Protective Effect of S-Nitrosylated $\alpha_1$-Protease Inhibitor on Hepatic Ischemia-Reperfusion Injury

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ABSTRACT

S-Nitrosylated compounds (nitrosothiols; RS-NOs) function as nitric oxide (NO) reservoirs and preserve the antioxidant activities of NO. We found remarkable cytoprotection by an S-nitrosylated protease inhibitor from human plasma, S-nitroso-$\alpha_1$-protease inhibitor (S-NO-$\alpha_1$-PI) that possesses a completely nitrosylated SH group, in hepatic ischemia-reperfusion injuries in rats. Liver ischemia was induced in rats by occluding both the portal vein and hepatic artery for 30 min and was followed by reperfusion. S-NO-$\alpha_1$-PI and control compounds such as native $\alpha_1$-PI, an NO synthase (NOS) inhibitor, and standard RS-NOs were given via the portal vein just after reperfusion was initiated. Liver injury was evaluated by measuring the extracellular release of liver enzymes (aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase). Infiltration of neutrophils and induction of apoptosis and heme oxygenase-1 (HO-1) in the liver were also examined. Maximal liver injury occurred at 3 h after reperfusion and then decreased gradually. Not only did S-NO-$\alpha_1$-PI treatment (0.1 $\mu$mol; 5.3 mg/rat) greatly reduce elevation of liver enzymes in plasma, as well as neutrophil accumulation and apoptotic change in liver, it also improved the impaired hepatic blood flow as assessed by laser Doppler flowmetry and potentiated the induction of HO-1 in the liver. Although native $\alpha_1$-PI moderately reduced liver injury, low molecular weight RS-NOs such as S-nitrosothiolate and S-nitroso-N-acetyl penicillamine produced no obvious protective effect. An NOS inhibitor exacerbated the hepatic ischemia-reperfusion injuries. These results suggest that S-NO-$\alpha_1$-PI exerts a potent cytoprotective effect on ischemia-reperfusion liver injury by maintaining tissue blood flow, inducing HO-1, and suppressing neutrophil-induced liver damage and apoptosis.

Nitric oxide (NO) produced in biological systems mediates a diverse array of physiological functions (Ignarro et al., 1988; Furchgott and Vanhoutte, 1989; Moncada and Higgs, 1993). In the vascular system, NO regulates organ blood flow, inhibits platelet aggregation, and attenuates neutrophil adherence (Ignarro et al., 1988; Kubes et al., 1991; Moncada and Higgs, 1993). A cytoprotective effect of NO has been reported for ischemia-reperfusion injuries in various organs (Tsao et al., 1990; Konorev et al., 1995; Liu et al., 1998; Ohmori et al., 1998; Cottart et al., 1999). Impairment of endothelial NO production may contribute to the pathogenesis of ischemia-reperfusion injuries, because a decrease in NO release can trigger neutrophil adherence and exude into the ischemic area, which exacerbates reperfusion injury.

It was recently proposed that various redox isoforms of NO have critical roles in the diverse physiological and pathophysiological events induced by NO. For example, biological S-nitrosation occurs via one-electron oxidation of NO catalyzed by heavy metal ions, such as copper and iron, and particularly by ceruloplasmin, which is a major multicopper-containing plasma protein in mammals (Inoue et al., 1999; Akaike, 2000). Sulphydryl-containing molecules such as glutathione are particularly susceptible to nitrosation; a nucleophilic attack by NO$^+$ results in S-nitrosothiol adducts (nitrosothiols; RS-NOs) (Stamler et al., 1992; Akaike, 2000). Nitrosothiols may function as endogenous NO reservoirs and preserve the antioxidant activities of NO (Ignarro et al., 1981; Stamler et al., 1992; Gaston et al., 1993; Rauhala et al., 1998).

