LIVER I/R INJURY IS IMPROVED BY THE ARGINASE INHIBITOR, N-OMEGA-HYDROXY-NOR-L-ARGININE (NOR-NOHA)

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ABSTRACT

Liver ischemia reperfusion (I/R) injury is associated with profound arginine depletion due to arginase release from injured hepatocytes. The purpose of this study was to determine if arginase inhibition with N^\text{\underline{o}}\text{-Hydroxy-nor-L-arginine} (nor-NOHA) would increase circulating arginine levels and decrease hepatic damage during liver I/R injury. The effects of nor-NOHA were initially tested in normal animals to determine in-vivo toxicity. In the second series of experiments, orthotopic syngeneic liver transplantation (OLT) was performed after 18 hour cold ischemia time (CIT) in Lewis rats. Animals were given nor-NOHA (100 mg/kg) or saline prior to and after graft reperfusion. In normal animals treated with nor-NOHA, there were no histopathologic changes to organs, liver enzymes, serum creatinine, or body weight. In the OLT model, animals treated with saline exhibited markedly elevated serum transaminases and circulating arginase protein levels. Nor-NOHA administration blunted the increase in serum arginase activity by 80%, and preserved serum arginine levels at 3 hours after OLT. Nor-NOHA treatment reduced post-OLT serum liver enzyme release by 50%. Liver histology (degree of necrosis) in nor-NOHA – treated animals was markedly improved compared to the saline-treated group. Furthermore, use of the arginase inhibitor nor-NOHA did not influence polyamine synthesis due to the decrease in ornithine levels. Arginase blockade represents a potentially novel strategy to combat hepatic I/R injury associated with liver transplantation.

Keywords: Liver transplantation, Arginine, Nitric Oxide, Preservation Injury
INTRODUCTION

Ischemia reperfusion (I/R) injury is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery to the compromised tissue. Transient episodes of hepatic ischemia occur during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes both direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damage resulting from activation of inflammatory pathways (3; 6; 8; 21; 26; 29). The injury that results from I/R after liver transplantation contributes to primary nonfunction in approximately 5-10% of liver grafts and delayed graft function in 15-30% of cases (9; 30).

Nitric oxide (NO) is known to have an important role in regulating liver physiology and blood flow. NO and citrulline are produced by the family nitric oxide synthases (NOS) from the substrate L-arginine(32). NO has been shown to exert protective effects in the liver by improving blood flow, antagonizing neutrophil activation and adhesion, neutralizing free radical injury, and eliciting anti-apoptotic effects (19; 23). The beneficial effects of the L-arginine-NO pathway have also been reported in liver transplantation models. Experiments using arginine supplementation and NO donors have shown that NO serves to improve liver ischemia injury, cardiac output, and hepatic blood flow after liver transplantation (7; 9; 15; 24; 27; 35). In addition, when NOS inhibitors were given in the post-liver transplant setting, the hepatic damage worsened, indicating the beneficial role of L-arginine/NO synthase pathway (13; 34; 35). Recently,
we reported that donor graft adenoviral iNOS gene transfer ameliorated liver transplant preservation injury and improved survival after prolonged graft cold storage (10).

Liver transplantation results in a profound depletion of serum arginine due to a massive release of arginase-I from injured liver parenchymal cells (25; 34). Arginase-I, found primarily in the cytosol of liver parenchymal cells, catabolizes the hydrolysis of L-arginine to produce L-ornithine and urea. With any traumatic event, including liver I/R injury, arginase is released into the bloodstream and acts to deplete arginine while increasing ornithine production (14; 20). While L-arginine supplementation in I/R models improves liver injury, the arginine depletion from the release of arginase is not completely reversed (2; 34). Moreover, Tenu et al. has previously reported that it would be more efficient to inhibit arginase than to provide supplemental L-arginine to support NO synthesis (28). Therefore, we hypothesized that an arginase inhibitor would increase arginine availability and improve liver injury by inhibiting the activity of circulating arginase in the transplanted animals. The naturally occurring arginase inhibitor is n-omega-Hydroxy-L-arginine (NOHA). It is produced as an intermediary step when L-arginine is metabolized by the NOS enzymes and is also a direct substrate for NOS (1; 16). Recently, a more potent synthetic analogue of NOHA was identified, n-omega-Hydroxy-nor-L-arginine (Nor-NOHA) (5; 16). This compound increases L-arginine availability by arginase blockade, but does not directly increase NO or citrulline production as it is not a substrate for NOS (16). We chose to utilize this compound because of its low IC₅₀, and due to the fact that it does not interfere with NOS activity when compared to NOHA (16; 33). In this study, we demonstrate that inhibition of
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arginase activity with nor-NOHA restores arginine availability and results in decreased hepatic injury in the liver transplant setting.

