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Mitochondrial Permeability Transition in Liver Ischemia and Reperfusion: Role of c-Jun N-Terminal Kinase 2

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Abstract

The mitochondrial permeability transition (MPT) mediates hepatic necrosis after ischemia and reperfusion (I/R). Here, we studied the role of c-Jun N-terminal kinase 2 (JNK2) in MPT-induced liver injury. Wildtype (WT) and JNK2 knockout (KO) mice underwent 70% liver ischemia for 1 hr followed by reperfusion for 8 hr, after which hepatocyte injury and animal survival was assessed. Compared with WT, JNK2 KO mice had 38% less alanine transaminase release and 39% less necrosis by histology. Survival out to 14 days was also greater in JNK2 KO mice (57% vs. 11%), and overall Kaplan-Meier survival was improved. No difference in apoptosis was observed. Intravital multiphoton microscopy of potential-indicating rhodamine 123 after reperfusion revealed depolarized mitochondria in 82% of WT hepatocytes, which decreased to 43% in JNK2 KO hepatocytes. In conclusion, JNK2 contributes to hepatocellular injury and death after I/R in association with increased mitochondrial dysfunction via the MPT.

Keywords

Apoptosis; c-Jun N-terminal kinase 2; Ischemia/reperfusion; Mitochondrial permeability transition; Necrosis

In liver and other organs, the mitochondrial permeability transition (MPT) plays an important role in the pathogenesis of injury after ischemia and reperfusion (I/R) (1–3). Opening of PT pores in the mitochondrial inner membrane causes the MPT (3). Permeability transition pores nonspecifically conduct low molecular weight solutes to cause mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude swelling. ATP depletion after uncoupling produces necrotic cell killing, whereas swelling leads to outer membrane rupture, release of proapoptotic proteins such as cytochrome *c* from the intermembrane space and ATP-dependent caspase activation (2). Thus, onset of the MPT leads to both oncotic necrosis from ATP depletion and caspase-dependent apoptosis if ATP depletion does not occur fully (2,3).

Previously, liver injury after warm I/R and graft failure after cold storage and transplantation were decreased by an inhibitor of c-Jun N-terminal kinase (JNK) (4,5). c-Jun N-terminal kinase is a stress-activated protein kinase, and two JNK isoforms, JNK1 and JNK2, are expressed in liver tissue (6). The JNK2 isoform promotes both acetaminophen-induced and TNF α -dependent liver injury (7,8). However, the role of JNK2 in MPT-dependent liver injury after warm I/R has not been determined.

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To assess the role of JNK2 in hepatic I/R injury, we clamped the hepatic artery and portal vein of the median and left lobes of livers of JNK2 knockout (KO) and wildtype (WT) mice (70% liver ischemia) for 1 hr followed by reperfusion. At 8 hr after sham operation, serum alanine transaminase (ALT) in WT and JNK2 KO mice was normal and comparable to unoperated mice (not shown). At 8 hr after I/R to WT mice, ALT increased to $23,188 \pm 2,494$ U/L. In JNK2 KO mice, ALT increased to $14,354 \pm 3,244$ U/L after I/R, which was 38% less than WT (Fig. 1A, $P < 0.05$).

Injury was also assessed histologically at 8 hr after surgery. In unoperated and sham-operated mice, liver histology was normal (not shown). By contrast after I/R, hepatic necrosis developed, which was greater in WT mice (Fig. 1C, dashed line) than in JNK2 KO mice (Fig. 1D, dashed line). The percent area of necrosis was $57\% \pm 10.4\%$ and $35\% \pm 2.9\%$ in WT and JNK2 KO mice, respectively (Fig. 1B, $P < 0.05$). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of liver sections was used to assess apoptosis in nonnecrotic liver areas. After sham operation to WT and JNK2 KO mice, TUNEL was 1.8 ± 0.1 and 2 ± 0.1 cells/high-power field (HPF), respectively (Fig. 2A, D, $P > 0.2$). After liver I/R, TUNEL was 2.5 ± 0.3 and 2.7 ± 0.6 cells/HPF in livers from WT and JNK2 KO mice, respectively (Fig. 2B–D, $P > 0.2$).

Long-term survival was assessed after liver I/R as described above except that the normoxic liver remnants were removed (30% partial hepatectomy) at the end of the ischemic period forcing reliance on the I/R-injured liver tissue (9). Of nine WT mice, eight died within 3 days resulting in 11% survival (Fig. 1E). In JNK2 KO mice, survival after I/R increased to 57% and Kaplan-Meier survival was improved ($P < 0.05$, Fig. 1E).

