Neutrophil elastase contributes to the development of ischemia-reperfusion-induced liver injury by decreasing endothelial production of prostacyclin in rats

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Because activated neutrophils have been shown to inhibit stress-induced increases in gastric NOS activities in rats (13) and NE has been shown to inhibit the endothelial production of PGI2 in vitro (22, 41), it is possible that activated neutrophils contribute to I/R-induced liver injury by inhibiting the endothelial production of NO and PGI2 through release of NE.

The purpose of the present study was to determine whether NE is involved in the I/R-induced reduction of hepatic tissue blood flow by decreasing endothelial production of NO and PGI2 in rats subjected to hepatic I/R. We investigated the effects of sivelestat sodium hydrochloride (sivelestat) and L-658,758 on I/R-induced changes in both hepatic tissue blood flow and hepatic tissue levels of 6-keto-PGF1α, a stable PGI2 metabolite, using a nonlethal rat model of I/R-induced liver injury. Furthermore, we attempted to determine whether inhibitors of NOS and indomethacin (IM), a nonselective inhibitor of COX, reverses protective effects induced by these two NE inhibitors.

MATERIALS AND METHODS

Materials. Pathogen-free male Wistar rats, weighing 220–280 g, were obtained from Nihon (Hamamatsu, Japan). The neutrophil elastase inhibitors sivelestat (18) and L-658,758 (40) were kindly supplied by Ono Pharmaceutical (Osaka, Japan) and Merck (Rahway, NJ), respectively. Iloprost was kindly provided by Eizai Pharmaceutical...
Nitro-1-arginine methyl ester (l-NAME), a nonselective inhibitor of NOS, aminoguanidine (AG), a selective inhibitor of inducible NOS (iNOS), IM, a nonselective inhibitor of COX, hexadecyltrimethylammonium bromide, and o-dianisidine were obtained from Sigma (St. Louis, MO). LPS (Escherichia coli, serotype 055:B5) was purchased from Difco (Detroit, MI). All other reagents used were of analytic grade.

**Animal model of hepatic I/R.** 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 24 h before each experiment. The hepatic I/R protocol was performed as described previously (8). In brief, after the induction of anesthesia, the liver of each was exposed through a midline laparotomy. 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. The right hepatic lobe was perfused to prevent intestinal congestion. After the period of ischemia, the ligatures around the left branches of the portal vein and hepatic artery were removed. 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 (15). The wound was closed with 3-0 silk. Sham-operated animals were similarly prepared except that no ligature was placed 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. No animals died until 12 h after reperfusion when serum levels of transaminases were determined.

**Administration of sivelestat and L-658,758.** Sivelestat was dissolved in normal saline and administered intravenously in a continuous fashion from the beginning of the ischemic period through 2 h of reperfusion as described previously (21). Control animals received normal saline in the same fashion. L-658,758 was dissolved in normal saline and administered subcutaneously 30 min before hepatic ischemia. Instead, the blood flow to the right lobe of the liver was occluded. No animals died until 12 h after reperfusion when serum levels of transaminases were determined.

**Measurement of hepatic blood flow.** Tissue blood flow at the surface of median lobe of the liver was measured using a laser-Doppler flowmeter (model ALF21; Advance, Tokyo, Japan) for 30 min after the ischemic period until 3 h after reperfusion as described previously (8). The flow is expressed as a percentage of the pres ischemic level.

**Measurement of hepatic tissue levels of 6-keto-PGF1α.** Hepatic 6-keto-PGF1α levels were determined before ischemia and 1, 2, and 3 h after reperfusion according to the methods described previously (8). In brief, the median lobe of the liver was weighed and homogenized in 5 ml 0.1 M phosphate buffer (pH 7.4) at 5°C. The homogenate was first centrifuged at 2,000 g for 10 min to remove minute amounts of solid tissue debris, and the supernatant was then acidified with 1 M HCl. 6-keto-PGF1α was extracted from the supernatant using columns packed with ethyl-bonded silica gel (C2 Ethyl; Amersham, Buckinghamshire, UK). The columns were prepared by washing them with 2 ml methanol, followed by 2 ml distilled water. The acidified supernatant was applied to the column, and this was followed by sequential washes with 5 ml distilled water, 5 ml 10% ethanol, and 5 ml hexane. The elution of the 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 assayed using a specific enzyme immunoassay kit (Amersham). The results are expressed as nanograms per gram of tissue.

