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WTC06 Endothelial Nitric Oxide Synthase Protects Transplanted Mouse Livers Against Storage/Reperfusion Injury: Role of Vasodilatory and Innate Immunity Pathways*

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Abstract

Endothelial nitric oxide synthase (eNOS) plays a role in microcirculatory and immunomodulatory responses after warm ischemia/reperfusion. We hypothesized that eNOS is essential to maintain microcirculation, attenuate macrophage infiltration and decrease graft injury after liver transplantation. Liver transplantation was performed after 18 h storage in cold UW solution from wildtype and eNOS deficient (B6.129P2-*Nos3^{tm1Unc}/J*) donor mice into wildtype mice. Serum ALT, necrosis by histology, apoptosis by TUNEL, and macrophage infiltration by immunostaining against F4/80 antigen were determined 2 to 8 h after implantation. Hepatic microcirculation was investigated after 4 h by intravital confocal microscopy following injection of fluorescein-labeled erythrocytes. After sham operation, livers of wildtype and eNOS deficient mice were not different in ALT, necrosis, apoptosis, macrophage infiltration, and microcirculation. After transplantation, ALT increased >3-times more after transplantation of eNOS deficient livers than wildtype livers. Necrosis was >4-times greater, and TUNEL and F4/80 immunostaining in non-necrotic areas were 2 and 1.5-times greater in eNOS deficient donor livers, respectively. Compared to wildtype and eNOS sham-operated mice, sinusoidal blood flow velocity increased 1.6-fold after wildtype transplantation, but sinusoidal diameter was not changed. After transplantation of eNOS deficient livers, blood flow velocity and sinusoidal diameter were decreased compared to transplanted wildtype livers. These results indicate that donor eNOS attenuates storage/reperfusion injury after mouse liver transplantation. Protection is associated with improved microcirculation and decreased macrophage infiltration. Thus, eNOS-dependent graft protection may involve both vasodilatory and innate immunity pathways.

Keywords

Apoptosis; microcirculation; monocyte/macrophage liver infiltration; mouse liver transplantation; storage; reperfusion injury

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List of Abbreviations

ALT, alanine aminotransferase; FITC, fluorescein isothiocyanate; H&E, hematoxylin and eosin; HPF, high power field; PTFE, polytetrafluoroethylene; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; UW, University of Wisconsin cold storage solution

Reperfusion of livers after longer periods of cold ischemic storage results in endothelial cell killing and microcirculatory disturbances leading to hepatocellular necrosis after liver transplantation (1). Similarly, microcirculatory disturbances and hepatocellular injury occurs after warm hepatic ischemia/reperfusion (2,3). Nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS) is an important vasodilatory mediator whose formation increases during ischemia/reperfusion and hemorrhagic shock. After warm hepatic ischemia/reperfusion, microcirculatory disturbances and necrosis are increased in eNOS knockout mice compared to wildtype mice (4). Moreover, NO donors added at reperfusion prevent cell killing after ischemia/reperfusion to cultured hepatocytes (5). eNOS-derived NO also plays a beneficial role in post-ischemic livers through inhibition of cytokine and oxidant release by Kupffer cells (4,6). However, the importance of eNOS for hepatic injury after cold storage/warm reperfusion in transplanted liver grafts is not known. Here, utilizing orthotopic mouse liver transplantation, we compare grafts from eNOS deficient donors to those from wildtype donors to show that eNOS deficient grafts manifest greater injury, increased microcirculatory disturbances and more marked macrophage infiltration.