At 4 hr after sham operation, intravital multiphoton microscopy showed bright fluorescence of Rh123 in hepatocytes whose punctate pattern denoted polarization of individual mitochondria (Fig. 3A). Cytosolic and nuclear areas had little fluorescence. PI labeling of nuclei after sham operation was very rare, indicating the absence of cell death. By contrast, at 4 hr after I/R to livers of WT mice, Rh123 staining became diffuse and dim in many hepatocytes. Additionally, PI positive red-fluorescing nuclei were observed (Fig. 3B, yellow arrow). PI labeling occurred in both the round nuclei of parenchymal cells and the flattened irregular nuclei of nonparenchymal cells. Overlays of the green and red channels revealed that all PI-labeled cells had diffuse or absent Rh123 staining. Thus, all nonviable cells had depolarized mitochondria. However, some parenchymal cells exhibited diffuse and dim Rh123 staining but did not label with PI, indicating mitochondrial depolarization before onset of cell death (Fig. 3B, white arrows).

After liver I/R in JNK2 KO mice, fewer hepatocytes displayed depolarized mitochondria (Fig. 3C, white arrows). PI labeling also decreased in this group. PI labeling and Rh123 punctate staining were scored for each liver. In sham-operated livers, virtually every hepatocyte contained polarized mitochondria, and no parenchymal and nonparenchymal cells were nonviable by the criterion of PI labeling. At 4 hr after liver I/R in WT mice, 82% of hepatocytes contained depolarized mitochondria but were still viable (diffuse Rh123 without nuclear PI) and 7.4% were nonviable (nuclear PI labeling). After liver I/R in JNK2 KO mice, viable hepatocytes with depolarized mitochondria decreased to 42% ($P < 0.05$), and nonviable hepatocytes decreased to 3.2% (Fig. 3D).

Liver damage caused by warm I/R often occurs in clinical settings of liver surgery, leading to adverse events. Previously, JNK2 deficiency was shown to decrease galactosamine/lipopolysaccharide and acetaminophen-induced liver injury by suppression of JNK2-dependent mitochondrial death pathways (7,8,10). Here, we demonstrate that JNK2 signaling also mediates necrotic liver damage after warm I/R (Fig. 1).

The specific mechanism by which JNK2 promotes liver injury has been unclear. Previous studies show that necrosis and apoptosis after I/R, acetaminophen, TNF α and a variety of other stressors are linked to the MPT (2,3,11–20). In the present work, warm I/R to the liver led to mitochondrial depolarization, which previous work shows to be because of onset of the MPT (20). Decreased depolarization in livers of JNK2 KO mice after I/R therefore signifies decreased onset of the MPT. Thus, JNK2 signaling is upstream to the MPT in the cell death-inducing pathway. However, as mitochondrial depolarization still occurred to a lesser extent in JNK2 KO mice, JNK2 signaling is not the only pathway inducing the MPT after I/R.

Whether the other JNK isoform expressed in liver, JNK1, also contributes to I/R injury is not addressed in the present study. However, on the basis of the earlier work in other models, the contribution of JNK1 to injury may be small (7,8). Because JNK1/JNK2 double knockouts are not viable, alternative approaches will be needed in future studies to address the role of JNK1 in liver I/R.

Although the MPT can induce both necrosis and apoptosis, in the present study, the predominant form of cell death after I/R was necrosis with very little increase in apoptosis. Previous work indicates that necrosis and apoptosis are not necessarily independent phenomena but can be intertwined and initiated by the same precipitating event, the MPT (1–3). The phenotype of death then depends on other factors, particularly ATP. ATP depletion as occurs after I/R promotes necrosis but suppresses apoptosis, whereas the presence of ATP prevents necrosis and promotes apoptosis (2,16). In our studies, overnight fasting causing hepatic glycogen depletion likely contributed to the severity of ATP depletion after I/R. Hence, activation of caspase 9/3, which requires ATP (or dATP), cannot occur. By contrast in other models where ATP depletion is not severe, JNK2 may open MPT pores to induce mitochondrial swelling, cytochrome *c* release and caspase activation, culminating in activation of mitochondrial pathways to apoptosis.

In conclusion, these findings indicate that JNK2 contributes to MPT-dependent I/R injury in liver. Thus, suppressing JNK2 activity might be beneficial in preventing mitochondrial dysfunction with subsequent liver injury after liver surgery and transplantation.

Materials and Methods

Experiments were conducted with male C57BL/6J and JNK2 deficient mice (B6.129*Mapk9*^{tm1Flv/J}) on a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME) weighing 24 to 29 g using protocols approved by the Institutional Animal Care and Use Committee. Under ether anesthesia, liver ischemia to 70% of the liver was induced by clamping the portal vein and hepatic artery branches to the median and left liver lobes, as described previously (21). After 1 hr, the clamp was removed to allow reperfusion. Surgery time averaged 1.5 hr.