**Determination of hepatic tissue levels of TNF-α.** Hepatic levels of TNF-α were determined as described previously (26). At the indicated times after reperfusion, the animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and exsanguinated via the abdominal aorta to separate circulating TNF-α from that in hepatic tissue. In brief, the medial hepatic lobe was weighed and then homogenized in 5 ml 0.1 M phosphate buffer (pH 7.4) containing 0.05% (vol/wt) of sodium azide at 5°C. The homogenate was first centrifuged at 2,000 g for 10 min to remove minute amounts of solid tissue debris. The supernatant was assayed using a rat TNF-α ELISA system (Amersham). This ELISA detects 31–2,500 pg/ml of TNF-α. The results were expressed as picograms per gram of tissue.

**Determination of hepatic tissue levels of cytokine-induced neutrophil chemoattractant.** Cytokine-induced neutrophil chemoattractant (CINC), which was measured in the liver in the present study, is also known as growth-regulated gene product/CINC-1 (29). CINC is produced by both leukocytes and endothelial cells (37). Hepatic levels of CINC were determined as previously described (8).

**Determination of hepatic tissue myeloperoxidase activity.** After the indicated period of reperfusion, the livers were quickly removed and the accumulation of leukocytes was assessed by measuring MPO activity, which reflects the tissue accumulation of neutrophils according to a previously described method (7).

**Measurement of serum levels of liver transaminases.** Blood samples were taken at 12 h after reperfusion to measure the level of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as previously described (8). These blood samples were collected into test tubes from the anesthetized animals via withdrawal from the abdominal aorta using a 22-gauge needle. ALT and AST levels were measured by standard clinical automated analysis, and the results were expressed as international units per liter.

**Determination of hepatic microvascular permeability.** Hepatic microvascular permeability was evaluated by measuring tissue accumulation of intravenously administered albumin using the Evans blue dye method as described previously (28) with some modification (24). In each case, Evans blue (1%, 0.5 ml/rat) was injected intravenously 30 min before the rats were killed. The hepatic vasculature was perfused via the main truncus of portal vein with 20 ml of normal saline. The liver was removed, and the amount of dye was measured as follows. The median lobe of the liver was weighed and put into a tube containing 5 ml dimethylsulfoxamide for 24 h. The concentration of Evans blue extracted in the supernatant was measured in a spectrophotometer (model DU-54; Beckman, Irvine, CA) at a wavelength of 610 nm and compared with the results of standard solutions of known concentrations.

**Monocyte preparation and incubation.** Peripheral blood mononuclear cells were isolated from buffy coats obtained from healthy volunteer blood donors as described previously (34). The cell preparations were >90% monocytes, as determined by May-Giemsa staining. Cell viability was >95%, as determined by trypan blue dye exclusion test. Mononuclear cells were adjusted to an appropriate volume and cultured in RPMI 1640 (Invitrogen, Grand Island, NY) plus 1% supplemented calf serum (Hyclone, Logan, UT) at 37°C in a humidified 5% CO2 incubator. NE inhibitors were added to cells 30 min before LPS (100 ng/ml) stimulation.
Measurement of TNF-α in vitro. Human monocytes (5 × 10⁵ cells/assay) were stimulated with LPS for 4 h in the presence or absence of two NE inhibitors. Concentrations of TNF-α in culture media were determined using an ELISA kit for human TNF-α (Bio-source International, Camarillo, CA). The minimum detectable concentration of TNF-α was 4 pg/ml. The coefficient of variation in this assay method was <3.9%.

**Statistical analysis.** Values are expressed as means ± SD. Data were analyzed using ANOVA followed by multiple-comparison test (Scheffé’s). A P value of <0.05 was considered to indicate a significant difference.

