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Role of Inducible Nitric Oxide Synthase in Mitochondrial Depolarization and Graft Injury after Transplantation of Fatty Livers

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Abstract

This study investigated the role of inducible nitric oxide synthase (iNOS) in failure of ethanol-induced fatty liver grafts. Rat livers were explanted 20 h after gavaging with ethanol (5 g/kg) and storing in UW solution for 24 h before implantation. Hepatic Oil-Red-O staining-positive areas increased from ~2% to ~33% after ethanol treatment, indicating steatosis. iNOS expression increased ~8-fold after transplantation of lean grafts (LG) and 25-fold in fatty grafts (FG). Alanine aminotransferase release, total bilirubin, hepatic necrosis, TUNEL-positive cells and cleaved caspase-3 were higher in FG than LG. A specific iNOS inhibitor 1400W (5 μ M in the cold-storage solution) blunted these alterations by >42% and increased survival of fatty grafts from 25% to 88%. Serum nitrite/nitrate and hepatic nitrotyrosine adducts increased to a greater extent after transplantation of FG than LG, indicating reactive nitrogen species (RNS) overproduction. Phospho-c-Jun and phospho-c-Jun *N*-terminal kinase-1/2 (JNK1/2) were higher in FG than in LG, indicating more JNK activation in fatty grafts. RNS formation and JNK activation were blunted by 1400W. Mitochondrial polarization and cell death were visualized by intravital multiphoton microscopy of rhodamine 123 and propidium iodide, respectively. After implantation, viable cells with depolarized mitochondria were 3-fold higher in FG than in LG and 1400W decreased mitochondrial depolarization in FG to the levels of LG. Taken together, iNOS is upregulated after transplantation of FG, leading to excessive RNS formation, JNK activation, mitochondrial

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dysfunction, and severe graft injury. The iNOS inhibitor 1400W could be an effective therapy for primary non-function of fatty liver grafts.

Keywords

ALCOHOL; INOS; LIVER TRANSPLANTATION; MITOCHONDRIAL DEPOLARIZATION; STEATOSIS

INTRODUCTION

The large discrepancy between the demand for, and availability of, donor livers is one of the greatest challenges that liver transplantation faces today. The number of patients on the waiting list for donor livers is 2–3 times greater than liver transplants performed each year [1]. Due to this severe shortage of donor livers, use of marginal livers, such as steatotic liver grafts, is increasing [1, 2]. Hepatic steatosis is highly prevalent in liver donors, and steatotic liver grafts represent the most common “extended criteria” organ. However, use of steatotic livers for transplantation remains problematic because these livers have higher graft nonfunction [3–5]. Therefore, approaches to prevent steatosis-induced primary graft failure are important for expansion of the usable donor pool and for improving liver transplantation safety.

Many factors, such as obesity and/or a high fat diet, cause hepatic steatosis. Alcohol consumption is also a well-known risk factor for causing steatosis. Unfortunately, many organ donors are accident victims where alcohol consumption is frequently involved [8]. Previous studies have shown that both acute and chronic alcohol hepatic steatosis increases graft failure after liver transplantation [6–8]. Higher susceptibility to ischemia/reperfusion (I/R) injury, increased toxic cytokine formation, and activation of Kupffer cells appear to play an important role [9, 10]. The impact of pro-inflammatory lipid mediators and microcirculatory impairment is also highlighted in fatty graft injury [5, 11]. Reactive oxygen species (ROS) increased substantially after transplantation of fatty livers, and antioxidants and gene delivery of superoxide dismutases improved the outcome of fatty liver transplantation [6, 7]. However, the role of reactive nitrogen species (RNS) remains unclear.

Mitochondrial function is essential for cell survival. The onset of the mitochondrial permeability transition (MPT) is an important step leading to cell death in I/R injury [12, 13]. Energetic status is compromised in fatty livers after I/R [14–16], suggesting poor mitochondrial function. Previous studies showed that NO directly interacts with mitochondrial targets and inhibits mitochondrial respiration [17, 18]. Inhibition of inducible nitric oxide synthase (iNOS) expression after I/R by a sphingosine kinase-2 inhibitor prevented the MPT onset and liver injury [19]. By contrast, other studies showed that NO donors protected cultured hepatocytes from I/R injury by prevention of MPT onset [20]. Endothelial nitric oxide synthase (eNOS)-deficiency increased whereas inhalation of NO decreased graft injury after transplantation of lean livers [21–23]. Thus, the role of RNS in liver graft injury is controversial. It is generally accepted that eNOS is cytoprotective while iNOS mediates cytotoxicity. NO donors, although producing NO in a short-term, also inhibit iNOS expression [24], making interpretation of the effects of NO donors somewhat problematic. Inhibition of iNOS improves the outcome after transplantation of small-for-size, non-alcoholic fatty liver grafts [25]. However, partial liver grafts are exposed to different stresses compared to full-size liver grafts. Partial liver grafts have smaller graft mass, increased metabolic burden, the necessity for liver regeneration, and altered hemodynamics. Full-size grafts, in contrast, usually experience longer cold storage time. Therefore, it remains unclear whether iNOS is beneficial or detrimental in full-size liver

transplantation, particularly in the presence of steatosis. Accordingly, the purpose of this study was to test the effects of 1400W, a specific iNOS inhibitor [24], on mitochondrial function and injury in fatty liver grafts from ethanol-treated donors.

