Danaparoid sodium reduces ischemia/reperfusion-induced liver injury in rats by attenuating inflammatory responses

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Summary

This study was undertaken to examine the mechanism by which danaparoid sodium (DS), a heparinoid that contains mainly heparan sulfate, prevents reperfusion-induced hepatic damage in a rat model of ischemia/reperfusion (I/R)-induced liver injury. Administration of DS significantly reduced liver injury and inhibited the decrease in hepatic tissue blood flow in rats. DS attenuated hepatic I/R-induced increases in hepatic tissue levels of tumor necrosis factor (TNF) and myeloperoxidase (MPO) *in vivo*. In contrast, neither monocytic TNF production nor neutrophil activation was inhibited by DS *in vitro*. DS enhanced I/R-induced increases in levels of calcitonin-gene related peptide (CGRP), a neuropeptide released from sensory neurons, and of 6-keto-prostaglandin (PG) $F_{1\alpha}$, a stable metabolite of PGI_2, in liver tissues. The therapeutic effects of DS were not seen in animals pretreated with capsazepine, an inhibitor of sensory neuron activation. The distribution of heparan sulfate in the perivascular area was significantly increased by DS administration in this rat model. DS significantly increased CGRP release from isolated rat dorsal root ganglion neurons (DRG) *in vitro*, while DX-9065a, a selective inhibitor of activated factor X, did not. DS enhanced anandamide-induced CGRP release from DRG *in vitro*. These observations strongly suggested that DS might reduce I/R-induced liver injury in rats by attenuating inflammatory responses. These therapeutic effects of DS might be at least partly explained by its enhancement of sensory neuron activation, leading to the increase the endothelial production of PGI_2.

Keywords

Capsaicin-sensitive sensory neurons, Calcitonin-gene related peptide, TNF

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Introduction

Danaparoid sodium (DS), a heparinoid that contains mainly heparan sulfate, has been used in the treatment of disseminated intravascular coagulation (DIC) in Japan (1). Since the anti-Xa /anti-thrombin activity of DS is higher than that of unfractionated heparin (UFH) (2), DS shows a lower frequency of bleeding than UFH in the treatment of DIC (1). The pathologic mechanisms leading to DIC vary depending on the nature of underlying diseases (3). Tumor necrosis factor (TNF), a pro-inflammatory cytokine that is elaborated by monocytes or macrophages, plays a pivotal role in the development of DIC associated with sepsis (4). TNF induces organ failure by activating neutrophils and endothelial cells as well as coagulation abnormalities in patients with sepsis (4). Thus, inhibition of TNF production as well as reduction of coagulation abnormalities might be critical for treating DIC associated with sepsis. However, whether DS inhibits neutrophil activation and TNF production is not known at present.

Ischemia/reperfusion (I/R) is an important pathologic process that leads to hepatic damage following circulatory shock and major hepatic surgery that frequently associates with DIC (5, 6). Pro-inflammatory cytokines such as TNF and other inflammatory mediators released from activated leukocytes play important roles in the development of I/R-induced liver injury (7, 8). Neutrophils activated by TNF have been shown to contribute to the development of I/R-induced liver injury by releasing neutrophil elastase and oxygen free radicals that are capable of damaging endothelial cells (9, 10).

In the present study, we analyzed the effect of DS on I/R-induced liver injury in rats and the mechanisms by which DS reduces I/R-induced liver injury. Since TNF, but not the coagulation abnormality, is critically involved in the development of I/R-induced liver damage in the rat model used in the present study (9), this model is useful to analyze whether anti-coagulants have anti-inflammatory properties.

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Materials and methods

Reagents

DS was kindly provided by Nippon Organon K.K. Co. (Osaka, Japan). DX-9065a, a selective activated factor X (Xa) inhibitor, was kindly provided by Daiichi Pharmaceutical Co. (Tokyo, Japan). Other materials obtained for the experiment included the following: capsaicin, capsazepine (CPZ; the vanilloid receptors antagonist), formyl-methionyl-leucyl-phenylalanine (fMLP), cytochalasin B, and N-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide (Sigma Chemicals Co., St. Louis, MO, USA); Heparin (Novo Nordisk A/G, Genotofte, Denmark); lipopolysaccharide (LPS, *Escherichia coli*, serotype 055:B5, Difco, Detroit, MI, USA); Nyco Prep 1.077 (Nycomed Pharma AS, Oslo, Norway); RPMI 1640 culture medium (Gibco, Gaithersburg, MD, USA). All other reagents were of analytical grade.

Animals

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. All rats were deprived of food, but not of water, for 12 hours (h) before each experiment.

Surgical procedure of hepatic I/R

The partial hepatic (median and left lobes) I/R protocol was performed according to our methods as described previously (10–13). During the period of hepatic ischemia, the animal's abdomen was covered with plastic wrap (Asahi Kasei Co, Osaka, Japan) to prevent dehydration.