**Results**

Effects of various doses of sivelestat and L-658,758 on changes in serum levels of transaminases after hepatic I/R. Serum levels of ALT and AST began to increase at 1 h after reperfusion, peaking at 12 h after reperfusion in animals subjected to hepatic I/R (26). We examined the effects of various doses of sivelestat (5, 15, and 50 mg·kg⁻¹·h⁻¹ for 3 h) and L-658,758 (1, 3, and 10 mg/kg iv) followed by infusions at various rates (0.5, 1.5, and 5 mg·kg⁻¹·h⁻¹, respectively) on hepatic I/R-induced increases in serum levels of transaminases. Sivelestat and L-658,758 at dosages of 50 mg·kg⁻¹·h⁻¹ and 10 mg/kg, respectively, significantly inhibited these increases in transaminases observed at 12 h after reperfusion (Fig. 1). Therefore, we used these doses of NE inhibitors in the present study.

Effects of sivelestat and L-658,758 on hepatic tissue levels of 6-keto-PGF₁α in rats subjected to hepatic I/R. Hepatic tissue levels of 6-keto-PGF₁α were increased after hepatic ischemia-reperfusion (I/R). Various doses of sivelestat (5, 15, and 50 mg·kg⁻¹·h⁻¹) were administered intravenously in continuous fashions from the beginning of the ischemia period through 2 h of reperfusion. Various doses of L-658,758 (1, 3, and 10 mg/kg) were administered intravenously immediately before ischemia, followed by infusions at various rates (0.5, 1.5, and 5 mg·kg⁻¹·h⁻¹, respectively) through 2 h of reperfusion. Control animals (Cont) received saline instead of neutrophil elastase inhibitors. Serum levels of aspartate aminotransferase (AST; A) and alanine aminotransferase (ALT; B) were measured 12 h after reperfusion. Each bar represents the mean ± SD derived from 5 animal experiments. *P < 0.05 vs. control.

![Fig. 1. Effects of various doses of sivelestat and L-658,758 on serum levels of aminotransferases in rats after hepatic ischemia-reperfusion (I/R). Various doses of sivelestat (5, 15, and 50 mg·kg⁻¹·h⁻¹) were administered intravenously in continuous fashions from the beginning of the ischemia period through 2 h of reperfusion. Various doses of L-658,758 (1, 3, and 10 mg/kg) were administered intravenously immediately before ischemia, followed by infusions at various rates (0.5, 1.5, and 5 mg·kg⁻¹·h⁻¹, respectively) through 2 h of reperfusion. Control animals (Cont) received saline instead of neutrophil elastase inhibitors. Serum levels of aspartate aminotransferase (AST; A) and alanine aminotransferase (ALT; B) were measured 12 h after reperfusion. Each bar represents the mean ± SD derived from 5 animal experiments. *P < 0.05 vs. control.](image)

![Fig. 2. Effects of sivelestat and L-658,758 on changes in hepatic tissue levels of 6-keto-PGF₁α in rats subjected to hepatic I/R. Animals were subjected to 60 min of hepatic ischemia followed by reperfusion as described in MATERIALS AND METHODS. Sham-operated animals were prepared in a similar manner, except that blood flow to the median and left hepatic lobes was not obstructed. Sivelestat was administered intravenously in a continuous fashion at the rate of 50 mg·kg⁻¹·h⁻¹ from the beginning of the ischemia period through 2 h of reperfusion. L-658,758 (10 mg/kg) was administered intravenously immediately before ischemia, followed by an infusion at a rate of 5 mg·kg⁻¹·h⁻¹ through 2 h of reperfusion. Control animals received saline instead of neutrophil elastase inhibitors. Hepatic tissue levels of 6-keto-PGF₁α were determined in sham-operated animals (open bar), in control animals (filled bar), and in those given sivelestat (hatched bar), and L-658,758 (gray bar). Data represent the means ± SD for 5 animals per group. *P < 0.05 vs. sham-operated animals; †P < 0.05 vs. control animals.](image)
Effects of sivelestat and L-658,758 on changes in hepatic microvascular permeability in rats subjected to hepatic I/R. Hepatic microvascular permeability was evaluated by measuring tissue accumulation of intravenously administered albumin using the Evans blue dye method. Hepatic microvascular permeability began to increase at 1 h after reperfusion and progressively increased to 3 h after reperfusion (Fig. 5). These increases were significantly higher than those seen in sham-operated animals (Fig. 5). Administration of sivelestat and L-658,758 inhibited the I/R-induced increases seen at 3 h after reperfusion (Fig. 6).