MATERIALS AND METHODS

Mouse liver transplantation

All experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee. Livers from male C57Bl/6 (wildtype) and eNOS deficient (B6.129P2-*Nos3^{tm1Unc}/J* on a C57Bl background) mice were transplanted into C57Bl/6J mouse recipients after 18 h of cold storage in University of Wisconsin (UW) solution. Donor and recipient mice weighed 20–25 g. For donor operations under ether anesthesia, livers were freed from peritoneal attachments, the gall bladders were removed, and the common bile ducts were cannulated with polytetrafluoroethylene (PTFE) tubing (0.2 mm inner diameter, Zeus, Inc., Orangeburg, SC) and divided. After portal infusion of ice cold UW solution (2 ml), the livers were excised, cuffs were placed on the portal veins and subhepatic inferior vena cava using 20-gauge and 18-gauge i.v. catheters, respectively (Braun Medical, Inc., Bethlehem, PA). Excised livers were immersed in UW solution inside plastic containers and stored in an ice-water bath until implantation. Time for the donor procedure averaged 23 min.

In the recipient operation, livers of recipient mice were removed after dividing the bile duct at the hilum and clamping and dividing the suprahepatic inferior cava, portal vein, and subhepatic inferior cava. Cold stored donor livers were then implanted by anastomosing the suprahepatic vena cava with a running 10-0 nylon suture and connecting the portal veins and infrahepatic vena cava by cuff insertion. Bile ducts were anastomosed over PTFE stents. The recipient operation averaged 45 min, and the portal vein clamp time averaged 15 min. For sham operations under ether anesthesia, wildtype and eNOS deficient mice were laparotomized. After 30 min, the abdomen was closed.

Measurements of injury

After ether anesthesia, blood samples were collected from the inferior vena cava at 2, 4, and 8 h after transplantation and analyzed for ALT by standard enzymatic methods. Liver grafts were fixed by immersion in 4% buffered paraformaldehyde at 8 h after liver transplantation and

embedded in paraffin, sectioned (4 μ m) and stained with H&E. Ten random fields were assessed for necrosis by standard morphologic criteria (*e.g.*, loss of architecture, vacuolization, karyolysis, increased eosinophilia), and the area percentage of necrosis was quantified (BioQuant BQ Nova Prime 6.7, R&M Biometrics, Nashville, TN). To assess apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on paraffin sections (Roche Diagnostics, Penzberg, Germany). TUNEL was counted in 10 randomly chosen high-power fields.

Detection of monocytes/macrophages

To assess infiltration of monocytes/macrophages into liver grafts, paraffin sections were immunostained against F4/80 antigen at 2, 4, and 8 h after implantation, using a rat anti-mouse antibody (Cl:A3-1, F4/80; Serotec, Inc., Raleigh, NC). The number of F4/80 positive cells was counted in 10 randomly selected non-necrotic high-power fields.

Microcirculation experiments

Erythrocytes from wildtype C57Bl/6 mice were isolated by differential centrifugation from sodium citrate anticoagulated whole blood. Erythrocytes were labeled by incubating the cells in phosphate buffered-saline containing 0.1 mg/ml fluorescein isothiocyanate (FITC) at room temperature for 2 h. Labeled erythrocytes were centrifuged at 400 g for 10 min and resuspended twice to a final concentration of 2×10^9 cells/ml. At 4 h after transplantation, mice were anesthetized with pentobarbital (50 mg/kg) and connected to a small animal ventilator via tracheostomy and a respiratory tube (20-gauge catheter). A catheter (0.4 mm inner diameter, Zeus, Inc., Orangeburg, SC) was inserted into the right carotid artery to inject FITC-labeled erythrocytes and monitor mean arterial pressure (Micro-Med, Inc., Louisville, KY) before experiments. After relaparotomy and prone positioning of the recipient, the anterior surface of the liver was gently withdrawn from the peritoneum and placed over a glass coverslip on the stage of a Zeiss Axiovert 100 microscope (Thornwood, NY) equipped with a spinning disk confocal imaging system (Attofluor CARV Optical Module, BD Bioimaging Systems, San Jose, CA). After infusion of 100 μ l FITC-labeled erythrocytes, 50 confocal images of each liver graft were taken in 10–15 random fields to measure blood flow. Ambient air temperature was kept at body temperature using an air stream incubator (Nicholson Precision Instruments, Bethesda, MD). Erythrocyte velocity and sinusoidal width were measured in the different groups.