Assessment of Injury and Survival

After reanesthesia, blood samples were collected from the inferior vena cava at 8 hr after reperfusion to measure ALT by a commercial kit (Pointe Scientific, Inc., Canton, MI). Liver tissue was also fixed in 4% paraformaldehyde, and 4- μ m paraffin sections were stained with hematoxylin and eosin (H&E). Ten random fields were assessed for necrosis by standard morphologic criteria (e.g., loss of architecture, vacuolization, karyolysis, increased eosinophilia) and quantified, as previously described (20). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on paraffin sections using an in situ cell death detection kit (Roche Diagnostics, Penzberg, Germany) and scored in nonnecrotic areas from 10 random high-power fields (HPF). To assess long-term survival, WT and JNK2 deficient mice underwent liver ischemia, as described above. Just before

reperfusion, nonischemic lobes were resected so that survival was dependent on the function of the liver tissue subjected to I/R, as described previously (9,22). Mice were then followed for 14 days after surgery.

Intravital Microscopy

At 4 hr after I/R, mice were anesthetized with pentobarbital (50 mg/kg) and connected to a small animal ventilator through a tracheostomy and respiratory tube (20-gauge catheter), as described previously (23). Through a catheter (0.4-mm inner diameter, Zeus, Inc., Orangeburg, SC) in the right carotid artery, membrane potential-indicating rhodamine 123 (Rh123, 2 μ mol/mouse) and cell death-indicating propidium iodide (PI, 20 nmol/mouse) were infused over 10 min. After re-laparotomy and prone positioning of the mouse, the liver was gently placed over a glass coverslip on the stage of a Zeiss LSM 510 NLO inverted laser scanning confocal/multiphoton microscope and imaged with a 63 $\times$ 1.3 NA water-immersion objective lens. Multiphoton excitation was 820-nm light from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent, Santa Clara, CA). Green Rh123 and red PI fluorescence was collected through 525 $\pm$ 25 nm and 700 $\pm$ 25 nm band pass filters, respectively. During image acquisition, the respirator was turned off for approximately 10 sec to eliminate breathing movement artifacts. In 20 fields per liver, parenchymal cells were scored for bright punctate green Rh123 fluorescence representing cells with polarized mitochondria or a dimmer diffuse cytosolic fluorescence representing cells with depolarized mitochondria. Nonviable PI positive cells, indicated by bright red nuclear fluorescence, were also counted. Image analysis was performed in a blinded fashion.

Statistical Analysis

Data are presented as means $\pm$ SEM. Statistical analysis was performed using the student's *t* test, analysis of variance, or Kaplan-Meier test, as appropriate, with *P*<0.05 as the criterion of significance.

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FIGURE 1.

Decreased liver injury and increased survival after ischemia and reperfusion to livers of JNK2 deficient mice. Ischemia for 1 hr to 70% of the liver or sham operation was performed in WT and JNK2 KO mice, as described in *Materials and Methods*. Serum ALT (A) and necrosis (B–D) were assessed after 8 hr. (B) Necrosis as percent area in liver sections. Dashed lines identify necrotic areas in H&E-stained sections of WT (C) and JNK2 KO (D) livers after I/R. Results are means $\pm$ SEM from 5 to 6 mice per group. (E) Survival was assessed after I/R with resection of nonischemic liver tissue just before reperfusion in 7 to 9 mice per experimental group. Bar is 50 μ m. * P < 0.05.

FIGURE 2.

No difference in apoptosis after ischemia and reperfusion to livers of JNK2 deficient and wildtype mice. TUNEL was assessed 8 hr after sham operation (A, only JNK2 KO sham is shown) or I/R to WT (B) and JNK2 KO (C) livers. Arrows denote TUNEL positive cells. (D) TUNEL positive cells per high power field (HPF). Results are means $\pm$ SEM from 5 to 6 mice per group. Bar is 50 μ m.

FIGURE 3.

Decreased mitochondrial depolarization after ischemia and reperfusion of livers of JNK2 deficient mice. Mitochondrial depolarization and cell death were assessed by intravital multiphoton microscopy at 4 hr postoperatively. Shown are representative overlay images of the fluorescence of membrane potential-indicating Rh123 (green) and cell death-indicating PI (red) in livers after sham operation (A, only JNK2 KO sham is shown) and after I/R to WT (B), and JNK2 KO (C). Punctate staining of Rh123 denoted polarization of individual mitochondria, whereas dim diffuse cellular staining indicated mitochondrial depolarization (white arrows). PI nuclear staining signified loss of cell viability (yellow arrow). (D) The number of viable parenchymal cells with depolarized mitochondria as a percentage of the total number of cells from 3 to 5 mice per group. Bar is 30 μ m. * P <0.05.