MATERIALS AND METHODS

Animals and Liver Transplantation

Female Lewis rats (230–250 g) were gavaged with ethanol (5 g/kg) or an equal volume of normal saline. Twenty hours later, donor rats were anesthetized with ether, and liver transplantation was performed using an arterialized two-cuff technique described elsewhere [6]. After explantation, each graft was stored in UW solution at 0–1°C for 24 h.

For implantation, the liver of the recipient was removed after clamping the suprahepatic and subhepatic vena cava and portal vein. After rinsing with 5 mL of lactated Ringer's solution, the explant was implanted by connecting the suprahepatic vena cava with a running suture, then inserting cuffs into the appropriate vessels and securing them with 6-0 silk suture. The hepatic artery and the bile duct were anastomosed with intraluminal splints. Portal vein clamping time was for 18 to 19 min, and implantation surgery required less than 50 min in total time. In some experiments, 1400W (*N*-[[3-(Aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride, Cayman Chemical Inc., Ann Arbor, Michigan) was added to the cold storage solution and the post-storage lactated Ringer's rinse solution at a final concentration of 5 μ M. Fatty livers were randomly assigned to the groups with or without 1400W treatment, and graft sizes were not statistically different between these two groups. For sham operation, ligaments around the liver were freed. Fifty minutes later, the abdominal wall was closed with running suture without transplantation. Rats were observed 7 days post-operatively for survival. Some rats were fed a choline-deficient diet (Dyets Co. Bethlehem, PA) for 2 weeks to induce expression of uncoupling protein-2 (UCP2) in the liver as a positive control for immunohistochemical staining. All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Measurement of Serum Alanine Aminotransferase, Total Bilirubin and Nitrite/Nitrate

To measure alanine aminotransferase (ALT) release and accumulation of total bilirubin due to liver injury, blood samples were collected from the inferior vena cava 8 h after implantation. Serum was obtained by centrifugation and stored at –80°C. ALT and total bilirubin were determined by analytical kits from Sigma Chemical (St. Louis, MO). To assess NO production, serum nitrite and nitrate levels were determined using an analytic kit from Cayman Chemical (Ann Arbor, MI).

Histology and Immunohistochemistry

Twenty hours after ethanol treatment, some livers from saline- and ethanol-treated rats were harvested and imbedded in Tissue-Tek OCT Compound (VWR Inc., West Chester, PA) and frozen-sectioned. Steatosis was visualized by Oil-Red-O staining. Images of ten random fields per slide were captured in a blinded manner using a Universal Imaging Image-1/AT image acquisition and analysis system (West Chester, PA) with an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) and a 20x objective lens. Hepatic steatosis was quantified by image analysis by dividing Oil-Red-O positive areas by total cellular area of the images. To detect iNOS, frozen sections were exposed to specific primary antibody to iNOS (Santa Cruz Biotech, Santa Cruz, CA) at a concentration of 1:250 in 0.1 M phosphate buffer-0.5% Tween 20 at room temperature for 1 h.

At 2 and 8 h after implantation, lean (LG) and fatty liver grafts (FG) were harvested, and slides were prepared as described elsewhere [6]. In sections stained with hematoxylin and eosin (H&E), ten random fields per slide were captured and analyzed as described above. Necrotic areas were quantified by image analysis using IP Lab 3.7v software (BD Biosciences, Rockville, MD) by dividing necrotic areas by total cellular area of the images [26]. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using an *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN) [26].

3-Nitrotyrosine adducts are a marker of peroxynitrite formation. After deparaffinization and rehydration, liver sections were immersed in 10 mM citrate acid (pH 6), heated in a microwave oven for antigen retrieval and then exposed to rabbit anti-nitrotyrosine polyclonal antibodies (Upstate, Lake Placid, NY) at a concentration of 1:150 in 0.1 M phosphate buffer-0.5% Tween 20 for 30 min at room temperature followed by a 20 min incubation with peroxidase-conjugated anti-rabbit IgG₁ antibody (DAKO Corp., Carpinteria, CA) at room temperature. 3,3'-Diaminobenzidine chromagen was then added as the peroxidase substrate. After the immunostaining procedure, a light counterstain of Meyer's hematoxylin was then applied. 3-Nitrotyrosine adducts were quantified by image analysis as described elsewhere [6]. Immunohistochemistry of UCP2 was performed using specific antibody (Santa Cruz Biotech. Santa Cruz, CA) at a dilution of 1:200 for 1 h at room temperature. To stain for cytochrome *c*, a primary antibody of 1:200 (BD Biosciences, Franklin Lakes, NJ) was applied at room temperature for 30 min after antigen retrieval as described above.