Statistics

Data are presented as means $\pm$ S.E.. Statistical analysis was performed by Student's t-test or ANOVA plus Student-Newman-Keuls test, as appropriate, using $p < 0.05$ as the criterion of significance.

RESULTS

After sham operation of wildtype and eNOS deficient mice, serum ALT did not increase and was comparable to unoperated mice (< 100 U/L, Fig. 1). By contrast after transplantation of wildtype livers, ALT increased to 3652 ± 407 , 5510 ± 2183 and 6283 ± 1470 U/L, respectively, at 2, 4 and 8 h after implantation. After transplantation of eNOS deficient livers, ALT increased to 6706 ± 1857 , 8739 ± 1990 and 19815 ± 3490 U/L after 2, 4, and 8 h, respectively, which was 2–3 fold times more ALT release than after transplantation of wild type livers ($p < 0.05$ at 8 h).

At 8 h after sham operation, liver histology was normal in control and eNOS deficient mice and indistinguishable from unoperated mice (Fig. 2). After transplantation of both wildtype and eNOS deficient livers, necrosis developed 8 h postoperatively with a predominately

pericentral and midzonal distribution. The area percentage of necrosis was 4-times greater in eNOS deficient grafts than wildtype grafts ($17.3 \pm 3\%$ v. $3.8 \pm 1.8\%$, $p < 0.01$, Fig. 2).

Apoptosis assessed by TUNEL was very rare 8 h after sham operation in both wildtype and eNOS knockout mice (< 0.05 cells/HPF, Fig. 3). At 8 h after liver transplantation, TUNEL increased to 6.6 ± 1.5 and 13.6 ± 2.1 cells/HPF, respectively, in wildtype and eNOS deficient liver grafts without apparent zonal localization ($p < 0.05$; Fig. 3). Thus, apoptosis as well as necrosis was increased after transplantation of eNOS deficient livers compared to wildtype livers.

Infiltration of monocytes/macrophages was assessed by F4/80 antigen immunostaining. At 8 h after sham operation and in unoperated wildtype and eNOS deficient, hepatic F4/80 staining was sinusoidal and distributed throughout the liver lobules. Since necrosis itself induces an inflammatory response, we only evaluated F4/80 immunostaining in non-necrotic areas of the liver. At 2 and 4 h after implantation of wildtype livers, positive staining increased slightly compared to sham operation and was distributed homogeneously through the liver lobules. At 8 h after implantation of wildtype grafts, F4/80 staining was increased 1.7-fold compared to sham. After transplantation of eNOS deficient livers, F4/80 staining at 2 h was similar to transplanted wildtype livers. However, at 4 and 8 h after implantation, F4/80 labeling increased markedly, and counting revealed increases of 1.7- and 2.2-fold compared to sham, respectively. Compared to wildtype transplanted livers, F4/80 staining increased 30 and 50% in eNOS deficient liver grafts at 4 and 8 h ($p < 0.05$, Fig. 4).

To characterize microcirculatory alterations after liver transplantation, intravital confocal microscopy was conducted at 4 h after implantation. Mean arterial blood pressure was comparable in all groups. To measure blood flow, FITC-labeled erythrocytes were injected into the carotid artery, and their movement through sinusoids was visualized with a spinning disk confocal fluorescence microscope. In wildtype and eNOS deficient sham-operated mice, sinusoidal blood flow velocity was 128 ± 26 $\mu\text{m/s}$ and 149 ± 26 $\mu\text{m/s}$, respectively. After transplantation of wildtype livers, flow velocity increased to 218 ± 31 $\mu\text{m/s}$ ($p < 0.05$, compared to sham, Fig. 5A). By contrast, after transplantation of eNOS deficient livers, flow velocity decreased to less than sham to 106 ± 15 $\mu\text{m/s}$ ($p < 0.05$, compared to wildtype transplant, Fig. 5A). From parenchymal autofluorescence, we could also measure sinusoidal diameter. In wildtype and eNOS deficient sham operated mice, sinusoidal diameter was 5.2 ± 0.6 μm and 5.4 ± 0.4 μm , respectively (Fig. 5B). At 4 h after transplantation of wildtype livers, sinusoidal width increased slightly to 5.9 ± 0.3 μm , but after transplantation of eNOS deficient liver grafts sinusoidal width decreased markedly compared to wildtype transplanted livers to 4.3 ± 0.3 μm ($p < 0.05$, Fig. 5B).

