containing 1.25 mg/ml o-dianisidine and 0.05% hydrogen peroxide. The assay was started by adding 0.1 ml of sample. The change in absorbance at 460 nm after 20 minutes was measured in a spectrophotometer (DU-54; Beckman, Irvine, CA) and the MPO activity in each sample was calculated using a standard curve prepared for purified MPO (Sigma). The results are expressed as units of MPO activity per gram of tissue.

**Statistical analysis**

All values are expressed as mean ± standard deviation. Survival rate was analyzed with Fisher’s exact probability test. Differences in motor scores were assessed using the nonpara-
Results

Effect of AT on survival and motor disturbances after spinal cord ischemia

Although 6 out of 9 animals (66.6%) in the I/R group died during the 21-day observation period, only one out of 8 animals (12.5%) died in the AT-treated group (Fig. 1). The difference of mortality was statistically significant (P< 0.04). From 1 to 7 days after the transient ischemia, neurological scores were significantly higher in AT-treated animals than in control animals (Fig. 2). The angle of the inclined plane was higher in animals given AT than that in control animals 24 hours after reperfusion (Fig. 3).

Effect of AT on the I/R-induced histopathologic changes

There were no histopathologic changes in sham-operated animals (Fig. 4A). Nissl’s substance could be seen in the cytoplasm of normal neurons in these animals (Fig. 4A). In contrast, spinal cord tissue sections from control animals showed neuronal injury in the ventral grey matter; i.e. many neurons were characterized by eosinophilic, structureless cytoplasm and loss of nuclear hematoxylin stainability (Fig. 4B). The tissue sections from animals given AT showed a marked reduction of these histopathologic changes (Fig. 4C). About 65% of motor neurons in the ventral grey matter were lost at 24 hours after the transient ischemia in control animals, while only about 30% of motor neurons were lost in animals treated with AT (Fig. 5).

Effect of AT on the microinfarction induced by spinal cord I/R

2, 3, 5, -Triphenyltetrazolium chloride (TTC) staining of the spinal cord tissue in sham-operated animals showed a deep red color (Fig. 6A). In contrast, spinal cord tissue in control animals was not stained by TTC, indicating the development of microinfarction in control animals (Fig. 6B). The infarced areas with no red staining were remarkably reduced in AT-treated animals (Fig. 6C).

Effects of Trp49-modified AT and indomethacin on motor disturbances

Since Trp49-modified AT which lacks affinity for heparin did not increase the endothelial production of PGI2 in rats given lipopolysaccharide (12), the interaction of AT with the endothelial cell surface heparin-like substances might be critical for promotion of the endothelial production of PGI2. To clarify whether promotion of the endothelial production of PGI2 might be important for the therapeutic effect of AT in this animal model of spinal cord ischemia, we examined the effects of Trp49-modified AT and indomethacin (IM) which inhibits prostaglandin synthesis on motor disturbances. As shown in Figure 3, neither Trp49-modified AT nor IM had any significant effects on motor disturbances. However, protective effects of AT on motor disturbances were abrogated when animals were pretreated with IM (Fig. 3).
Effects of AT, Trp49-modified AT and IM on spinal cord tissue levels of 6-keto-PGF1α

Spinal cord tissue levels of 6-keto-PGF1α were significantly increased after reperfusion, peaking at 3 hours after reperfusion compared with those of sham-operated animals (Fig. 7). To determine whether AT promoted the endothelial release of PGI2,
in the spinal cord after transient ischemia by interacting with GAGs on the endothelial cell surface, we examined the effects of AT, Trp49-modified AT, and IM on the tissue level of 6-keto-PGF1α at 3 hours after reperfusion. Administration of AT significantly enhanced the I/R-induced increase in the tissue level of 6-keto-PGF1α (Fig. 8), whereas the administration of Trp49-modified AT did not affect the tissue level of 6-keto-PGF1α (Fig. 8). Pretreatment with IM inhibited the I/R-induced increase in the tissue levels of 6-keto-PGF1α and it reversed these effects of AT on the tissue level of 6-keto-PGF1α (Fig. 8).

