Contribution of capsaicin-sensitive sensory neurons to antithrombin-induced reduction of ischemia/reperfusion-induced liver injury in rats

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Summary

We previously reported that antithrombin (AT) reduced ischemia/reperfusion (I/R)-induced liver injury in rats by increasing endothelial production of prostacyclin (PGI_2). However, the mechanism(s) underlying this phenomenon remains to be fully elucidated. We also demonstrated that activation of capsaicin-sensitive sensory neurons increased endothelial production of PGI_2 by releasing calcitonin gene-related peptide (CGRP) in rats subjected to hepatic I/R. In the present study, we investigated whether AT increases endothelial production of PGI_2 through activation of the sensory neurons in rats subjected to hepatic I/R. AT significantly enhanced the I/R-induced increases in hepatic tissue levels of CGRP in rats. Increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, the increase in hepatic-tissue blood flow, and attenuation of both hepatic local inflammatory responses and liver injury in rats administered AT were completely reversed by administration of capsazepine, an inhibitor of sensory neuron activation and CGRP(8–37), a CGRP antagonist. AT did not show any protective effect on liver injury in animals undergoing functional denervation by administration of a large amount of capsaicin. AT significantly increased CGRP release from cultured dorsal root ganglion neurons isolated from rats in the presence of capsaicin. Taken together, these observations strongly suggested that AT might increase hepatic tissue levels of PGI_2 via enhancement of hepatic I/R-induced activation of capsaicin-sensitive sensory neurons, thereby reducing liver injury in rats. In this process, CGRP-induced activation of both endothelial nitric oxide synthase and cyclooxygenase-1 might be critically involved.

Keywords

Antithrombin, ischemia/reperfusion, capsaicin-sensitive sensory neurons, calcitonin gene-related peptide, prostacyclin

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Introduction

Antithrombin (AT) is an important serine protease inhibitor that regulates the coagulation cascade (1). Inhibition of coagulation factors, such as thrombin by AT, is markedly accelerated by its interaction with glycosaminoglycans on the endothelial cell surface (1). The clinical relevance of AT as an important natural anticoagulant has been well illustrated by severe thrombosis in patients with congenital AT deficiency, and in patients with variant AT molecules that lack affinity for heparin (2, 3).

Ischemia/reperfusion (I/R)-induced liver injury is an important pathologic condition that often follows liver surgery, hepatic transplantation, and circulatory shock (4). Activated neutrophils have been shown to be involved in the development of I/R-induced liver injury (4). We have previously reported that AT reduced ischemia/reperfusion (I/R)-induced liver injury in rats by inhibiting neutrophil activation through promotion of endothelial release of prostacyclin (PGI_2) (5). PGI_2 has been shown to exert anti-inflammatory activities by inhibiting neutrophil activation directly or by inhibiting monocytic production of tumor necrosis factor-α (TNF-α) which potently activates neutrophils (6, 7). However, since AT did not directly increase the endothelial release of PGI_2 *in vitro* (8), the mechanism(s) by which AT promotes the endothelial release of PGI_2 *in vivo* might involve unknown factors, other than endothelial cells.

Capsaicin-sensitive sensory neurons are nociceptive neurons that are activated by a wide variety of noxious physical and chemical stimuli (9). Since ablation of the sensory fibers can re-

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sult in a marked increase in the severity of inflammation (10), the sensory neurons have been shown to play a role in the maintenance of tissue integrity by regulating local inflammatory responses. On activation, the sensory neurons release calcitonin gene-related peptide (CGRP) that can increase the endothelial production of PGI_2 *in vitro* (11). Since various noxious stimuli that activate the sensory neurons to release CGRP are capable of inducing tissue damage (12), the CGRP-induced increase in the endothelial production of PGI_2 might contribute to attenuation of local inflammatory responses, thereby reducing tissue damage. Consistent with this hypothesis, we previously reported that capsaicin-sensitive sensory neurons were activated during the hepatic I/R, leading to an increase in the endothelial production of PGI_2 via activation of both endothelial nitric oxide synthase (NOS) and cyclooxygenase (COX)-1 (13). Such activation of the sensory neurons contributed to the reduction of I/R-induced liver injury by attenuating inflammatory responses (13). A similar important role of the nervous system in the regulation of inflammatory responses was also demonstrated for the vagus nerve (14).

Based on these observations, we hypothesized that AT might promote the endothelial release of PGI_2 by enhancing the activation of capsaicin sensitive-sensory neurons, thereby exerting anti-inflammatory activity. In the present study, we examined this possibility using a rat model of hepatic I/R.

Methods

Reagents

AT was kindly provided by Mitsubishi Welpharma Pharmaceutical Co. (Osaka, Japan). AT was purified from heat-treated, pooled human plasma by adsorption on fixed heparin according to a modified version of a previously described method (5). The AT concentrate used in the experiments revealed a single band in response to polyacrylamide gel electrophoresis with sodium dodecyl sulfate. Capsaicin, capsazepine (CPZ)(an antagonist of capsaicin), L-nitro-arginine-methyl-ester (L-NAME) (a non-selective inhibitor of NOS), and indomethacin (a non-selective inhibitor of COX) were purchased from Sigma Chemical Co (St. Louis, MO). N-(3-(aminomethyl)benzyl) acetamidine (1400W) (a selective inhibitor of iNOS) was purchased from Cayman Chemical (Ann Arbor, MI). NS-398 (a selective inhibitor of COX-2) was a generous gift from Taisho Pharmaceutical Co (Saitama, Japan). Human CGRP (8–37)(an antagonist of CGRP) was purchased from Peptide Institute (Osaka, Japan). All other reagents were of analytical grade.