**MATERIALS AND METHODS**

*Materials.* \(\text{N}^\text{G}\)-omega-hydroxy-nor-L arginine was purchased from Bachem Bioscience, Inc. King of Prussia, PA.

*Orthotopic liver transplantation.* Male Lewis rats (LEW, RT1), weighing 220-320 gm were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained in a laminar-flow, specific-pathogen-free atmosphere at the University of Pittsburgh. The basic techniques of liver harvesting and orthotopic transplantation of syngeneic grafts (LEW to LEW) without hepatic artery reconstruction were performed according to the method previously described by Kamada (11). The donor liver was flushed in situ with 20 ml University of Wisconsin (UW) solution through the aorta, followed by infusion from the hepatic artery (2 ml) and portal vein (4 ml). The donor liver was kept in a bath of UW solution at 4°C for a total preservation period of 18 hours. Livers were flushed with cold lactated ringer’s solution immediately prior to transplantation to syngeneic recipients.

*Experimental design.* Nor-NOHA (100 mg/kg) was given to normal animals by intravenous penile vein injection 15 minutes prior to arginase injection. For the liver transplant group, nor-NOHA (100 mg/kg) was given by intravenous penile vein injection to recipient animals immediately prior to hepatectomy and 15 minutes after graft reperfusion. Control animals received intravenous saline injections at matched time
points. Animals were sacrificed at 3, 6 and 24 hours after transplantation for serum and tissue samples.

**Sodium Dodecyl Sulfate/Polyacrylamide Agrose Gel Electrophoresis.** Cytosolic liver proteins were prepared as described (18) and quantitated with bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL). Western blot analysis for arginase I protein was performed using 50 µg of serum protein on 13% SDS-PAGE as described (18). Primary polyclonal chicken antibody to rat arginase I (1:50,000; a generous gift from the Sidney M. Morris Jr., PhD, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine) and secondary peroxidase-conjugated rabbit anti-chicken IgY (1:5,000; Jackson ImmunoResearch Laboratories, West Grove, PA) were used for the arginase I Western blot. Membranes were developed with the Super Signal West Pico chemiluminescent kit (Pierce, Rockford, IL) and exposed to film.

**Arginase Activity Assay.** L-arginine is hydrolyzed to ornithine and urea. Based on this principle, a colorimetric assay in which ornithine gives a color product with ninhydrin. Arginase activity is defined as the amount of Mn$^{2+}$ activated enzyme that produces 1µmol of ornithine /min at 37°C (4; 12)

**Histopathology.** Formalin fixed liver samples were embedded in paraffin and cut to 6 µm thick sections. Tissues were stained with hematoxylin and eosin and slides were assessed for inflammation and tissue damage.

**Liver function tests.** To assess hepatic function and cellular injury following rat liver transplantation, serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) levels were measured using the Opera Clinical Chemistry
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System (Bayer Co., Tarry Town, NY).

**Serum Arginine Analysis.** To assess serum amino acid levels following rat liver transplantation, serum samples were deproteinized using 1.5M HClO₄ and 2M K₂CO₃. Arginine, ornithine, citrulline, and polyamine levels were measured using HPLC analysis as previously described (31). To assess tissue arginine levels following rat liver transplantation, 250 mg of tissue was homogenized in 1.5M HClO₄ and neutralized with 2M K₂CO₃. The extracts were used for amino acid analysis by HPLC (31).

**NO Synthesis.** NO production was determined by measuring serum nitrite and nitrate levels using a commercially available kit (Oxford Biomedical Research Inc., Oxford, MI).