Measurement of serum liver enzymes

Blood samples were taken 12 h after reperfusion to measure the level of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as previously described (13). Serum levels of ALT and AST after reperfusion were elevated in sham-operated animals (14). Since the right branches of the portal vein and hepatic artery were ligated in sham-operated animals 1 h after a midline laparotomy, the consequent increase in hepatic blood flow in the median and left lobes might induce a reperfusion-like phenomenon, thereby increasing serum levels of liver enzymes in sham-operated animals.

Measurement of hepatic tissue blood flow

Hepatic tissue blood flow was measured using a laser-Doppler flowmeter (ALF21N, Advance, Tokyo, Japan) 3 h after reperfusion, as described previously (13). The results are expressed as % of pre-ischemia levels.

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

Hepatic 6-keto-$PGF_{1\alpha}$ levels were determined in animals subjected to I/R before and after 1 h of reperfusion according to the methods described previously (11). The results are expressed as ng of 6-keto-$PGF_{1\alpha}$ per g of tissue.

Determination of hepatic levels of TNF

Hepatic levels of TNF were determined by a modification of a previously described method (15). The results are expressed as pg of TNF per g of tissue.

Determination of hepatic myeloperoxidase (MPO) activity

After the indicated period of reperfusion, the livers were quickly removed, and accumulation of leukocytes was assessed by measuring MPO activity in the liver according to a previously described method (16). Results are expressed as units of MPO activity per g tissue.

Immunohistochemical staining of CGRP and heparan sulfate (HS) in the liver

The peroxide-antiperoxide technique was used for immunohistochemical staining of liver tissue preparations with anti-CGRP or anti-HS antibody according to the methods described previously but with slight modifications (17). The unfixed tissue blocks of rat liver were frozen in dry ice-cooled OCT compound (Tissue Tek; Miles, Elkhart, IN, USA). Tissue sections (6–8 μm thick) were mounted on glass slides, immersed in absolute acetone at –20°C for 5 minutes (min), rinsed in phosphate buffered saline (PBS) five times for 5 min each, and then incubated for 20 min with 10% porcine serum in PBS at room temperature. They were incubated for 1 h at 37°C with rabbit anti-rat CGRP or anti-human HS polyclonal antibody at 1:100 dilution. After five rinses in PBS, the sections were treated with horseradish peroxide-conjugated anti-rabbit antibody (MBL Co. Nagoya, Japan) at a 1:2,000 dilution for 1 h at 37°C. Reaction products were developed by immersing the sections in 3'3-diaminobenzidine tetrahydrochloride solution containing 0.03% hydrogen peroxide. As control for immunostaining, non-immune rabbit serum, was used at the first step in place of primary antiserum, and the first step or use of the first antiserum preabsorbed with an excess of the homologous antigen was used. Samples were mounted with Entellan onto glass slides, examined and photographed under light microscope.

DS administration

DS (300 U/kg) was dissolved in normal saline and administered intravenously immediately prior to ischemia induction. Control animals received the same volume of normal saline.

CPZ administration

CPZ was dissolved in 10% Tween 20/10% ethanol (10%) normal saline. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to hepatic ischemia as described previously (18).

Five separate groups of animals were used to assess liver enzyme levels, hepatic tissue blood flow, hepatic 6-keto-$PGF_{1\alpha}$ levels, hepatic TNF levels, and hepatic MPO activity as described earlier. We carefully avoided problems associated with excessive blood loss due to blood sampling or those associated with fluid loss due to blood flow measurement during the experiments.

In-vitro experiments

Preparation of neutrophils from rats

Heparinized venous blood obtained from rats was mixed with an equal volume of 2% dextran solution and allowed to stand for

30 min to permit erythrocyte sedimentation. The supernatant was centrifuged, and the precipitate fraction was collected. Neutrophils were isolated by a Nycodenz gradient method using Nyco Prep 1.077 (19). Contaminated erythrocytes were removed by hemolysis with 0.2% saline for 25 seconds. The resulting preparation, which contained more than 95% neutrophils, was washed twice with phosphate-buffered saline. Cell viability of 95% or higher was confirmed by the trypan blue dye exclusion test. Cells were suspended in phosphate-buffered saline in a volume of 5,000/µl.

Release of neutrophil elastase from neutrophils

The neutrophil suspension (5,000/µl) in phosphate-buffered saline was mixed with 5 µg/ml of fMLP, 5 µg/ml of cytochalasin B, and 2 mM of $CaCl_2$ in the presence or absence of DS. After incubation for 30 min at 37°C, neutrophil suspensions were centrifuged at 5,000 x g for 10 min at 4°C. Neutrophil elastase activity in supernatants was measured using a chromogenic substrate, N-(Methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide, according to a previously described method (20).

Determination of TNF release from mononuclear cells in rats

Isolated rat mononuclear cells (5.0 x 10^5 /ml in RPMI 1640) were stimulated with LPS (100 ng/ml) for 4 h at 37°C in a humidified 5% CO_2 incubator in the presence or absence of various concentrations of DS as described previously (21). Levels of TNF in supernatant fractions were determined using an ELISA kit for rat TNF (Genzyme, Cambridge, MA, USA).

