0148-6071/01/2503-0142$03.00/0
JOURNAL OF PARENTERAL AND ENTERAL NUTRITION

Vol. 25, No. 3
Printed in U.S.A.

Protective Role of the L-Arginine-Nitric Oxide Synthase Pathway on Preservation Injury after Rat Liver Transplantation

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ABSTRACT. *Background:* A major problem complicating liver transplantation is the preservation injury that results from cold storage and subsequent ischemia/reperfusion injury after organ revascularization. The L-arginine—nitric oxide (NO) pathway has been recognized to play critical roles during infection, inflammation, organ injury, and transplant rejection. Recent data indicates that NO synthesis has beneficial effects in several models of liver injury. The purpose of this study is to examine the role of the L-arginine—NO pathway on preservation injury in an experimental model of rat liver transplantation. *Methods:* Orthotopic liver transplantation was performed in syngeneic (LEW to LEW) rats. Liver preservation injury was determined by measuring serum liver function tests 6 to 48 hours after transplantation. In some experiments, rats received L-arginine supplementation 0 to 24 hours after transplantation. In other experiments, NO synthase inhibitors (L-NAME or L-NIL) were injected at the time of isograft revascularization. *Results:* L-Arginine supplementation decreased hepatic transaminase levels at all time points examined (6–48 hours). L-Arginine produced a significant improvement in liver preservation injury by 12 hours after reperfusion. The NO synthase inhibitor L-NAME caused a significant increase in liver injury 24 hours after injection. The inducible NO synthase (iNOS)-specific inhibitor L-NIL had no significant effect on liver injury. *Conclusions:* The results show that L-arginine supplementation and NO synthesis improve hepatic injury and have a protective role in the transplanted liver graft. The protective effect may be mediated by low-level cNOS-derived NO. (*Journal of Parenteral and Enteral Nutrition* **25:**142–147, 2001)

Liver graft preservation injury results from a cold ischemia storage period followed by reperfusion injury after graft revascularization. All donor organs experience some degree of ischemia/reperfusion injury. Pathologic features include sinusoidal endothelial cell damage, Kupffer cell activation, and hepatocellular edema.[1,2] Complement activation and adhesion of platelets and leukocytes has also been documented. Mediators implicated in the pathophysiology of liver preservation injury include cytokines, reactive oxygen intermediates (ROIs), and eicosanoid products.[3]

Due to the tremendous shortage of liver organ donors, there has been a recent trend among major liver transplant centers to expand the organ donor pool with the use of "marginal" donors. Although not ideal, the fact that over 16,000 patients are currently on the UNOS wait list for liver transplantation with the supply being limited to 5000 liver organ donors per year in the U.S. indicates that such an approach is warranted. This has resulted in a delayed or primary non-function rate of 5% to 10% for liver grafts due to severe preservation injury. Early graft non-function contributes to significant morbidity and mortality and often requires emergency re-transplantation. Thus elucidating the molecular and cellular mechanisms responsible for graft preservation injury may lead to strategies to reduce the organ damage.

Nitric oxide (NO) is a potent mediator which has triggered an explosion of studies over the last decade, resulting in the 1998 Nobel Prize in Medicine being awarded for the discovery of NO. This research has identified diverse physiologic and pathophysiologic roles for NO as a vasodilator, neurotransmitter, antimicrobial effector molecule, and immunomodulator. Within cells, NO is produced from the amino acid L-arginine by a family of NOS enzymes (Fig. 1). Cloning studies have identified three distinct NOS isoforms.[4] Two of the NOS genes encode constitutive NOS (cNOS) enzymes and are expressed continuously. One cNOS isoform has been cloned from endothelial cells and serves as a normal regulator of vascular tone. The second cNOS isoform has been cloned from neurons and has been localized to cells in the central/peripheral nervous system. Both cNOS isoforms are calcium/calmodulin-dependent and can be immediately activated upon stimulation to produce small amounts of NO. The two cNOS proteins participate in physiologic "normal" cell-to-cell signaling and are not up-regulated as part of inflammatory responses. This is in marked contrast to the third NOS isoform, inducible NOS (iNOS), which is not normally present in resting cells. iNOS expression must first be induced by inflammatory cytokines.[5,6] When activated, the iNOS gene is