We recently found that $\alpha_1$-protease inhibitor ($\alpha_1$-PI) from human plasma is readily S-nitrosated under physiological conditions and that its nitrosylation is 10 times more effi-

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ABBREVIATIONS: NO, nitric oxide; RS-NO, nitrosothiol; S-NO-$\alpha_1$-PI, S-nitroso-$\alpha_1$-protease inhibitor; TUNEL, terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end-labeling; NOS, NO synthase; HO-1, heme oxygenase-1; L-NAME, N-nitro-L-arginine methyl ester; GS-NO, S-nitrosothiolate; SNAP, S-nitroso-N-acetyl penicillamine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.
cient than nitrosylation of bovine serum albumin and glutathione (Miyamoto et al., 2000a,b). α₁-PI, which is the most abundant serine protease inhibitor in human plasma (30–60 μM), is known to be an important defense-oriented acute phase protein (Heidtmann and Travis, 1986). α₁-PI (mol. wt., 53,000) has no intramolecular disulfide bridge but does have a single Cys residue at position 232. In our earlier study, α₁-PI was nitrosylated with isomynitrite at this single −SH, yielding 100% S-nitrosylated α₁-PI (S-NO-α₁-PI), which exhibits discrete homogeneity. More importantly, S-NO-α₁-PI has multiple biological functions, including potent antimicrobial activity and inhibition of cysteine protease (Miyamoto et al., 2000a,b). In the present report, we describe a clear protective effect of S-NO-α₁-PI on hepatic ischemia-reperfusion injury in rats.

**Experimental Procedures**

**Materials.** α₁-PI, provided by Chemo-Sero-Therapeutic Institute (Kumamoto, Japan), was purified from human plasma as described previously (Miyamoto et al., 2000a). S-NO-α₁-PI was prepared by S-nitrosation (100%) of a single −SH group of α₁-PI with isomynitrite and was purified to homogeneity by gel filtration column chromatography (Miyamoto et al., 2000a,b). N²-Nitro-I-arginine methyl ester (l-NAME) was purchased from Sigma Chemical Co. (St. Louis, MO). S-Nitrosoglutathione (GS-NO) and S-nitroso-N-acetyl penicillamine (SNAP) were from Dojindo Laboratories (Kumamoto, Japan).

**Animals.** Male Wistar rats were obtained from a commercial supplier (Kyudo, Inc., Kumamoto, Japan). All animals were maintained under standard conditions and were fed water and rodent chow ad libitum. The animals weighed between 200 and 230 g.

**Experimental Protocol.** Guidelines of the Center for Animal Resource and Development, Kumamoto University, were followed for anesthesia during the operative procedure and subsequent postoperative care. The animals were fasted for 9 h before surgery but were allowed access to water. Rats were anesthetized with ether during the operation. After the abdomen was shaved and disinfected with 70% ethanol, a complete midline incision was made. The portal vein and hepatic artery were exposed and cross-clamped for 30 min with a razor blade. The tissue blocks were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) and were immediately frozen in dry ice-acetone. Frozen sections, 6 μm thick, were prepared with a cryostat, and cryosections were air-dried overnight. After fixation in pure acetone for 10 min at room temperature, the cryosections were stained with an antileukocyte monoclonal antibody (Serotec Inc., Raleigh, NC) and were visualized by use of an indirect immunoperoxidase method, with 3,3′-diaminobenzidine as a substrate (Doi et al., 1999). Neutrophil infiltration was also analyzed histochemically with an esterase stain, according to a method reported earlier (Molony et al., 1960).

In addition, apoptotic change in the liver occurring during ischemia-reperfusion was analyzed in situ by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method (Negoescu et al., 1996) with use of an apoptosis detection kit (TACS; Trevigen, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. Briefly, after the sections prepared as just described were fixed with 4% formaldehyde and endogenous peroxidase activity was inhibited, TdT-mediated biotin nick labeling was performed, followed by a streptavidin-labeled peroxidase reaction with TACS Blue Label for blue coloration. In some experiments, the sections were first immunostained with antileukocyte antibody, and then apoptotic cells were detected in situ by the TUNEL method.