Isolation and culture of dorsal root ganglion (DRG) neurons and measurement of CGRP in culture

DRG neurons from the lumbar, cervical and thoracic region were dissected from rats as described previously (18). In brief, DRG neurons were placed in ice-cold sterile calcium- and magnesium-free Dulbecco's phosphate buffered saline (CMF-Dulbecco's PBS) (Gibco, Grand Island, NY, USA). 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, USA). 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 2,000 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 2,500 x g for 5 min, the resultant pellet was washed twice in serum free Ham's F-12 medium (Hyclone, Logan, UT, USA). Cells were plated on 60-mm polystyrene dishes precoated with Vitrogen (Cohesion Technologies, Palo Alto, CA, USA) 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 Biotechnology, Lake Placid, NY, USA). After 24 h, the culture medium was removed and replaced every 2 days. After 5 days in culture, the medium was gently aspirated, and the cells were washed with serum free Ham's F-12 medium. Cells were incubated with DS (0.1, 1, or 10 U/ml) or DX-9065a (0.1, 1, or 10 µM), and anandamide (10 µM) or DS (1 U/ml) in combination with anandamide (10 µM) for 30 min in Ham's F-12 medium containing 1% SCS without NGF. After incubation, media were sampled and stored at –20°C until assayed for CGRP using a rat CGRP enzyme immunometric assay kit (SPI-bio, Massey Cedex, France).

Statistical analysis

Data are expressed as the mean ± SD. The results were compared using ANOVA followed by Scheffé's post-hoc test. The level of significance was set at $p \leq 0.05$.

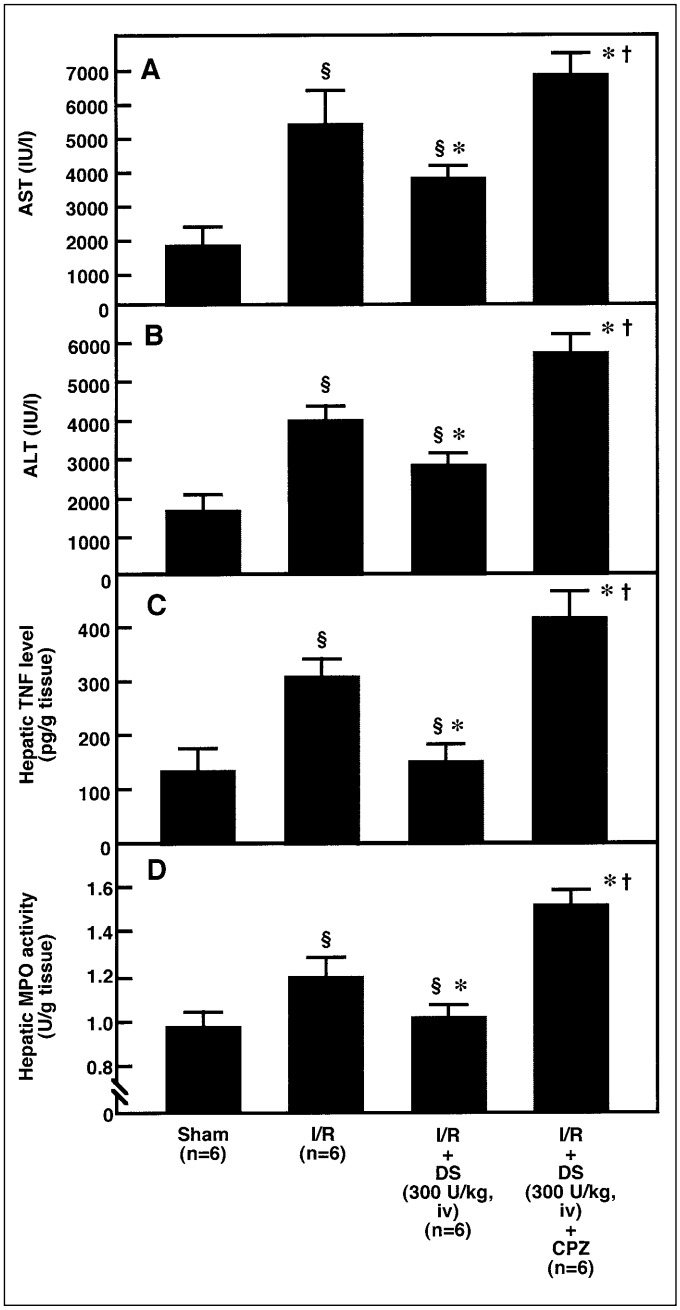

Figure 1: Effect of danaparoid sodium (DS) alone or combined with capsazepine (CPZ) on serum levels of transaminases (A and B), hepatic levels of TNF (C), and hepatic MPO activity (D) in rats subjected to hepatic I/R. DS (300 U/kg) was injected intravenously immediately before hepatic ischemia. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to hepatic ischemia. Each column and bar represent the mean value ± SD. §, $p<0.01$ vs. sham; *, $p<0.01$ vs. I/R; †, $p<0.01$ vs. DS-treated I/R animals.

