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Award Number: W81XWH-09-1-0484

TITLE: Mitochondrial Permeability Transition in Pathogenesis of Hemorrhagic Injury: Targeted Therapy with Minocycline

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REPORT DATE: March 2012

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2012	Revised Final	1 July 2009 - 31 December 2011

4. TITLE AND SUBTITLE

Mitochondrial Permeability Transition in Pathogenesis of Hemorrhagic Injury: Targeted Therapy with Minocycline

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0484

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Medical University of South Carolina
Charleston, SC 29425-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Patients that initially survive hemorrhage and resuscitation may develop a systemic inflammatory response syndrome (SIRS) that leads to injury and dysfunction of vital organs (multiple organ dysfunction syndrome, MODS), particularly to the liver and kidney. SIRS and MODS may involve mitochondrial dysfunction. Minocycline and doxycycline are tetracycline derivatives that are cytoprotective to liver, brain and other organs in various models of hypoxic, ischemic and oxidative stress, which may act by preserving mitochondrial function. We determined whether minocycline and doxycycline protect liver and kidney in a mouse model of hemorrhage and resuscitation. Minocycline and doxycycline each decreased liver enzymes and creatinine in the blood after hemorrhage/resuscitation compared to vehicle. Minocycline and doxycycline also significantly decreased liver necrosis and liver and kidney apoptosis. Lastly, minocycline protected against mitochondrial inner membrane permeabilization (mitochondrial permeability transition) occurring after HS/R and improved survival. In conclusion, minocycline and doxycycline when administered only after resuscitation decrease liver and kidney injury after hemorrhage/resuscitation and improve survival. These safe and widely used agents might be useful clinically to prevent SIRS and MODS after hemorrhagic shock.

15. SUBJECT TERMS
Doxycycline, hemorrhage, kidney, liver, minocycline, mitochondria, permeability transition,resuscitation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	23	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Despite improvements in evacuation, 35% of deaths from far forward battlefield injuries occur prior to arrival to a field hospital. Even after successful hemorrhagic resuscitation, late deaths from multiple organ failure occur in up to 50% of victims of severe multiple trauma and hemorrhage. Early intervention is therefore needed and must be applied in a far forward setting to help stabilize injured combatants and to lessen the likelihood of unsuccessful resuscitation and late development of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS).

We hypothesize that mitochondrial dysfunction is central to the pathobiology leading to these deaths and that onset of the mitochondrial permeability transition is the fundamental pathophysiological event leading to mitochondrial failure and its dire bioenergetic consequences. Accordingly, we will make direct measurements by intravital multiphoton microscopy to determine whether onset of the mitochondrial permeability transition and resultant mitochondrial dysfunction does occur in the livers and kidneys of mice subjected to hemorrhage and resuscitation. Furthermore, we will assess the ability of minocycline, an agent that prevents the mitochondrial permeability transition by blocking mitochondrial calcium uptake, to decrease liver and kidney injury and improve overall survival after hemorrhage and resuscitation. Recently, we have discovered that doxycycline, another tetracycline derivative, is even more strongly cytoprotective in models of hypoxic and ischemic injury. Accordingly, we have added an examination of minocycline to our originally planned experiments.

Body

Minocycline Decreases Liver Injury after Hemorrhagic Shock and Resuscitation in Mice. Patients that initially survive hemorrhage and resuscitation may develop a systemic inflammatory response syndrome (SIRS) that leads to injury and dysfunction of vital organs (multiple organ dysfunction syndrome, MODS). SIRS and MODS may involve mitochondrial dysfunction. Under pentobarbital anesthesia, C57BL6 mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution containing minocycline (10 mg/kg body weight), tetracycline (10 mg/kg body weight) or vehicle. Serum alanine aminotransferase (ALT), necrosis, apoptosis and oxidative stress were assessed 6 h after resuscitation. Mitochondrial polarization were assessed by intravital microscopy. After H/R with vehicle or tetracycline, ALT increased to 4538 U/L and 3999 U/L, respectively. Minocycline treatment decreased ALT to 1763 U/L ($p<0.01$). Necrosis and TUNEL also decreased from 24.5 % and 17.7 cells/field, respectively, after vehicle to 8.3% and 8.7 cells/field after minocycline. Tetracycline failed to decrease necrosis (23.3%) but decreased apoptosis to 9 cells/field ($p<0.05$). Additionally, minocycline (67%) and tetracycline (77%) decreased caspase-3 activity in liver homogenates ($p<0.05$). Lipid peroxidation was decreased after resuscitation with minocycline about 70% but not after resuscitation with tetracycline ($p<0.05$). Intravital microscopy showed that minocycline preserved mitochondrial polarization after H/R ($p<0.05$). Minocycline decreases liver injury after hemorrhage and resuscitation by preventing MPT-dependent mitochondrial dysfunction . Minocycline might be useful clinically after hemorrhage shock and resuscitation to prevent SIRS and MODS.

Doxycyline as Well as Minocycline Decrease Liver and Kidney Injury after Hemorrhagic Shock and Resuscitation in Mice. Patients that initially survive hemorrhage and resuscitation may develop a

systemic inflammatory response syndrome (SIRS) that leads to injury and dysfunction of vital organs (multiple organ dysfunction syndrome, MODS), particularly to the liver and kidney. SIRS and MODS may involve mitochondrial dysfunction. Minocycline and doxycycline are tetracycline derivatives that are cytoprotective to liver, brain and other organs in various models of hypoxic, ischemic and oxidative stress and which may act by preserving mitochondrial function. Here, our **Aim** was to determine whether minocycline and doxycycline protect liver and kidney in a mouse model of hemorrhage and resuscitation. **Methods**: Under pentobarbital anesthesia, C57BL6 mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution containing minocycline (10 mg/kg body weight), doxycycline (5 mg/kg body weight) or vehicle. Serum alanine aminotransferase (ALT), serum creatinine and urea, and caspase-3 activity were assessed 6 h after resuscitation. Liver and kidney histology and immunochemistry were also assessed at 6 h after the resuscitation. **Results**: After resuscitation with vehicle, ALT increased to 1988 U/L. Minocycline and doxycycline decreased ALT to 857 U/L and 789 U/L, respectively ($p<0.001$). After resuscitation with vehicle, blood creatinine increased to 273 μM. Minocycline and doxycycline treatment decreased blood creatinine to 109 μM and 92 μM, respectively ($p<0.05$). Minocycline and doxycycline treatment also significantly decreased liver necrosis and liver and kidney apoptosis. Caspase-3 activity in liver homogenates was decreased by 52% ($p<0.01$). **Conclusion**: Minocycline and doxycycline even when administered only after resuscitation decrease liver and kidney injury after hemorrhage/resuscitation. These safe and widely used agents might be useful clinically to prevent SIRS and MODS after hemorrhagic shock and other systemic stresses.

Liver injury following hemorrhagic shock/resuscitation: mechanisms and targeted therapy with minocycline. Here, our aim was to determine by intravital multiphoton microscopy whether the mitochondrial permeability transition (MPT) is involved in HS/R-induced liver injury and whether minocycline, an MPT inhibitor, can decrease that injury. Under anesthesia, C57BL6 mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution with and without minocycline or tetracycline (10 mg/kg). In some experiments, mice were pretreated with minocycline and tetracycline instead. Serum ALT, liver necrosis and apoptosis were assessed at 6 h after HS/R. To investigate if onset of MPT takes place after HS/R, intravital multiphoton imaging of tetramethylrhodamine methylester (TMRM) and calcein acetoxymethyl ester was performed at 4 h after HS/R. Minocycline decreased ALT release, hepatic necrosis and apoptosis after HS/R and also improved survival. Minocycline administered in Ringer's solution at the end of resuscitation was as protective as minocycline given 1 h before hemorrhage. In sham-operated mice, red TMRM fluorescence was punctate in virtually all hepatocytes, indicating normal mitochondrial polarization. Green calcein fluorescence outlined mitochondria as dark voids. In contrast at 4 h after HS/R, mitochondria did not take up TMRM in many hepatocytes, indicating mitochondrial depolarization, and dark voids of calcein fluorescence disappeared, indicating mitochondrial inner membrane permeabilization and onset of the MPT. Minocycline prevented these changes in most cells. In conclusion, the MPT plays a major role in liver injury after HS/R. Minocycline blocks the onset of MPT after HS/R, protects against hepatic injury and improves survival even when administered at the end of resuscitation. Minocycline, an MPT blocker, is a safe, clinically used, FDA-approved drug that might therefore be used to mitigate liver injury after HS/R.

Key Research Accomplishments

We have shown that minocycline and doxycycline even when administered only after resuscitation each decrease liver and kidney injury after hemorrhage/resuscitation. Minocycline also protects against mitochondrial inner membrane permeabilization (MPT) occurring after HS/R and improves survival. These safe and widely used agents might be useful clinically to prevent SIRS and MODS after hemorrhagic shock in civilian and military medicine.