DISCUSSION

Here utilizing wildtype and knockout mice, we investigated the importance of eNOS on graft storage/reperfusion injury after liver transplantation. We found that eNOS deficiency in liver grafts exacerbated storage/reperfusion injury, as shown by elevated ALT, increased necrosis and apoptosis, disrupted microcirculation, and enhanced graft infiltration of monocytes/macrophages, which we observed as early as 4 h after liver transplantation.

As demonstrated by intravital confocal microscopy, a 1.5 to 1.7-fold increase of sinusoidal flow velocity was measured from visualization of the movement of FITC-labeled erythrocytes in transplanted wildtype liver grafts compared to livers of sham operated wildtype and eNOS deficient mice. This increase in velocity appears to be the consequence of eNOS-mediated vasodilatation, since both flow velocity and sinusoidal diameter were diminished after transplantation of eNOS deficient liver grafts. Although no marked sinusoidal vasodilatation

was observed after wildtype transplantation compared to sham operation, small increases in diameter might result in potentiated changes in blood flow according to Hagen-Poiseuille's law. Alternatively, vasodilatation may be occurring in presinusoidal vessels to promote increased blood flow. Microcirculatory changes were related to the time of cold ischemic storage (18 h), since after shorter storage times (2–8 h), flow velocity did not increase in either wildtype or eNOS deficient liver grafts, and damage to the grafts was minimal (data not shown). Since graft injury increased in eNOS deficient compared to wildtype liver grafts after 18 h of storage, the hyperemia observed after 18 h storage in wildtype grafts most likely represent a protective adaptation to storage/reperfusion stress. Our findings after cold ischemia/reperfusion are in accordance with previous studies of warm ischemia/reperfusion to livers from wildtype and eNOS deficient mice in which eNOS deficiency increased ALT release and Kupffer cell formation of reactive oxygen species (4,7). Previous work using Doppler flow measurements did not find microcirculatory differences after warm ischemia/reperfusion to livers of wildtype versus eNOS deficient mice. Thus, after warm ischemia/reperfusion, eNOS-dependent microcirculatory adaptations may not be as important for minimization of injury as after cold storage/reperfusion.

Previous work also shows that postischemic infiltration of leukocytes acts to enhance and extend injury after both warm and cold hepatic ischemia/reperfusion (8,9). Infiltration of monocytes/macrophages into liver grafts after cold storage/reperfusion may be suppressed, in part, by eNOS, since F4/80 staining of eNOS deficient liver grafts was increased compared to wildtype grafts. Increased staining in eNOS deficient liver grafts may be the direct consequence of loss of an eNOS-dependent inhibitory signal on monocyte/macrophage infiltration or an indirect effect mediated through alterations of the microcirculation caused by eNOS deficiency. Recently, macrophage derived eNOS has been implicated in pathophysiological responses, since decreased upregulation of pro-inflammatory proteins and a more stable hemodynamic profile was observed in eNOS deficient mice after sepsis (10). However, in our setting of transplantation, the presence of eNOS in donor grafts led to less injury than in eNOS deficient grafts. Thus, a differential effect of eNOS in the donor (protective) versus eNOS in the recipient (possibly injurious) may be occurring, as recently reported when tumor necrosis factor receptor 1 deficient and wildtype mice were cross-transplanted in all four possible combinations (11). Further investigations will be needed to address the *in vivo* role of recipient eNOS in graft injury after transplantation implementing cross-transplantation of wildtype and eNOS deficient mice livers.