Results

Effect of DS on serum levels of transaminases and hepatic tissue blood flow in rats subjected to hepatic I/R

Serum levels of AST and ALT were significantly increased after reperfusion compared with those of sham-operated animals, peaking at 12 h after reperfusion (14). Intravenous administration of DS significantly inhibited the I/R-induced increase of transaminases at 12 h after reperfusion at the dose of 300 U/kg, but not at 50 or 150 U/kg (Fig. 1A, B). Hepatic tissue blood flow after hepatic I/R was significantly increased in animals given DS (300 U/kg) compared with control animals (Fig. 2).

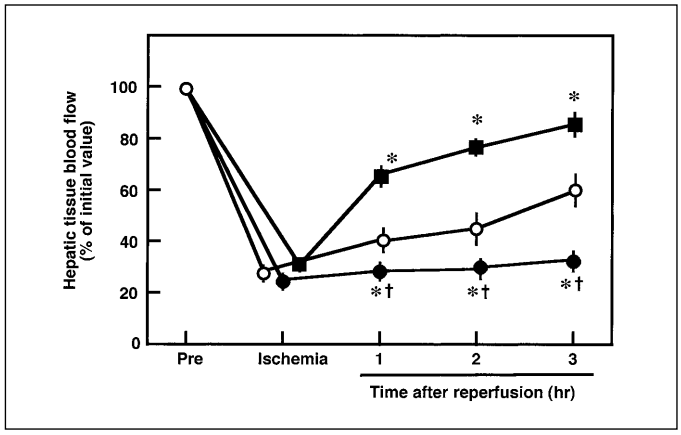

Figure 2: Effect of danaparoid sodium (DS) alone or combined with capsazepine (CPZ) on hepatic tissue blood flow in rats subjected to hepatic I/R. DS (300 U/kg) was injected intravenously immediately before hepatic ischemia. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to hepatic ischemia. Each symbol and bar represent the mean value ± SD from five animals. Open circles; I/R, closed squares; I/R + DS, closed circles; I/R + DS + CPZ. *, p<0.01 vs. I/R; †, p<0.01 vs. DS-treated I/R animals.

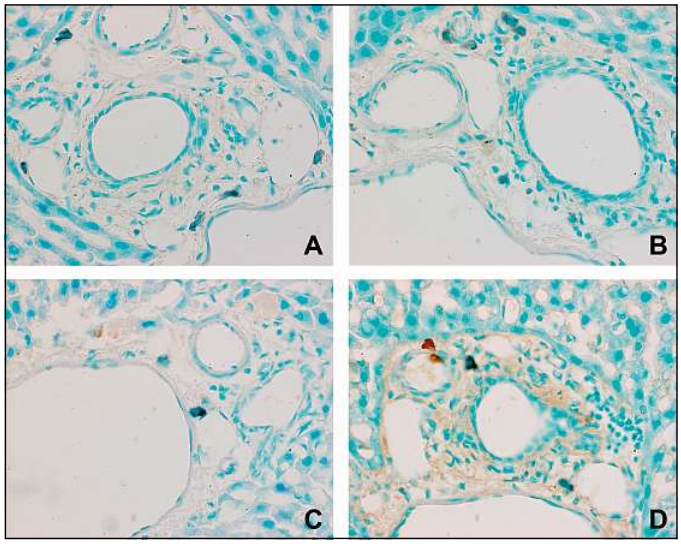

Figure 3: Effect of danaparoid sodium (DS) on immunohistochemical staining of CGRP in the liver of rats subjected to I/R. Immunohistochemical CGRP staining of frozen sections of the liver was performed using anti-rat CGRP antibody in sham-operated animals (A and B) and in I/R animals 1 h after reperfusion (C and D) (original magnification x 100). DS (300 U/kg) was injected intravenously immediately before hepatic ischemia. A, sham-operation; B, sham + DS; C, I/R; D, I/R + DS. Five animals were examined in each group. Typical results are shown.

Effects of DS on I/R-induced increases in hepatic tissue levels of TNF and MPO

Hepatic tissue levels of TNF and MPO were markedly increased after reperfusion, peaking at 1 and 6 h after reperfusion, respectively (13). DS (300 Ukg) significantly inhibited I/R-induced increases of TNF and MPO (Fig. 1C, D).

Effect of DS on neutrophil elastase release from rat neutrohils stimulated with fMLP *in vitro*

DS (0.1, 1, and 10 U/ml) did not inhibit the increase in neutrophil elastase release from rat neutrophis stimulated with fMLP (data not shown).

Effect of DS on TNF production by rat monocytes stimulated with LPS *in vitro*

DS (0.1, 1, and 10 U/ml) did not inhibit the increase in TNF production in rat monocytes stimulated with LPS (data not shown).

