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

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Okajima, Kenji, Naoaki Harada, Mitsuhiro Uchiba, and Masakazu Mori. 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 287: G1116–G1123, 2004. First published July 8, 2004; doi:10.1152/ajpgi.00061.2004.—We previously reported that nitric oxide (NO) derived from endothelial NO synthase (NOS) increased endothelial prostacyclin (PGI2) production in rats subjected to hepatic ischemia-reperfusion (I/R). The present study was undertaken to determine whether neutrophil elastase (NE) decreases endothelial production of PGI2, thereby contributing to the development of I/R-induced liver injury by decreasing hepatic tissue blood flow in rats. Hepatic tissue levels of 6-keto-PGF1α, a stable metabolite of PGI2, were transiently increased and peaked at 1 h after reperfusion, followed by a gradual decrease until 3 h after reperfusion. Sivelestat sodium hydrochloride and L-658,758, two NE inhibitors, reduced I/R-induced liver injury. These substances inhibited the decreases in hepatic tissue levels of 6-keto-PGF1α, at 2 and 3 h after reperfusion but did not affect the levels at 1 h after reperfusion. These NE inhibitors significantly increased hepatic tissue blood flow from 1 to 3 h after reperfusion. Both hepatic I/R-induced increases in the accumulation of neutrophils and the microvascular permeability were inhibited by these two NE inhibitors. Protective effects induced by the two NE inhibitors were completely reversed by pretreatment with nitro-L-arginine methyl ester, an inhibitor of NOS, or indomethacin. Administration of iloprost, a stable derivative of PGI2, produced effects similar to those induced by NE inhibitors. These observations strongly suggest that NE might play a critical role in the development of I/R-induced liver injury by decreasing endothelial production of NO and PGI2, leading to a decrease in hepatic tissue blood flow resulting from inhibition of vasodilation and induction of activated neutrophil-induced microvascular injury.

endothelial cells; tumor necrosis factor; activated leukocytes

ISCHEMIA-REPERFUSION (I/R)-induced liver injury is an important pathological condition that often follows liver surgery, hepatic transplantation, and circulatory shock (12, 19). Activated neutrophils have been shown to be involved in the development of I/R-induced liver injury (15, 16). Activated neutrophils release various inflammatory mediators, such as proteases and reactive oxygen species, that are capable of damaging endothelial cells (30, 38, 39). Neutrophil elastase (NE), a protease released from activated neutrophils, damages endothelial cells because it is most active at neutral pH and has a broad substrate specificity (31, 33). We previously demonstrated that two NE inhibitors (sivelestat sodium hydrochloride, formerly called ONO-5046, and L-658,758) protected the liver from I/R injury in rats (21, 26). Although NE appeared in our previous reports to contribute to I/R-induced liver injury by inducing hepatic microcirculatory disturbance, the precise mechanism remains to be elucidated.

Prostacyclin (PGI2), synthesized in endothelial cells, is an important PG that has been shown to be a powerful vasodilator and a potent inhibitor of neutrophil activation (1, 17, 25). We previously demonstrated that iloprost, a stable derivative of PGI2, protected the liver from I/R-induced liver injury by inhibiting the decrease in hepatic tissue blood flow (8). Therefore, endothelial PGI2 might play an important role in preventing I/R-induced hepatic injury by maintaining proper hepatic microcirculation and inhibition of activated neutrophil-induced endothelial cell injury. We also demonstrated that nitric oxide (NO), a potent vasodilator and an inhibitor of neutrophil activation, derived from endothelial NO synthase (eNOS) increased PGI2 production by activating cyclooxygenase (COX)-1 in rats subjected to hepatic I/R (10).

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 PG12 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 PG12 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 Eisai Pharmaceutical.
Administration of L-NAME, AG, indomethacin, and iloprost.

LNAME (5 mg/kg) and AG (40 mg/kg) were dissolved in normal saline and administered subcutaneously 30 min before hepatic ischemia as described previously (10). IM (20 mg/kg) was suspended in bicarbonate-buffered saline and administered subcutaneously 30 min before ischemia. Control animals received the same volume of bicarbonate-buffered saline instead of IM. Iloprost was dissolved in saline and continuously infused (100 ng/kg) during anesthesia. Control animals received normal saline in the same fashion.