**Effects of AT, Trp49-modified AT, and IM on spinal cord tissue levels of TNF-α, rat IL-8 and MPO**

Spinal cord tissue levels of TNF-α, rat IL-8 and MPO at the lumbar-sacral spinal cord segments were measured during the reperfusion period (Fig. 9). Tissue levels of TNF-α and rat IL-8 were increased after reperfusion, peaking at 3 and 6 hours after reperfusion, respectively, compared with those of the sham-operated animals (Figs. 9A, 9B). Administration of AT inhibited these I/R-induced increases in spinal cord tissue levels of TNF-α and rat IL-8 at each peak time after reperfusion (Figs. 10A, 10B). In contrast, neither Trp49-modified AT nor IM had any effect on these increases (Figs. 10A, 10B). These inhibitory effects of AT on the I/R-induced increases in tissue levels of TNF-α and rat IL-8 were not observed when animals were pretreated with IM (Figs. 10A, 10B).

Accumulation of neutrophils in the postischemic spinal cord tissue was evaluated by measuring MPO activity. The MPO activity at the lumbar-sacral spinal cord segments in control animals increased over time after reperfusion, peaking at 24 hours after reperfusion (Fig. 9C). AT significantly inhibited this increase in MPO activity 24 hours after reperfusion (Fig. 10C), while neither Trp49-modified AT nor IM had any effect (Fig. 10C). AT did not inhibit this increase in MPO activity in animals pretreated with IM (Fig. 10C).

**Discussion**

In the present study, AT reduced the mortality and motor disturbances in rats subjected to transient spinal cord ischemia. Since TNF-α might be an important causal substance in the development of I/R-induced spinal cord damage (30), AT might reduce spinal cord damage by inhibiting TNF-α production in the present study. TNF-α activated endothelial cells and neutrophils, thereby inducing the endothelial cell injury (31). The vascular permeability was markedly increased as a consequence of the activated neutrophil-induced endothelial cell damage, leading to spinal cord ischemia (32). Such microcirculatory disturbances might lead to microinfarction of the spinal cord and reduction of the number of motor neurons, thereby inducing motor disturbances (19). Thus, it is possible that AT inhibits microinfarction and the decrease in the number of motor neurons by inhibiting the activated neutrophil-induced

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**Figure 7:** Changes in tissue levels of 6-keto-PGF1α, a stable metabolite of PGI2, in the spinal cord tissue over time after transient ischemia. The levels were measured in the lumbar-sacral spinal cord segments before and 1.5, 3, 6, 12 and 24 hours after transient ischemia. Open circles indicated sham-operated animals and closed circles indicated I/R animals. Data are expressed as mean ± standard deviation from seven animals. Comparisons with the I/R group were made by Student's t-test. *, P<.01 vs. sham-operated animals.

**Figure 8:** Comparisons on levels of 6-keto-PGF1α, in the spinal cord tissue 3 hours after transient ischemia. AT (250 U/kg) or Trp49-modified AT (250 U/kg) was intravenously administered 30 minutes before ischemia. Indomethacin (IM, 20 mg/kg) was administered subcutaneously 1 hour before ischemia. Data are expressed as mean ± standard deviation from six animals. *, P<.01 vs. sham-operated animals; †, P<.01 vs. I/R animals; §, P<.01 vs. I/R+AT animals.
endothelial cell damage through inhibition of TNF-α production, leading to the reduction of mortality and motor disturbances in this rat model of spinal cord damage.

Although an inactive derivative of F.Xa, which selectively inhibits thrombin generation, inhibited renal I/R-induced coagulation abnormalities to the same extent as native AT (11), it failed to reduce the motor disturbances seen after transient ischemia of the spinal cord (19). These observations suggested that the therapeutic effect of AT observed in the present study might not mainly be mediated via inhibition of thrombin.

Figure 9: Changes of levels of TNF-α (A), rat IL-8 (B), and MPO (C) in the spinal cord tissue over time after transient ischemia. The levels were measured before and 1.5, 3, 6, 12 and 24 hours after transient ischemia. Open circles indicated sham-operated animals and closed circles indicated I/R animals. Data are expressed as mean ± standard deviation from seven animals. Comparisons with the I/R group were made by Student’s t-test. *, P<.01 vs. sham-operated animals.

