NIM811, a Mitochondrial Permeability Transition Inhibitor, Prevents Mitochondrial Depolarization in Small-for-Size Rat Liver Grafts

Z. Zhong, T. P. Theruvatha, R. T. Currinc, P. C. Waldmeier and J. J. Lemasters

Introduction

Due to shortage of donor organs, living donor and split liver transplantation has developed rapidly in recent years (1–3). Adult-to-adult living donor and split liver transplantation result in small-for-size liver grafts. Such small-for-size grafts are associated with increased mortality and decreased graft function (1,4). Mechanisms underlying dysfunction and failure of small-for-size grafts remain unclear. Our previous study showed that ATP production decreases markedly in small-for-size liver grafts after transplantation, which was associated with increased graft injury, suppressed regeneration and higher mortality (5,6). Energy supply is critical for cell survival and proliferation. Since mitochondria are the major source of ATP in highly aerobic hepatocytes, mitochondrial dysfunction may contribute to failure of small-for-size liver grafts after transplantation.

Mitochondrial oxidative phosphorylation is the major generator of ATP in aerobic metabolism (7). Transport of electrons through the respiratory chain (Complexes I–IV) creates an electrochemical gradient of H+ ions across the mitochondrial inner membrane comprised mostly of an electrical potential gradient. The energy of this proton gradient drives ATP synthesis via the mitochondrial ATP synthase (Complex V). When it occurs, mitochondrial membrane depolarization compromises energy production profoundly and leads to cell injury and death. Factors that cause mitochondrial depolarization include respiratory inhibition, protonophoric uncoupling, increased expression of uncoupling proteins (UCPs) and onset of the mitochondrial permeability transition (MPT). The last of these, the MPT, is caused by opening of permeability transition (PT) pores in the mitochondrial inner membrane that nonspecifically transport aqueous solutes up to a molecular weight of about 1500 Da (8,9).

Cyclosporin A (CsA) specifically inhibits PT pores by binding to cyclophilin D in the matrix and on the inner surface of the inner membrane (10–13). N-Methyl-4-isoleucine cyclosporin (NIM811) is a nonimmunosuppressive derivative of CsA that does not inhibit calcineurin but nonetheless inhibits the MPT in cultured hepatocytes and isolated liver mitochondria (14). Growing evidence implicates a critical role of the MPT in necrosis and apoptosis (15–17). Therefore, the MPT might also play a role in injury to small-for-size liver grafts.

ATP decreases markedly in small-for-size liver grafts. This study tested if the mitochondrial permeability transition (MPT) underlies dysfunction of small-for-size livers. Half-size livers were implanted into recipients of about twice the donor weight, resulting in quarter-size liver grafts. NIM811 (5 µM), a nonimmunosuppressive MPT inhibitor was added to the storage solutions. Mitochondrial polarization and cell death were assessed by confocal microscopy of rhodamine 123 (Rh123) and propidium iodide (PI), respectively. After quarter-size transplantation, alanine aminotransferase (ALT), serum bilirubin and necrosis all increased. NIM811 blocked these increases by >70%. After 38 h, BrdU labeling, a marker of cell proliferation and graft weight increase were 3% and 5%, respectively, which NIM811 increased to 30% and 42%. NIM811 also increased survival of quarter-size grafts. In sham-operated livers, hepatocytes exhibited punctate Rh123 fluorescence. By contrast, in quarter-size grafts at 18 h after implantation, mitochondria of most hepatocytes did not take up Rh123, indicating mitochondrial depolarization. Nearly all hepatocytes not taking up Rh123 continued to exclude PI at 18 h, indicating that depolarization preceded cell death. NIM811 and free radical-scavenging polyphenols strongly attenuated mitochondrial depolarization. In conclusion, mitochondria depolarized after quarter-size liver transplantation. NIM811 decreased injury and stimulated regeneration, probably by inhibiting free radical-dependent MPT onset.

Key words: Confocal microscopy, liver graft failure, liver regeneration, mitochondrial permeability transition, partial liver transplantation

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liver grafts, leading to mitochondrial depolarization and graft dysfunction. Accordingly, we investigated whether MPT blockade with NIM811 could alleviate injury and improve outcome after transplantation of small-for-size liver grafts.