Perhaps most relevant to the success and function of the liver grafts, eNOS deficiency increased both necrosis and apoptosis after liver transplantation, also described as necrapoptosis (12). Necrosis assessed by histology and serum ALT and apoptosis assessed by TUNEL were 2–3 fold greater in eNOS deficient grafts than wildtype grafts. Previously after liver transplantation in rats, recipient supplementation with the NO precursor L-arginine in the peritransplantation period decreased necrotic and apoptotic injury, whereas inhibition of NOS with L-NAME increased necrosis and apoptosis (3). Both cell death pathways can occur through a mitochondrial pathway involving the opening of permeability transition pores in the mitochondrial inner membrane causing the mitochondrial permeability transition (MPT) (12). After warm ischemia/reperfusion to cultured rat hepatocytes, NO signaling suppresses mitochondrial permeability pore opening after reperfusion by stimulating guanylyl cyclase, increasing intracellular cGMP and activating a cGMP-dependent protein kinase (5). Since eNOS deficiency increased both necrosis and apoptosis, graft injury and cell death after storage/reperfusion may be largely a necrapoptotic phenomenon, indicating a potentially important role of mitochondrial dysfunction in its pathogenesis.

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Figure 1. eNOS decreases ALT release after mouse liver transplantation

Serum ALT was assessed at 2 (n = 2), 4 (n = 4), and 8 h (n = 6) after transplantation of wildtype and eNOS deficient liver grafts into wildtype mouse recipients following 18 h of cold storage in UW solution. The 0 h time point represents the ALT levels 8 h after sham operation (n = 5). Grafts were stored for 18 h in UW before transplant. * $p < 0.05$, compared to wildtype liver transplant at 8 h.

Figure 2. eNOS decreases necrotic liver injury on histology

Livers were harvested from wildtype and eNOS deficient mice and stored in cold UW solution for 18 h before implantation. Hematoxylin-eosin staining was performed on wildtype sham-operated, eNOS deficient sham-operated (eNOS^{-/-}), wildtype transplanted (LT), and eNOS deficient transplanted livers at 8 h after surgery. The bottom panel depicts the area percentage of necrosis in the different groups. Bar is 100 μ m. * p <0.01 compared to wildtype LT (n = 5 per group).

Figure 3. eNOS decreases apoptosis after mouse liver transplantation

Livers were harvested from wildtype and eNOS deficient mice and treated as described in Fig. 2. At 8 h after implantation or sham operation, liver sections were examined for TUNEL. Representative low-power fields are shown. Quantification was performed with a high power objective, and TUNEL positive cells were counted in 10 high power fields (HPF) per section. Bar is 100 μ m. * p <0.05, compared to wildtype LT (n = 5 per group).

Figure 4. eNOS decreases monocyte/macrophage infiltration early after reperfusion

Livers were harvested from wildtype and eNOS deficient mice and treated, as described in Fig. 1. Liver sections were immunostained for F4/80 antigen at 2 (n = 2), 4 (n = 4), and 8 h (n = 5) after liver transplantation. Positive cells were counted in 10 HPF per section. Results are depicted as fold change compared to untreated and sham-operated livers that averaged 32.6 ± 1.5 positive cells/HPF. * $p < 0.05$ compared to wildtype LT at same times of reperfusion.

Figure 5. eNOS improves microcirculation at 4 h after mouse liver transplantation

Livers were harvested from wildtype and eNOS deficient mice and implanted into wildtype mice after 18 h of cold storage. At 4 h after implantation, microcirculation was assessed after injection of FITC-labeled erythrocytes. Intravital confocal microscopy was performed to determine flow velocity (**A**) and sinusoidal width (**B**). ^{*} $p < 0.05$ compared to wildtype liver transplantation (LT); [#] $p < 0.05$, compared to sham operations (n = 5–7 per group).