Administration of various agents

We have previously reported that the plasma concentration of 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, begins to rise 30 min after the intravenous (iv) administration of AT (250 U/kg) in intact rats (15). According to these findings, AT (250 U/kg) was given 30 min prior to reperfusion in this study.

CPZ was dissolved in 10% Tween 20/10% ethanol (10%) with normal saline. CPZ (15 mg/kg) was injected subcutaneously (sc) 30 min prior to hepatic ischemia as described previously (16). L-NAME (5 mg/kg) was dissolved in normal saline, and administered sc 30 min prior to hepatic ischemia as described previously (17). 1400W (5 mg/kg) was dissolved in normal saline, and injected intravenously (iv) just before the hepatic ischemia as described previously (18). CGRP (10 μg/kg) and CGRP(8–37) (100 μg/kg) were dissolved in sterile distilled water and injected iv just before the hepatic ischemia as described previously (19). Indomethacin (20 mg/kg) was suspended in 5% sodium bicarbonate-buffered saline and injected sc 30 min prior to hepatic ischemia as described previously (5). NS-398 (30 mg/kg) was suspended in 0.5% carboxymethyl cellulose aqueous suspension and administered orally 1 hr prior to hepatic ischemia as described previously (20). Solutions were prepared immediately before the experiments.

Animal model of hepatic I/R

Adult, pathogen-free, male Wistar rats (Nihon SLC, Hamamatsu, Japan), 220–280 g, were used in each experiment. The care and handling of the animals were in accordance with the National Institute of Health guidelines. All experimental procedures described below were approved by the Kumamoto University Animal Care and Use Committee. The hepatic I/R protocol was performed as described previously (21, 22). Complete ischemia of the median and left hepatic lobes was produced by clamping the left branches of the portal vein and the hepatic artery for 60 min. In order to accurately evaluate blood flow of the median and left hepatic lobes after ischemia, the right branches of the portal vein and the hepatic artery were ligated to prevent shunting to the right lobe after reperfusion (21). Sham-operated animals were similarly prepared except that no clamping was made to obstruct the blood flow to the left and median hepatic lobes. Instead the blood flow to the right lobe of the liver was occluded.

Denervation of primary sensory nerves by capsaicin

Ablation of hepatic visceral afferent nerves was accomplished by high-dose capsaicin administration as previously described (19, 23). Rats received a total dose of 125 mg/kg capsaicin administered subcutaneously in divided doses over two days. Two weeks after treatment with high-dose capsaicin, animals were subjected to stress. To determine the effectiveness of sensory afferent nerve denervation procedure, a drop of 0.001% capsaicin in saline was instilled into either eye of the rats, and their protective wiping movements were observed. Capsaicin-treated rats that showed any wiping movement were excluded from the study. Control animals were injected subcutaneously 1 ml of 10% Tween 20/10% ethanol (10%) with normal saline.

Determination of hepatic CGRP level

Hepatic levels of CGRP were determined in animals by modification of the methods as described previously (24). The concentration of CGRP was assayed by using a specific enzyme immunoassay kit (SPI-BIO, Massey Cedex, France). Results are expressed as μg of CGRP per gram of tissue.

Immunohistochemical staining of CGRP, AT, and iNOS in the liver

The peroxide-antiperoxide technique was used for immunohistochemical staining of the liver with anti-CGRP antibody, anti-AT antibody, or anti-iNOS antibody according to the methods as described previously with slight modification (25–27). The un-

fixed tissue blocks of rat liver were frozen in dry ice-cooled OCT compound (Tissue Tek; Miles, Elkhart, IN). Sections (6–8 μm thick) were mounted on glass slides, immersed in absolute acetone at –20°C for 5 min, rinsed in phosphate buffered saline (PBS) five times for 5 mins each, and then incubated for 20 min with 10% porcine serum in PBS at room temperature. They were incubated for 1 hr at 37°C with rabbit anti-CGRP polyclonal antibody, sheep anti-AT polyclonal antibody, or rabbit anti-iNOS polyclonal antibody at 1:100 dilution. After five rinses in PBS, the sections were treated with horseradish peroxide-conjugated anti-rabbit or anti-sheep IgG, respectively (MBL Co. Nagoya, Japan) at 1:2000 dilution for 1 hr at 37°C. Reaction products were developed by immersing the sections in 3'3-diaminobenzidine tetrahydrochloride solution containing 0.03% hydrogen peroxide. The control for immunostaining was performed by non-immune rabbit or sheep serum, respectively, as first step in place of primary antiserum, and omission of the first step or use of the first antiserum preabsorbed with an excess of the homologous antigen. Samples were mounted with Entellan onto glass slides, examined and photographed under light microscope.

Determination of hepatic 6-keto-PGF$_{1\alpha}$ levels

Since we have previously demonstrated that hepatic 6-keto-PGF11$_{1\alpha}$ levels are increased after I/R, peaking at 1 hr of reperfusion (5), we measured the hepatic 6-keto-PGF11$_{1\alpha}$ levels 1 hr after reperfusion in this study according to the methods described previously (5). The concentration of 6-keto-PGF11$_{1\alpha}$ was assayed using a specific enzyme immunoassay kit (Amersham, Buckinghamshire, UK). The results are expressed as ng of 6-keto-PGF11$_{1\alpha}$ per g of tissue.