Methods

Animals and liver transplantation

Male Lewis rats (170–200 g) were used for orthotopic liver transplantation, as described elsewhere (18). Briefly, livers were flushed in situ with 5 mL University of Wisconsin (UW) cold storage solution (0–1°C, Barr Laboratories, Inc., Pomona, NY) via the portal vein and explanted. Cuffs prepared from 14-gauge i.v. catheters were placed over the subhepatic vena cava and the portal vein. Liver mass was then reduced by about 50% by removing the left lateral lobe, the left portion of the median lobe and the anterior and posterior caudate lobes after ligation with 4-0 silk suture (18). Explants were stored in UW solution at 0–1°C for 6 h and rinsed with room temperature lactated Ringer’s solution (Abbott Laboratories, North Chicago, IL) just prior to implantation. NIM811 (5 μM, Novartis Pharma Ltd., Switzerland) was added to the storage and rinse solutions. Our previous study showed that free radical production increased markedly after transplantation of small-for-size liver grafts (6) and reactive oxygen species could cause onset of the MPT (13,19–21). To investigate if the MPT in small-for-size liver grafts is a free radical-dependent event, a potent free radical-scavenging polyphenol-enriched aqueous extract (20 μg/mL) from Camellia sinensis (Taiyo Kagaku Co., Yokkaichi, Mie, Japan) was added to the storage and rinse solutions. The polyphenol composition of the extract is described elsewhere (22).

Reduced-size liver explants were implanted into recipients of similar (170–200 g) or greater body weight (350–420 g), which results in a graft weight/recipient weight ratio of ~25% (quarter-size) and ~50% (half-size), respectively. In addition, unreduced livers were implanted into recipients of similar body weights (170–200 g) as full-size controls. Our previous study showed that under these conditions, 2-week survival was 100% in full-size grafts, 80% in half-size grafts and 30% in quarter-size grafts (6). The graft weight/standard body weight was 24.8 ± 0.8% in the quarter-size group and 25.5 ± 3.1% for quarter-size grafts treated with NIM811 (p > 0.1). Graft weight was measured just prior to cold storage and after recovering at 38 h after implantation. Animals were observed 1 week for survival after transplantation in this study. All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Serum alanine aminotransferase (ALT) and total bilirubin

To access liver graft injury and function, serum ALT and total bilirubin were measured from blood samples collected from the tail vein at 18, 24 and 38 h after implantation using analytical kits from Sigma Chemical (St. Louis, MO). Serum ALT is affected by the size of graft, which releases ALT and total bilirubin (27). To adjust for graft size and recipient body weight, serum ALT was normalized by multiplying by the recipient’s standard body weight (6.4% of body weight) and dividing by graft weight (23). Peak ALT and bilirubin levels were the average of the highest values in these time points.

Histology and Immunohistochemical Staining for TUNEL, 5-Bromo-2′-deoxyuridine (BrdU), Proliferating Cell Nuclear Antigen (PCNA) and UCP2

Rats were anesthetized with pentobarbital (60 mg/kg, i.p.) at various times after implantation and the portal veins were cannulated with 24-gauge i.v. catheters. The livers were infused with 10 mL normal saline followed by 10 mL of 4% paraformaldehyde (Sigma) in Dulbecco’s phosphate-buffered saline (Invitrogen Corp., Grand Island, NY). Tissue blocks were imbedded in paraffin after immersion in 4% paraformaldehyde for 48 h. In sections stained with hematoxylin-eosin, necrotic areas were quantified by image analysis using a Universal Imaging Image-1 AT image acquisition and analysis system (West Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) and a 10× objective lens.

Apoptosis was assessed by labeling DNA strand breaks immunohistochemically using an in situ Cell Death Detection Kit with fluorescein isothiocyanate conjugated to alkaline phosphatase (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer’s instructions. Alkaline phosphatase activity was revealed with fast red chromagen (0.25 mg/mL in 0.1 M Tris-HCl buffer, pH 8.2, Roche Diagnostics Corp.) for 9 min at room temperature. TUNEL-positive and negative cells were counted in 10 randomly selected fields using a 40× objective lens.