Received for publication, December 13, 2000.
Accepted for publication, February 5, 2001.
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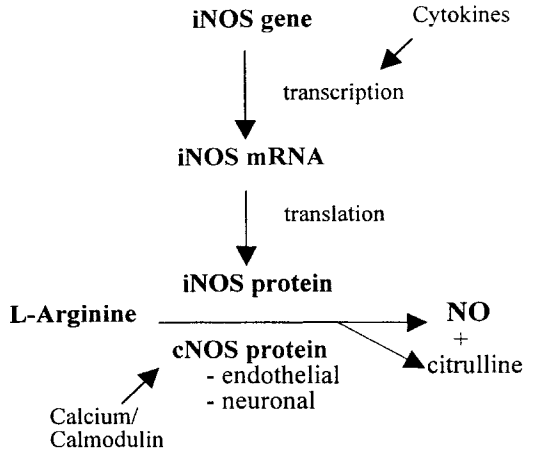

FIG. 1. L-Arginine—NO synthase pathway. Nitric oxide (NO) is produced from the amino acid L-arginine by a family of NO synthase (NOS) enzymes yielding L-citrulline as by-product. Cloning studies have identified three distinct NOS isoforms. Two of the NOS genes encode constitutive NOS (cNOS) enzymes and are regulated post-translationally by intracellular calcium/calmodulin. The cNOS enzymes were isolated from neuronal and endothelial cells. The third NOS gene, inducible NO synthase (iNOS), is controlled primarily by transcriptional mechanisms and is activated by inflammatory cytokines.

transcribed to mRNA and then translated to protein, resulting in large amounts of NO production.

In a rodent model of warm liver ischemia-reperfusion injury, NOS inhibition decreased hepatic blood flow and increased liver damage.[7] In addition, both L-arginine supplementation and NO donors improved blood flow and attenuated hepatic damage in rodent, canine, and porcine models of liver ischemia-reperfusion injury.[8–13] Figure 2 is a list summarizing the beneficial effects of NO in the liver. In experimental endotoxemia, iNOS is widely expressed in many tissues. The large quantity of NO produced by iNOS has important antimicrobial functions, and NO can inhibit the growth of viruses, parasites, and certain bacteria.[14] In the immune system, NO reduces both neutrophil adhesion[15] and lymphocyte activation.[16] NO is a potent vasodilator and anticoagulant that minimizes organ ischemia. The induced NO synthesis that occurs in vascular beds during sepsis is protective in solid organs such as the liver, kidney, and intestine.[17–20] In the liver, the protective effect is mediated in part by the capacity of NO to prevent platelet thrombi and neutralize toxic oxygen radicals.[17,18] Although this is an adaptive response aimed at maximizing organ perfusion, severe hypotension can result in fatal consequences. Furthermore, excessive NO production can react with superoxide to generate peroxynitrite which can have cytotoxic effects. Thus induced NO synthesis can have both beneficial and detrimental effects.

Another major mechanism of NO-mediated protection in the liver is the antiapoptotic effects of NO. Adenoviral iNOS gene transfer protected rat hepatocytes from spontaneous and TNF+Actinomycin-D-induced apoptosis *in vitro*.[21] *In vivo*, a NO donor decreased liver damage and blocked apoptosis in an animal model of acute liver failure,[22] and NOS inhibitors increased apoptosis in LPS-challenged animals.[23] NO blocked apoptosis by inhibiting caspase-3-like protease activity by several mechanisms.[24,25] One mechanism is a direct inhibition of caspase-3–like activity by S-nitrosylation, and another is indirect inhibition of caspase-3–like activity mediated by a cGMP-dependent mechanism.[24] NO also suppressed apoptosis by inhibiting Bcl-2 cleavage and cytochrome c release.[26]

The aim of this study was to examine the role of the L-arginine—NO synthase pathway in mediating liver transplant preservation injury. The results indicate that augmenting the L-arginine—NO synthase pathway with exogenous L-arginine can ameliorate hepatic injury, whereas blockade of the NO synthase pathway with a cNOS inhibitor worsens the ischemia/reperfusion injury after rat liver transplantation.