Measurement of hepatic tissue blood flow. Blood flow at the surface of median lobe of the liver was measured using a laser-Doppler flowmeter (model ALF21; Advance, Tokyo, Japan) from 30 min before the ischemic period until 3 h after reperfusion as described previously (8). The flow was expressed as a percentage of the preschismic 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 TNFα. Hepatic levels of TNFα 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 TNFα 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 TNFα ELISA system (Amersham). This ELISA detects 31–2,500 pg/ml of TNFα. The results were expressed as picograms of TNFα 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 dimethylformamide 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 (Biosource 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 tests (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 (Fig. 1). 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 reperfusion, compared with sham-operated animals, peaking at 1 h after reperfusion followed by a gradual decrease until 3 h after reperfusion (Fig. 2). Although administration of sivelestat and L-658,758 did not affect the peak values of hepatic tissue levels of 6-keto-PGF₁α, these NE inhibitors significantly inhibited subsequent decreases in hepatic tissue levels of 6-keto-PGF₁α seen at 2 and 3 h after reperfusion (Fig. 2)

Effects of sivelestat and L-658,758 on changes in hepatic tissue blood flow in rats subjected to hepatic I/R. During hepatic ischemia, hepatic tissue blood flow decreased to ~30% of the preischemia level and then gradually increased to ~60% of the preischemia level at 3 h after reperfusion (Fig. 3A). Administration of sivelestat and L-658,758 significantly increased hepatic tissue blood flow from 1 to 3 h after reperfusion (Fig. 3A).

Effects of sivelestat and L-658,758 on I/R-induced hepatic inflammatory responses. Hepatic inflammatory responses were evaluated in terms of changes in hepatic tissue levels of TNF-α, CINC, and MPO after reperfusion. Hepatic tissue levels of TNF-α, CINC, and MPO were increased after reperfusion, peaking at 1, 2, and 6 h after reperfusion, respectively (26). Administration of sivelestat and L-658,758 significantly inhibited these increases in animals subjected to hepatic I/R (Fig. 4).
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-PGF$_1$α 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
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 (10) and activated neutrophils have been shown to decrease gastric NOS activities in rats subjected to water-immersion restraint stress (8), it is possible that NE inhibits endothelial production of NO as well as PGI2, thereby decreasing hepatic tissue blood flow. Pretreatment with L-NAME reversed the increase in hepatic tissue blood flow in rats subjected to hepatic I/R but treated with NE inhibitors, whereas that with AG did not (Fig. 3B). Inhibition of I/R-induced increases in hepatic inflammatory responses and hepatic microvascular permeability seen in animals given two NE inhibitors was also completely reversed by pretreatment with L-NAME but not by pretreatment with AG (Figs. 5 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 L-NAME (Fig. 7).

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 consistent with these in vivo observations are in vitro findings reported the accumulation of neutrophils (37). Thus it is possible that PGI₂ production of CINC, a member of the IL-8 family that promotes and endothelial cells (4). TNF-α/H9251 inhibitory activities on neutrophil activation and monocytic pros.

**DISCUSSION**

The present study demonstrated that sivelestat and L-658,758, two NE inhibitors, inhibited decreases in both hepatic tissue levels of 6-keto-PGF₁₀α, a stable metabolite of PGI₂, and hepatic tissue blood flow in rats subjected to hepatic I/R, suggesting that NE might contribute to the I/R-induced decrease in hepatic tissue blood flow by inhibiting endothelial production of PGI₂. Consistent with these in vivo observations are in vitro findings reported by LeRoy et al. (22) and Weksler et al. (40), demonstrating that NE inhibited PGI₂ production by cultured endothelial cells. Because PGI₂ is a potent vasodilator (1), inhibition of the decrease in endothelial production of PGI₂ by NE inhibitors might contribute to maintenance of hepatic tissue blood flow in animals subjected to hepatic I/R. In addition to vasodilatory activity, PGI₂ has potent inhibitory activities on neutrophil activation and monocyte production of TNF-α (17, 24), which potently activates neutrophils and endothelial cells (4). TNF-α has been shown to enhance the production of CINC, a member of the IL-8 family that promotes the accumulation of neutrophils (37). Thus it is possible that PGI₂ inhibits both accumulation and activation of neutrophils in inflamed tissue by inhibiting TNF-α production. Because activated neutrophils have been shown to decrease tissue blood flow by increasing microvascular permeability (35), 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-PGF₁₀α 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 tissue levels of TNF-α by NE inhibitors might contribute to the 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