**Statistical analysis.** Results of serum liver injury tests are expressed as the mean ± standard error of the mean (SEM). Group comparisons were performed using the student's t-test or ANOVA. Differences were considered significant at p<0.05.
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RESULTS

Arginase activity peaks three hours after graft reperfusion. To confirm that arginase is released into the serum after liver transplantation, western blot was performed for arginase I protein on serum obtained from animals after OLT. Arginase I protein was not detected in normal serum, and was markedly increased at 3 hrs after OLT (Figure 1A). This was associated with a dramatic fall in serum arginine concentrations and an associated 4-fold increase in serum ornithine levels at 3 hrs after transplant (Figure 1A).

To show that the serum arginine depletion was due to increased serum arginase activity, serum arginase assay was performed from 1- 48 hrs after transplant. After liver graft reperfusion, arginase activity in the serum peaked at 3 hours after transplant and remained elevated until 48 hours where its activity began to decrease (Figure 1B).

Nor-NOHA does not exhibit toxicity in normal animals. To determine if the arginase inhibitor nor-NOHA exhibited in vivo toxicity, normal animals were injected with 400-800 mg/kg of nor-NOHA intravenously. There were no changes observed in serum creatinine (mg/dL), AST, ALT (IU/L), or body weight in the animals (Table I). Gross and histological analysis of liver, heart, lung, kidney, spleen and pancreas did not reveal any structural abnormalities (data not shown). Further, the animals did not show any subjective signs of distress.

Nor-NOHA reverses arginase-induced arginine depletion in normal animals. To determine the ability of nor-NOHA to inhibit arginase activity in vivo, animals were injected with exogenous arginase protein to mimic the hepatic arginase release that
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occurs in the post-transplant setting. Systemic injection of arginase rapidly produced a marked depletion in serum arginine concentration (Figure 2). Arginine levels remained low for 1-3 hrs after arginase injection, and then returned to baseline by 24 hrs. In those animals treated with nor-NOHA and arginase, arginine levels were only partially depleted at 1 hr, and were completely restored by 3 hrs after arginase injection (Figure 2). There was no change in arginine levels when nor-NOHA was given alone. These results indicate that nor-NOHA is able to counteract exogenous arginase-mediated depletion of circulating arginine.

Nor-NOHA reverses arginine depletion in transplanted animals. Since hepatocellular arginase release depleted serum arginine after liver transplantation, we injected nor-NOHA (100 mg/kg) in recipient animals immediately prior to hepatectomy and 15 minutes after graft reperfusion. Control animals received intravenous saline injections at matched time points. Saline-treated control animals showed marked elevation of serum arginase activity 3 hrs after transplant while nor-NOHA treated animals demonstrated significantly lower serum arginase activity (Figure 3A). Nor-NOHA treatment also resulted in a ~10-fold increase in circulating serum arginine (Figure 3B). The beneficial effect of nor-NOHA treatment on arginase activity was diminished by 6 hrs, suggesting that repeat dosing may be required due to the relatively short-term effect of nor-NOHA. The blunted arginase activity seen in nor-NOHA treated animals at 3 hrs did result in a significant rise in serum arginine concentration compared to saline-injected animals (Figure 3B). The nor-NOHA induced partial restoration of serum arginine towards
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normal levels was lost at 6 hrs and is consistent with the elevated arginase activity at this time point.

*Liver injury is improved in transplanted animals treated with nor-NOHA.* To determine the effects of nor-NOHA on liver injury after transplantation, we analyzed serum transaminases and graft histology for saline- and nor-NOHA treated animals at 3, 6 and 24 hours after reperfusion. The nor-NOHA treated animals had a marked improvement in liver damage with serum transaminases showing a 50% reduction compared to saline treated animals (*Figure 4*). Liver histology confirmed this observation with absent to very minimal necrosis in the nor-NOHA-treated animals (*Figure 5*).

*Serum arginase protein levels are decreased in transplanted animals treated with nor-NOHA.* Since nor-NOHA treatment during liver transplantation partially inhibited circulating serum arginase activity leading to increased arginine levels and less liver graft injury, we next sought to determine the effect of nor-NOHA on serum arginase protein levels. In the saline-treated control animals, serum arginase protein is detected as early as three hours after reperfusion and remains elevated up to 24 hrs after transplant (*Figure 6*). Animals treated with nor-NOHA exhibited minimal arginase release at 3 hrs after transplant, and showed significantly decreased levels at 6 and 24 hours after reperfusion when compared to saline-injected controls (*Figure 6*). These results suggest that nor-NOHA reduces liver graft injury leading to decreased hepatocellular arginase release.
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*Nor-NOHA does not change global NO synthesis post-transplant.*

To determine the effect of arginase blockade on NO synthesis, serum nitrite+nitrate levels were measured with saline or nor-NOHA injection in control animals and 6 hrs after transplant (Table 2). Although arginase blockade increased arginine availability, it does not appear that there is a significant difference in total NO production at the 6 hr time point when comparing transplant alone to transplant+nor-NOHA. It is possible that any change in eNOS-derived NO synthesis is masked by any small change in iNOS levels.