Effects of DS on hepatic tissue expression of CGRP and 6-keto-$PGF_{1\alpha}$ in rats subjected to hepatic I/R

Prostacyclin (PGI_2) is a potent inhibitor of both monocytic TNF production and neutrophils activation (22, 23). We previously reported that CGRP, a neuropeptide released from sensory neurons, increased hepatic tissue levels of PGI_2, thereby inhibiting TNF production in rats subjected to hepatic I/R (24). Since DS did not directly inhibit TNF production, it is possible that DS reduces I/R-induced liver damage by increasing hepatic PGI_2 production through the increase of CGRP in rats subjected to hepatic I/R. To examine this possibility, we investigated the effects of DS on I/R-induced increases in hepatic tissue expression of CGRP and 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2, in rats. Hepatic tissue levels of both CGRP and 6-keto-$PGF_{1\alpha}$ were increased after reperfusion, peaking at 1 h after reperfusion (18). Administration of DS to sham-operated animals did not increase hepatic tissue CGRP expression as determined by immunohistochemical staining (Fig. 3A, B), while it significantly increased the expression of CGRP in animals subjected to hepatic I/R at 1 h after reperfusion (Fig. 3C, D). DS significantly enhanced I/R-induced increases in hepatic tissue levels of CGRP and 6-keto-$PGF_{1\alpha}$ at 1 h after reperfusion (Fig. 4).

Effect of DS on the perivascular distribution of heparan sulfate (HS) in rats subjected to hepatic I/R

To know whether administered DS gains access to sensory neurons in the liver, we examined the effect of DS on the perivascular distribution of HS in rats subjected to hepatic I/R. Immunohistochemical staining of HS was slightly increased on the sinusoidal surface after DS administration in sham-operated animals (Fig. 5A, B), while it was significantly increased both on the sinusoidal surface and in the perivascular area of the liver of animals subjected to hepatic I/R (Fig. 5C, D).

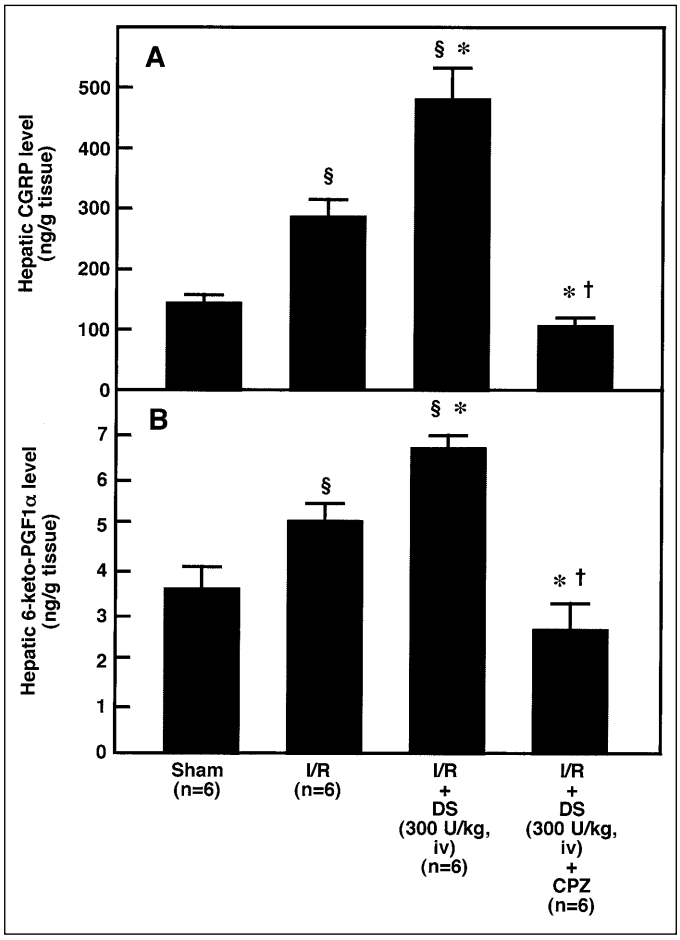

Figure 4: Effect of danaparoid sodium (DS) alone or combined with capsazepine (CPZ) on hepatic tissue levels of CGRP (A) and 6-keto-PGF$_{1\alpha}$ (B) in rats subjected to hepatic I/R. DS (300 U/kg) was injected intravenously immediately before hepatic ischemia. CPZ (15 mg/kg) was injected subcutaneously 30 min prior to hepatic ischemia. Each column and bar represent the mean value ± SD. §, p<0.01 vs. sham; *, p<0.01 vs. I/R; †, p<0.01 vs. DS-treated I/R animals.