The sections were examined by light microscopy; photomicrographs were taken at a magnification of 20×. A total of 100 photomicrographs for each group were subjected to morphometrical analysis. The number of infiltrated neutrophils was then counted and expressed per square millimeter of liver section.

**Detection of Heme Oxygenase-1 mRNA Expression in the Liver after Ischemia-Reperfusion.** Heme oxygenase-1 (HO-1) expression was analyzed by Northern blotting as described previously (Doi et al., 1999). Briefly, liver specimens obtained from animals exanguinated at various time points after reperfusion were frozen quickly in liquid nitrogen and were stored at −80°C until RNA extraction. Total RNA was extracted from the liver after ischemia-reperfusion by using the guanidine thiocyanate lysis method with Trizol reagent (Life Technologies, Gaithersburg, MD). Each RNA sample (20 μg) underwent electrophoresis on agarose gel and was transferred to the Hybond-N nylon membrane, followed by hybridization of a DNA probe for rat HO-1. The DNA probe was radiolabeled by the random primer technique using [³²P]dCTP and the Megaprime labeling system (Amersham Pharmacia Biotech, Buckinghamshire, UK). An HO-1 cDNA fragment of 882 base pairs was used for hybridization as reported previously (Doi et al., 1999), and a cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase was used as a control for gene expression. The HO-1 mRNA signals were quantified by densitometric analysis, after normalization with glyceraldehyde-3-phosphate dehydrogenase mRNA signals, using a Macintosh computer with an image scanner (GT6500; Epson Co., Ltd., Tokyo, Japan) and the public domain IMAGE program (National Institutes of Health).

**Statistical Analysis.** Statistical difference was determined by the two-tailed unpaired t test. Dose-response data were evaluated by two-way ANOVA. A P value of <.05 was considered statistically significant.

**Results**

**Ischemia-Reperfusion Liver Injury Assessed by Measurement of Liver Enzymes in Plasma.** Liver injury in this model was evaluated by measuring the extracellular release of the liver enzymes AST, ALT, and LDH in the plasma. These enzymes increased to a maximum at 3 h after reperfusion and then decreased gradually during 24 h (Fig. 1). When S-NO-α₁-PI was administered just after initiating
reperfusion, the increased levels of both ALT and AST were markedly reduced at almost all time points except for 24 h after reperfusion (Fig. 1A). S-NO-α1-PI treatment also reduced the elevated LDH levels in the plasma at 3 h after reperfusion (Fig. 1B). The elevated liver enzyme activities were lowered by S-NO-α1-PI in a dose-dependent manner, as assessed at 3 h after reperfusion (Fig. 2, A and B). Native α1-PI slightly reduced the levels of all these enzymes (Fig. 2, C and D). However, significant changes in ALT and AST levels were not observed after treatment with the same dose of GS-NO and SNAP (Fig. 3, A and B). L-NAME administration tended to exacerbate the ischemia-reperfusion injury (Fig. 3, A and B). A similar trend of exacerbation of liver damage as assessed by LDH levels was observed for groups treated with either GS-NO or SNAP (Fig. 3B).

To determine whether the protective effect of S-NO-α1-PI

**Fig. 1.** Time profile of changes in serum levels of AST (A), ALT (A), and LDH (B) after hepatic ischemia-reperfusion in rats. Ischemia was induced by occluding both the portal vein and the hepatic artery for 30 min followed by reperfusion. Vehicle (saline) and S-NO-α1-PI (0.1 μmol; 5 mg/rat) were administered via the portal vein immediately after reperfusion was initiated. Data are means ± S.E.M. (n = 5 at each time point). *P < .05, **P < .01, versus the vehicle-treated group.