Effects of indomethacin pretreatment on various hepatic I/R-induced events in animals given sivelestat and L-658,758. Hepatic tissue levels of 6-keto-PGF₁α, after reperfusion in animals pretreated with IM were significantly lower than those seen in animals without IM pretreatment (8). Pretreatment with IM completely reversed inhibitory effects of sivelestat and L-658,758 on the I/R-induced decrease in hepatic tissue blood flow (Fig. 3A). Inhibition of I/R-induced increases in both hepatic inflammatory responses and hepatic microvascular permeability seen in animals given two NE inhibitors were also

Fig. 3. Effects of sivelestat, L-658,758, indomethacin, iloprost, and nitric oxide synthase (NOS) inhibitors on hepatic tissue blood flow in rats subjected to hepatic I/R. Administration of sivelestat and L-658,758 was performed as in Fig. 1. Indomethacin (20 mg/kg) was administered subcutaneously 30 min before ischemia. Iloprost was continuously infused (100 ng·kg⁻¹·min⁻¹) via the right jugular vein. This infusion began before the onset of hepatic ischemia and continued until the rats were killed 3 h after onset of reperfusion. Nitro-L-arginine methyl ester (L-NAME; 5 mg/kg) and aminoguanidine (AG; 40 mg/kg) were dissolved in normal saline and administered subcutaneously 30 min before hepatic ischemia. Hepatic tissue blood flow was measured as described in MATERIALS AND METHODS. A: hepatic tissue blood flow in animals with I/R (controls); I/R + sivelestat; I/R + L-658,758; I/R + indomethacin; I/R + sivelestat + indomethacin; I/R + L-658,758 + indomethacin; I/R + iloprost. Each point represents the mean ± SD from 5 animal experiments. *P < 0.05 vs. controls.

Fig. 4. Effects of sivelestat, L-658,758, indomethacin, iloprost, and NOS inhibitors on hepatic tissue levels of TNF-α, cytokine-induced neutrophil chemoattractant (CINC), and MPO in rats subjected to hepatic I/R. Hepatic tissue levels of TNF-α, CINC, and MPO were increased after reperfusion, peaking at 1, 2, and 6 h after reperfusion, respectively. Administration of sivelestat (S), L-658,758 (L), indomethacin (IM), L-NAME, and AG was performed as in Fig. 3. Iloprost (I) was continuously infused (100 ng·kg⁻¹·min⁻¹) via the right jugular vein as described in MATERIALS AND METHODS. Animals given neither of these neutrophil elastase inhibitors were shown as N. Each bar represents the mean ± SD from 5 animal experiments. *P < 0.05 vs. N; †P < 0.05 vs. S; ‡P < 0.05 vs. L.
completely reversed by IM pretreatment (Figs. 4 and 6). Inhibition of hepatic I/R-induced increases in serum levels of transaminases by these two NE inhibitors was not seen in animals pretreated with IM (Fig. 7).

Effect of iloprost, a stable derivative of PGI2, on various hepatic I/R-induced events. When intravenously injected, iloprost, a stable derivative of PGI2, significantly increased hepatic tissue blood flow after hepatic I/R in rats (8). Both increases in the hepatic microvascular permeability and inflammatory responses including increases in hepatic tissue levels of TNF-α, CINC, and MPO in animals subjected to hepatic I/R were significantly inhibited by administration of iloprost (Figs. 5 and 6). Hepatic I/R-induced increases in serum levels of transaminases seen at 12 h after reperfusion were inhibited in rats given iloprost (Fig. 7).