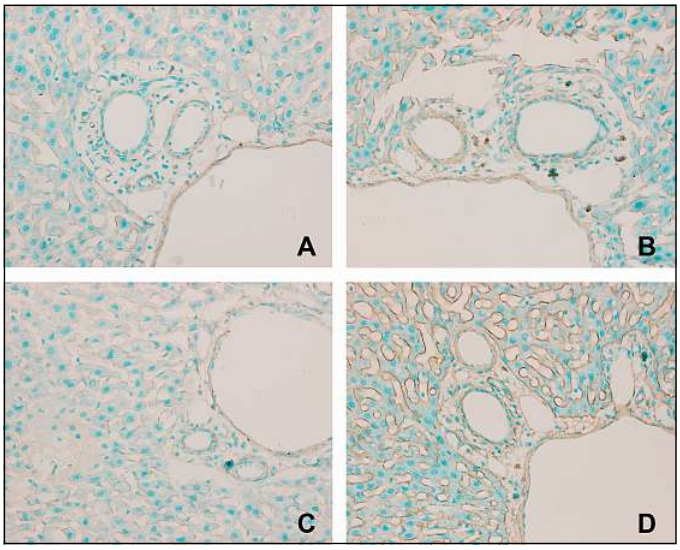

Figure 5: Effect of danaparoid sodium (DS) on immunohistochemical staining of heparan sulfate (HS) in the liver of rats subjected to I/R. Immunohistochemical HS staining of frozen sections of the liver was performed using anti-human HS antibody in sham-operated animals (A and B) and in I/R animals 1 h after reperfusion (C and D) (original magnification x 100). DS (300 U/kg) was injected intravenously immediately before hepatic ischemia. A, sham-operation; B, sham + DS; C, I/R; D, I/R + DS. Five animals were examined in each group. Typical results are shown.

Effect of CPZ pretreatment on therapeutic effects of DS in rats subjected to hepatic I/R

We previously demonstrated that CPZ alone significantly inhibits I/R-induced increase in hepatic levels of CGRP and 6-keto-PGF$_{1\alpha}$, thereby worsened the I/R-induced liver injury by increasing hepatic levels of TNF and MPO in rats (18, 24). To examine whether the therapeutic effects of DS were mediated by sensory neuron activation, we investigated the effect of CPZ, an inhibitor of sensory neuron activation (18), in rats treated with DS after hepatic I/R. As shown in Figure 1, pretreatment with CPZ completely prevented the therapeutic effect of DS on liver injury (Fig. 1A, B), hepatic tissue blood flow (Fig. 2), hepatic inflammatory responses (Fig. 1C, D), and hepatic tissue levels of CGRP and 6-keto PGF$_{1\alpha}$ (Fig. 4) in animals subjected to hepatic I/R, but given DS.

Effect of DS or DX-9065a, and effects of DS and/or anandamide on CGRP release from dorsal root ganglion (DRG) isolated from rats *in vitro*

To determine whether DS directly increases CGRP release from sensory neurons, we measured CGRP released from DRG isolated from rats. As shown in Figure 6, DS itself increased CGRP release from isolated DRG at concentrations of 1 and 10 U/ml. DX-9065a, a specific inhibitor of Xa, at concentrations of 0.5, 1, and 10 μM did not increase CGRP release from isolated DRG (Fig. 6). Anandamide is an endogenous activator of sensory neurons (25) and it significantly increased CGRP release from isolated DRG (Fig. 6B). DS (1 U/ml) significantly enhanced anandamide-induced CGRP release from isolated DRG (Fig. 6).

Discussion

In the present study, we demonstrated that DS reduced I/R-induced liver injury and the associated inflammatory responses including increases in hepatic tissue levels of TNF and MPO. Since anti-rat TNF antibody reduced the liver injury (9), it is likely that DS reduced the I/R-induced liver injury by inhibiting TNF production in the present study. Since neither DEGR-F.Xa, a selective inhibitor of thrombin generation, nor DX-9065a, a selective inhibitor of factor Xa, reduced the liver injury despite their potent anti-coagulant effects in this rat model of hepatic I/R (9), the therapeutic effects of DS cannot be explained by its anti-coagulant effects.

Activated neutrophils play critical roles in the development of I/R-induced liver injury by releasing various inflammatory mediators that are capable of damaging endothelial cells (9, 10). However, DS did not directly inhibit neutrophil elastase release from isolated neutrophils *in vitro*. Since TNF plays a fundamental role in the accumulation of neutrophils at the site of inflammation (26), DS might prevent I/R-induced liver injury by inhibiting neutrophils activation through the inhibition of TNF production. However, DS did not inhibit TNF production by LPS-stimulated