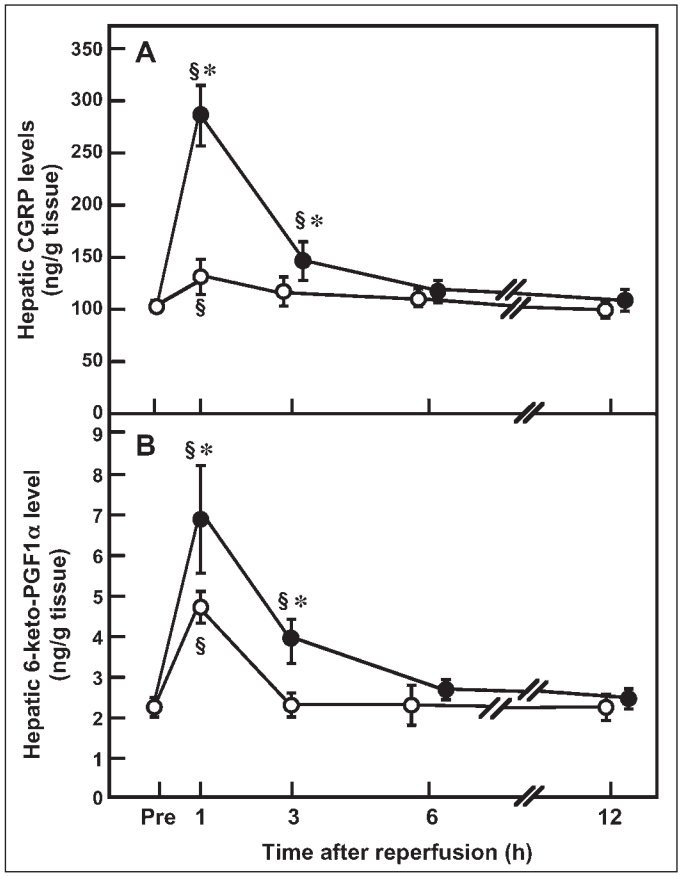

Figure 1: Changes in hepatic tissue levels of CGRP (A) and 6-keto-PGF$_{1\alpha}$ (B) in rats subjected to hepatic I/R. Values are expressed as the means ± SD derived from five animal experiments. Open circles; sham operated animals, closed circles; I/R animals. §, p<0.01 vs. pre-ischemia, *, p<0.01 vs. sham.

Measurement of hepatic tissue blood flow

Hepatic tissue blood flow was measured by laser-Doppler flowmeter (ALF21N, Advance, Tokyo, Japan) for 3 hr after reperfusion, as described previously (5). The results are expressed as % of pre-ischemia levels.

Determination of hepatic levels of TNF-α

Hepatic levels of TNF-α were determined using a rat or mouse TNF-α ELISA system (Amersham, Buckinghamshire, UK) as described previously (28). The results are expressed as pg of TNF-α per g of tissue.

Determination of hepatic myeloperoxidase activity

After the indicated period of reperfusion, the livers were quickly removed, and accumulation of leukocytes was assessed by measuring myeloperoxidase (MPO) activity in the liver according to a previously described method (29). Results are expressed as units of MPO activity per g tissue.

Measurement of serum liver enzymes

Blood samples were taken 12 hr after reperfusion to measure the level of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as previously described (30). ALT and AST levels were measured by standard clinical automated analysis and the results are expressed in IU/l.

Isolation and culture of dorsal root ganglion neurons

Dorsal root ganglion neurons from the lumbar, cervical and thoracic region were dissected from specific pathogen free male Wistar rats (body weight, 220–280 g; Nihon SLC, Hamamatsu, Japan) as described previously (31). In brief, dorsal root ganglia were placed in ice-cold sterile calcium-, and magnesium-free Dulbecco's phosphate buffered saline (CMF-Dulbecco's PBS) (Gibco, Grand Island, NY). Ganglia were chopped and incubated at 37°C for 15 min in CMF-Dulbecco's PBS containing 20 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ). The tissue was then incubated at 37°C for 15 min in CMF-Dulbecco's PBS containing 4 mg/ml collagenase type II (Worthington Biochemical Corporation). The tissue was incubated for a further 30 min in CMF-Dulbecco's PBS containing 2000 U/ml dispase I (Godo Shusei, Tokyo, Japan) at 37°C. Individual cells were then dissociated by trituration through a fire-polished Pasteur pipette. After centrifugation at 250 *g* for 5 min, the resultant pellet was washed twice in serum free Ham's F-12 medium (Hyclone, Logan, UT). Cells were plated on 60 mm polystyrene dish precoated with Vitrogen (Cohesion Technologies, Palo Alto, CA) in Ham's F-12 medium containing 10% supplemented calf serum (SCS), 2 mM glutamine and 50 ng/ml mouse 2.5S nerve growth factor (NGF) (Upstate Biotecnology, Lake Placid, NY). After 24 h, the culture medium was removed and replaced every 2 days.