BrdU was injected (100 mg/kg i.p.) 1 h prior to liver recovering to detect cells synthesizing DNA. At 18, 24 and 38 h after implantation, BrdU incorporation in liver sections was determined by immunohistochemical staining. Briefly, sections were deparaffinized with xylene (Mallinckrodt Baker, Paris, KY) and taken through a graded series of alcohol/water mixtures to rehydrate the tissue. Sections were incubated with 4 N HCl for 20 min and then incubated with pepsin reagent (DAKO, Carpinteria, CA) at 37°C for 15 min to retrieve antigen. Sections were then exposed to mouse anti-BrdU monoclonal antibody (1:200 dilution) in 0.1 M phosphate buffer (pH 7.1) containing 0.1% Tween-20 and 1% bovine albumin (Sigma) for 10 min at room temperature. Peroxidase-conjugated anti-mouse IgG1 antibody (DAKO) was applied and then 3,3′-diaminobenzidine chromogen was added as the peroxidase substrate. A light counterstain of modified Mayer’s hematoxylin (American Master Tech Scientific, Lodi, CA) was applied for identification of unlabeled nuclei. Immunohistochemistry of PCNA, another marker of cell proliferation, was performed with an antibody against PCNA (DAKO) at a dilution of 1:250 for 30 min at room temperature. For BrdU and PCNA staining procedures, positive and negative cells were counted in 10 randomly selected fields under the light microscope using a 20× objective lens. Immunohistochemistry of UCP2 was performed with an antibody against UCP2 (Santa Cruz Biotecnology, Santa Cruz, CA) at a dilution of 1:200 for 1 h at room temperature.

Liver perfusion and confocal microscopy

Rhodamine 123 (Rh123, Sigma) was used to detect mitochondrial polarization after transplantation. Rh123 is a cationic fluorophore that is taken up by polarized mitochondria in response to their negative membrane potential. When mitochondria depolarize, Rh123 is released and fluorescence declines. At 18 h after transplantation, full-size and partial liver grafts were isolated and perfused via a cannula inserted into the portal vein with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with O2:CO2 (95:5) in a noncirculating system at a flow rate of ~4 mL/min (24). Rh123 (2 μM) and propidium iodide (PI, 0.5 μM, Sigma), which labels nuclei of non-viable cells, were infused into the livers for 15 min. Fluorescent imaging of the perfused livers was performed using a CARV spinning disk confocal microscopic system (ATTO Bioscience, Rockville, MD) with a 40× water immersion objective lens (Zeiss C-Apochromat, 1.2 NA) using excitation wavelengths of 488 and 555 nm, respectively and a multilength emission filter.

Statistical analysis

Groups were compared using ANOVA plus a Student-Newman-Keuls post hoc test. Data shown are means ± SEM (4–6 livers in each group). Differences were considered significant at p < 0.05.
Results

**NIM811 decreases transaminase release and prevents hyperbilirubinemia after transplantation of small-for-size liver grafts**

Graft injury after transplantation was assessed from serum ALT measured 18, 24 and 38 h postoperatively. Before implantation, serum ALT was about 0.05 U/g. At 18 h after implantation of quarter-size grafts, ALT increased, peaked at about 18 h and then decreased slightly (Figure 1A). Peak ALT reached 1.4 and 18.5 U/g in rats receiving full-size and quarter-size grafts, respectively (Figure 1B). NIM811, a specific inhibitor of the MPT, decreased peak ALT to 4.8 U/g in rats implanted with quarter-size liver grafts, which reflected a ∼75% decrease compared to quarter-size grafts without NIM811 treatment (Figure 1B). These results indicated that NIM811 markedly reduces injury to quarter-size grafts.

Increased serum bilirubin indicates poor liver function. After transplantation of quarter-size grafts, serum bilirubin increased and peaked at 38 h (Figure 1C). By contrast, after transplantation of quarter-size grafts pretreated with NIM811, bilirubin increased initially and peaked at 18 h but decreased afterward (Figure 1C). Peak bilirubin increased from 0.003 mg/g liver in sham-operated rats to 0.43 mg/g liver in rats receiving quarter-size grafts (Figure 1D). NIM811 decreased peak bilirubin by 72% in rats receiving quarter-size liver grafts (Figure 1D).