**Fig. 2.** Effect of various doses of S-NO-α1-PI (A and B) and native α1-PI (C and D) on hepatic ischemia-reperfusion injury in rats. S-NO-α1-PI was given to the animals in a manner similar to the protocol described in Fig. 1. The plasma levels of ALT and AST (A and C) and LDH (B and D) were measured at 3 h after reperfusion was initiated. Each experimental group consisted of five animals. Data are means ± S.E.M. *P < .05, **P < .01, versus the vehicle-treated group.
is due to enhanced bioactivity (or bioavailability) of the protein nitrosothiol or whether it is simply conferred by the additive effect of $\alpha$-PI and nitrosothiol, we examined the dose-responses to GS-NO with or without $\alpha$-PI and S-NO-$\alpha$-PI over the dose-response range shown in Fig. 2. In this experiment, we used only AST values for estimation of the liver injury, because other liver enzymes showed a tendency to increase by the treatment with GS-NO as just described. As demonstrated in Fig. 3C, a strong synergy was found in the hepatoprotective effect of S-NO-$\alpha$-PI compared with that of native $\alpha$-PI plus GS-NO, as evidenced by two-way ANOVA of their dose-response curves ($P < .01$).

These results suggest that NO is beneficial for ischemia-reperfusion injury in the liver, and S-NO-$\alpha$-PI as an NO donor has a remarkable protective effect in the liver. It is notable that simple NO (NO$^+$) donors such as GS-NO and SNAP do not necessarily work well; among various nitrosothiols, S-NO-$\alpha$-PI appears to be exceptionally effective in preventing liver damage.

**Hepatic Tissue Blood Flow.** Hepatic blood flow before and after ischemia-reperfusion was measured by using a laser Doppler flowmeter (Fig. 4). Blood flow decreased immediately after ischemia and did not change during reperfusion; it remained significantly lower in the vehicle-treated group at 30 and 60 min after reperfusion was initiated compared with that before ischemia. In contrast, with S-NO-$\alpha$-PI, the impaired hepatic blood flow almost completely recovered after ischemia-reperfusion: recovery was 95.2% at 30 min and 100.8% at 60 min after reperfusion. GS-NO and SNAP at the same dose did not affect the reduction of hepatic blood flow induced by ischemia-reperfusion. Inhibition of NO production by L-NAME treatment caused a greater decrease in hepatic blood flow after ischemia-reperfusion.

Injection of S-NO-$\alpha$-PI (0.1 $\mu$mol; 5 mg) into rats with...
hepatic ischemia-reperfusion injury produced a transient decline in mean arterial pressure for about 5 min, followed by recovery to the normal blood pressure (data not shown).

Infiltration of Neutrophils and Apoptotic Change in the Liver after Ischemia-Reperfusion. Immunostaining with an antineutrophil antibody revealed a large number of neutrophils in the exudate in the sinusoidal areas of the liver after ischemia-reperfusion: 122.1 ± 24.7/mm² and 114.3 ± 6.9/mm² in vehicle-treated and native β1-PI-treated rats, respectively, at 3 h after reperfusion (Fig. 5). Neutrophil infiltration was also identified by an esterase stain, which revealed a similar distribution of neutrophils in the liver (data not shown). The number of neutrophils increased in a time-dependent manner after reperfusion was initiated, with a peak at 12 h after reperfusion. S-NO-α1-PI treatment remarkably reduced neutrophil accumulation in the liver: to 53.3 ± 7.8/mm² at 3 h after reperfusion and throughout the observation period up to 24 h after ischemia-reperfusion.

The increased number of apoptotic cells caused by ischemia-reperfusion in the liver was clearly observed at 3 h after reperfusion was initiated, remained high until 12 h after reperfusion, and declined thereafter until 24 h (Fig. 6). The time profile of the apoptotic change correlated quite well with that of the neutrophil infiltration. As seen with neutrophil
infiltration in the liver, S-NO-α1-PI treatment led to significant reduction in production of apoptotic cells throughout the course of reperfusion. Native α1-PI, however, caused moderate improvement of apoptotic change in the liver caused by ischemia-reperfusion injury (Fig. 6, C and D). As described above, native α1-PI treatment produced a similar trend of slight reduction liver injury, as assessed by the release of liver enzymes into the plasma (Fig. 3).