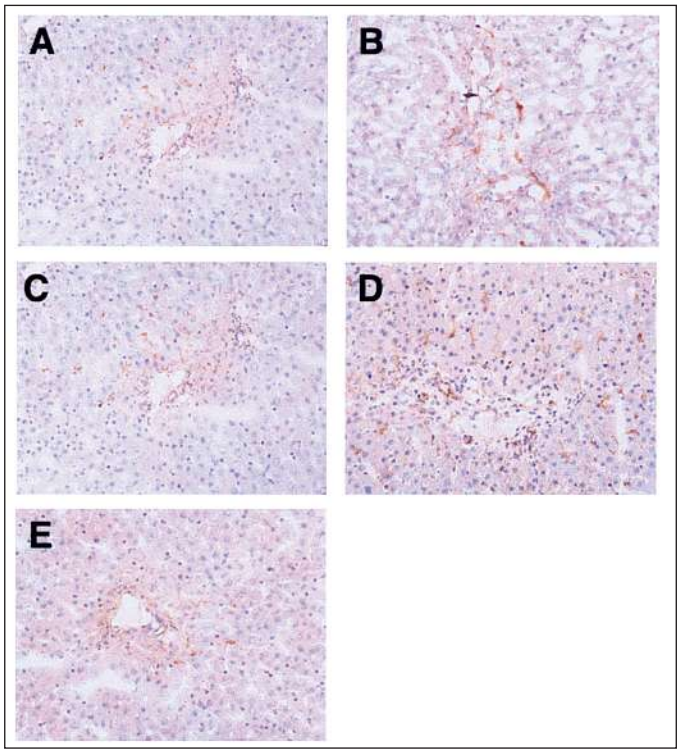

Figure 2: Effect of AT on immunohistochemical staining of CGRP in the liver of rats subjected to I/R. Immunohistochemical CGRP staining of frozen sections of the liver was determined by anti-rat CGRP antibody in sham-operated animals (A) and in I/R animals 1 hr after reperfusion (B, C, D and E) (original magnification x 100). AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to ischemia. A, sham-operation; B, I/R; C, I/R+CPZ; D, I/R+AT; E, I/R+AT+CPZ. Administration of the vehicle did not affect the I/R-induced immunohistochemical staining of CGRP. Five animals in each group were examined: typical results are shown.

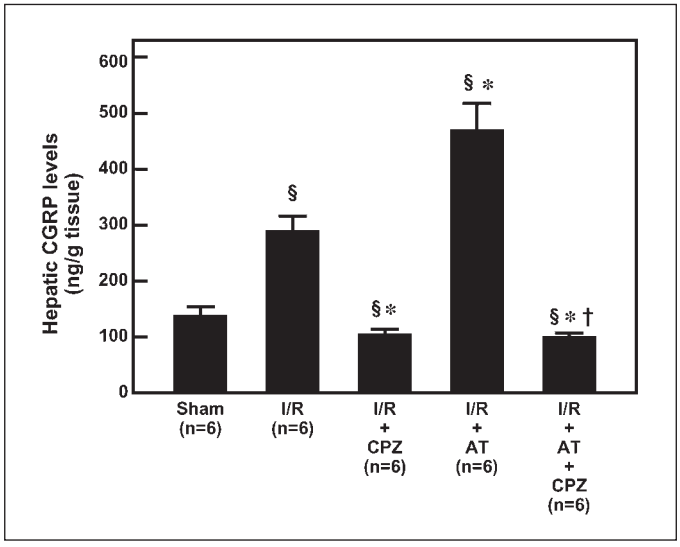

Figure 3: Effects of AT and CPZ on I/R-induced increases in hepatic CGRP levels after 1 hr of reperfusion. AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to ischemia. Values are expressed as means ± SD. *, p<0.01 vs. I/R; †, p<0.01 vs. I/R + AT.

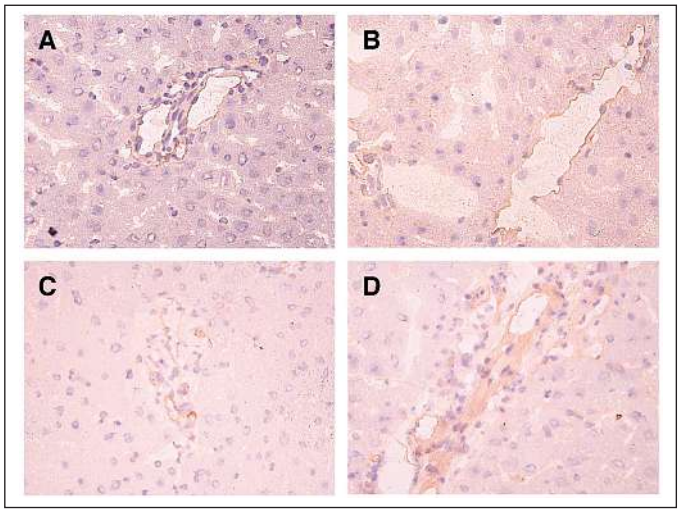

Figure 4: Effect of AT on immunohistochemical staining of AT in the liver of rats subjected to I/R. Immunohistochemical AT staining of frozen sections of the liver were determined by anti-human AT antibody in sham-operated animals (A, B) and in I/R animals 1 hr after reperfusion (C, D) (original magnification x 100). AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to ischemia. A, sham-operation; B, sham-operation + AT; C, I/R; D, I/R + AT. Administration of the vehicle did not affect the I/R-induced immunohistochemical staining of AT. Five animals in each group were examined: typical results are shown.

Release of CGRP from dorsal root ganglion neurons in culture

After 5 days in culture, the medium was aspirated gently and washed with serum free Ham's F-12 medium. Cells were incubated with capsaicin (50 μM) or AT (5 U/ml) or capsaicin in combination with AT (1, 2, or 5 U/ml) for 30 min in Ham's F-12 medium containing 1% SCS without NGF. After incubation, supernatants were sampled and stored at –20°C for CGRP measurement. CGRP levels were determined using a commercial rat CGRP enzyme immunometric assay kit (SPI bio, Massy, France).

Statistical analysis

Data are expressed as the mean ± SD. The results were compared using either an analysis of variance followed by Scheffé's post hoc test or an unpaired Student's t-test. A level of $p \leq .05$ was considered statistically significant.

Results

Effects of CPZ and/or AT on I/R-induced increases in hepatic tissue levels and the immunohistochermical staining of CGRP

Hepatic tissue levels of CGRP were increased after hepatic I/R, peaking at 1 hr after reperfusion (Fig. 1A). The levels at 1 hr after reperfusion in animals subjected to hepatic I/R were significantly higher than those of sham-operated animals (Fig. 1A). Immunohistochemical staining of CGRP in the liver at 1 hr after reperfusion was increased in animals subjected to hepatic I/R compared with that of sham-operated animals (Fig. 2, A and B).