*Nor-NOHA does not affect polyamine synthesis post-transplant.*

A potential adverse effect of nor-NOHA treatment is altered polyamine synthesis due to effective arginase inhibition and decreased ornithine levels. We thus measured serum polyamine levels in saline and nor-NOHA treated animals using HPLC. We did not observe any significant effect of nor-NOHA on polyamine synthesis as measured by serum putrescine, spermidine, or spermine levels (*Figure 7*).

**DISCUSSION**

The damage to the liver caused by I/R continues to be an important limiting factor in many clinical settings such as liver surgery, transplantation, and low flow states. Liver I/R injury is associated with profound arginine depletion due to arginase release from injured hepatocytes. The depletion of arginine has been shown to decrease tissue arginine availability with subsequent down regulation of NO (17; 25). Since the L-arginine/NOS pathway has been recognized to play critical roles during inflammation, the purpose of this study was to determine if arginase inhibition with nor-NOHA would increase
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circulating arginine levels and decrease hepatic damage during liver I/R injury. The major and novel findings of this investigation are: 1) arginase protein levels and enzyme activity are increased after OLT in rats; 2) treatment of animals with nor-NOHA partially restored serum arginine levels and resulted in a marked improvement in liver damage after OLT; 3) there was no significant effect of nor-NOHA on polyamine synthesis as a result of arginase inhibition.

The L-arginine/nitric oxide synthase (NOS) pathway has been recognized to play important roles during infection, inflammation, organ injury, and transplant rejection. NO is known to exert protective effects after I/R by improving blood flow, antagonizing neutrophil activation and adhesion, neutralizing free radical injury and eliciting anti-apoptotic effects (19; 23). We have previously shown that supplemental arginine, the substrate for NOS, improves hepatic injury after OLT (34). However, although arginine supplementation provided a modest improvement in liver damage, we did not observe any appreciable increases in circulating arginine levels. The lack of response to supplemental arginine was most likely due to the presence of high levels of arginase protein released from injured hepatocytes after I/R that counteracted our attempts to increase serum arginine levels.

In our current work, we describe a novel method of decreasing liver I/R injury by using a potent inhibitor of arginase, nor-NOHA. We hypothesized that the mechanism of protection with nor-NOHA against liver I/R injury is its effect on the conversion of arginine to NO. In animals treated with nor-NOHA, circulating levels of arginine are increased while ornithine, a product of arginase enzyme activity, is decreased. This suggests that nor-NOHA treatment results in the preferential use of arginine by NOS.
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instead of arginase. However, treatment of animals with nor-NOHA only resulted in decreased arginase activity associated with increased serum arginine levels 3 hrs after transplantation. The beneficial effect of nor-NOHA treatment on arginase activity was diminished by 6 hrs, suggesting that nor-NOHA may have a relatively short window of activity. An interesting next step would be to analyze the effect of nor-NOHA on liver I/R injury in conjunction with exogenous arginine delivery. In a study by Pastor et al, intracellular arginine alone was not sufficient to achieve maximal NO production by NOS. However, with exogenous arginine delivery, NO production could be further increased (22). We speculate that the best strategy to increase arginine levels may be to combine arginase blockade with low-dose arginine supplement, especially if nor-NOHA treatment is unable to completely inhibit the arginase activity.

Although our data show a beneficial effect for arginase blockade by nor-NOHA, there may be a concern for possible adverse effects of excessive arginase blockade. Since arginase is a key enzyme in the urea cycle, nor-NOHA inhibition may result in hyperammonemia. However, we did not observe any rise in ammonia levels after treatment of animals with NOHA (data not shown). Another potential detrimental effect on the blockade of arginase may be a decrease in plasma ornithine levels. Ornithine can be metabolized to polyamines by ornithine decarboxylase or to proline by ornithine aminotransferase. Both of these pathways are important in the host adaptive response for cellular proliferation and tissue repair after liver injury. Following liver transplant, wound healing and hepatic parenchymal reconstruction from the I/R injury are vital processes. However, our data demonstrate that the plasma levels of proline and polyamines are
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preserved and not affected by the short-term manipulation of the arginase pathway with nor-NOHA.