**NIM811 attenuates hepatic necrosis and apoptosis after transplantation of small-for-size liver grafts**

No pathological changes were observed in liver tissue at 38 h after sham operation (Figure 2, upper left panel). Necrosis and eosinophilic inclusion bodies developed after implantation of quarter-size liver grafts, mainly in the periportal and midzonal regions of the liver lobule (Figure 2, lower left panel). By contrast, necrosis and eosinophilic inclusion bodies were rarely present in full-size grafts (Figure 2, upper right panel). Necrotic areas, as determined by image analysis, constituted 17% of liver sections in quarter-size grafts, compared to about 2% in full-size grafts (Figure 3A). NIM811 decreased necrotic areas in quarter-size grafts to 4%, which was not statistically different from untreated full-size grafts (Figure 2, lower right panel; Figure 3A).

Apoptosis in liver sections was detected by TUNEL labeling of DNA. TUNEL-positive cells were rare (0.32 ± 0.046%, mean ± SEM, n = 4) in livers from sham-operated animals. Thirty-eight hours after implantation of full-size liver grafts, TUNEL-positive cells were 0.57 % ± 0.07%, (n = 4, p > 0.05 vs. sham). After quarter-size liver transplantation, TUNEL-positive cells were 1.2 ± 0.17% without NIM811 treatment (n = 4, p < 0.05 vs. sham and full-size grafts). NIM811 decreased TUNEL labeling to 0.37 ± 0.05% (n = 4, p < 0.05 vs. quarter-size grafts without...
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Figure 3: NIM811 protects against necrosis and increases survival after transplantation of small-for-size liver grafts. Thirty-eight hours after implantation, liver grafts were fixed, sectioned, and stained with hematoxylin and eosin. Necrotic areas (A) were quantified by image analysis, as described in ‘Methods’. Group sizes are four livers per group. (A) p < 0.05 vs. sham operation; (B) p < 0.05 vs. full-size (100%); (C) p < 0.05 vs. quarter-size (25%). Rats were observed 1 week after transplantation for survival (B). Survival of quarter-size grafts treated with NIM811 was significantly greater than untreated quarter-size grafts (p < 0.05 by Fisher’s exact test).

NIM811 reverses suppression of liver regeneration after transplantation of small-for-size liver grafts

Our previous study showed that liver regeneration is suppressed in small-for-size liver grafts (5). Therefore, we investigated if NIM811 improves liver regeneration after transplantation of small-for-size liver grafts. Liver regeneration was evaluated by BrdU incorporation, expression of PCNA and increases of graft weight. BrdU-positive cells were barely detectable 38 h after sham operation. After implantation of half-size livers, increased BrdU labeling began after 18 h in both perportal and midzonal regions of the liver lobule and continued to increase up to 38 h (data not shown). At this stage, proliferating cells were distributed throughout the liver lobules with highest concentration in midzonal regions (data not shown). Proliferating cells were predominantly hepatocytes, consistent with our previous report (5).

Cell proliferation in different treatment groups was compared at 38 h after implantation. BrdU-positive cells were 0.23% and 2.3% in sham-operated livers and full-size grafts, respectively, at 38 h after transplantation (Figure 4, upper left and right panels; Figure 5A). BrdU labeling increased sharply to 16.4% in half-size grafts but was only 2.9% in quarter-size grafts (Figure 4, middle left and right panels; Figure 5A), indicating suppression of cell proliferation in quarter-size grafts. NIM811 did not alter cell proliferation in full-size graft (data not shown) but increased cell proliferation in quarter-size grafts to 30.9%, which was nearly double the proliferation observed in untreated half-size liver grafts (Figure 4, lower left panel; Figure 5A). PCNA staining, another indicator of cell proliferation, showed similar alterations (data not shown).

Increases of graft weight reflect both cell proliferation and hypertrophy. Thirty-eight hours after implantation, graft weight did not increase in full-size grafts but increased 39% in half-size grafts (Figure 5B). After quarter-size transplantation, graft weight did not increase. By contrast with NIM811 treatment, graft weight increased 42% after quarter-size transplantation (Figure 5B).