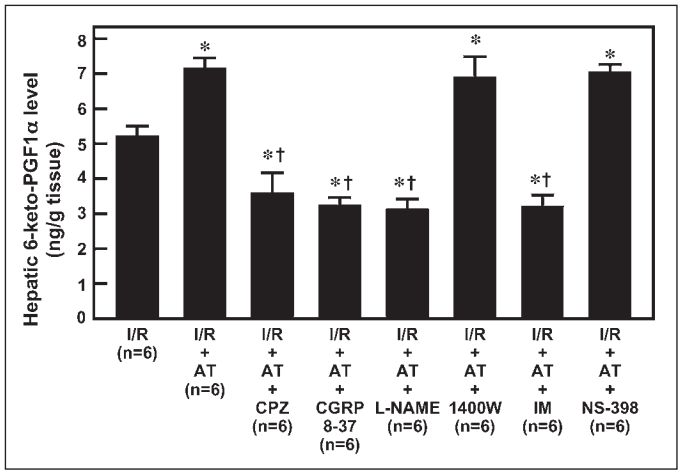

Figure 5: Effects of AT and various agents on I/R-induced increases in hepatic levels of 6-keto-$PGF_{1\alpha}$. AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. Animals were either injected sc with CPZ (15 mg/kg), L-NAME (5 mg/kg), or indomethacin (IM; 20 mg/kg) 30 min prior to hepatic ischemia. 1400W (5 mg/kg) and CGRP (8–37) (100 μg/kg) were injected intravenously immediately prior to AT administration. NS-398 (30 mg/kg) was administered orally 1 hr prior to hepatic ischemia. Each bar represents the mean ± SD. * p<0.01 vs. I/R; †, p<0.01 vs. I/R + AT.

Pretreatment with CPZ (15 mg/kg, sc), an antagonist of capsaicin, inhibited both the increase in hepatic tissue levels of CGRP (Fig. 3) and the immunohistochemical staining of CGRP (Fig. 2C) seen 1 hr after reperfusion. AT (250 U/kg, iv) significantly enhanced the I/R-induced increases in both hepatic tissue levels (Fig. 3) and immunohistochemical staining of CGRP in the liver (Fig. 2D) at 1 hr after reperfusion. Pretreatment with CPZ completely inhibited these AT-induced increases in hepatic tissue levels (Fig. 3) and immunohistochemical staining of CGRP (Fig. 2E) at 1 hr after reperfusion in rats subjected to hepatic I/R.

Tissue distribution of intravenously administered AT

To determine whether intravenously administered AT can gain access to sensory nerve endings localized in the perivascular space (31), we analyzed the hepatic tissue distribution of intravenously administered AT by immunohistochemical staining using anti-human AT antibody (Fig. 4). Although AT was not administered, it was shown immunohistochemically using anti-human AT antibody mainly in the endothelial surface of the liver in sham-operated animals (Fig. 4A) and in those subjected to hepatic I/R (Fig. 4C). This might be due to the cross-reactivity of rat AT with anti-human AT antibody (32). In sham-operated animals administered AT, AT was also shown to be distributed mainly at the endothelial surface (Fig. 4B). However, AT was demonstrated immunohistochemically in the perivascular space as well as the endothelial surface in AT-treated animals subjected to hepatic I/R (Fig. 4D). These observations suggested that intravenously administered AT might extravasate at the site of endothelial injury where vascular permeability is increased.

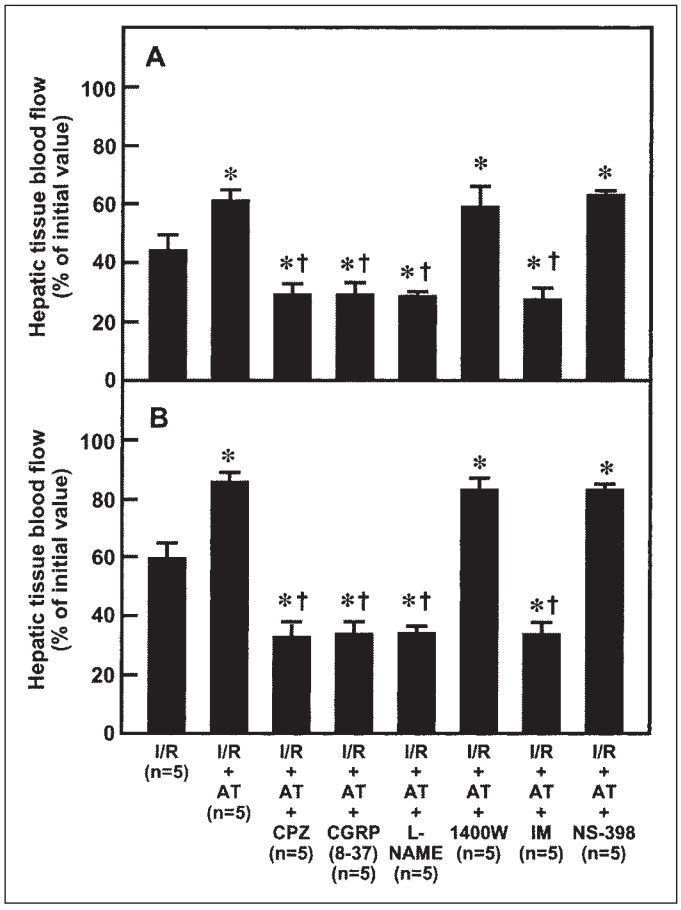

Figure 6: Effects of AT and various agents on the changes in hepatic tissue blood flow 1 h (A) and 3 h (B) after reperfusion in rats subjected to hepatic I/R. AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. Animals were either injected sc with CPZ (15 mg/kg), L-NAME (5 mg/kg), or indomethacin (IM; 20 mg/kg) 30 min prior to hepatic ischemia. 1400W (5 mg/kg) and CGRP (8–37) (100 μg/kg) were injected intravenously immediately prior to AT administration. NS-398 (30 mg/kg) was administered orally 1 hr prior to hepatic ischemia. Each value represents the mean ± SD deprived from 5 animals. *, p<0.01 vs. I/R; †, p<0.01 vs. I/R + AT.