Effects of the NOS inhibitors, L-NAME, and AG, on protective effects induced by NE inhibitors. Because NO derived from endothelial NOS increases hepatic PGI2 production by activating COX-1 in rats subjected to hepatic I/R, administration of S, L, IM, L-NAME, and AG was performed as in Fig. 3. Animals given neither of these neutrophil elastase inhibitors were shown as N. Serum levels of ALT and AST were measured 12 h after reperfusion. *P < 0.05 vs. N; †P < 0.05 vs. S; ‡P < 0.05 vs. L.

Effects of the NOS inhibitors, L-NAME, and AG, on protective effects induced by NE inhibitors. Because NO derived from endothelial NOS increases hepatic PGI2 production by activating COX-1 in rats subjected to hepatic I/R, administration of S, L, IM, L-NAME, and AG was performed as in Fig. 3. Animals given neither of these neutrophil elastase inhibitors were shown as N. Serum levels of ALT and AST were measured 12 h after reperfusion. *P < 0.05 vs. N; †P < 0.05 vs. S; ‡P < 0.05 vs. L.

Effects of sivelestat and L-658,758 on TNF-α production by monocytes stimulated with LPS in vitro. To determine whether these two NE inhibitors inhibit TNF-α production by monocytes in vitro, we examined effects of sivelestat and L-658,758 on TNF-α production from isolated monocytes stimulated with LPS. L-658,758 at concentrations ranging from 10^-6 to 10^{-3} M did not inhibit TNF-α production by isolated monocytes stimulated with LPS, whereas sivelestat only at a concentration of 10^{-3} M inhibited such production (Fig. 8).
NE inhibited PGI2 production by cultured endothelial cells. By LeRoy et al. (22) and Weksler et al. (40), demonstrating that in vivo observations are in vitro findings reported the accumulation of neutrophils (37). Thus it is possible that PGI2 might contribute to maintenance of tissue blood flow by inhibiting neutrophil activation. Consistent with this hypothesis, these two NE inhibitors inhibited hepatic I/R-induced increases in both microvascular permeability and hepatic tissue levels of TNF-α, CINC, and MPO in the present study. The inhibitory activities of these two NE inhibitors on the decrease in hepatic tissue blood flow and increase in microvascular permeability and inflammatory responses in animals subjected to hepatic I/R were completely abrogated by pretreatment with IM, which inhibits the synthesis of PGI2. Furthermore, iloprost, a stable derivative of PGI2, produced effects similar to those induced by these two NE inhibitors in the present study. Reduction of I/R-induced liver injury by the two NE inhibitors, as evidenced by inhibition of hepatic I/R-induced increases in serum levels of transaminases, was also reversed by IM pretreatment and mimicked by iloprost administration. These observations strongly suggested that effects induced by these two NE inhibitors might be mediated by the action of endogenous PGI2.

In the present study, although NE inhibitors did not affect I/R-induced increases in hepatic tissue levels of 6-keto-PGF1α at 1 h after reperfusion, hepatic tissue blood flow was significantly higher from 1 to 3 h after reperfusion in animals given NE inhibitors than in controls. This finding suggested that NE inhibitors might increase hepatic tissue blood flow at 1 h after reperfusion by mechanism(s) other than inhibition of a decrease in PGI2 production. We previously reported (10) in rats that the I/R-induced increase in endothelial production of PGI2 in the liver seen at 1 h after reperfusion was a consequence of the activation of capsaicin-sensitive sensory neurons. Activation of the sensory neurons increased the release of CGRP from the nerve endings, thereby increasing endothelial production of PGI2 through activation of eNOS (11). Hisanaga et al. (13) reported that FK506, an immunosuppressive agent that is capable of inhibiting TNF-α-induced neutrophil activation (5), reduced water immersion-restraint stress-induced gastric mucosal injury by inhibiting the decrease in gastric NOS activity. These observations strongly suggested that activated neutrophils might be involved in the development of stress-induced gastric mucosal injury in rats by decreasing eNOS activity. Consistent with this hypothesis are the observations in the present study demonstrating that protective effects of NE inhibitors, including the maintenance of hepatic tissue blood flow at 1 h after reperfusion, were completely reversed by l-NAME but not by AG. Thus NE might contribute to induction of I/R-induced liver injury by inhibiting the sensory neuron-mediated increase in endothelial production of NO and PGI2, leading to a decrease in hepatic tissue blood flow.