Immunoblotting

Liver tissue was homogenized in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS, 1% IGEPal, 1% protease and 1% phosphatase inhibitor cocktails (Sigma, St. Louis, MO), and centrifuged at $14,000 \times g$ for 15 min at 4°C. Aliquots of supernatant (40 µg of protein) were separated on NuPAGE 4–12% Bis-Tris gels for c-Jun, phosphorylated c-Jun (p-c-Jun), c-Jun *N*-terminal kinase (JNK), phosphorylated JNK1/2 (p-JNK1/2), ATP synthase-β (AS-β), NADH dehydrogenase-3 (ND3), and cytochrome *c* oxidase-IV (COX-IV) and on 10% Bis-Tris gels for iNOS, transferred onto nitrocellulose membranes and immunoblotted with primary antibodies specific for c-Jun, p-c-Jun, JNK1/2, p-JNK1/2, COX-IV, ND3 (Invitrogen Inc., Carlsbad, California), AS-β (Abcam Inc. Cambridge, MA), iNOS (Santa Cruz Biotech) at 1:1000 and actin (ICN, Costa Mesa, CA) at 1:3000 over night at 4°C. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was by chemiluminescence (Pierce Biotech., Rockford, IL).

Intravital Multiphoton Microscopy

Hepatic mitochondria polarization in living rats was detected using a cationic fluorophore rhodamine 123 (Rh123, Sigma, St. Louis, MO). Two hours after implantation or sham operation, rats were anesthetized with pentobarbital (50 mg/kg, *i.p.*) and connected to a small animal ventilator via a respiratory tube (14-gauge catheter) after tracheotomy, as described [27]. Rh123 (6 µmol/rat), which labels polarized mitochondria, and propidium iodide (PI, 0.16 µmol/rat), which labels nuclei of non-viable cells, were infused via polyethylene (PE50) tubing inserted into the carotid artery over 10 min.

To perform intravital multiphoton microscopy, laparotomized rats were put in a prone position over a coverslip mounted on the stage of a Zeiss LSM 510 NLO microscope (Thornwood, NY) equipped with a 25x water-immersion objective lens. Green Rh123 and red PI fluorescence excited with infrared light of 800 nm was imaged through 500–550 nm and 650–710 nm bandpass emission filters, respectively, in ~10 random fields per liver.

Parenchymal cells with bright punctate Rh123 fluorescence representing cells with polarized mitochondria or a dimmer diffuse cytosolic fluorescence representing cells with depolarized mitochondria were counted. Nonviable cells, indicated by red nuclear PI fluorescence, were also counted in the same fashion.

Statistical Analysis

Groups were compared using the Kaplan-Meier test or ANOVA plus Student-Newman-Keuls posthoc test as appropriate. Values shown are means $\pm$ SEM. There were 8 transplantations per group in the survival study and 4 transplantations per group in other parameters as indicated in figure legends. Differences were considered significant at $p < 0.05$.

RESULTS

iNOS is Upregulated in Ethanol-Induced Fatty Liver Grafts after Transplantation

Twenty hours after acute ethanol treatment, Oil-Red-O stained fat droplets (red) were detected in ~60% of parenchymal cells of livers from rats treated acutely with ethanol (Fig. 1A). Fat droplets were largely microvesicular with some macrovesicular. Oil-Red-O staining-positive areas, as assessed by image analysis, increased from 2% in livers from the rats without ethanol treatment to 33% in livers from ethanol-treated rats, indicating that ethanol caused steatosis in the liver as previously described [6].

Expression of iNOS was detected by immunohistochemical staining (Fig. 1B) and Western blotting (Fig. C and D). In preliminary experiments, we observed that iNOS expression did not increase during cold storage (data not shown). iNOS was undetectable in livers from saline-treated rats but increased slightly after acute ethanol treatment alone or 2 h after transplantation of LG. After transplantation of LG, expression of iNOS increased mainly in non-parenchymal cells. After transplantation of FG, expression of iNOS increased ~25-fold compared to untransplanted lean livers, indicating that ethanol treatment and transplantation synergistically upregulated iNOS. Expression of iNOS increased not only in non-parenchymal cells but also overtly in parenchymal cells.

1400W Decreases Cell Death after Transplantation of Fatty Liver Grafts

Since iNOS increased substantially after transplantation of ethanol-induced fatty livers, 1400W, a highly specific inhibitor of iNOS [28], was used to investigate the role of iNOS in liver injury after transplantation. No overt histological difference was observed between fatty livers cold-stored in UW solution with or without 1400W before implantation (not shown). No pathological changes were observed in lean livers 8 h after sham operation (Fig. 2A and I). After acute ethanol treatment and sham-operation, no necrosis was observed. After transplantation of LG, necrotic areas were only 2% (Fig. 2B). By contrast, after transplantation of FG, large focal necrosis was observed (Fig. 2C), reaching 21%. Necrosis was largely localized to pericentral and midzonal regions. Inhibition of iNOS with 1400W decreased necrotic areas to ~2% in FG (Fig. 2D).

Apoptosis was detected by TUNEL staining (Fig. 2E–H). TUNEL-positive cells (with red nuclear staining) were rare in lean livers after sham operation. After ethanol treatment alone apoptosis was not significantly increased (data not shown). After transplantation of LG, apoptosis increased modestly. By contrast, apoptotic cells increased markedly after transplantation of FG and treatment with 1400W decreased apoptosis in FG. Cleaved caspase-3 was barely detectable by immunoblotting in sham-operated lean livers and increased only slightly after ethanol treatment alone, but increased more in implanted LG (Fig. 2J). After transplantation of FG, cleaved caspase-3 increased to a higher level than LG, consistent with more apoptosis. 1400W decreased caspase-3 activation in FG.