Apoptotic cells and neutrophil infiltration were found in completely different locations (Fig. 7). The apoptotic cells appear to be hepatocytes rather than exudate inflammatory cells (neutrophils), on the basis of their morphological and histological properties.

**HO-1 Induction in the Liver after Ischemia-Reperfusion.** HO-1 mRNA transcript was induced as early as 1 h after perfusion and peaked sharply at 3 h. It was sustained at a moderately elevated level for 24 h after reperfusion (Fig. 8A). S-NO-α1-PI administration significantly potentiated HO-1 mRNA induction obtained at 3 h after initiating reperfusion, but native α1-PI had no effect (Fig. 8B). The induction of HO-1 by S-NO-α1-PI was also confirmed by elevation of enzyme activity determined as described previously (Doi et al., 1999) (data not shown). In contrast, GS-NO treatment did not affect HO-1 induction in the liver, at least at the same dose as that of S-NO-α1-PI.

**Discussion**

The data presented here demonstrate that S-NO-α1-PI exerts potent protective effects against hepatic ischemia-reperfusion injury as evidenced by decreased levels of plasma liver enzymes, improvement of impaired of hepatic blood flow, inhibition of neutrophil infiltration and apoptosis, and enhanced induction of HO-1 in the liver after ischemia-reperfusion.

Local excessive formation of NO has pro-inflammatory effects such as edema formation due to enhanced vascular permeability, inflammatory cell infiltration, and cytotoxicity (possibly through its conversion to peroxynitrite and other reactive nitrogen oxides) (Beckman and Koppenol, 1996; Rubbo et al., 1996; Akaike and Maeda, 2000). However, NO also has completely opposite physiological functions, i.e., anti-inflammatory activities, such as inhibition of platelet aggregation and of neutrophil adherence to endothelium, anti-apoptotic effects, and inhibition of lipid peroxidation (Kubes et al., 1991; De Caterina et al., 1995; Rubbo et al., 1996; Ogura et al., 1997; Mannick et al., 1999). A number of reports show a beneficial effect of NO on hepatic microcirculation and liver injury as evidenced by cytoprotective effects of NO donors and detrimental consequences of NOS inhibition (Liu et al., 1998; Ohmori et al., 1998; Cottart et al., 1999). The present experiments support these earlier observations, because the novel NO donor S-NO-α1-PI had a remarkable ameliorating effect on such injury and the NOS inhibitor L-NAME exacerbated the injury.

Typical biological actions of NO include vasodilatation
vascular smooth muscle relaxation) and inhibition of platelet aggregation, which are mediated partly through elevation of intracellular cGMP produced by soluble guanylate cyclase activated by NO (Rapoport and Mura, 1983; Furchgott and Vanhoutte, 1989; Radomski et al., 1990; Moncada and Higgs, 1993; Ignarro et al., 1988). Some part of the endothelium-dependent vasodilation and inhibition of platelet aggregation is reported to be cGMP-independent (Bolotina et al., 1994; Pawloski et al., 1998). In addition, NO has inhibitory effects on endothelium-neutrophil interaction, partly via NF-kB-mediated down-regulation of the expression of adhesion molecules on both endothelium and neutrophils (Kubes et al., 1991; Gauthier et al., 1994; De Caterina et al., 1995; Liu et al., 1998) and via suppression of cytokine production by inflammatory cells (De Caterina et al., 1995). Also, NO causes hepatic sinusoidal dilatation and improves liver microcirculation by altering the morphofunctional activity of fat-storing (Ito cells (Kawada et al., 1993). In this context, the potent therapeutic effect of S-NO-α1-PI on hepatic ischemia-reperfusion injury might be produced by NO supplied to vascular systems to prevent neutrophil-mediated endothelial damage and sustain microcirculation in the liver. In fact, only a small amount of NO, as low as a nanomolar concentration, was produced in our ischemia-reperfusion model, as identified in an ex vivo perfusion system of the liver with ischemia-reperfusion injury (data not shown). However, given the profound effect of S-NO-α1-PI on the microcirculation in the liver, the reduced neutrophil accumulation may be a consequence of the improved microcirculation and not a direct effect of NO.