Mitochondrial depolarization occurs after transplantation of small-for-size liver grafts: prevention by NIM811

If the MPT occurs after liver transplantation, mitochondria will depolarize. To monitor mitochondrial depolarization after transplantation, liver grafts were infused with Rh123, a green-fluorescing fluorophore that is taken up by polarized mitochondria. Additionally, red-fluorescing PI was infused to detect nonviable cells. Overlays of green and red treatment). Although apoptosis increased in small-for-size liver grafts, only small number of hepatocytes (~1%) underwent apoptosis. Therefore, increased cell killing after quarter-size liver transplantation was mainly due to necrosis rather than apoptosis.

Clinically, small-for-size liver transplantation is associated with a higher probability of graft failure. Therefore, we evaluated the effect of NIM811 on survival of quarter-size liver grafts. Survival rates were 100% after sham operation. After transplantation of quarter-size grafts, survival decreased significantly to 30% (Figure 3B). Death occurred in the first 3 days after implantation. Importantly, NIM811 significantly increased survival of quarter-size grafts to 81% (Figure 3B).

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Figure 4: NIM811 reverses suppression of cell proliferation in small-for-size liver grafts after transplantation. Livers were recovered at 38 h after sham operation or transplantation. BrdU (100 mg/kg, i.p.) was injected 1 h before recovery and BrdU incorporation was detected immunohistologically. Panels are: upper left, liver from a sham-operated rat; upper right, full-size graft (100%); middle left, half-size graft (50%); middle right, quarter-size graft (25%); lower left, quarter-size graft treated with NIM811. Bar is 50 μm.

Confocal fluorescent images allowed identification of viable cells (no red fluorescence) with polarized mitochondria (intracellular punctate green fluorescence), viable cells with depolarized mitochondria (no or diffuse green fluorescence) and nonviable cells (red nuclear fluorescence). In nonviable cells, mitochondria were consistently depolarized.

In sham-operated rats, green Rh123 fluorescence was punctate in virtually all hepatocytes, indicating mitochondrial polarization (Figure 6, first row) and red PI labeling of nuclei was rare (Figure 6, upper middle). After full-size liver transplantation, mitochondrial depolarization was also infrequent (3.5 ± 0.13 cells/hpf, mean ± SEM, second row, Figure 6) but greater than after sham operation (0.13 ± 0.13 cells/hpf, p < 0.05 vs. full-size grafts). After quarter-size transplantation, many more hepatocytes contained depolarized mitochondria that did not accumulate Rh123 (16 ± 0.78 cells/hpf; Figure 6, third row). Some parenchymal and nonparenchymal cells also labeled with PI. PI-labeled hepatocytes never contained polarized mitochondria, whereas many hepatocytes with depolarized mitochondria excluded PI. Mitochondrial polarization could not be reliably assessed in nonparenchymal cells because of their scant cytoplasm. In some hepatocytes, Rh123 fluorescence was diffuse but relatively bright. This may indicate the recent depolarization of mitochondria and release of mitochondrial Rh123 into the cytosol. Overall, the results indicated that at 18 h after implantation of quarter-size liver grafts, mitochondrial depolarization occurred in many hepatocytes and that mitochondrial depolarization preceded hepatocyte death.

To determine whether NIM treatment affected mitochondrial depolarization, quarter-size liver grafts after NIM811 treatment were also perfused and loaded with Rh123 and PI. NIM811 blocked mitochondrial depolarization by 66% (5.3 ± 0.86 cells/hpf, p < 0.05, Figure 6, fourth row). NIM811 did not significantly decrease PI uptake because cell death was still very low (<1 cell/hpf) at this relatively early stage after transplantation.

Figure 5: Liver regeneration is suppressed after transplantation of small-for-size liver grafts: reversal by NIM811. Full-size and reduced-size rat livers were transplanted and recovered in comparison to sham operation, as described in Figure 4. Liver grafts were weighed after recovered and at 38 h after implantation and BrdU incorporation was detected immunohistologically. In (A) the percentage BrdU labeling in hepatocytes is plotted. In (B) increases of graft wet weight are plotted. Group sizes are four livers per group. (A) p < 0.05 vs. sham; (B) p < 0.05 vs. 100%; (C) p < 0.05 vs. 50%, (D) p < 0.05 vs. 25%.