1400W Decreases Injury and Improves Function and Survival of Fatty Liver Grafts

Graft injury was also assessed by ALT release in the blood 8 h after liver transplantation. Serum ALT levels were ~50 U/L in rats receiving sham-operation (Fig. 3A). Ethanol treatment alone increased ALT to 120 U/L. After implantation of LG or FG, ALT increased 28- and 81-fold, respectively. 1400W decreased ALT to a 47-fold increase after FG transplantation.

Total bilirubin, an indicator of graft function, was 0.12 mg/dL in rats subjected to sham-operation, and ethanol treatment alone had no effect (Fig. 3B). Bilirubin increased 2- and 16-fold after LG and FG transplantation, respectively. 1400W almost completely reversed the accumulation of bilirubin after transplantation of FG (Fig. 3B). Only 25% of recipients of FG survived 7 days after transplantation while 1400W treatment increased survival after fatty liver transplantation to ~88% (Fig. 3C).

Increases in Reactive Nitrogen Species Formation after Transplantation of Fatty Liver Grafts: Prevention by 1400W

Because iNOS increased substantially in fatty livers after transplantation, we investigated whether inhibition of iNOS blocks RNS formation in FG. Production of NO was evaluated by nitrite/nitrate in sera. Ethanol treatment alone and transplantation of LG increased serum nitrite/nitrate slightly (Fig. 4A). By contrast, nitrite/nitrate increased ~4 fold after transplantation of FG over the basal levels. Elevation of nitrite/nitrate in sera was blunted by 1400W.

3-Nitrotyrosine adducts, an indicator of peroxynitrite formation, were barely detectable in livers from rats treated with saline and increased slightly after ethanol treatment alone and after transplantation of LG (Fig. 4B, C and D). After transplantation of FG, 3-nitrotyrosine adducts increased markedly in the liver and adduct-positive areas increased by 20-fold (Fig. 4B and E). Formation of 3-nitrotyrosine adducts in FG was largely blocked by 1400W (Fig. 4B and F). Taken together, production of RNS increased substantially after transplantation of FG, which was blocked by 1400W.

Mitochondrial Depolarization Occurs in Fatty Liver Grafts: Prevention by 1400W

Mitochondria depolarize when the MPT occurs and leads to cell death. We performed intravital multiphoton microscopy in living rats at 2 h after transplantation to evaluate mitochondrial polarization status of hepatocytes using Rh123, a fluorophore that is taken up by polarized mitochondria. In the livers of sham-operated rats, punctate green Rh123 fluorescence was observed in virtually all hepatocytes, indicating mitochondrial polarization (Fig. 5A). Cells with depolarized mitochondria were only 1.3 cells/high power field (hpf) at 20 h after ethanol treatment alone and 3.9 cells/hpf after transplantation of LG (Fig. 5B and E). By contrast, mitochondria in many hepatocytes (12 cells/hpf) of FG did not take up Rh123 after transplantation (Fig. 5C and E). Despite the absence of mitochondrial polarization, the majority of hepatocytes lacked nuclear PI staining (red), indicating that they maintained membrane integrity. Only a small number of parenchymal and nonparenchymal cells (~1 cell/hpf) were not viable (Fig. 5C). In some cells, Rh123 fluorescence was relatively bright but diffuse, possibly indicating recent mitochondrial depolarization and release of Rh123 into the cytosol. These results reveal that at an early stage after transplantation of FG, mitochondrial depolarization had occurred in most hepatocytes and that this event preceded hepatocyte death. Importantly, mitochondrial depolarization was low in FG treated with 1400W (Fig. 5D and E).

Mitochondrial Cytochrome c is Released after Transplantation of Fatty Liver Grafts: Prevention by 1400W

Onset of the MPT causes large amplitude mitochondrial swelling, outer membrane rupture and release of cytochrome *c* from the intermembrane space into cytosol, which could trigger apoptosis [29, 30]. Therefore, we investigated if 1400W prevents cytochrome *c* release after transplantation. Cytochrome *c*, detected immunohistochemically in liver sections, was punctate in hepatocytes from sham-operated rats (Fig. 6A), consistent with mitochondrial localization. At 8 h after transplantation of LG, cytochrome *c* staining was diffuse in only a few cells (Fig. 6B). However, after transplantation of FG, cytochrome *c* staining became diffuse in many hepatocytes, indicating release of cytochrome *c* into the cytosol (Fig. 6C). Release of mitochondrial cytochrome *c* in FG was largely prevented by 1400W (Fig. 6D).