Reportable Outcomes

Papers:

1. Czerny, C., A. Kholmukhamedov, T.P. Theruvath, E. Maldonado, V.K. Ramshesh, M. Lehnert, I. Marzi, Z. Zhong and J.J. Lemasters (2012) Minocycline decreases liver injury after hemorrhagic shock and resuscitation in mice. *HPB Surgery* **2012**:259512.
2. Czerny, C., T.P. Theruvath, E. Maldonado, M. Lehnert, I. Marzi, Z. Zhong and J.J. Lemasters (2012) c-Jun N-terminal kinase 2 promotes liver injury via the mitochondrial permeability transition after hemorrhage and resuscitation. *HPB Surgery* **2012**:641982.

Abstracts:

3. Kholmukhamedov, A., C. Czerny, J. Hu, J. Schwartz and J.J. Lemasters (2010) Minocycline decreases liver and kidney injury after hemorrhagic shock and resuscitation in mice: comparison of minocycline pre- and post-treatment. In *Program Book and Abstracts:* Southeastern Society of Toxicology Fall Meeting, Athens, GA, October 11-12, 2010.
4. Kholmukhamedov, A., C. Czerny, J. Hu, J. Schwartz and J.J. Lemasters (2010) Minocycline and doxycycline decrease liver and kidney injury after hemorrhagic shock/resuscitation in mice. *Hepatology* **52** (Suppl.), 588A.
5. Kholmukhamedov A.,C. Czerny, J. Hu, J. Schwartz and and J.J. Lemasters (2011) Minocycline and doxycyline, but not tetracycline, decrease liver and kidney injury after hemorrhagic shock and resuscitation (HS/R) in mice. *Toxicol. Sci.* **120** (Suppl. 2), 354-355.
6. Kholmukhamedov, A., C. Czerny, J. Hu, J. Schwartz and J.J. Lemasters (2011) Liver and kidney injury after the hemorrhagic shock and resuscitation: Protection by minocycline and doxycycline. *Program Book and Abstracts, Department of Pharmaceutical and Biomedical Sciences Graduate Student Retreat,* Charleston, SC, September 23, 2011.
7. Kholmukhamedov, A., X. Zhang, J. Schwartz and J.J. Lemasters (2012) Lysosomal iron release promotes hemorrhage/resuscitation-induced liver injury. *Toxicological Sciences*, in press (abstract).
8. Kholmukhamedov, A., C. Czerny and J.J. Lemasters (2012) Liver injury following hemorrhagic shock/resuscitation: mechanisms and targeted therapy with minocycline. *Shock*, in press (abstract).

Conclusion

Minocycline and doxycycline are safe and widely used agents that might be useful clinically to prevent SIRS and MODS after hemorrhagic shock in civilian and military medicine.

References

None.

Appendices

Two published papers are attached.

Hindawi Publishing Corporation
HPB Surgery
Volume 2012, Article ID 259512, 9 pages
doi:10.1155/2012/259512

Research Article

Minocycline Decreases Liver Injury after Hemorrhagic Shock and Resuscitation in Mice

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Received 27 January 2012; Accepted 21 March 2012

Academic Editor: Peter Schemmer

Patients that survive hemorrhage and resuscitation (H/R) may develop a systemic inflammatory response syndrome (SIRS) that leads to dysfunction of vital organs (multiple organ dysfunction syndrome, MODS). SIRS and MODS may involve mitochondrial dysfunction. Under pentobarbital anesthesia, C57BL6 mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution containing minocycline, tetracycline (both 10 mg/kg body weight) or vehicle. Serum alanine aminotransferase (ALT), necrosis, apoptosis and oxidative stress were assessed 6 h after resuscitation. Mitochondrial polarization was assessed by intravital microscopy. After H/R with vehicle or tetracycline, ALT increased to 4538 U/L and 3999 U/L, respectively, which minocycline decreased to 1763 U/L ($P < 0.01$). Necrosis and TUNEL also decreased from 24.5% and 17.7 cells/field, respectively, after vehicle to 8.3% and 8.7 cells/field after minocycline. Tetracycline failed to decrease necrosis (23.3%) but decreased apoptosis to 9 cells/field ($P < 0.05$). Minocycline and tetracycline also decreased caspase-3 activity in liver homogenates. Minocycline but not tetracycline decreased lipid peroxidation after resuscitation by 70% ($P < 0.05$). Intravital microscopy showed that minocycline preserved mitochondrial polarization after H/R ($P < 0.05$). In conclusion, minocycline decreases liver injury and oxidative stress after H/R by preventing mitochondrial dysfunction.

1. Introduction

Trauma and surgical procedures, including gastrointestinal and hepatobiliary surgery, can lead to severe hemorrhage and hypovolemic shock. Fluid resuscitation after less than one hour of severe hemorrhagic shock restores hemodynamics and typically leads to full recovery. By contrast although restoring hemodynamics, resuscitation after greater than an hour may lead instead to multiple organ dysfunction syndrome (MODS), which is associated with mortality of 30% [1]. Effective strategies to extend this golden hour for resuscitation are therefore needed to improve the treatment of hemorrhagic shock and decrease the incidence of MODS and its lethal consequence. Hemorrhage/resuscitation (H/R) is an example of ischemia/reperfusion (I/R) and hypoxia/reoxygenation injuries, for which mitochondrial dysfunction plays a major pathophysiological role [2–4]. Moreover, the liver with its crucial involvement in metabolism and homeostasis is among the most frequently affected organs after hemorrhage-induced hypotension in humans [5].

I/R injury leads to both necrotic cell death and apoptosis. A common pathway for hepatic apoptosis and necrosis after I/R is the mitochondrial permeability transition (MPT) [6]. Opening of permeability transition (PT) pores in the mitochondrial inner membrane causes the MPT with

consequent mitochondrial depolarization and uncoupling of oxidative phosphorylation. ATP depletion after uncoupling produces necrotic cell killing, the main pathway of cell death after I/R, whereas cytochrome *c* release due to MPT-driven mitochondrial swelling induces caspase-dependent apoptosis. Previously, experimental strategies to inhibit the MPT after liver transplantation in rats improved survival and decreased mitochondrial dysfunction [7].

Minocycline is a semisynthetic tetracycline antibiotic, which is protective against neurodegenerative disease, trauma, and hypoxia/ischemia [8–12]. Mechanisms by which minocycline exerts neuroprotection include inhibition of apoptotic pathways, decreased mitochondrial release of proapoptotic factors like cytochrome *c*, and upregulation of antiapoptotic Bcl-2 and inhibitor of apoptosis proteins (IAPs) [13, 14]. In orthotopic rat liver transplantation, minocycline cytoprotection against storage/reperfusion injury is mediated by suppression of the MPT through inhibition of the mitochondrial calcium uniporter [7]. Here, we investigated whether resuscitation with minocycline also decreases liver injury after H/R.

2. Materials and Methods

2.1. Chemicals and Reagents. Minocycline, tetracycline, rhodamine 123, and other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals. Male C57BL/6J mice (8–10 wk of age, 23–27 g) were obtained from Jackson Laboratory (Bar Harbor, ME). Animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

2.3. Hemorrhagic Shock and Resuscitation. After an overnight fast, mice were anesthetized with pentobarbital sodium (80 mg/kg body weight). Under spontaneous breathing, both femoral arteries were exposed and cannulated with polyethylene-10 catheters (SIMS Portex). The catheters were flushed with normal saline containing heparin (100 IU/l) before insertion. One catheter was connected via a transducer to a pressure analyzer (Micro-Med; Louisville, KY), and blood was withdrawn via the second catheter into a heparinized syringe (10 units) over 5 min to a mean arterial pressure of 30 mm Hg. This pressure was maintained for 3 h by withdrawal or reinfusion of shed blood [15]. Body temperature was monitored and maintained at 37°C. After 3 h, mice were resuscitated with a syringe pump over 30 min with shed blood followed by a volume of lactated Ringer's solution corresponding to 50% of the shed blood volume [16, 17]. As indicated, the resuscitating Ringer's solution contained minocycline (10 mg/kg body weight), tetracycline (10 mg/kg), or vehicle. Doses of minocycline and tetracycline were based on a prior study [7]. Adequacy of resuscitation was determined by the restoration of blood pressure. Catheters were then removed, the vessels were ligated, and the groin incisions were closed. Sham-operated animals underwent the same surgical procedures, but hemorrhage was not carried out. No mortality in any group occurred over the course of the experiments.

For the determination of hemorrhage-/resuscitation-dependent liver damage, mice were anesthetized and killed by exsanguination 6 h after the end of resuscitation. For each mouse, the two right dorsal liver lobes were snap-frozen in liquid nitrogen. The remaining liver was flushed with normal saline, infused and fixed with 4% buffered paraformaldehyde through the portal vein, and embedded in paraffin sections.