MATERIALS AND METHODS

Materials

L-NAME and L-NIL were purchased from Alexis (San Diego, CA). L-Arginine was from Sigma Chemical Co (St. Louis, MO).

Orthotopic liver transplantation

Male Lewis rats (LEW, RT1[l]), weighing 200 to 300 g 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.[27] The donor liver was flushed *in situ* with University of Wisconsin (UW) solution through the aorta. 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 before transplantation.

Experimental design

In the first series of experiments, exogenous L-arginine (300 mg/kg) was given by IV or intraperitoneal injection at 0, 0.5, 2, 12, and 24 hours after graft reperfusion. Control animals received a saline injection. Serum hepatocellular enzymes were measured 6 to 48 hours after liver transplantation and compared with control animals that did not receive supplemental L-arginine. In the second series of experiments, NO synthase inhibitors L-NAME (50 mg/kg IV) or L-NIL

1. Antimicrobial agent
2. Improves blood flow
   - vasodilator
   - anti-platelet effect
3. Inhibits PMN activation
4. Neutralizes free radicals
5. Anti-apoptotic effect

FIG. 2. Beneficial effects of nitric oxide in the liver. Nitric oxide (NO) has multiple beneficial actions in the liver.

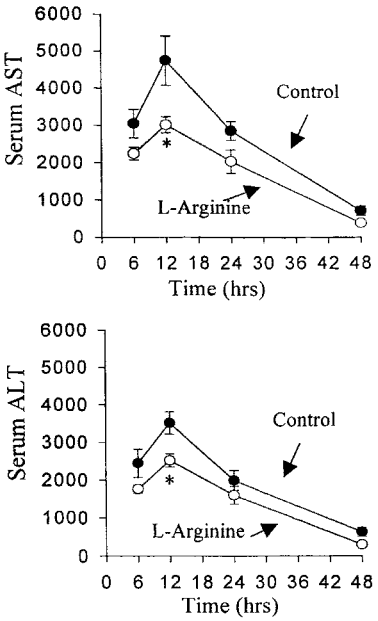

FIG. 3. Protective effect of L-arginine on liver transplant preservation injury. L-Arginine (300 mg/kg) was given by IV injection at 0, 0.5, and 2 hours, and by intraperitoneal injection at 12 and 24 hours, after graft reperfusion. Serum transaminase levels were significantly decreased 12 hours after transplantation in the liver grafts supplemented with L-arginine *vs* control grafts.

(60 mg/kg IV) were given to the rats at the time of graft reperfusion and liver enzyme injury was determined 24 hours after transplantation.

Liver function tests

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

Statistical analysis

Results of serum liver injury tests are expressed as the means ± standard error of the mean (SEM). Group comparisons were performed using the student's t test or ANOVA. Differences were considered significant at $p < .05$.

RESULTS

L-Arginine supplementation improves hepatic injury after rat liver transplantation

Increased NO production has been reported in humans after liver transplantation.[28,29] Because NO is protective in several models of liver injury, it was reasonable to hypothesize that L-arginine supplementation and NO synthesis would also exert protective effects on hepatic preservation injury in liver transplantation. The need for arginine supplementation is especially important given that L-arginine deficiency has been documented after human orthotopic liver transplantation due to increased arginase efflux from the allograft.[30] To determine if L-arginine supplementation conferred a protective effect on rat liver isograft preservation injury, exogenous L-arginine was given at 0, 0.5, 2, 12, and 24 hours after graft reperfusion. Serum transaminase levels were significantly decreased 12 hours after transplantation in the liver grafts supplemented with L-arginine *vs* control grafts (Fig. 3).

To determine if the L-arginine supplementation increased circulating NO levels, plasma nitrite + nitrate levels were measured. Although there was a trend for increased circulating nitrite + nitrate levels at 12 and 24 hours after transplant in the rats that received L-arginine, the results were not significantly different from control animals (data not shown).