Effects of pretreatment with CPZ, CGRP, NOS inhibitors and COX inhibitors on AT-induced increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ and hepatic tissue blood flow

Hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ were increased after hepatic I/R, peaking at 1 hr after reperfusion and these levels were significantly higher in animals subjected to hepatic I/R than in sham-operated animals (Fig. 1B). Although administration of capsaicin and CGRP enhanced increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ at 1 hr after reperfusion and increased hepatic tissue blood flow after reperfusion, pretreatment with CPZ (15 mg/kg, sc), CGRP (8–37) (100 μg/kg, iv), L-NAME (a non-selective inhibitor of NOS) (5 mg/kg, sc), and indomethacin (a non-selective inhibitor of COX) (20 mg/kg, sc) inhibited these increases and decreased hepatic tissue blood flow (13). However, neither 1400W (a selective inhibitor of iNOS)(5 mg/kg, iv) nor NS-398 (a selective inhibitor of COX-2) (30 mg/kg, po) had any effect on these increases and hepatic tissue blood flow (13). In-

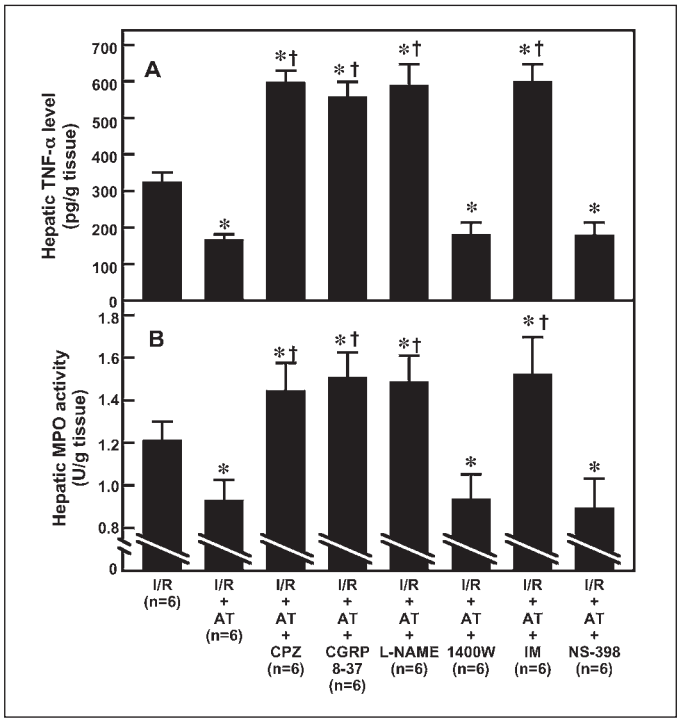

Figure 7: Effects of AT and various agents on I/R-induced increases in hepatic levels of TNF-α (A) and hepatic MPO activity (B). AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. Animals were either injected sc with CPZ (15 mg/kg), L-NAME (5 mg/kg), or indomethacin (IM; 20 mg/kg) 30 min prior to hepatic ischemia. 1400W (5 mg/kg) and CGRP (8–37) (100 μg/kg) were injected intravenously immediately prior to AT administration. NS-398 (30 mg/kg) was administered orally 1 hr prior to hepatic ischemia. Each bar represents the mean ± SD. * p<0.01 vs. I/R; †, p<0.01 vs. I/R + AT.

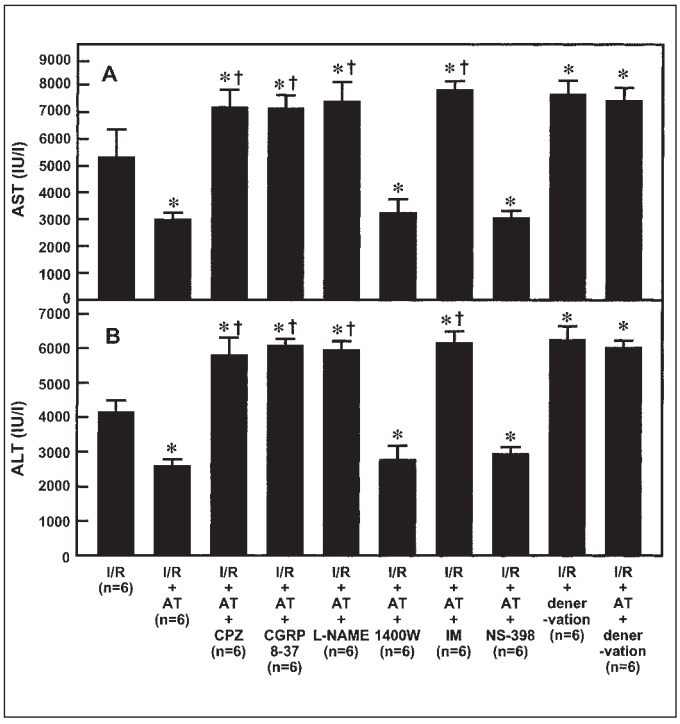

Figure 8: Effects of AT and various agents on I/R-induced increases in serum AST (A) and ALT (B) level. AT (250 U/kg) was injected intravenously 30 min prior to reperfusion. Animals were either injected sc with CPZ (15 mg/kg), L-NAME (5 mg/kg), or indomethacin (IM; 20 mg/kg) 30 min prior to hepatic ischemia. 1400W (5 mg/kg) and CGRP (8–37) (100 μg/kg) were injected intravenously immediately prior to AT administration. NS-398 (30 mg/kg) was administered orally 1 hr prior to hepatic ischemia. Each bar represents the mean ± SD. * p<0.01 vs. I/R; †, p<0.01 vs. I/R + AT.