2.4. Alanine Aminotransferase (ALT). Blood samples to measure ALT were collected from the inferior vena cava 6 h after H/R for analysis by standard methods.

2.5. Histology. Sections (4 μm) 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). Images were captured by an image analysis system (Olympus BH-2 Microscope; Micropublisher 5.0 RTV, Center Valley, PA), and the area percentage of necrosis was quantified using a computer program (BioQuant BQ Nova Prime 6.7, R&M Biometrics, Nashville, TN).

2.6. TUNEL. 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). TUNEL-positive cells were counted by light microscopy in 10 random high-power fields (HPF).

2.7. Caspase-3. Liver tissue (~100 mg) was homogenized (Polytron PT-MR2100, Kinematica, Luzern, Switzerland) in 1 mL of lysis buffer containing 0.1% 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid, 2 mM EDTA, 5 mM dithiothreitol, 1 mM Pefabloc, 10 ng/mL pepstatin A, 10 ng/mL aprotinin, 20 μg/mL leupeptin, and 10 mM HEPES buffer, pH 7.4. The lysate was centrifuged at 15,000 rpm for 30 min. Activity of caspase-3 in the supernatant was determined using a Caspase-3 Colorimetric Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Activity was normalized to protein concentration of each sample and expressed as fold increase compared to sham.

2.8. 4-Hydroxynonenal. Paraffin-embedded sections were deparaffinized, rehydrated, and incubated with polyclonal antibodies against 4-hydroxynonenal (4-HNE, Alpha Diagnostics; San Antonio, TX) in PBS (pH 7.4) containing 1% Tween 20 and 1% bovine serum albumin. Peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit, DAKO) were used to detect specific binding. A Universal Imaging Metamorph image acquisition and analysis system (Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss; Thornwood, NY) was used to capture and analyze the immunostained tissue sections at 40x magnification. The extent of labeling was determined in randomly selected fields as the percentage of area within

a preset color range determined by the software. Data from each tissue section (10 fields/section) were pooled to determine means, as described previously [18].

2.9. Intravital Multiphoton Microscopy. At 4 h after H/R, mice were anesthetized with pentobarbital (50 mg/kg) and connected to a small animal ventilator via a tracheostomy and respiratory tube (22-gauge catheter), as described previously [19]. Laparotomy was performed, and a polyethylene-10 catheter was inserted into the distal part of the right colic vein. Using a syringe pump, rhodamine 123 (1 μmol/mouse), a membrane potential-indicating fluorophore, was infused via the catheter over 10 min. After prone positioning of the mouse, the liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the microscope stage. Rhodamine 123 fluorescence was excited with 820 nm light from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent, Santa Clara, CA) and imaged with a Zeiss LSM 510 NLO inverted laser scanning confocal microscope using a 63x 1.3 NA water-immersion objective lens. Green rhodamine 123 fluorescence was collected through a 525 ± 25 nm band pass filter. During image acquisition, the respirator was turned off for ~5 sec to eliminate movement artifacts from breathing. In 20 fields per liver, parenchymal cells were scored for bright punctate rhodamine 123 fluorescence representing hepatocytes with polarized mitochondria or dimmer diffuse cytosolic fluorescence representing hepatocytes with depolarized mitochondria. Image analysis was performed in a blinded fashion.

2.10. Statistical Analysis. Data are presented as means ± S.E., unless otherwise noted. Statistical analysis was performed by ANOVA plus Student-Newman-Keuls test, as appropriate, using $P < 0.05$ as the criterion of significance.

3. Results

3.1. Decreased ALT Release and Liver Necrosis after Resuscitation with Minocycline. C57BL6 mice were hemorrhaged for 3 h and resuscitated with shed blood followed by half the volume of lactated Ringer solution, containing minocycline (10 mg/kg), tetracycline (10 mg/kg), or vehicle. As described previously [20], resuscitation restored mean arterial pressure to ~80 mm Hg, which was nearly identical to blood pressure before hemorrhage (data not shown). At 6 h postoperatively, sham-operated mice had serum ALT of 105 ± 15 U/L (Figure 1). After H/R, ALT after vehicle treatment increased to 4538 U/L ± 557 U/L, which decreased to 1763 ± 213 U/L after resuscitation with minocycline ($P < 0.01$). Identical treatment with tetracycline did not cause a statistically significant change of serum ALT (3999 ± 491 U/L) compared to vehicle (Figure 1).

Liver injury was also assessed histologically at 6 h postoperatively. In sham-operated mice, liver histology was normal and indistinguishable from untreated mice (Figure 2(a) and data not shown). After H/R with vehicle and tetracycline treatments, large areas of necrosis developed 6 h

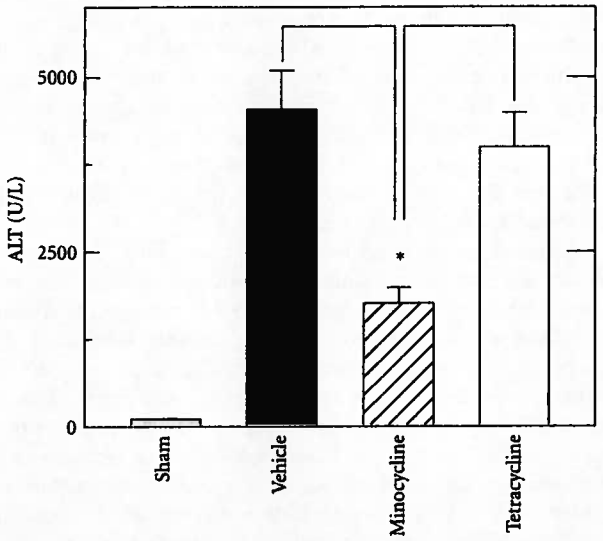

FIGURE 1: Minocycline decreases ALT release after hemorrhage and resuscitation. Mice were resuscitated with shed blood and then half the volume of lactated Ringer's solution containing tetracycline or minocycline (10 mg/kg body weight) or vehicle, as described in materials and methods. Serum ALT was assessed 6 h after resuscitation. Group sizes were sham, 4; vehicle, 7; minocycline, 7; tetracycline, 7. *$P < 0.01$ versus vehicle and tetracycline.

postoperatively with a predominately pericentral and midzonal distribution, which was decreased after resuscitation with minocycline (Figures 2(b)–2(d)). Resuscitation with minocycline decreased hepatic necrosis from 24.5 ± 1.5% after vehicle to 8.3 ± 1.4% ($P < 0.05$) (Figure 2(e)). By contrast, resuscitation with tetracycline did not decrease liver necrosis after H/R (23.3 ± 1.5%) in comparison to vehicle treatment. Overall, minocycline treatment decreased hepatic necrosis by nearly two-thirds.

3.2. Decreased Liver Apoptosis after Resuscitation with Minocycline and Tetracycline. TUNEL was performed on tissue sections to assess double-stranded DNA breaks that are characteristic of apoptosis. TUNEL-positive parenchymal cells were rare after sham operation, averaging less than one cell per high power field (HPF). At 6 h after H/R with vehicle, TUNEL of parenchymal cells in nonnecrotic areas increased to 17.7 ± 3.2 cells/HPF (Figure 3). Treatment with minocycline decreased TUNEL by half to 8.7 ± 1.7 cells/HPF ($P < 0.05$ compared to vehicle, Figure 3). After resuscitation with tetracycline, TUNEL-positive cells in nonnecrotic areas decreased to 9 ± 2.2 cells/HPF ($P < 0.05$ compared to vehicle, Figure 3) as well.

To further investigate the extent of apoptosis after minocycline and tetracycline treatment, caspase-3 activity was measured in liver extracts at 6 h after resuscitation with vehicle, minocycline and tetracycline in comparison to sham operation (Figure 4). After sham operation, caspase-3 activity was very low. After H/R with vehicle, caspase-3 activity increased 8.6-fold, which decreased to 2.8-fold