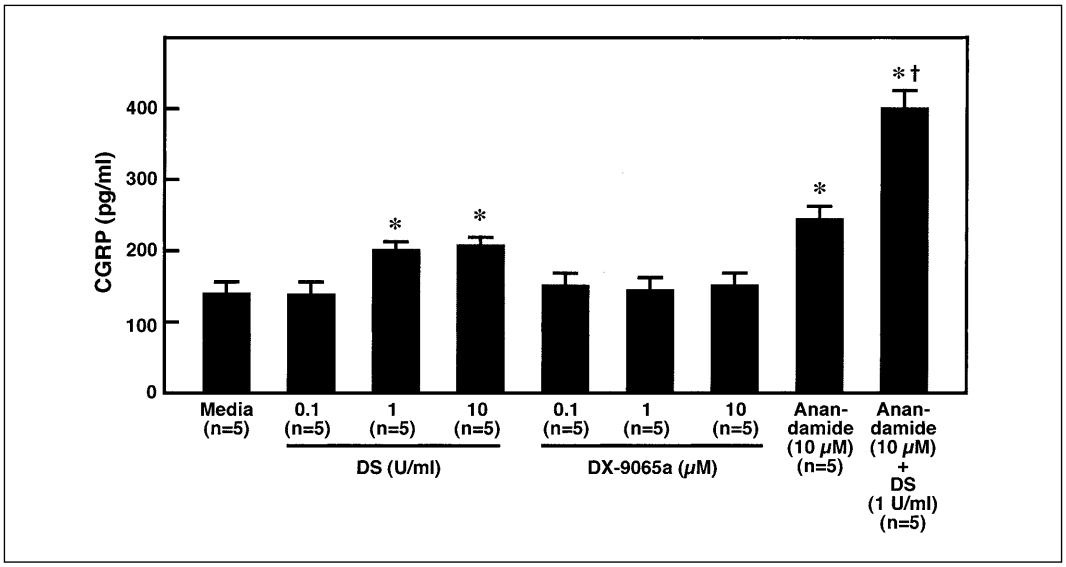

Figure 6: Effect of danaparoid sodium (DS) or DX-9065a and effects of DS and/or anadamide on CGRP release from dorsal root ganglion isolated from rats. Dorsal root ganglion neurons were incubated with DS (0.1, 1, or 10 U/ml) or DX-9065a (0.1, 1, or 10 μM), and DS (1 U/ml) and/or anandamide (10 μM) for 30 min. Supernatants were sampled and CGRP levels were measured by enzyme immunoassay. Each column and bar represent the mean value ± SD from five animals. *, $p<0.01$ vs. media; †, $p<0.01$ vs. anandamide.

monocytes *in vitro*. These observations suggested that DS might not directly inhibit leukocyte activation *in vivo*.

We previously demonstrated that I/R-induced increase in the production of PGI_2 in the hepatic tissue contributed critically to the reduction of liver injury by attenuating inflammatory responses (11). Furthermore, we demonstrated that CGRP released from sensory neurons increases the endothelial production of PGI_2 by activating endothelial nitric oxide synthase and cyclooxygenase-1 in rats subjected to hepatic I/R (24). As shown in the present study, DS significantly enhanced both the hepatic tissue expression of CGRP and the increase of CGRP and 6-keto-$PGF_{1\alpha}$ at 1 h after reperfusion. Furthermore, all the effects of DS observed in animals subjected to hepatic I/R were completely reversed by pretreatment with CPZ, an inhibitor of sensory neurons activation. These observations strongly suggested that DS might reduce I/R-induced liver injury by enhancing sensory neurons activation in rats. Consistent with these in-vivo observations, DS increased CGRP release from DRG *in vitro* as shown in the present study. Since DX-9065a, a specific inhibitor of Xa, did not increase CGRP release from DRG as shown in the present study, DS might increase CGRP release from sensory neurons independent of its anti-Xa activity. Furthermore, DS enhanced anandamide-induced increase in CGRP release from DRG, suggesting that DS might enhance hepatic I/R-induced activation of sensory neurons *in vivo*, thereby reducing I/R-induced liver injury by increasing the endothelial production of PGI_2 in rats. Anandamide activates sensory neurons by phosphorylation of vanilloid receptor-1 through activation of cAMP-dependent protein kinase A (27). Since DS enhanced anandamide-induced increase in CGRP release from sensory neurons as shown in the present study, it is possible that DS enhances the activation of sensory neurons by increasing intracellular levels of cAMP. We are currently investigating this possibility.

Although microthrombus formation is not critically involved in the development of liver injury in rats induced by 60 min-hepatic ischemia and the subsequent reperfusion (9), it contributes to the development of liver injury by enhancing hepatic inflammatory responses when rats were subjected to 120 min-hepatic ischemia and the subsequent reperfusion (28). Thus, anti-coagulant activities of DS might be useful in reducing liver injury when hepatic ischemic period is prolonged to induce microthrombus formation.

References

1. Fujishima Y, Yokota K, Sukamoto T. The effect of danaparoid sodium (danaparoid) on endotoxin-induced experimental disseminated intravascular coagulation (DIC) in rats. Thromb Res 1998; 91: 221–7.

2. Ishida M, Nakada Y, Horiuchi M, et al. Pharmacokinetics of danaparoid sodium, dalteparin sodium and heparin determined by inhibitory effect on the activated coagulation factor X activity after single intravenous administration in rabbits. Arzneimittelforschung 1998; 48: 818–21.

3. Okajima K, Sakamoto Y, Uchiba M. Heterogeneity in the incidence and clinical manifestations of disseminated intravascular coagulation: a study of 204 cases. Am J Hematol 2000; 65: 215–22.

4. Okajima K. Regulation of inflammatory responses by natural anticoagulants. Immunol Rev 2001; 184: 258–74.

5. Hasselgren PO. Prevention and treatment of ischemia of the liver. Surg Gynecol Obstet 1987; 164: 187–96.

6. Keller GA, West MA, Cerra FB, et al. Macrophage-mediated modulation of hepatic function in multi-system failure. J Surg Res 1985; 39: 555–63.