travenous administration of AT significantly enhanced increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ at 1 hr after reperfusion (Fig. 5) and increased hepatic tissue blood flow after reperfusion (Fig. 6). Pretreatment with CPZ, CGRP (8–37), L-NAME, and indomethacin significantly inhibited AT-induced increases in both hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ at 1 hr after reperfusion (Fig. 5) and hepatic tissue blood flow (Fig. 6), but neither 1400W nor NS-398 had any effect (Figs. 5 and 6).

Effects of pretreatment with CPZ, CGRP, NOS inhibitors, and COX inhibitors on AT-induced inhibition of increases in hepatic tissue levels of TNF-α and MPO

Hepatic tissue levels of TNF-α and MPO were markedly increased after I/R, peaking at 1 and 6 hr after reperfusion, respectively, and these levels were significantly higher than those of sham-operated animals (32). Hepatic I/R-induced increases in hepatic tissue levels of TNF-α and MPO were significantly inhibited by administration of capsaicin and CGRP, but were enhanced by administration of CPZ, CGRP (8–37), L-NAME, and indomethacin (13). Neither pretreatment with 1400W nor that with NS-398 showed any effects on these levels (13). Intravenous administration of AT significantly inhibited these I/R-induced increases (Fig. 7, A and B). Although pretreatment with CPZ (15 mg/kg, sc), CGRP (8–37) (100 μg/kg, iv), L-NAME (5 mg/kg, sc), and indomethacin (20 mg/kg, sc) completely abrogated the AT-induced reduction of hepatic tissue levels of TNF-α and MPO after reperfusion, neither pretreatment with 1400W (5 mg/kg, iv) nor that with NS-398 (30 mg/kg, po) had any effect (Fig. 7, A and B).

Effects of pretreatment with CPZ, functional denervation of capsaicin-sensitive sensory neurons, CGRP, NOS inhibitors and COX inhibitors on AT-induced reduction of liver injury

Serum levels of transaminases were increased after hepatic I/R, peaking at 12 hr after reperfusion (29). These increases were inhibited by administration of capsaicin and CGRP, while these were enhanced by administration of CPZ, CGRP (8–37), L-NAME, and indomethacin (13). Neither pretreatment with 1400W nor that with NS-398 showed any effects on these levels (13). AT significantly inhibited the I/R-induced increases in serum levels of transaminases (Fig. 8, A and B). Reduction by AT of I/R-induced liver injury was not observed in animals pretreated with CPZ (15 mg/kg, sc) and in animals undergoing functional denervation by administration of a large amount of capsaicin (Fig. 8, A and B). Pretreatment with CGRP (8–37) (100 μg/kg, iv), L-NAME (5 mg/kg, sc), and indomethacin (20 mg/kg, sc) reversed the AT-induced inhibition, but neither 1400W (5 mg/kg, iv) nor that with NS-398 (30 mg/kg, po) had any effect (Fig. 8, A and B).

Effect of AT on the expression of iNOS in the liver after hepatic I/R

To determine whether iNOS is involved in reduction of I/R-induced liver injury in animals given AT, we examined the effect of AT on the expression of iNOS in the liver using immunohistochemical staining of iNOS at 1 hr after reperfusion. Expression of iNOS was not observed in the liver at 1 hr after reperfusion in animals without AT administration (data not shown). Administration of AT did not induce the expression of iNOS in the liver at 1 hr after reperfusion (data not shown).

Effects of AT and/or capsaicin on CGRP release from dorsal root ganglion neurons isolated from rats

To determine whether AT directly promotes CGRP release from the sensory neurons, we analyzed the effect of AT on CGRP release from dorsal root ganglion neurons isolated from rats. As shown in figure 9, although AT (5 U/ml) itself did not increase CGRP release from dorsal root ganglion neurons, it significantly enhanced CGRP release in the presence of capsaicin (50 μM) at 30 min after incubation. However, AT, at concentrations lower than 5 U/ml, did not enhance CGRP release even in the presence of capsaicin at 30 min after incubation (Fig. 9).

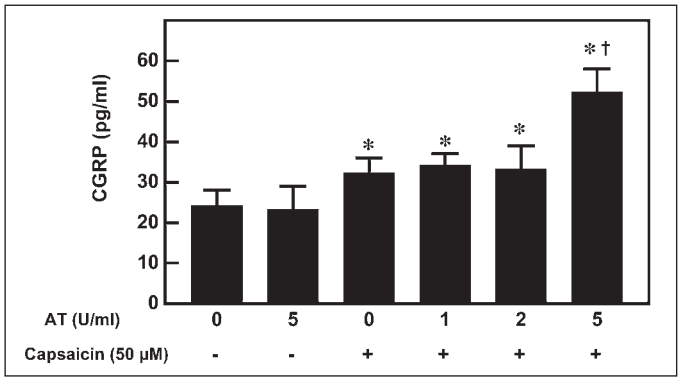

Figure 9: Effect of AT on CGRP release by capsaicin-stimulated dorsal root ganglion neurons. Dorsal root ganglion neurons were incubated with capsaicin (50 μM) or AT (5 U/ml) or capsaicin in combination with AT (1, 2 or 5 U/ml) for 30 min. Supernatants were sampled and CGRP levels were measured by enzyme immunoassay. Each value represents the mean ± SD deprived from 5 animals. *, $p < 0.01$ vs. capsaicin(-)/AT(0); †, $p < 0.01$ vs. capsaicin(+)/AT(0).