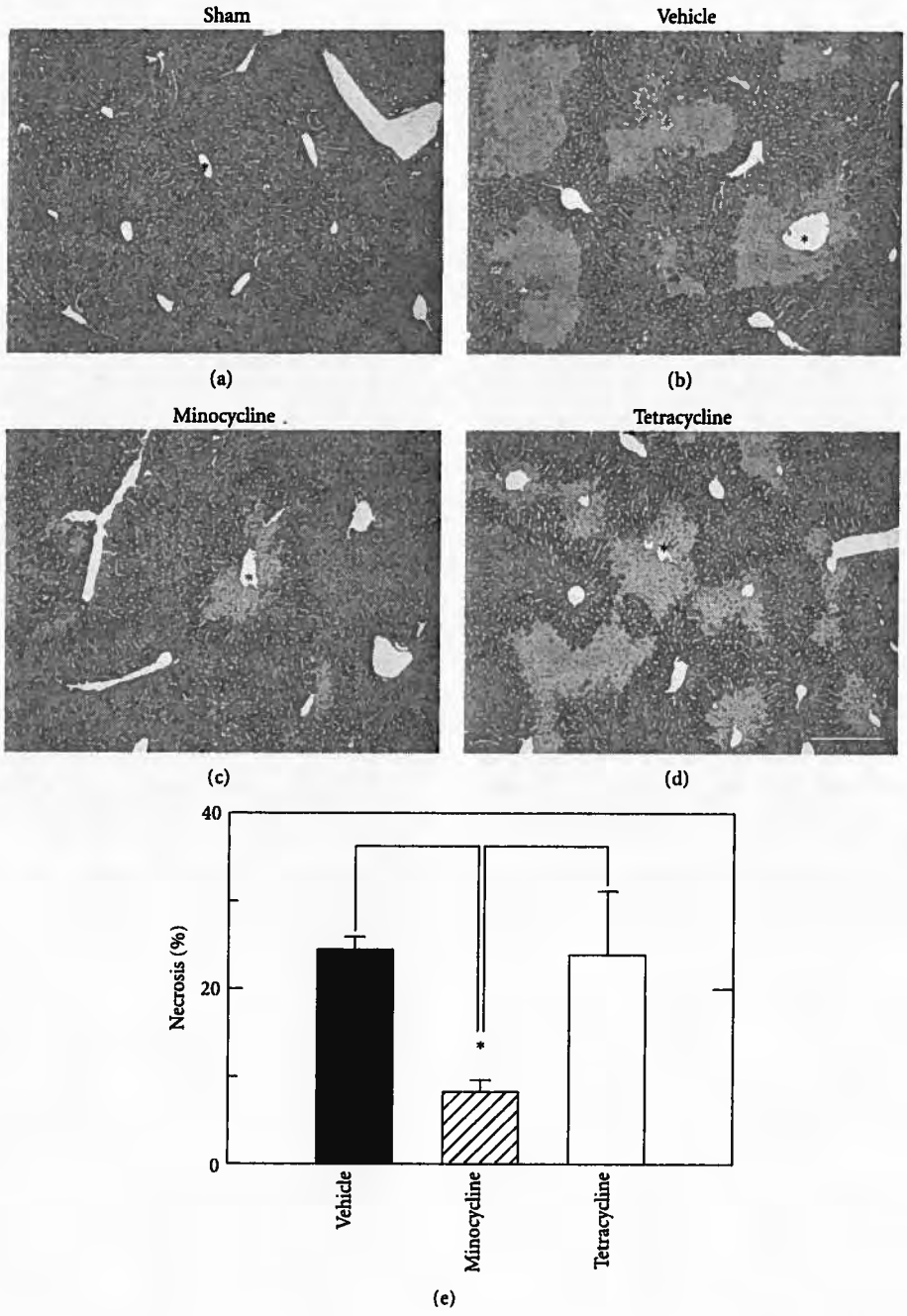

FIGURE 2: Minocycline decreases necrosis after hemorrhage and resuscitation. H/R was performed, as described in Figure 1. Necrosis was assessed by H&E histology at 6 h after sham operation (a) or resuscitation with vehicle, minocycline, or tetracycline (b–d). In (e), necrosis as percent area in liver sections was averaged from 5 livers per treatment group. Necrosis in sham-operated mice was absent and not plotted. *, central vein. Bar is 100 μm. * $P < 0.05$ versus vehicle and tetracycline.

after minocycline and to 2-fold after tetracycline ($P < 0.05$ compared to vehicle, Figure 4).

3.3. Decreased Oxidative Stress after Resuscitation with Minocycline. We used 4-HNE immunohistochemistry to evaluate oxidative stress in livers 6 h after hemorrhage and resuscitation. HNE is an aldehyde product of lipid peroxidation that forms covalent adducts with proteins that are recognized by anti-HNE antibodies. After sham operation, the brown reaction product of HNE immunohistochemistry was virtually undetectable (Figure 5(a)). By contrast at 6 h after resuscitation with vehicle or tetracycline, wide confluent areas of HNE immunoreactivity developed in pericentral

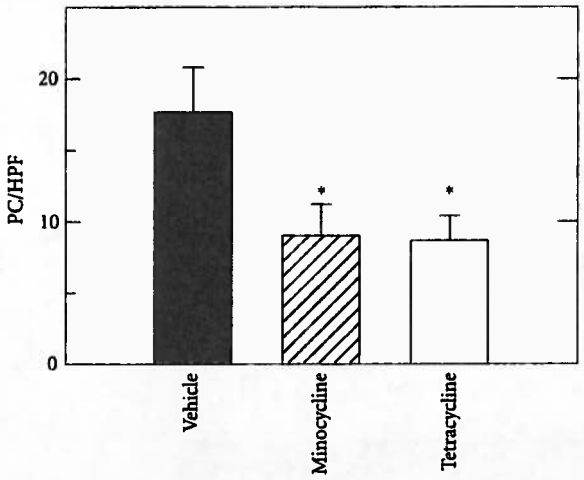

FIGURE 3: Minocycline and tetracycline decrease apoptosis after hemorrhage and resuscitation. H/R was performed, as described in Figure 1. Apoptosis of parenchymal cells was assessed by TUNEL in nonnecrotic areas at 6 h after sham operation or resuscitation with vehicle, minocycline, or tetracycline. The average number of TUNEL positive cells is plotted for each treatment group. TUNEL for sham was virtually zero and is not plotted. Bar is 50 μm. *P < 0.05 versus vehicle.

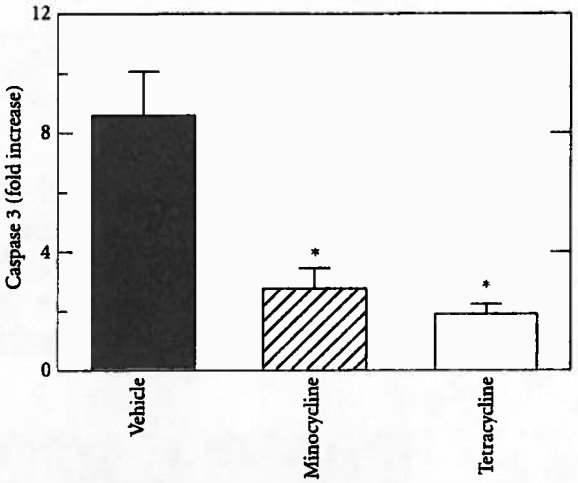

FIGURE 4: Minocycline and tetracycline decrease caspase 3 activation after hemorrhage and resuscitation. H/R was performed, as described in Figure 1, and caspase 3 activity was assessed in liver homogenates after sham operation or resuscitation with vehicle, minocycline, or tetracycline. Activity is expressed as fold increase over sham-operated mice. *P < 0.05 versus vehicle.

and midzonal areas with relative sparing the periportal regions (Figures 5(b) and 5(d)). However, after H/R with minocycline, HNE immunoreactivity was decreased about 70% compared to vehicle and tetracycline treatments. HNE staining with minocycline was confined mostly to pericentral regions. (P < 0.05 compared to vehicle and tetracycline, Figures 5(c) and 5(e)).

3.4. Mitochondrial Dysfunction In Vivo after Hemorrhage and Resuscitation: Protection by Minocycline. At 4 h after sham operation, intravital multiphoton microscopy revealed bright fluorescence of rhodamine 123 in virtually all hepatocytes whose punctate pattern signified polarization of individual mitochondria and normal mitochondrial function (Figure 6(a)). Cytosolic and nuclear areas had little fluorescence. By contrast at 4 h after H/R with vehicle treatment, rhodamine 123 staining became diffuse and dim in many hepatocytes (Figure 6(b)), which indicated mitochondrial depolarization and dysfunction. Similar to the necrosis and HNE immunoreactivity that became present at 6 h after H/R (see Figures 2 and 5), mitochondrial depolarization after 4 h had a predominantly pericentral and midzonal distribution (data not shown). After H/R with minocycline, fewer hepatocytes contained depolarized mitochondria (Figure 6(c)), whereas mitochondrial depolarization after tetracycline treatment was indistinguishable from vehicle-treated liver after H/R (Figure 6(d)). At 4 h postoperatively, livers were scored and counted for rhodamine 123 staining (Figure 6(e)). In sham-operated mice, 0.05 ± 0.002 hepatocytes/HPF contained depolarized mitochondria. After H/R with vehicle treatment, 12.2 ± 0.9 hepatocytes/HPF contained depolarized mitochondria, which corresponded to depolarization of 57.8 ± 5.2% of hepatocytes. After H/R with minocycline treatment, hepatocytes with depolarized mitochondria decreased to 5.4 ± 0.7 hepatocytes/HPF (P < 0.05 versus vehicle and tetracycline). By contrast, after H/R with tetracycline, 12.7 ± 0.9 hepatocytes/HPF contained depolarized mitochondria, which was not different from vehicle treatment (Figure 6(e)).