NO synthase inhibitors worsen hepatic injury after rat liver transplantation

A further protective role for NO was shown when the non-selective NOS inhibitor L-NAME significantly increased liver damage (Fig. 4). The iNOS selective inhibitor L-NIL did not modify graft injury, suggesting that the protective effect was mediated by cNOS-generated NO rather than iNOS-mediated production. Taken together, these results support a beneficial for the L-arginine—NO pathway in reducing liver transplant preservation injury.

DISCUSSION

The role of NO in transplant immunology was first defined by Hoffman and colleagues where cytotoxic T lymphocyte activity and proliferation was increased by inhibition of NO synthesis.[16,31,32] Induced NO synthesis was then shown in rejecting rat cardiac allografts by electron paramagnetic resonance (EPR) spectroscopy[33] and by measuring elevated NO end-products in rat orthotopic liver and small bowel transplants.[34] Plasma NO metabolites were suggested to be a predictive parameter of acute allograft rejection in human liver transplants.[29] Since these early reports, there have been dozens of studies investigating the role of NO in mediating acute cellular rejection, chronic allograft rejection, and organ preservation injury. Due to the complex regulation of NO synthesis by three dis-

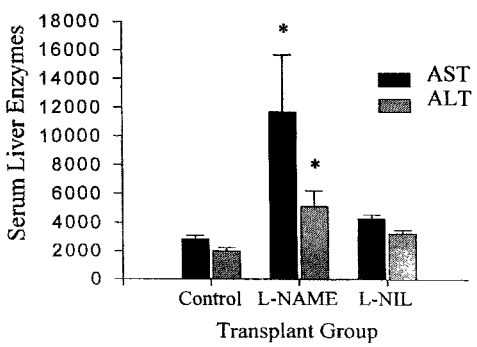

FIG. 4. Role of NOS inhibitors L-NAME or L-NIL on liver transplant preservation injury. NO synthase inhibitors L-NAME (50 mg/kg IV) or L-NIL (60 mg/kg IV) were given to the rats at the time of graft reperfusion and liver enzyme injury was determined 24 hours after transplantation. The non-selective NOS inhibitor L-NAME significantly increased liver damage, whereas the iNOS selective inhibitor L-NIL did not.

tinct human NOS genes,[4] a true paradox exists where NO can elicit either beneficial or detrimental effects depending on the site, timing, redox state, and quantity of NO produced.

The findings in the current study show a protective role for the L-arginine—NO synthase pathway in rat liver transplant preservation injury. The ischemia/reperfusion injury assessed by liver enzymes was significantly improved by arginine supplementation in the recipient animals. Inhibition of the NO synthase pathway with the NOS inhibitor L-NAME worsened the liver injury, further implicating a beneficial role for NO synthesis in ameliorating liver injury after cold preservation. The predominant change in liver injury was seen with L-NAME which is a non-selective NOS inhibitor (with greater cNOS activity) compared with the iNOS-specific inhibitor L-NIL that had no effect on hepatic transaminase release. These results suggest that low-level cNOS-derived NO is responsible for mediating the beneficial effects in liver injury. It is possible that the improvement in liver injury seen with L-arginine administration is mediated by increased cNOS activity. This is especially likely given that arginine deficiency has been shown after human orthotopic liver transplantation due to increased arginase efflux from the allograft.[29]