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Our previous study showed that reactive oxygen species (ROS) formation increases after quarter-size liver transplantation (6). Since oxidative stress causes the MPT leading to mitochondria depolarization (13,19–21), we investigated the role of oxidative stress by determining whether free radical scavenging by green tea polyphenols protected against mitochondrial depolarization after quarter-size liver transplantation. As shown in Figure 6 (fifth row), polyphenol treatment also blunted mitochondrial depolarization in quarter-size grafts, indicating that mitochondrial depolarization in quarter-size grafts is a free radical-dependent event.

**Expression of UCP2 was not increased in hepatocytes after transplantation of small-for-size liver grafts**

Increased expression of UCP2 might promote mitochondrial depolarization after quarter-size liver transplantation. Accordingly, UCP2 was detected immunocytochemically in liver sections. UCP2 was virtually undetectable in livers after sham operation and in full-size liver grafts (Figure 7, upper panels). After half-size and quarter-size liver transplantation, UCP2 protein increased in some nonparenchymal cells, but was not detectable in virtually all hepatocytes (Figure 7, lower panels).

**Discussion**

**Mitochondrial dysfunction occurs after transplantation of small-for-size liver grafts**

Transplantation of small-for-size liver grafts is associated with increased mortality and impaired graft function (1,4). Small-for-size liver grafts experience a higher metabolic burden than full-size liver grafts. In addition to the higher demand for ATP to maintain physiological and metabolic functions, increased energy supply is also required for liver regeneration in partial liver grafts. ATP not only serves as the energy supply for regeneration but also affects signal transduction. ATP activates JNK through P2 purinergic receptors (25) and is a required substrate for activation of JNK and its upstream kinases such as MKK4 and MKK7 (26). Other events involved in the G1-S transition necessary for regeneration, including activation of CyD1/cdk protein complexes, ion channels and enzymes (ornithine decarboxylase, thymidine kinase and chromatin remodeling enzymes), also require ATP (27). Therefore, a deficiency of ATP in small-for-size liver grafts both limits energy supply for biogenesis of new liver cells and inhibits regeneration signaling (5). No doubt, proper mitochondrial function is essential for survival of partial liver grafts after liver transplantation.
Despite increased energy requirements after partial liver transplantation, defective energy supply appears to occur after transplantation of small-for-size liver grafts. After living donor liver transplantation, alteration of arterial ketone body ratios, a measure of hepatic mitochondrial functioning, is an early indicator of partial graft dysfunction (28). In our previous study, graft ATP decreased \( \sim 70\% \), after quarter-size rat liver transplantation and compromised intracellular energy supply was associated with increased graft injury, poorer regeneration and higher mortality (5). In the present study, we show that compromised energy supply in quarter-size liver grafts was associated with mitochondrial depolarization in \( >75\% \) of hepatocytes (Figure 6). This mitochondrial dysfunction occurred prior to cell death and was accompanied by graft injury (ALT release and necrosis), poor liver function (hyperbilirubinemia), suppression of liver regeneration and decreased survival (Figures 1–5). These data suggest that mitochondrial depolarization and dysfunction are likely responsible, at least in part, for poor outcomes after transplantation of small-for-size grafts.

**Mitochondrial depolarization in hepatocytes is not due to overexpression of UCP2**

Several mechanisms may contribute to mitochondrial depolarization after transplantation of small-for-size liver grafts. Maintaining the mitochondrial membrane potential is critical for proper mitochondrial function. UCPs are proton transporters that are capable of dissipating the mitochondrial membrane potential and pH gradient and decreasing the efficiency of ATP synthesis (29,30). In normal adult rat liver, only nonparenchymal cells, particularly Kupffer cells, express UCP2 at low levels (29,30). Oxidative stress, cholestasis, endotoxemia, lipids, fish oil and fibrate compounds increase UCP2 activity and expression in hepatocytes (29–35). Increased UCP2 also occurs in the fatty livers from ob/ob mice and, to a lesser extent, in mice treated chronically with ethanol (34,36,37). Thus, mitochondrial depolarization in small-for-size liver grafts might be due to increased expression of UCP2. However, we found that although UCP2 expression increased in small-for-size liver grafts, UCP2 expression remained almost exclusively in nonparenchymal cells (Figure 7). Therefore, mitochondrial depolarization in hepatocytes is unlikely due to increased UCP2 expression.