C-Jun N-Terminal Kinase is Activated after Transplantation of Fatty Liver Grafts: Prevention by 1400W

Previous studies showed that JNK activation after warm ischemia/reperfusion and cold storage/liver transplantation leads to MPT onset [31, 32]. Therefore, we investigated JNK activation after transplantation. Expression of c-Jun was not altered by ethanol treatment alone, transplantation alone or the combination of both (Fig. 7A and B). Phosphorylated c-Jun was barely detectable in sham-operated lean livers and was not altered by ethanol treatment alone. Phosphorylated c-Jun increased 2-fold at 2 h after transplantation of LG and was 4-fold higher in FG, indicating higher JNK activity. JNK1/2 and p-JNK1/2 levels followed the same pattern as c-Jun and p-c-Jun (Fig. 7A). Phospho-JNK2 was higher than phospho-JNK1 (Fig. 7A). 1400W largely blocked JNK1/2 activation, indicating that RNS play important role in activation of JNK in FG.

Uncoupling Protein-2 is not Increased after Transplantation of Lean and Fatty Liver Grafts

We also investigated whether UCP2 was up regulated after transplantation. UCP2, the major subtype of UCP in the liver, was undetectable in sham-operated lean livers (Fig. 8A). UCP2 did not increase following acute ethanol treatment alone (Fig. 8B) or transplantation of LG or FG (Fig. 8C and D). In contrast, UCP2 increased overtly in fatty livers caused by feeding of choline-deficient diet (positive control; Fig. 8E)

Oxidative Phosphorylative Proteins are not Decreased after Transplantation of Lean and Fatty Liver Grafts

Our previous study showed that inhibition of iNOS blunted decreases of mitochondrial oxidative phosphorylative proteins after transplantation of steatotic partial liver grafts [25]. Substantial decreases of oxidative phosphorylative proteins might lead to mitochondrial dysfunction. Therefore, we examined expression of oxidative phosphorylative proteins. Ethanol treatment alone did not alter either the nuclear DNA-encoded mitochondrial proteins AS- β and COX-IV or the mitochondrial DNA-encoded mitochondrial protein ND3. At 2 h after transplantation when mitochondrial depolarization became overt in fatty liver grafts, AS- β , COX-IV and ND3 were not significantly decreased in either lean or fatty liver grafts (Fig. 9).

DISCUSSION

Overproduction of RNS Contributes to Failure of Fatty Liver Grafts

Fatty liver, the most common hepatic disorder in humans, was found in 26% to 50% of potential liver donors [33, 34]. In general, transplantation centers reject severely steatotic grafts for transplantation since steatosis markedly increases the risk of primary graft non-function [3–5, 35]. Hepatic steatosis is often associated with obesity; therefore, high body

mass index is used as an indicator for rejection of potential donors for living liver donation [36]. Alcohol consumption, a known risk factor of steatosis, is frequent in accident victims, a major population of liver donors [8]. Therefore, hepatic steatosis is an important factor that limits the usable donor pool for transplantation. However, recent studies suggested that steatotic grafts should no longer be discarded due to the urgent needs of liver grafts [37]. Thus, how to improve the outcome of fatty liver transplantation remains a crucial issue.

Here we investigated whether iNOS plays a role in the failure of FG. iNOS expression increased slightly after transplantation of LG or ethanol treatment alone (Fig. 1). However, after transplantation of ethanol-induced fatty livers, iNOS expression increased 25-fold. Consistent with the upregulation of iNOS, nitrite/nitrate increased in the serum after transplantation of FG, indicating formation of NO. NO reacts with superoxide radicals to form peroxynitrite, a highly reactive and toxic species that causes nitration of proteins. Our previous study showed that formation of ROS also increased after transplantation of ethanol-induced fatty livers [6], indicating the availability of superoxide radicals. 3-Nitrotyrosine adducts, an indicator of peroxynitrite formation, increased markedly in FG after transplantation. Together, these data indicate that steatosis and transplantation synergistically increase iNOS formation, resulting in excessive RNS formation.

Mechanisms leading to higher expression of iNOS in FG remain unclear. TNF α and ROS production increase in fatty livers and by obesity [38, 39]. Liver transplantation also increases TNF α and ROS production, possibly due to Kupffer cell activation [10, 40]. Moreover, ROS formation was higher after transplantation of FG than LG [41]. TNF α through NF κ B signaling upregulates iNOS expression [42, 43]. Therefore, steatosis and liver transplantation could additively or synergistically increase TNF α and ROS formation and stimulate iNOS expression.

In this study increased iNOS expression and RNS in FG was associated with more severe graft injury, poorer liver function and lower survival after transplantation (Fig. 1–4). Importantly, 1400W, which has substantially higher selectivity to iNOS than nNOS and eNOS (5000:25:1) compared to other iNOS inhibitors, decreased RNS production and improved the outcomes of fatty liver transplantation. These data are consistent with the hypothesis that overproduction of RNS by iNOS plays an important role in failure of FG.