Numerous studies have shown a protective role for exogenous L-arginine or NO donors in animal models of warm hepatic ischemia-reperfusion injury.[7–12] With regards to liver transplantation (cold ischemia-reperfusion injury), Yin has shown that ischemic preconditioning protects the liver graft from cold preservation-reperfusion injury in a rat liver transplant model.[35] The beneficial effect of ischemic preconditioning was attenuated by a NO synthase inhibitor and improved by L-arginine, suggesting that the protective effect may be mediated in part by endogenous NO production. Kuo reported that blockade of NO synthesis in a spontaneous hyporesponsive rat liver transplant model increased serum transaminase levels and was associated with an increased periportal inflammatory infiltrate.[36] Another study showed that an extended graft preservation time was associated with increased hepatic iNOS protein levels, although the precise role of NO in this setting was not established.[37] Langle demonstrated that an IV infusion of L-arginine during the early reperfusion period in pig liver transplantation improved cardiac output and hepatic blood flow and reduced pulmonary vascular resistance.[13] Recently, Rodriguez showed that sodium nitroprusside (an NO donor) added to University of Wisconsin solution during prolonged cold storage improved bile production, histology, and intrahepatic circulation in the isolated perfused liver model.[38] Taken together, these studies show a beneficial effect for L-arginine and NO in the liver transplant setting. Ongoing studies will clarify the optimal conditions required to maximize the protective effect of L-arginine and NO on liver graft preservation injury.

The role of the L-arginine—NO synthase pathway has also been examined in transplantation of extra-hepatic organs. In an experimental model of rat lung transplantation, Pinksy reported that augmenting the NO/cGMP pathway in the preservation solution improved graft function and increased survival.[39] In another study, inhaled NO attenuated reperfusion injury and improved survival in pig lung transplants from non-heart beating donors.[40] In a rat model of acute renal allograft rejection, Cattell localized iNOS expression to infiltrating macrophages in the kidney graft, but the functional significance of induced NO synthesis was not determined.[41] In rodent models of renal ischemia-reperfusion injury, administration of L-arginine or NO improved renal function.[42,43] Along similar lines, the NO donors are protective in ischemia/reperfusion injury of the pancreas[44] and small intestine.[45] In a heterotopic rat small bowel transplant model, Mueller reported that graft function and survival were inferior when NO synthase inhibitors were given, indicating a beneficial role for NO.[46]

In cardiac transplantation, the role of NO is more controversial and most studies have focused on the relationship between NO production and acute cellular rejection. Excessive NO has been shown to inhibit cardiac contractility,[47] and cytokines are released during acute rejection that could induce iNOS. Induced NO synthesis was first shown in rejecting cardiac allografts by EPR-NO signal.[33] Using a heterotopic rat heart transplant model, Yang demonstrated iNOS mRNA, protein, and enzyme activity expression in rejecting allografts.[48] More recently, Lewis has shown that iNOS mRNA was expressed in human heart transplant biopsies and was associated with contractile dysfunction of the left ventricle.[49] Szabolcs reported a close relationship between apoptosis and increased iNOS expression in human cardiac allograft rejection.[50] These findings support the hypothesis that NO contributes to the tissue injury and alloimmune response during acute rejection and resulted in many experimental investigations to modify acute rejection by blocking induced NO synthesis. Depending on the animal model or selectivity of iNOS inhibitor used, NOS inhibition either prolonged allograft survival, had no effect on graft survival, or caused a decrease in graft survival.[51–54] Moving away from acute cellular rejection, Lowenstein's group identified iNOS mRNA and protein expression in coronary arteries of transplanted human hearts with accelerated graft arteriosclerosis.[55] Although the role of NO in the pathogenesis of accelerated graft arteriosclerosis (transplant vasculopathy) is unknown, Shears has shown that adenoviral iNOS delivery suppressed the development of allograft arteriosclerosis in rat aortic allografts.[56]

In summary, the L-arginine—NO synthase pathway is important in the transplant setting and has many clinical implications. For preservation injury of the liver and some extra-hepatic organs, addition of L-arginine or NO is clearly beneficial. Further studies are required in large animals to define the ideal delivery protocol that optimizes the protective effects for each organ. For acute and chronic rejection of solid organs after transplantation, the role of L-arginine and NO is not as clear and requires a better understanding of the molecular mechanisms involved before deciding if modulation of the NO pathway is useful.

ACKNOWLEDGMENTS

This research was supported by NIH grants R01-GM52021 (D.A.G.), the George H. A. Clowes, Jr., MD, FACS, Memorial Research Career Development Award of the American College of Surgeons (D.A.G.), and the Howard Hughes Medical Institute Research Training Fellowship for Medical Students (G.P.Y.).

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