It is well documented that the inflammatory response involving neutrophil infiltration is generally elicited during reperfusion of various ischemic organs and that neutrophils are the major effector cells contributing to tissue injury in ischemia-reperfusion (Jordan et al., 1999; Lentsch et al., 1999). These neutrophils can induce reperfusion injury of endothelial cells and hepatocytes by releasing a variety of cytotoxic substances, including proteases such as neutrophil elastase and matrix metalloproteases, cytokines, leukotrienes, cationic proteins, and reactive oxygen and nitrogen species, all of which can cause tissue damage (Beckman and Koppenol, 1996; Rubbo et al., 1996; Akaike and Maeda, 2000), apparently contradictory but important function of NO is its antioxidant potential, observed, for example, in lipid peroxidation (Rubbo et al., 1996) and iron-overload-induced neurotoxicity (Rauhala et al., 1998). Nitrosothiol formation may be critically involved in such antioxidant and cytoprotective actions of NO (Konorev et al., 1995; Rauhala et al., 1998; Akaike, 2000): NO is protected from reaction with superoxide after thiol-adduct formation of NO, and thus production of the potent cytotoxic peroxynitrite is attenuated. It is thought that S-NO-α1-PI could act as a nitroso donor rather than a pure NO donor after in vivo administration. The cytoprotective effect of S-NO-α1-PI obtained in the present study, therefore, might also be attributable to antioxidant activity of nitrosothiols introduced by S-NO-α1-PI.

Another important finding is that administration of S-NO-α1-PI potentiated induction of HO-1 in the liver after ischemia-reperfusion; its effect was greater that that of vehicle and native α1-PI. Lancaster’s group reported that pretreatment of rat hepatocytes with a low dose of NO donor conferred resistance to oxidative damage of the cell, possibly through induction of HO-1 (Kim et al., 1995). We confirmed a similar cytoprotective function of HO-1 in our recent in vivo study using a solid tumor model (rat hepatoma cells, AH136B) (Doi et al., 1999). HO-1, which catalyzes the conversion of heme to biliverdin and CO, has been shown to be constitutively expressed in the liver and spleen (Maires, 1997). Furthermore, HO-1 is readily induced by heme compounds, heavy metals, UV irradiation, and various oxidative stresses (Kim et al., 1995; Maires, 1997). One possible mechanism of the protective effect of HO-1 induction is thought to be the antioxidant action of bilirubin (Doré et al., 1999), which is generated by reduction of biliverdin, a product of the enzymatic reaction of HO-1 using heme as a substrate. In addition, ferritin, whose expression was triggered by iron release during heme breakdown catalyzed by HO-1, has been proposed to protect against oxidative stress by sequestering iron (Kim et al., 1995). On the basis of these findings, the beneficial effect of S-NO-α1-PI on ischemia-reperfusion injury of the liver seems to result from its direct antioxidant activity and indirectly induced antioxidant levels produced by HO-1 in the liver after ischemia-reperfusion injury.

In conclusion, the present results suggest that S-NO-α1-PI has a potent cytoprotective effect on hepatic ischemia-reperfusion injury, possibly by maintaining tissue blood flow, suppressing neutrophil-induced liver damage, and inducing HO-1. The beneficial effect of S-NO-α1-PI was found to be far superior to that of other nitrosothiols such as GS-NO, SNAP, and S-NO-albumin (data not shown), and thus the multiple biological activities of S-NO-α1-PI (protease inhibitory and nitrosothiol-mediated actions) may contribute to its potent cytoprotective activity. Another advantage of using S-NO-α1-PI as an NO donor is that S-NO-α1-PI can be readily formed from the endogenous α1-PI that exists at a high level in human plasma, so that clinical application of this compound should be more feasible than that of other synthetic NO donors.

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References