4. Discussion

Hemorrhage is a risk of trauma and major surgery, particularly gastrointestinal and hepatobiliary surgery, and tissue damage after hemorrhage and resuscitation is a variant of ischemia/reperfusion injury. Despite advances in medical and surgical treatment, the golden hour for resuscitation remains a time limit and barrier to effective treatment of hemorrhagic shock. Moreover, the liver is among the most frequently affected organs after hemorrhage-induced hypotension in humans [5]. Here in a mouse model of H/R, we show that minocycline substantially decreases hepatic injury after resuscitation following 3 h of profound hemorrhagic hypotension. Specifically after 3 h of hemorrhage followed by resuscitation with shed blood and then lactated Ringers solution, hepatic necrosis, apoptosis, and enzyme release decreased by 50% or more after minocycline treatment (Figures 1–4). Minocycline also improved mitochondrial function as assessed by intravital multiphoton imaging of the fluorescence of the mitochondrial membrane potential-indicating fluorophore, rhodamine 123 (Figure 6). Notably, minocycline protected even when used after blood resuscitation as a component of Ringer's solution.

Previous studies show cytoprotection by minocycline in a variety of settings, including rat liver transplantation, ischemic renal injury, and various injuries to the central

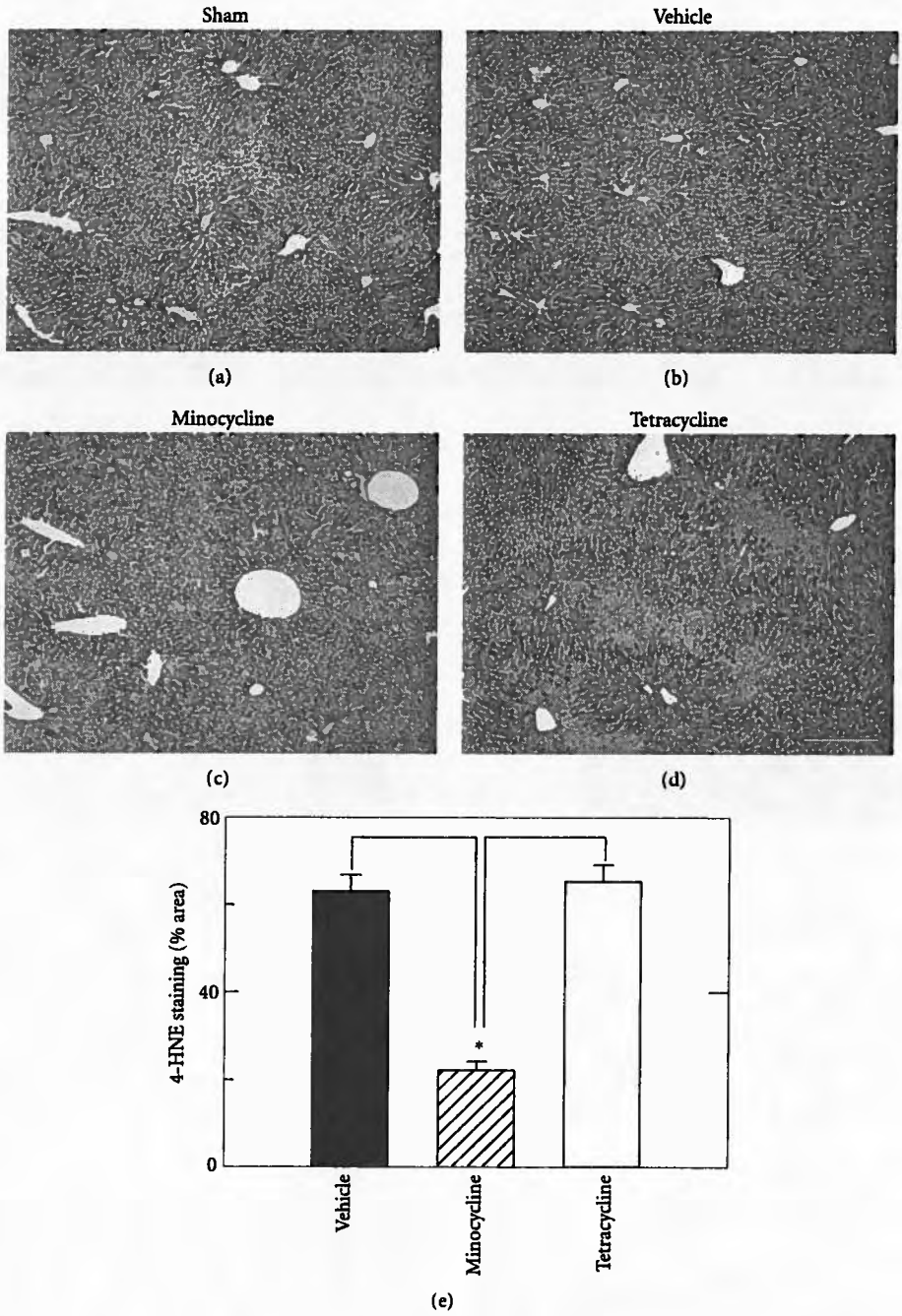

FIGURE 5: Minocycline decreases oxidative stress after hemorrhage and resuscitation. H/R was performed, as described in Figure 1, and immunohistochemical staining was performed for 4-HNE adducts at 6 h after sham operation (a) or resuscitation with vehicle, minocycline, or tetracycline (b–d). In (e), HNE staining as percent area in liver sections was averaged from 5 livers per group. HNE in sham-operated livers was virtually zero and not plotted. Individual group size was 5. Bar is 50 μm. $^*P < 0.05$ versus vehicle.

nervous system [7–12]. In our model of mouse H/R, minocycline protected even when used late during resuscitation after the initial blood resuscitation. Hepatic necrosis assessed by ALT and histology decreased by half at 6 h after H/R with minocycline treatment, and apoptosis assessed by TUNEL and caspase-3 activity also decreased by more than half (Figures 1–4). Necrosis represents the predominant mode of cell death in the setting of hepatic I/R with apoptosis contributing to a lesser extent [21, 22]. However, both modes of cell death, namely, apoptosis progressing to necrosis, can occur through a common mitochondrial pathway involving the MPT, a phenomenon of necrapoptosis [23–25].