**Role of the MPT in failure of small-for-size grafts**

Alternatively, onset of the MTP could cause mitochondrial depolarization. The MPT is caused by opening of nonselective, highly conductive PT pores in the mitochondrial inner membrane (38). The exact molecular composition of PT pores remains unclear. In one model, PT pores are composed of the voltage-dependent anion channel (VDAC) from the outer membrane, the adenine nucleotide translocator (ANT) from the inner membrane, cyclophilin D and other molecular chaperones (39). When the MPT occurs, the mitochondrial membrane potential collapses, leading to failure of oxidative phosphorylation and necrotic cell death (15,16,40). In addition, the MPT causes large amplitude swelling, outer membrane rupture and release of cytochrome c from the intermembrane space, which triggers activation of caspases and apoptosis (19,40). Growing evidence supports a critical role of the MPT in cell necrosis and apoptosis in ischemia/reperfusion injury (15,16).

After transplantation of small-for-size grafts, increased metabolic burden on partial liver grafts may make mitochondria more prone to damage. After 70% partial hepatectomy, electron microscopy shows swollen mitochondria in regenerating livers and after the MPT isolated mitochondria swell in a fashion blocked by CsA (17). Accordingly, we tested the hypothesis that the MPT occurs after small-for-size liver transplantation and that inhibition of the MPT protects against graft injury and improves regeneration. In support of the hypothesis, we found that mitochondria of reduced size liver grafts became depolarized in situ 18 h after transplantation. Moreover, NIM811, a nonimmunosuppressive inhibitor of the MPT, substantially decreased mitochondrial depolarization (Figure 6), alleviated graft injury (Figures 1–3), improved graft function (Figure 1), enhanced liver regeneration (Figures 4 and 5) and increased survival (Figure 3) after small-for-size liver transplantation. These results indicate that the MPT likely plays a critical role in the failure of partial liver grafts. NIM811 is a nonimmunosuppressive derivative of CsA. Both inhibit the MPT by binding to cyclophilin D. NIM811 is equipotent to CsA in inhibition of onset of the MPT in cultured cells (14). Clinically, CsA is given at doses of about 4 mg/kg/day to graft recipients, starting several hours or even 1 day after liver transplantation and resulting a trough level in the blood of about 200 ng/mL (~0.17 \( \mu \)M). However, in this study, NIM811 was added to the cold storage solution and post-storage rinse solution at 5 \( \mu \)M. Due to the differences in method, dose and time frames of application, estimation of the extent that immunosuppressive concentrations of CsA would reproduce the findings of the current study is difficult.

ROS formation can also damage mitochondria and our previous study showed that ROS production increases markedly after transplantation of small-for-size liver grafts (6). ROS also trigger opening of MPT pores (13,19–21), whereas uncoupling of oxidative phosphorylation caused by the MPT further increases oxidative stress (13,41), thus causing a vicious cycle. In the present study, we found that free radical-scavenging polyphenols largely blocked mitochondrial depolarization after small-for-size liver grafts (Figure 6). These findings are consistent with the conclusion that increased ROS formation by small-for-size grafts...
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promotes MPT onset and subsequent graft dysfunction, injury and failure to regenerate.

Taken together, the results show that mitochondrial depolarization and dysfunction occur after transplantation of small-for-size liver grafts, which are due, at least in part, to onset of the MPT. Of potential therapeutic significance, inhibition of the MPT with the nonimmunosuppressive CsA analog, NIM811, protected against injury, improved function and restored regeneration of small-for-size liver grafts. Such interventions to prevent the MPT might improve outcomes in clinical living donor and split liver transplantation.

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