Figure 10: Comparisons on levels of TNF-α (A), rat IL-8 (B), and MPO (C) at each peak time point. AT (250 U/kg) or Trp49-modified AT (250 U/kg) was intravenously administered 30 minutes before ischemia. Indomethacin (IM, 20 mg/kg) was administered subcutaneously 1 hour before ischemia. Spinal cord tissue levels of TNF-α, rat IL-8, and MPO were determined at 3, 6 and 24 hrs after reperfusion, respectively. Data are expressed as mean ± standard deviation from five animals. *, P<.01 vs. sham-operated animals; †, P<.01 vs. I/R animals; §, P<.05 vs. I/R+AT animals.
However, we recently reported that microthrombus formation might play a precipitating role in the development of I/R-induced tissue injury in rats by further increasing TNF-α production (33, 34). Taken together, these observations strongly suggested that the therapeutic effect of AT in this animal model of spinal cord injury might involve both its anti-inflammatory and antithrombotic effects.

We previously demonstrated that AT reduced endotoxin-induced hypotension by inhibiting TNF-α production through promotion of endothelial production of PGI₂ in rats (13), suggesting that antiinflammatory effects of AT might be mediated mainly by PGI₂. Neuroprotective effects of AT might also be mediated by promotion of endothelial production of PGI₂ for the following reasons; [1] AT significantly enhanced the spinal cord I/R-induced increases in the spinal cord tissue levels of 6-keto-PGF₁α; [2] Trp⁹⁹-modified AT that failed to promote the endothelial production of PGI₂ due to the lack of the heparin affinity (12) did not reduce the spinal cord injury; and [3] neuroprotective effects of AT were significantly reversed by pretreatment with IM.

Although spinal cord tissue levels of 6-keto-PGF₁α were lower in animals with IM pretreatment than those without IM pretreatment (Fig. 8), the severity of the motor disturbance was apparently less in the former than the latter (Fig. 3). Thromboxane A₂ causes vasoconstriction, thereby contributing to the development of spinal cord injury in rats subjected to spinal cord trauma by inducing spinal cord ischemia (35). Since IM inhibits the synthesis of thromboxane A₂, pretreatment with IM might contribute to reduce the motor disturbance in this rat model of spinal cord injury. This notion might explain why the motor disturbance was apparently less in IM-pretreated animals than control animals despite the lower tissue levels of 6-keto-PGF₁α in the former than the latter.

Furthermore, although spinal cord tissue levels of 6-keto-PGF₁α were lower in animals pretreated with IM and AT than animals pretreated with IM alone (Fig. 8), the motor disturbance was apparently less in the former than the latter (Fig. 3). With respect to the mechanism of antiinflammatory effects of AT, Oelschlager et al. (36) demonstrated that AT directly inhibited monocyctic production of TNF-α by inhibiting NF-kB activation in vitro. These observations suggested that, although the anti-inflammatory activity of AT might be mainly mediated by PGI₂ produced by endothelial cells, antiinflammatory activity of AT itself might also contribute to the reduction of the spinal cord injury in the present study.

The molecular mechanism by which AT increases endothelial production of PGI₂ remains to be fully elucidated. We demonstrated that AT did not increase the production of PGI₂ directly in cultured endothelial cells (37), suggesting that some factors other than endothelial cells might be involved in the AT-induced endothelial production of PGI₂ in vivo. We recently demonstrated that activation of capsaicin-sensitive sensory neurons led to an increase in endothelial production of PGI₂ in rats subjected to hepatic I/R (38). We also reported briefly that AT reduced I/R-induced liver injury by increasing endothelial production of PGI₂ through an increase in the hepatic tissue levels of calcitonin-gene related peptide (CGRP), a neuropeptide released from capsaicin-sensitive sensory neurons in rats (39). Recently AT-deficient mice (AT +/- mice) was generated through gene targeting (40). Susceptibility to thrombus formation induced by lipopolysaccharide or restraint stress in AT +/- mice was significantly increased compared with that in wild type mice (41). Preliminary experiments using AT +/- mice demonstrated that I/R-induced increases in the hepatic tissue levels of CGRP and 6-keto-PGF₁α in AT +/- mice were about half of those in wild type mice and administration of AT significantly increased the tissue levels of these substances in AT +/- mice to levels comparable to those of wild type mice. These observations support the hypothesis described above.

References