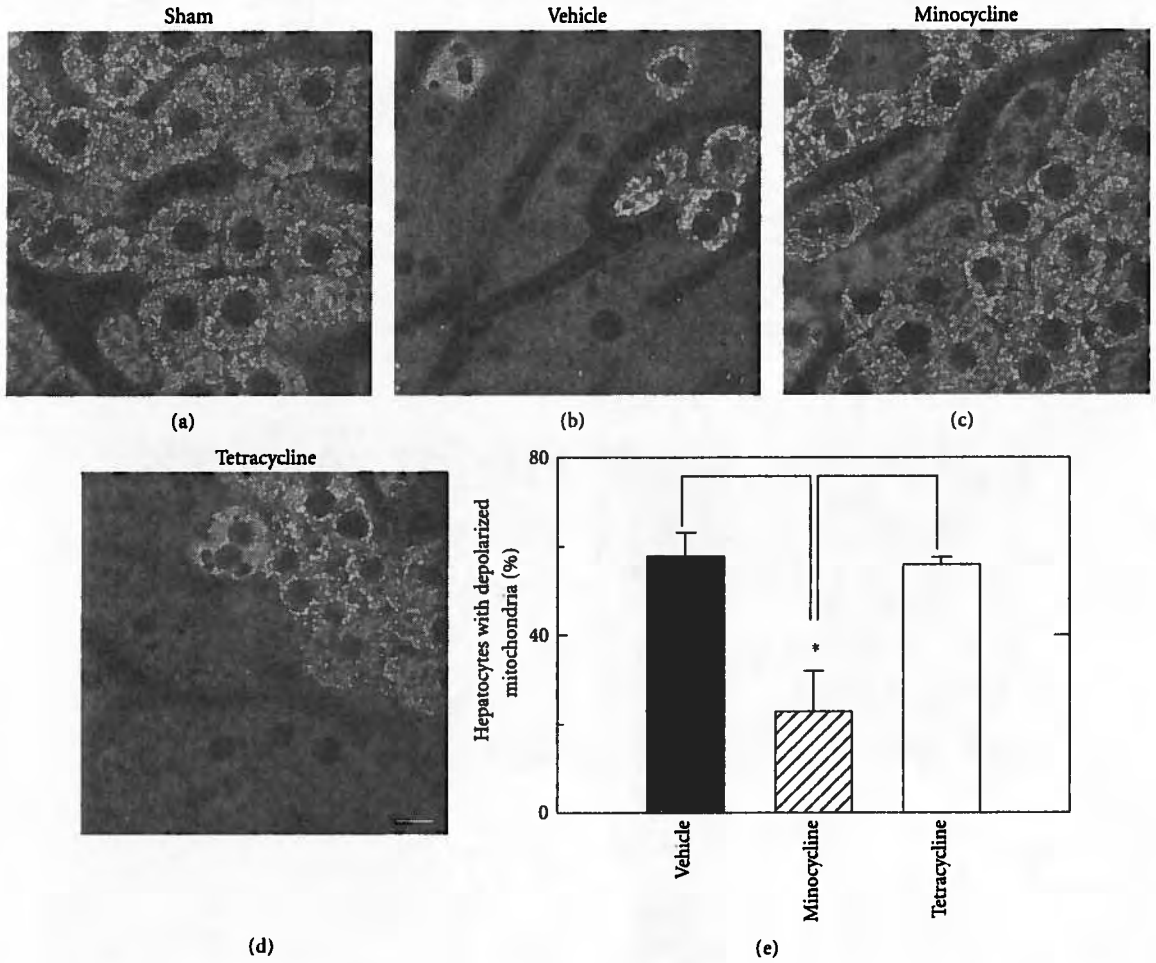

FIGURE 6: Minocycline decreases mitochondrial depolarization after hemorrhage and resuscitation. H/R was performed, as described in Figure 1, and intravital multiphoton microscopy of rhodamine 123 fluorescence was performed 4 h after sham operation (a) or resuscitation with vehicle, minocycline, or tetracycline (b–d), as described in materials and methods. Punctate staining of rhodamine 123 denoted polarization of individual mitochondria, whereas dim diffuse cellular staining indicated mitochondrial depolarization. In (e), the average percentage of hepatocytes with depolarized mitochondria is plotted for each H/R treatment group. Mitochondrial depolarization in sham-operated livers was virtually zero and not plotted. Size of individual groups was 5. Bar is 30 μm. * $P < 0.05$ versus vehicle.

After orthotopic rat liver transplantation, minocycline cytoprotection against hepatic necrosis, apoptosis, and enzyme release is virtually identical to the cytoprotection of *N*-methyl-4-isoleucine cyclosporin (NIM811), a specific inhibitor of the MPT [7]. Minocycline also inhibits calcium-induced MPT onset in isolated mitochondria. Unlike NIM811 which inhibits the MPT pore component, cyclophilin D, minocycline prevents MPT onset by blocking electrogenic calcium uptake by the mitochondrial calcium uniporter. Since H/R caused mitochondrial depolarization that was virtually identical to mitochondrial depolarization after liver transplantation, and since minocycline protected against this depolarization (Figure 6), it is likely the minocycline protects against hepatic injury after H/R by blocking MPT onset. Importantly, minocycline-sensitive mitochondrial depolarization signifying the MPT preceded necrotic cell death and thus was not a consequence of cell death, since after 4 h few cells labeled with propidium iodide, a marker of nonviable cells, as described previously [7]. Tetracycline, which did not decrease hepatic necrosis and ALT release after H/R, did not prevent mitochondrial depolarization after H/R (Figure 6). Because minocycline protected against mitochondrial depolarization, necrosis, and apoptosis, liver damage after H/R would appear to be largely a necroapoptotic phenomenon [26].

In storage/reperfusion injury during liver transplantation and in isolated mitochondria, tetracycline does not protect against hepatic damage, mitochondrial depolarization, and onset of the MPT [7]. Similarly in the present work, tetracycline did not protect against hepatic necrosis, enzyme release, and mitochondrial depolarization after H/R (Figures 1, 2 and 6). By contrast, tetracycline protected similarly to minocycline against apoptosis, as assessed by TUNEL and caspase 3 (Figures 3 and 4). This finding suggests

different protective actions—one unique to minocycline and another shared by both tetracycline and minocycline. One shared action is that minocycline and tetracycline are both calcium chelators [27, 28], although only minocycline blocks mitochondrial calcium uptake [7]. Thus, suppression of apoptosis by tetracycline and minocycline might be due to calcium chelation. Alternatively in necrapoptosis, apoptosis progresses to necrosis with increasing severity of an inducing stress. Consequently, protective strategies may revert necrosis to apoptosis, such that protected necrotic areas begin toshow apoptosis. Accordingly, protection against apoptosis by an agent like minocycline may be offset in part by increased apoptosis in areas that otherwise would have become necrotic. Tetracycline, by contrast, did not decrease necrosis, and tetracycline may simply represent a much weaker protective agent than minocycline that protects partially against apoptosis but not at all against necrosis. Future studies will be needed to distinguish between these possibilities.

In I/R, oxidative stress after reperfusion promotes the MPT, and antioxidants are protective. After H/R in our mouse model, 4-HNE immunostaining increased substantially as an indicator of lipid peroxidation and oxidative stress (Figure 5). Minocycline decreased this 4-HNE staining after H/R. Since minocycline is not an antioxidant, decreased HNE staining by minocycline suggests that oxidative stress is occurring as a consequence of the MPT and cell death. However, much HNE staining occurred in regions that had not yet become necrotic, and this oxidative stress might nonetheless be contributing to the progression of injury.

Endotoxin acting through lipopolysaccharide-binding protein contributes to H/R injury to liver [15]. As an antibiotic, minocycline might alter intestinal flora and hence endotoxemia after H/R. However, tetracycline is also a broad spectrum antibiotic, and tetracycline did not protect after H/R. The danger of bacterial infection necessitates prophylactic use of antibiotics, such as broad spectrum cephalosporins, after multiple trauma and in advance of major surgery [29–31]. Since minocycline is a broad spectrum antibiotic with an excellent safety record, one-time treatment of hemorrhagic shock patients with minocycline would be consistent with current clinical practice and has the additional benefit of decreasing injury from H/R and the subsequent development of MODS. Future studies will be needed to determine what benefit, if any, minocycline might have in a clinical setting of hemorrhagic shock and resuscitation.

List of Abbreviations

4HNE: 4-Hydroxynonenal
ALT: Alanine aminotransferase
ATP: Adenosine triphosphate
H/R: Hemorrhage and resuscitation
H&E: Hematoxylin and eosin
HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPF: High power field
MODS: Multiple organ dysfunction syndrome
MPT: Mitochondrial permeability transition
NIM811: *N*-Methyl-4-isoleucine cyclosporin
PT: Permeability transition
SIRS: Systemic inflammatory response syndrome
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Acknowledgments

This work was supported, in part, by Grants DK37034 and DK073336 from the National Institutes of Health and Grant W81XWH-09-1-0484 from the Department of Defense. Imaging facilities for this research were supported, in part, by Cancer Center Support Grant P30 CA138313 to the Hollings Cancer Center, Medical University of South Carolina, with animal facility support from Grant C06 RR015455. Portions of this work were presented at the International Shock Congress, Cologne, Germany, June 28–July 2, 2008.

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Hindawi Publishing Corporation
HPB Surgery
Volume 2012, Article ID 641982, 9 pages
doi:10.1155/2012/641982

Research Article

C-Jun N-Terminal Kinase 2 Promotes Liver Injury via the Mitochondrial Permeability Transition after Hemorrhage and Resuscitation

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Received 16 February 2012; Accepted 24 March 2012

Academic Editor: Peter Schemmer

Hemorrhagic shock leads to hepatic hypoperfusion and activation of mitogen-activated stress kinases (MAPK) like c-Jun N-terminal kinase (JNK) 1 and 2. Our aim was to determine whether mitochondrial dysfunction leading to hepatic necrosis and apoptosis after hemorrhage/resuscitation (H/R) was dependent on JNK2. Under pentobarbital anesthesia, wildtype (WT) and JNK2 deficient (KO) mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution. Serum alanine aminotransferase (ALT), necrosis, apoptosis and oxidative stress were assessed 6 h after resuscitation. Mitochondrial polarization was assessed by intravital microscopy. After H/R, ALT in WT-mice increased from 130 U/L to 4800 U/L. In KO-mice, ALT after H/R was blunted to 1800 U/l ($P < 0.05$). Necrosis, caspase-3 activity and ROS were all substantially decreased in KO compared to WT mice after H/R. After sham operation, intravital microscopy revealed punctate mitochondrial staining by rhodamine 123 (Rh123), indicating normal mitochondrial polarization. At 4 h after H/R, Rh123 staining became dim and diffuse in 58% of hepatocytes, indicating depolarization and onset of the mitochondrial permeability transition (MPT). By contrast, KO mice displayed less depolarization after H/R (23%, $P < 0.05$). In conclusion, JNK2 contributes to MPT-mediated liver injury after H/R.

1. Introduction

Multiple trauma is the principal cause of hemorrhagic shock and is typically the consequence of traffic accidents, falls, and, in time of war, casualties of combat [1, 2]. After hemorrhagic shock, resuscitation can lead to multiple organ dysfunction syndrome (MODS), which remains the most significant contributor to late mortality and intensive care unit resource utilization in critical care medicine [3, 4]. The liver is quite vulnerable to injury after ischemia and reperfusion (I/R). After I/R, hepatic necrosis is the predominant mode of cell death, whereas apoptosis is of less importance [5–7]. However, apoptosis and necrosis share common pathways, particularly the mitochondrial permeability transition (MPT) [8].