Several studies show that inhibition of iNOS decreases hepatic IR injury [21, 44–46]. By contrast, other studies suggested that NO is protective in I/R and transplantation [20–23, 47]. Whether NO production is protective or detrimental may depend on the context of the physiological or pathological process, the intensity and nature of stresses, the amount, timing and location of RNS production and the co-existence with other risk/protective factors (e.g. ROS). For example, increased NO production by eNOS maybe cytoprotective whereas increased iNOS activity mediates cytotoxicity [48]. Similarly, reperfusion with NO donors is protective after warm ischemia to cultured hepatocytes [20]. However, protection is only seen at lower concentrations. At higher concentrations, NO donors aggravate injury. Other factors such as use of non-selective/low specificity NOS inhibitors could result in blockage of not only iNOS but also eNOS. NO donors, although producing NO in the short term, also inhibit iNOS expression in the longer term [24]. Lastly, the role of RNS may also be organ specific. The present work using a highly selective iNOS inhibitor supports the hypothesis that excessive RNS have injurious effects in FG. Whether 1400W protects against injury in LG will be investigated in the future.

Role of Mitochondrial Depolarization in Fatty Liver Grafts

Mitochondrial function is essential for graft survival after liver transplantation [13, 26]. We explored mitochondrial dysfunction in FG using intravital multiphoton microscopy, a

technique that allows us to visualize mitochondria in living animals. We observed more extensive mitochondrial depolarization in FG than in LG (Fig. 5), most likely due to the MPT onset. Opening of highly conductive, nonselective “megachannels” in the mitochondrial membranes, namely MPT pores, leads to collapse of the inner membrane potential and failure of oxidative phosphorylation [49]. In addition, the MPT causes mitochondrial swelling and release of cytochrome *c* from the inter-membrane space, which triggers apoptosis [30]. Many *in vitro* studies showed that MPT onset is a common pathway of cell necrosis and apoptosis in I/R injury [30, 50]. Recent studies also showed that the MPT onset plays a role in liver injury after I/R and transplantation *in vivo* [12, 13, 26, 31]. Blockade of MPT onset with cyclosporin A and NIM811 attenuated mitochondrial depolarization after transplantation [13, 26], indicating MPT onset plays essential roles in mitochondrial depolarization after transplantation. In this study, inhibition of iNOS prevented the loss of mitochondrial membrane potential and cytochrome *c* release (Fig. 5–6).

Mitochondrial depolarization could also be due to upregulation of UCPs, the proton transporters in mitochondria that are capable of dissipating the mitochondrial membrane potential [51, 52]. In normal adult rat liver, only non-parenchymal cells, particularly Kupffer cells, express UCP2 at low levels [51, 52]. Oxidative stress, endotoxemia, hepatic steatosis and chronic ethanol increase UCP2 activity and expression in hepatocytes [51, 53–55]. Although we detected overt UCP2 expression in fatty livers from rats fed a choline-deficient diet, we did not observe up-regulation of UCP2 after acute ethanol treatment or transplantation of LG or FG (Fig. 8). This is not surprising given the short course of acute ethanol exposure. Thus, mitochondrial depolarization in LG and FG was not due to UCP2 expression. Decreases of respiratory proteins might also lead to mitochondrial dysfunction. However, changes of respiratory protein expression were not observed after transplantation of steatotic grafts at time points where mitochondria depolarized (Fig. 9).

Role of Reactive Nitrogen Species in Mitochondrial Dysfunction Remains Controversial

A NO donor prevented MPT onset in cultured hepatocytes after I/R [20], and this protection was not mediated by PKG but by PKA [56]. Similarly, NO donors and cardiac myocyte-specific expression of iNOS protected cardiac myocytes from the MPT onset [47, 57]. By contrast, other studies showed that NOS inhibitors prevented the MPT onset in astrocytes caused by IR [58]. RNS inhibits cytochrome *c* oxidase and mitochondrial respiration [18, 48], and the nNOS inhibitor 7-nitroindazole prevents acetaminophen-induced mitochondrial depolarization in cultured hepatocytes [59]. Stimulation of iNOS expression by pro-inflammatory cytokines or adenoviral delivery of the iNOS gene causes mitochondrial damage and cell death [42], and iNOS-deficiency blocks sepsis-induced mitochondrial RNS production and mitochondrial function inhibition [60, 61]. Finally, high fat diet and diabetes induce iNOS expression, leading to protein nitration, suppression of mitochondrial respiration and decreased mitochondrial membrane potential in the liver [62, 63]. Together, these studies suggest that RNS play detrimental role in mitochondria.

In this study, we observed more severe mitochondrial depolarization and cytochrome *c* release in FG than LG (Fig. 5 and 6) in association with more iNOS expression and RNS formation (Fig. 1 and 4). Inhibition of iNOS largely prevented mitochondrial depolarization in FG. Furthermore, mitochondrial dysfunction in fatty livers was associated with more necrotic and apoptotic cell death and failure of FG (Fig. 2–3). Blockade of mitochondrial depolarization by inhibition of RNS formation improved the outcome of fatty liver transplantation. Therefore, iNOS generated RNS-induced mitochondrial dysfunction plays a crucial role in FG failure.