Discussion

In the present study, AT significantly enhanced I/R-induced increases in both hepatic tissue levels and immunohistochemical staining of CGRP in rats. These increases were inhibited by pretreatment with CPZ, a vanilloid receptor-1 antagonist. Pretreatment with CPZ and CGRP (8–37), an antagonist of CGRP, inhibited AT-induced increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ in rats subjected to hepatic I/R. Reduction by AT of I/R-induced liver injury was not observed in animals pretreated with CPZ and CGRP (8–37) and in animals whose sensory neurons were functionally denervated. Taken together, these observations strongly suggest that AT might increase the endothelial production of PGI_2 mainly by enhancing the activation of capsaicin sensitive sensory neurons in the liver of rats subjected to hepatic I/R, thereby reducing liver injury.

Since sensory nerve endings are located at the perivascular area of various tissues (31), the question arises as to how intravenously administered AT gains access to nerve endings to stimulate the sensory neurons. As shown in the present study, AT was found in the extravascular space as well as on the endothelial surface of the liver of AT-treated animals subjected to hepatic I/R, but it was not demonstrated in the extravascular space in AT-treated animals undergoing sham-operations. Activated neutrophils have been shown to increase microvascular permeability by interacting with endothelial cells (33). Consistent with this notion is the observation from our preliminary experiments, demonstrating that ONO-5046, a neutrophil elastase inhibitor (29), significantly inhibited the I/R-induced increase in hepatic microvascular permeability in rats. Thus, it is possible that intravenously administered AT might extravasate at the site where activated neutrophils increase vascular permeability in the liver of animals subjected to hepatic I/R. Consistent with this hypothesis, Koj et al. (34) previously demonstrated that extravasation of AT was observed in rabbits at the site of local inflammation where vascular permeability was significantly increased. These observations suggest that intravenously administered AT might interact with sensory nerve endings at the site of inflammation in the liver of animals subjected to hepatic I/R.

AT-induced increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ were inhibited by L-NAME and indomethacin, but not by 1400W and NS-398 as shown in the present study, suggesting that AT-induced increases in the endothelial production of PGI_2 might involve the activation of endothelial NOS (eNOS) and COX-1 in rats subjected to hepatic I/R. Consistent with this hypothesis is the observation in the present study demonstrating that the expression of iNOS was not observed in the liver of rats given AT. We previously demonstrated that activation of both eNOS and COX-1 was closely involved in sensory neuron-mediated increases in hepatic tissue levels of 6-keto-$PGF_{1\alpha}$ in rats subjected to hepatic I/R (13), supporting the hypothesis that AT increases PGI_2 production by enhancing activation of sensory neurons in rats subjected to hepatic I/R.

Since CGRP and PGI_2 are both potent vasodilators (35, 36), AT-induced increases in both release of CGRP and production of PGI_2 in the liver may increase the hepatic tissue blood flow. Consistent with this hypothesis is the observation in the present study that AT-induced increases in hepatic tissue blood flow after reperfusion were significantly inhibited by pretreatment with CGRP (8–37) and indomethacin. Neutrophils activated by TNF-α might be involved in the development of I/R-induced liver injury through a decrease in hepatic tissue blood flow (37). Since PGI_2 is a potent inhibitor of neutrophil activation (38), AT might increase hepatic tissue blood flow by vasodilation and by inhibition of neutrophil activation through activation of sensory neurons.

In the present study, although AT itself did not increase CGRP release from dorsal root ganglion neurons isolated from adult rats *in vitro*, it significantly enhanced CGRP release from dorsal root ganglion neurons in the presence of capsaicin. Since capsaicin is not an endogenous agonist for the activation of sensory neurons, AT might promote CGRP release in the presence

of other endogenous activators for sensory neurons such as anandamide (33), TNF-α (39) and (H^+) (40) in the liver of rats subjected to hepatic I/R. These observations suggest that AT distributed in the perivascular space in the liver after reperfusion might sensitize the sensory neurons in the presence of some endogenous agonists that are capable of activating the sensory neurons, thereby increasing CGRP release. The detailed molecular mechanism(s) by which AT enhances CGRP release from dorsal root ganglion neurons needs to be elucidated by further investigations.

In contrast to our reports, Oelschlager et al. (41) demonstrated that AT inhibits monocytic TNF-α production directly by inhibiting NF-κB activation *in vitro*. However, anti-inflammatory activity of AT was not observed in rats pretreated with indomethacin in our rat model of sepsis (42, 43) and in rats subjected to hepatic (5) and renal I/R (44), suggesting that AT-induced increases in endothelial production of PGI_2 might play more important roles in the anti-inflammatory properties of AT than its direct anti-inflammatory activity *in vivo*.

We demonstrated that interaction of AT with endothelial surface-glycosaminoglycans was critical for the promotion by AT of endothelial production of PGI_2 in rats (15). However, how interaction of AT with glycosaminoglycans contributes to stimulation by AT of the sensory neurons remains to be determined. Further investigations are necessary to explore more detailed molecular mechanism(s) of the interaction of AT with sensory neurons using dorsal root ganglion neurons *in vitro*.

Taken together, observations in the present study strongly suggested that AT might exert anti-inflammatory activity by enhancing the activation of the sensory nerve-mediated anti-inflammatory mechanism, an important connection between the nervous system and the immune system.

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