The MPT is caused by opening of high conductance MPT pores in the mitochondrial inner membrane, which leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude mitochondrial swelling [9]. The MPT plays a prominent role in the pathogenesis of cell death after I/R injury and a variety of other stresses [9–12]. After onset of the MPT, necrotic cell killing (oncosis) can occur as a consequence of ATP depletion, whereas swelling of mitochondria after the MPT leads to rupture of the outer membrane and release of proapoptotic proteins like cytochrome c. The extent of ATP depletion is crucial to

whether necrosis or apoptosis occurs, since caspase-dependent apoptosis requires ATP, and necrosis does not occur until ATP is depleted by more than 85%.

c-Jun N-terminal kinase (JNK) is a stress-activated protein kinase that becomes activated after stresses like ultraviolet (UV) radiation, I/R and inflammation [13–16]. JNK-dependent phosphorylation of the transcription factor c-Jun/AP-1 promotes gene expression for an enhanced immune response [17]. JNK can also induce apoptosis via JNK-mediated phosphorylation of proapoptotic Bcl2 family proteins, such as Bim and Bmf, leading to mitochondrial outer membrane permeabilization, release of cytochrome c, and caspase activation [18, 19]. Moreover, translocation of activated JNK to mitochondria promotes the MPT [20, 21]. JNK becomes activated after experimental liver transplantation, warm hepatic I/R and hemorrhage/resuscitation (H/R), and pharmacological inhibition of JNK decreases liver injury, improves liver function, and increases survival in these settings [14, 15, 22–25]. Liver expresses two isoforms of JNK—JNK1 and JNK2 [26]. In models of acetaminophen hepatotoxicity, TNFα-dependent hepatic injury, warm I/R to liver and liver transplantation, JNK2 deficient mice are relatively protected against injury compared to wildtype mice [27–30].

Another organ vulnerable to injury during H/R is the gut. H/R compromises the barrier function of the gut, causing toxins and bacterial products like lipopolysaccharide (LPS) to enter the liver via the portal vein [31]. LPS and other gut-derived toxins entering the liver after H/R stimulate free radical generation and proinflammatory cytokine release by Kupffer cells to contribute to hepatic injury and increased cytokines in the blood stream [32–35]. Since JNK2 is also associated with the loss of barrier function of the gut [36, 37], we hypothesized that JNK2 is important for promotion of liver injury after H/R. Here, we test this hypothesis and show that liver injury decreases and hepatic function improves after H/R to JNK2 deficient mice in comparison to wildtype mice. These improvements are associated with improved mitochondrial function.

2. Materials and Methods

2.1. Chemicals and Reagents. Rhodamine 123 (Rh123) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals. Experiments were performed using protocols approved by the Institutional Animal Care and Use Committee. C57BL/6 (wildtype) and JNK2-deficient (B6.129S2-Mapk9tm1Flv/J on a C57BL background) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice used were males of 8 to 10 weeks of age and weighing 21–25 g.

2.3. Hemorrhagic Shock and Resuscitation. After an overnight fast, mice were anesthetized with sodium pentobarbital (80 mg/kg body weight). Under spontaneous breathing, the left and right femoral arteries were exposed and cannulated with polyethylene-10 catheters (SIMS Portex), as described [15]. Before insertion, the catheters were flushed with normal saline containing heparin (100 IU/l). One catheter was connected via a transducer to a pressure analyzer (Micro-Med; Louisville, KY, USA), and blood was withdrawn over 5 min via the second catheter into a heparinized syringe (10 units) to a mean arterial pressure of 30 mm Hg. This pressure was maintained for 3 h by the reinfusion or withdrawal of shed blood. An animal temperature controller was used to maintain rectal temperature between 36.6 and 37.3°C. After 3 h, mice were resuscitated with the shed blood followed by lactated Ringer's solution corresponding to 50% of the shed blood volume infused with a syringe pump over 30 min. Adequacy of resuscitation was determined by the restoration of blood pressure to ~80 mm Hg. After resuscitation, the catheters were removed, the vessels were ligated, and the groin incisions were closed. Sham-operated animals underwent the same surgical procedures without hemorrhage. In sham-operated mice, pentobarbital anesthesia lasted up to 120 min before the animals began to awaken, and a second injection was required to continue the anesthesia. In mice undergoing H/R, a second injection of pentobarbital was not necessary to maintain anesthesia, most likely due to decreased pentobarbital metabolism by the hypoperfused liver. Over the course of the experiments, no mortality in any group occurred. For the determination of H/R-dependent liver damage, mice were anesthetized, and the two right dorsal liver lobes were snap frozen in liquid nitrogen. The remaining liver was flushed with saline through the portal vein, fixed by infusion of 4% buffered paraformaldehyde, and embedded in paraffin.

2.4. Alanine Aminotransferase (ALT). Blood samples to measure ALT were collected from the inferior vena cava 6 h after H/R for analysis using a kit (Sigma Chemical, St. Louis, MO, USA).

2.5. Histology. Necrosis was evaluated 6 h after H/R in 4-μm paraffin sections stained with hematoxylin and eosin (H&E). Necrosis was identified by standard morphologic criteria (e.g., loss of architecture, karyolysis, vacuolization, increased eosinophilia). Areas of necrosis were outlined in 10 random fields for each liver. Images were captured (Olympus BH-2 Microscope; Micropublisher 5.0 RTV, Center Valley, PA, USA), and the area percentage of necrosis was quantified using a computer program (BioQuant BQ Nova Prime 6.7, R&M Biometrics, Nashville, TN, USA).

2.6. Caspase-3. Liver tissue (~100 mg) was homogenized (Polytron PT-MR2100, Kinematica, Luzern, Switzerland) in 1 mL of lysis buffer containing 0.1% 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid, 5 mM DTT, 2 mM EDTA, 1 mM pefabloc, 10 ng/mL pepstatin A, 10 ng/mL aprotinin, 20 μg/mL leupeptin and 10 mM HEPES buffer, pH 7.4. After centrifugation at 15,000 rpm for 30 min, activity of caspase-3 in the supernatant was determined using a Caspase-3 Colorimetric Assay Kit (R&D Systems,

Minneapolis, MN) according to the manufacturer's instructions. Activity was normalized to protein concentration and expressed as fold increase compared to sham.

2.7. 4-Hydroxynonenal. Paraffin sections were deparaffinized, rehydrated, and incubated with polyclonal antibodies against 4-hydroxynonenal (4-HNE, Alpha Diagnostics; San Antonio, TX, USA) in PBS (pH 7.4) containing 1% Tween 20 and 1% bovine serum albumin. Peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit, DAKO) were used to detect specific binding.

2.8. Intravital Microscopy. At 4 h after H/R, mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and connected to a small animal ventilator via a tracheostomy and respiratory tube (22-gauge catheter), as described [29]. Laparotomy was performed, and a polyethylene-10 catheter was inserted into the distal right colic vein. Using a syringe pump, a membrane potential indicating fluorophore, Rh123 (1 μmol/mouse), was infused via the catheter over 10 min. After prone positioning of the mouse, the liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the stage of an inverted microscope. Rh123 fluorescence was excited with 820 nm light from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent, Santa Clara, CA, USA) and imaged with a Zeiss LSM 510 NLO laser scanning confocal microscope using a 63 × 1.3 NA water-immersion objective lens. Green Rh123 fluorescence was collected through a 525 ± 25 nm band pass filter. During image acquisition, the respirator was turned off for ~5 sec to eliminate breathing movement artifacts. In 20 fields per liver, hepatocytes were scored for bright punctate Rh123 fluorescence signifying polarized mitochondria or a dimmer diffuse cytosolic fluorescence denoting depolarized mitochondria. Image analysis was performed in a blinded manner.

2.9. Statistical Analysis. Data are presented as means ± S.E., unless noted otherwise. Statistical analysis was performed by ANOVA with Student-Newman-Keuls test, as appropriate, using $P < 0.05$ as the criterion of significance.

3. Results

3.1. Decreased ALT Release and Liver Necrosis after Hemorrhage and Resuscitation of JNK2-Deficient Mice. After sham operation, serum ALT averaged 112 ± 15 U/L in wildtype and JNK2 deficient mice (Figure 1). After H/R, ALT increased to 4860 ± 538 U/L 6 h after resuscitation in wildtype mice compared to 1806 ± 126 U/L in JNK2-deficient mice ($P < 0.001$, Figure 1).

In sham-operated wildtype and JNK2-deficient mice, liver histology was normal and indistinguishable from untreated mice (Figure 2(a) and data not shown). At 6 h after H/R to wildtype mice, large areas of hepatic necrosis developed with a predominantly pericentral and midzonal distribution (Figure 2(b)). In JNK2-deficient mice, hepatic necrosis after H/R decreased from 24.5 ± 1.5% in wildtype mice to 6.6 ± 1.5% ($P < 0.05$, Figures 2(c) and 2(d)).