Role of Reactive Nitrogen Species in JNK Activation in Fatty Liver Grafts

JNK is activated in response to a variety of physiological and pathophysiological stresses [64–68] and plays an important role in both the intrinsic and extrinsic apoptotic pathways [69] as well as in toxic cytokine and ROS-induced necrotic cell death [70]. Inhibition of JNK prevents preservation-induced graft injury and improves the outcome of non-heartbeating liver transplantation [46, 71]. ROS and RNS formation, calcium mobilization, TNF α formation, Bax translocation, and JNK activation promote MPT onset [31, 72, 73]. In this study, we observed more extensive mitochondrial depolarization in FG than in LG, and this effect was associated with more JNK activation (Fig. 5 and 6), which was blocked by 1400W. Thus, activation of JNK likely mediates MPT onset in fatty liver grafts. Consistent with this hypothesis, generation of peroxynitrite by SIN-1 results in protein nitration and activation of tyrosine kinase and Rac, leading to increased JNK activities [74]. Moreover, iNOS-deficiency attenuated activation of JNK after IR [75], indicating iNOS could control JNK activation.

Calcium is also a known trigger of the MPT, but the importance of calcium in MPT induction varies with circumstances [76]. In some circumstances (e.g. Reye-related agents' cytotoxicity and storage-reperfusion injury to liver grafts), calcium appears to be permissive for MPT onset. By contrast, calcium overload after I/R is the consequence of bioenergetic failure after the MPT in cardiac myocytes [76]. Thus, the exact role of calcium for MPT onset and cell death depends on the particular biologic setting. Future studies will be needed to investigate the relation of RNS and calcium homeostasis in the mitochondrial dysfunction of fatty liver grafts.

Conclusion

In summary, this study shows that hepatic steatosis and cold storage/liver transplantation synergistically induces iNOS and increases RNS production, which leads to JNK activation, mitochondrial damage and FG failure. Inhibition of iNOS effectively prevents excessive RNS production and decreases injury of fatty grafts. Therefore, iNOS inhibitors could be a promising therapy to improve the outcome of fatty liver transplantation.

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ABBREVIATIONS

ALT	alanine aminotransferase
eNOS	endothelial nitric oxide synthase
FG	fatty liver grafts
hpf	high power field
iNOS	inducible nitric oxide synthase
I/R	ischemia/reperfusion
JNK	c-Jun <i>N</i> -terminal kinase
LG	lean liver grafts
MPT	mitochondrial permeability transition

NOS	nitric oxide synthase
NO	nitric oxide
PI	propidium iodide
Rh123	rhodamine 123
RNS	reactive nitrogen species
ROS	reactive oxygen species
TNFα	tumor necrosis factor- α
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
TX	liver transplantation
UCP	uncoupling protein
UW solution	University of Wisconsin cold storage solution

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HIGHLIGHTS

- This study investigates the role of iNOS in fatty liver graft failure.
- iNOS expression and reactive nitrogen species increase markedly in fatty grafts.
- iNOS inhibition attenuates injury and improves survival of fatty liver grafts.
- iNOS inhibition prevents JNK activation and mitochondrial depolarization.
- Mitochondrial depolarization in fatty liver grafts is not due to UCP2 upregulation.

Fig. 1. Inducible Nitric Oxide Synthase is Upregulated after Transplantation of Fatty Liver Grafts from Ethanol-Treated Rats

A: Livers were harvested 20 h after saline (**left**) or ethanol treatment (**right**) (without implantation) for Oil-Red-O staining to evaluate steatosis. **B:** Livers were harvested at 2 h after transplantation (TX) or sham-operation for inducible nitric oxide synthase (iNOS) immunohistochemical staining. Panels are: **upper left**, lean liver from sham-operated rat receiving saline; **upper right**, fatty liver from sham-operated rat receiving ethanol; **lower left**, implanted lean graft from rat receiving saline; **lower right**, implanted fatty graft from rat receiving ethanol. Bar is 20 μ m. iNOS and actin were detected by immunoblotting in liver tissue harvested 2 h after implantation or sham-operation. Representative immunoblot images are shown in **C**, and images were quantified by densitometry (**D**). a, $p < 0.05$ vs the lean livers from sham-operated rats receiving saline; b, $p < 0.05$ vs the fatty livers from sham-operated rats receiving ethanol; c, $p < 0.05$ vs the implanted lean grafts from rats receiving saline. Group sizes were 4 in each group.

Fig. 2. 1400W Decreased Necrosis and Apoptosis in Fatty Liver Grafts after Transplantation
 Livers were collected at 8 h after sham operation (Sham) or liver transplantation (TX). Sections were stained with hematoxylin-eosin (**A–D**, arrows identify necrotic areas) or by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL, **E–H**). Rows are: **1st**, lean liver from sham-operated rat receiving saline; **2nd**, implanted lean graft from rat receiving saline; **3rd**, implanted fatty graft from rat receiving ethanol; **4th**, implanted fatty graft from rat receiving ethanol + 1400W treatment. Bar is 100 μ m. Necrotic areas (**I**) were quantified by image analysis of 10 random fields per liver. a, $p < 0.05$ vs lean livers from sham-operated rats receiving saline; b, $p < 0.05$ vs implanted lean grafts from rats receiving saline; c, $p < 0.05$ vs fatty livers from sham-operated rats receiving ethanol; d, $p < 0.05$ vs implanted fatty grafts from rats receiving ethanol. Cleaved caspase-3 and actin were detected by immunoblotting and representative gels are shown in **J** ($n = 4$ per group).