In summary, the purpose of this study was to determine if enhancement of arginine availability through arginase blockade can protect against hepatic I/R injury. The results demonstrate that nor-NOHA, an inhibitor of arginase, is capable of partially reversing the arginine depletion seen in I/R injury and improving the histopathological damage that is observed after transplantation. Interventions using arginase blockade may be effective in ischemic liver injury by minimizing organ damage and may be useful in other clinical settings associated with inflammation and cellular necrosis.

ACKNOWLEDGEMENTS:

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GRANTS

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**FIGURE LEGENDS:**

**Figure 1. Serum arginase activity and arginine levels after liver transplantation.** A) Western blot analysis of serum arginase I protein expression 3 hours post orthotopic liver transplantation. Serum amino acid arginine (solid bars) and ornithine (open bars) concentrations were measured by HPLC analysis. Blot shown is representative of 3 separate experiments. B) Time course of serum arginase activity in animals undergoing liver transplantation. Data represents means ± SE, n = six rats per group. * indicates p<0.05 vs normal.

**Figure 2. Effect of nor-NOHA exogenous arginase-induced arginine depletion.** Animals were treated with nor-NOHA after exogenous arginase injection. Arginase administration (open squares) rapidly depleted serum arginine compared to normal control animals (solid squares). Nor-NOHA (solid circles) reverses arginine depletion achieved by exogenous arginase delivery. Nor-NOHA alone (open circles) had no effect. Data represents means ± SE, n = six rats per group.

**Figure 3. Effect of nor-NOHA treatment on serum arginase activity and arginine levels after liver transplantation.** A) Serum arginase activity in nor-NOHA (100 mg/kg) or saline-treated animals undergoing OLT. Normal (NL) serum arginase activity are shown as control. Data represents means ± SE, n = six rats per group. * P<0.05 versus control saline treated rats three hours post reperfusion. B) Serum arginine levels in nor-NOHA or saline-treated animals undergoing OLT. Data represents means ± SE, n = six rats per group. * p<0.05 versus control saline treated rats three hours post reperfusion.
Figure 4. Effect of nor-NOHA on serum transaminase levels following liver transplantation. Animals treated with nor-NOHA (open squares) or control saline (closed squares) underwent OLT with various times of reperfusion. Serum AST and ALT levels were analyzed as a measure of hepatocellular injury. Data represents means ± SE, n = four to six rats per group. * p<0.05 versus nor-NOHA- treated rats.

Figure 5. Histopathology of liver sections from nor-NOHA and saline-treated animals. Hematoxylin-eosin-stained liver sections from animals treated with saline or nor-NOHA undergoing OLT with 24 hours of reperfusion (Original magnification x 100). Decreased hepatic necrosis is noted in the nor-NOHA group (small arrow) when compared to saline-treated animals (large arrows). Images are representative liver sections from four rats per group.

Figure 6. Western blot analysis for serum arginase I in nor-NOHA and saline treated animals. Animals treated with saline or nor-NOHA underwent OLT with various times of reperfusion. Serum was obtained at the time points shown. Blot shown is representative of three similar experiments.

Figure 7. Nor-NOHA does not affect polyamine synthesis post-transplant. Animals treated with saline or nor-NOHA underwent OLT with serum levels of polyamines measured with HPLC at 3 hours after reperfusion. Values of polyamines in normal animals without OLT are shown. Data represents means ± SE, n = six rats per group.
<table>
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<th>AST</th>
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Table 2. Serum total nitrite + nitrate production in nor-NOHA versus saline treated animals at 6 hrs post-injection

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<th>Treatment</th>
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<td>Control + nor-NOHA</td>
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<tr>
<td>Transplant + saline</td>
<td>19.02 ± 1.19</td>
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<tr>
<td>Transplant + nor-NOHA</td>
<td>17.89 ± 0.85</td>
</tr>
</tbody>
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Figure 1

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