7. Campbell EJ, Senior RM, McDonald JA, et al. Proteolysis by neutrophils. Relative importance of cell-substrate contact and oxidative inactivation of proteinase inhibitors *in vitro*. J Clin Invest 1982; 70: 845–52.

8. Carlos TM, Harlan JM. Leukocyte-endothelial adhesion molecules. Blood 1994; 84: 2068–101.

9. Okajima K, Harada N, Kushimoto S, et al. Role of microthrombus formation in the development of ischemia/reperfusion-induced liver injury in rats. Thromb Haemost 2002; 88: 473–80.

10. Okajima K, Harada N, Uchiba M, et al. Neutrophil elastase contributes to the development of ischemia-reperfusion-induced liver injury by decreasing endothelial production of prostacyclin in rats. Am J Physiol Gastrointest Liver Physiol 2004; 287: G1116–23.

11. Harada N, Okajima K, Kushimoto S, et al. Antithrombin reduces ischemia/reperfusion injury of rat liver by increasing the hepatic level of prostacyclin. Blood 1999; 93: 157–64.

12. Hayashi H, Chaudry IH, Clemens MG, et al. Hepatic ischemia models for determining the effects of ATP-MgCl2 treatment. J Surg Res 1986; 40: 167–75.

13. Harada N, Okajima K, Kushimoto S. Gabexate mesilate, a synthetic protease inhibitor, reduces ischemia/reperfusion injury of rat liver by inhibiting leukocyte activation. Crit Care Med 1999; 27: 1958–64.

14. Kushimoto S, Okajima K, Uchiba M, et al. Role of granulocyte elastase in ischemia/reperfusion injury of rat liver. Crit Care Med 1996; 24: 1908–12.

15. Shito M, Wakabayashi G, Ueda M, et al. Interleukin 1 receptor blockade reduces tumor necrosis factor production, tissue injury, and mortality after hepatic ischemia-reperfusion in the rat. Transplantation 1997; 63: 143–8.
16. Warren JS, Yabroff KR, Remick DG, et al. Tumor necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. J Clin Invest 1989; 84: 1873–82.
17. Akiyoshi H, Gonda T, Terada T. A comparative histochemical and immunohistochemical study of aminergic, cholinergic and peptidergic innervation in rat, hamster, guinea pig, dog and human livers. Liver 1998; 18: 352–9.
18. Harada N, Okajima K, Yuksel M, et al. Contribution of capsaicin-sensitive sensory neurons to antithrombin-induced reduction of ischemia/reperfusion-induced liver injury in rats. Thromb Haemost 2005; 93: 48–56.
19. Watanabe K, Konishi K, Fujioka M, et al. The neutrophil chemoattractant produced by the rat kidney epitheloid cell line NRK-52E is a protein related to the KV/gro protein. J Biol Chem 1989; 264: 19559–63.
20. Nakajima K, Powers JC, Ashe BM, et al. Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha 1-protease inhibitor reactive site. J Biol Chem 1979; 254: 4027–32.
21. Molor-Erdene P, Okajima K, Isobe H, et al. Urinary trypsin inhibitor reduces LPS-induced hypotension by suppressing tumor necrosis factor-α production through inhibition of Egr-1 expression. Am J Physiol Heart Circ Physiol 2005; 288: H1265–71.
22. Eisenhut T, Sinha B, Grottrup-Wolfers E, et al. Prostacyclin analogs suppress the synthesis of tumor necrosis factor-α in LPS-stimulated human peripheral blood mononuclear cells. Immunopharmacology 1993; 26: 259–64.
23. Kainoh M, Imai R, Umetsu T, et al. Prostacyclin and beraprost sodium as suppressors of activated rat polymorphonuclear leukocytes. Biochem Pharmacol 1990; 39: 477–84.
24. Harada N, Okajima K, Uchiba M, et al. Ischemia/reperfusion-induced increase in the hepatic level of prostacyclin is mainly mediated by activation of capsaicin-sensitive sensory neurons in rats. J Lab Clin Med 2002; 139: 218–26.
25. Tognetto M, Amadesi S, Harrison S, et al. Anandamide excites central terminals of dorsal root ganglion neurons via vanilloid receptor-1 activation. J Neurosci 2001; 21: 1104–1109
26. Sedgwick JD, Riminton DS, Cyster JG, et al. Tumor necrosis factor: a master-regulator of leukocyte movement. Immunol Today 2000; 21: 110–3
27. Okajima K, Harada N. Regulation of inflammatory responses by sensory neurons: molecular mechanism (s) and possible therapeutic applications. Curr Med Chem 2006; 13: 2241–51.
28. Okajima K, Harada N, Uchiba M. Microthrombus formation enhances tumor necrosis factor-α production in the development of ischemia/reperfusion-induced liver injury in rats. J Thromb Haemost 2003; 1: 1316–7.