Fig. 3. 1400W Improved Function and Survival of Fatty Liver Grafts

Blood was collected 8 h after sham operation or transplantation (TX). ALT (**A**) and total bilirubin (**B**) in sera were measured. a, $p < 0.05$ vs lean livers from sham-operated rats receiving saline; b, $p < 0.05$ vs implanted lean grafts from rats receiving saline; c, $p < 0.05$ vs fatty livers from sham-operated rats receiving ethanol; d, $p < 0.05$ vs implanted fatty grafts from rats receiving ethanol ($n = 4-5$ per group). Rats were observed 7 d after TX for survival (**C**), $p = 0.002$ by Kaplan-Meier test ($n = 8$ per group).

Fig. 4. 1400W Decreased Reactive Nitrogen Species Formation in Fatty Liver Grafts

Livers and blood were collected at 2 h after transplantation (TX) or sham-operation. Nitrite and nitrate in sera were detected (A). 3-Nitrotyrosine adducts in liver were detected immunohistochemically (C–F). Panels are: C, lean liver from sham-operated rat receiving saline; D, implanted lean graft from rat receiving saline; E, implanted fatty graft from rat receiving ethanol; F, implanted fatty graft from rat receiving ethanol+1400W treatment; Bar is 50 μm. Nitrotyrosine adduct-positive areas were quantified by image analysis (B). a, $p < 0.05$ vs lean livers from sham-operated rats receiving saline; b, $p < 0.05$ vs implanted lean grafts from rats receiving saline; c, $p < 0.05$ vs fatty livers from sham-operated rats receiving ethanol; d, $p < 0.05$ vs implanted fatty grafts from rats receiving ethanol (n=4 per group).

Fig. 5. Mitochondrial Depolarization Occurs after Transplantation of Fatty Liver Grafts: Prevention by 1400W

Intravital confocal/multiphoton microscopy was performed at 2 h after transplantation. Typical intravital multiphoton fluorescence images are shown. Panels are: **A**, sham-operated liver from saline-treated rat; **B**, implanted lean graft from saline-treated rat; **C**, implanted fatty graft from ethanol-treated rat; **D**, implanted fatty graft from ethanol-treated rat+1400W treatment; Bar is 10 μ m. Hepatocytes with depolarized mitochondria were counted in 10 random fields per rats (**E**). hpf: high power field; a, $p < 0.05$ vs lean livers from sham-operated rats receiving saline; b, $p < 0.05$ vs implanted lean grafts from rats receiving saline; c, $p < 0.05$ vs fatty livers from sham-operated rats receiving ethanol; d, $p < 0.05$ vs implanted fatty grafts from rats receiving ethanol (n=4 in each group).

Fig. 6. Cytochrome c Release from Mitochondria after Transplantation of Fatty Liver Grafts: Prevention by 1400W

Livers grafts were harvested for cytochrome *c* immunohistochemistry at 8 h after transplantation. Representative images are shown (n=4 per group). Panels are: **A**, sham-operated liver from saline-treated rat; **B**, lean liver graft from saline-treated donor; **C**, fatty liver graft from ethanol-treated donor; **D**, fatty liver graft from ethanol-treated donor with 1400W treatment. Bar is 50 μ m.

Fig. 7. 1400W Inhibited c-Jun N-Terminal Kinase Activation after Transplantation of Fatty Liver Grafts

Livers were harvested at 2 h after sham operation or transplantation (TX). Phospho-c-jun (p-c-Jun), c-jun, phospho-c-Jun *N*-terminal kinase (p-JNK1/2), JNK1/2 and actin were detected by immunoblotting. Panel **A** shows representative gels. Panel **B** plots mean p-c-Jun/actin ratios. a, $p < 0.05$ vs lean livers from sham-operated rats receiving saline; b, $p < 0.05$ vs fatty livers from sham-operated rats receiving ethanol; c, $p < 0.05$ vs implanted lean grafts from rats receiving saline; d, $p < 0.05$ vs implanted fatty grafts from rats receiving ethanol ($n = 4$ per group).

Fig. 8. Uncoupling Protein-2 did not Increase after Transplantation of Lean and Fatty Liver Grafts

Livers were harvested at 2 h after sham operation or transplantation (TX). Some livers harvested from rats fed choline-deficient diet (CDD) for 2 weeks (no transplantation) were used as positive control. UCP-2 was detected by immunohistochemistry. Shown are representative images (n=4 per group). **A**, sham-operated lean liver; **B**, sham-operated fatty liver from an ethanol-treated rat; **C**, implanted lean graft; **D**, implanted fatty graft from an ethanol-treated rat; **E**, liver from a rat fed a choline-deficient diet (n=4 per group). Bar is 50 μ m.

Fig. 9. Oxidative Phosphorylation Proteins were not Significantly Decreased at Early Stage after Transplantation of Lean and Fatty Liver Grafts

Livers were harvested at 2 h after sham operation or transplantation (TX). ATP synthase-β (AS-β), cytochrome c oxidase-IV (COX-IV), NADH dehydrogenase-3 (ND3), and actin were detected by immunoblotting. Shown are representative gels (n=4 per group).