Pretreatment with IM also reversed the effect of NE inhibitors on hepatic tissue blood flow at 1 h after reperfusion when NO derived from eNOS plays a central role in maintaining the hepatic tissue blood flow, suggesting that PGI2 might contribute to NO production at 1 h after reperfusion. Consistent with this hypothesis is a previous report (2) demonstrating that PGI2 enhanced sensory neuron activation leading to NO production. This might explain why pretreatment with IM reversed the effect of NE inhibitors on hepatic tissue blood flow at 1 h after reperfusion.

In the pathological process leading to I/R-induced liver injury, Kupffer cells were activated at the early phase of reperfusion, and they might damage endothelial cells by generating proinflammatory cytokines such as TNF-α (14, 36).
Thus, if these two NE inhibitors inhibit Kupffer cell activation, they might reduce liver injury by inhibiting TNF-α production. However, this possibility seems less likely, because L-658,758 did not inhibit TNF-α production by isolated monocytes stimulated with LPS, despite its protection against I/R-induced liver injury.

PGI₂ is mainly synthesized in endothelial cells and regulates various physiological processes occurring at the interface between the blood and endothelium (6), suggesting that changes in hepatic tissue levels of 6-keto-PGF₁α, a stable metabolite of PGI₂, determined in liver homogenates might mainly reflect changes in endothelial production of PGI₂. However, Levine (23) demonstrated that rat liver cells were capable of synthesizing PGI₂, suggesting that 6-keto-PGF₁α present in liver homogenates might not necessarily be derived from PGI₂ synthesized by endothelial cells but from that produced by liver cells. However, this possibility seems less likely, because CGRP (8–37), a peptide competing with CGRP for CGRP receptors located on endothelial cells, almost completely reversed I/R-induced increases in hepatic tissue levels of 6-keto-PGF₁α (10). Thus a major part of 6-keto-PGF₁α determined in liver homogenates in the present study might be derived from PGI₂ synthesized by endothelial cells.

We previously demonstrated (26) that anti-rat TNF-α antibody reduced I/R-induced liver injury using this rat model, suggesting that TNF-α plays a causative role in the development of I/R-induced liver injury. Thus inhibition of I/R-induced increases in hepatic tissue levels of TNF-α by NE inhibitors might contribute to reduction of I/R-induced liver injury in the present study. I/R of the liver has been shown to promote TNF-α production by Kupffer cells and circulating monocytes (27, 36). Because we determined tissue levels of TNF-α in liver homogenates in the present study, we could not completely separate TNF-α levels in the circulation from those in the hepatic tissue. Thus the elevation of hepatic tissue TNF-α levels after I/R might be a consequence of the production of TNF-α by both Kupffer cells and circulating monocytes.

TNF-α contributes to the development of I/R-induced liver injury by activating neutrophils and endothelial cells (3, 4). We determined hepatic tissue levels of MPO as an indicator of hepatic accumulation of neutrophils in the present study. Because the liver contains some peroxidases (20), MPO activity might reflect not only neutrophils but also other hepatic peroxidases. We previously reported (9) that increases in hepatic MPO activities were closely correlated with the increase in the number of infiltrated polymorphonuclear leukocytes evaluated by staining using the naphthol AS-D chloroacetate esterase technique. Furthermore, iloprost, which inhibits endothelial adhesion of neutrophils, inhibited the I/R-induced increase in hepatic MPO activity as shown in the present study. These observations strongly suggested that hepatic MPO activity determined in the present study might mainly reflect the hepatic accumulation of neutrophils.

Taken together, observations in the present study strongly suggested that NE inhibitors might be useful in reduction of liver injury seen in clinical settings such as liver transplantation and liver trauma. Because sivelestat is now being used for the treatment of acute respiratory failure associated with the systemic inflammatory response syndrome (32), usefulness of this agent in the treatment of liver injury should also be examined in the clinical setting in the near future.

REFERENCES