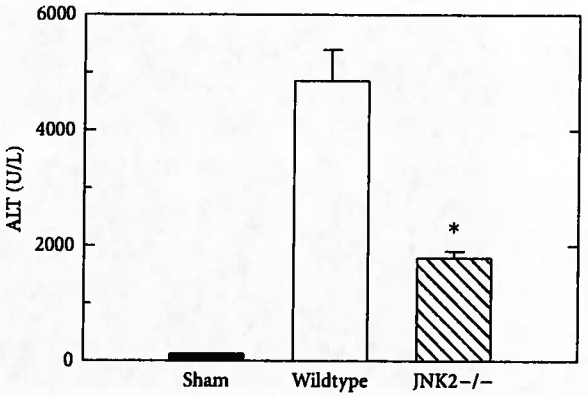

FIGURE 1: Decreased alanine aminotransferase (ALT) release after hemorrhage/resuscitation in JNK2-deficient mice. Wildtype and JNK2-deficient (JNK2−/−) mice were subjected to sham operation or bled to a mean arterial pressure of 30 mm Hg and resuscitated after 3 h, as described in Section 2. Blood was collected at 6 h after resuscitation for ALT measurement. Group sizes were 5-6 mice/group. *$P < 0.05$ versus wildtype. Average ALT values of wildtype and JNK2 deficient mice after sham operation were not statistically significantly different and are pooled.

Thus, hepatic necrosis in JNK2-deficient mice after H/R was decreased by more than two-thirds in comparison to wildtype mice (Figure 2(d)).

3.2. Decreased Apoptosis after Hemorrhage and Resuscitation of JNK2-Deficient Mice. Caspase 3 activity was measured in liver extracts at 6 h after H/R of wildtype- and JNK2-deficient mice in comparison to sham-operated mice. After sham operation, caspase 3 activity in the liver was nearly undetectable (Figure 3). After H/R of wildtype mice, caspase 3 activity increased significantly by 7.6-fold. By contrast after H/R of JNK2-deficient mice, hepatic caspase 3 activity increased only 2.6-fold ($P < 0.05$ versus wildtype, Figure 3).

3.3. Improved Mitochondrial Function In Vivo after Hemorrhage and Resuscitation of JNK2-Deficient Livers. Intravital multiphoton microscopy revealed bright fluorescence of Rh123 in hepatocytes at 4 h after sham operation. The punctate pattern denoted polarization of individual mitochondria. No differences in Rh123 fluorescence were observed between livers of wildtype- and JNK2-deficient mice (Figure 4(a) and data not shown). We then imaged Rh123 fluorescence at 4 h after H/R. This time point was selected because previous studies of liver transplantation after cold ischemic storage showed that 4 h after reperfusion was a time point where mitochondrial dysfunction could be detected prior to onset of cell death [38]. At 4 h after H/R in wildtype mice, Rh123 staining became diffuse and dim in many hepatocytes indicative of depolarized mitochondria (Figure 4(b)). By contrast, after H/R of JNK2-deficient mice, mitochondria depolarized in fewer hepatocytes than in wildtype mice (Figure 4(c)). Rather, most hepatocytes

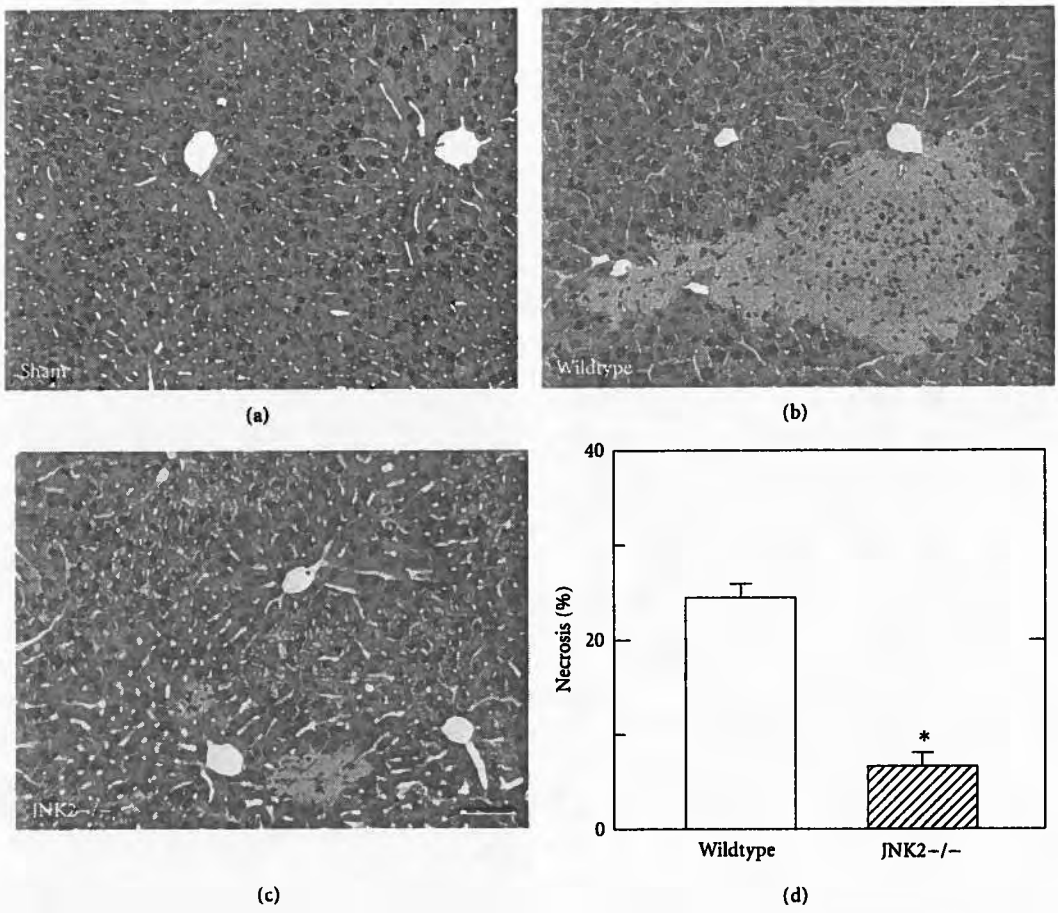

FIGURE 2: Decreased necrosis after hemorrhage and resuscitation in JNK2 deficient mice. At 6 h after resuscitation, necrosis was assessed by H&E in livers from sham-operated wildtype mice (a) and from wildtype- (b) and JNK2-deficient (c) mice after H/R. Bar is 50 μm. In (d), the percent area of necrosis is averaged from 5 livers per group. Necrosis was not present after sham operation of either wildtype- or JNK2 deficient mice and is not plotted. $^*P < 0.05$.

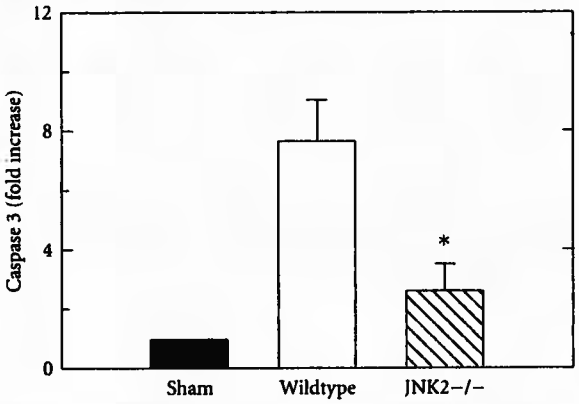

FIGURE 3: Decreased caspase 3 activation after hemorrhage and resuscitation of JNK2-deficient mice. At 6 h postoperatively, caspase 3 activity was assessed after sham operation and after H/R of wildtype and JNK2-deficient (JNK2−/−)mice, as described in Section 2. $P < 0.05$ versus wildtype, $n = 5$ per group.

exhibited bright, punctate staining by Rh123 in JNK2-deficient mice. In these experiments, hepatocytes were scored for Rh123 staining. In sham-operated mice, virtually no hepatocytes contained depolarized mitochondria. At 4 h after H/R of wildtype mice, 58% of hepatocytes contained depolarized mitochondria (Figure 4(d)). By contrast, at 4 h after H/R of JNK2-deficient mice, hepatocytes with depolarized mitochondria became 23%, less than half of that in wildtype mice ($P < 0.05$ versus wildtype, Figure 4(d)).

3.4. Decreased Oxidative Stress after Hemorrhage and Resuscitation of JNK2-Deficient Mice. We used 4-HNE immunohistochemistry to evaluate oxidative stress in mouse livers 6 h after H/R. 4-HNE is a product of lipid peroxidation that forms protein adducts that are recognized by anti-4-HNE antibodies. After sham operation, the brown reaction product of 4-HNE immunohistochemistry was virtually undetectable (Figure 5(a)). By contrast at 6 h after H/R of wildtype mice, wide confluent areas of HNE

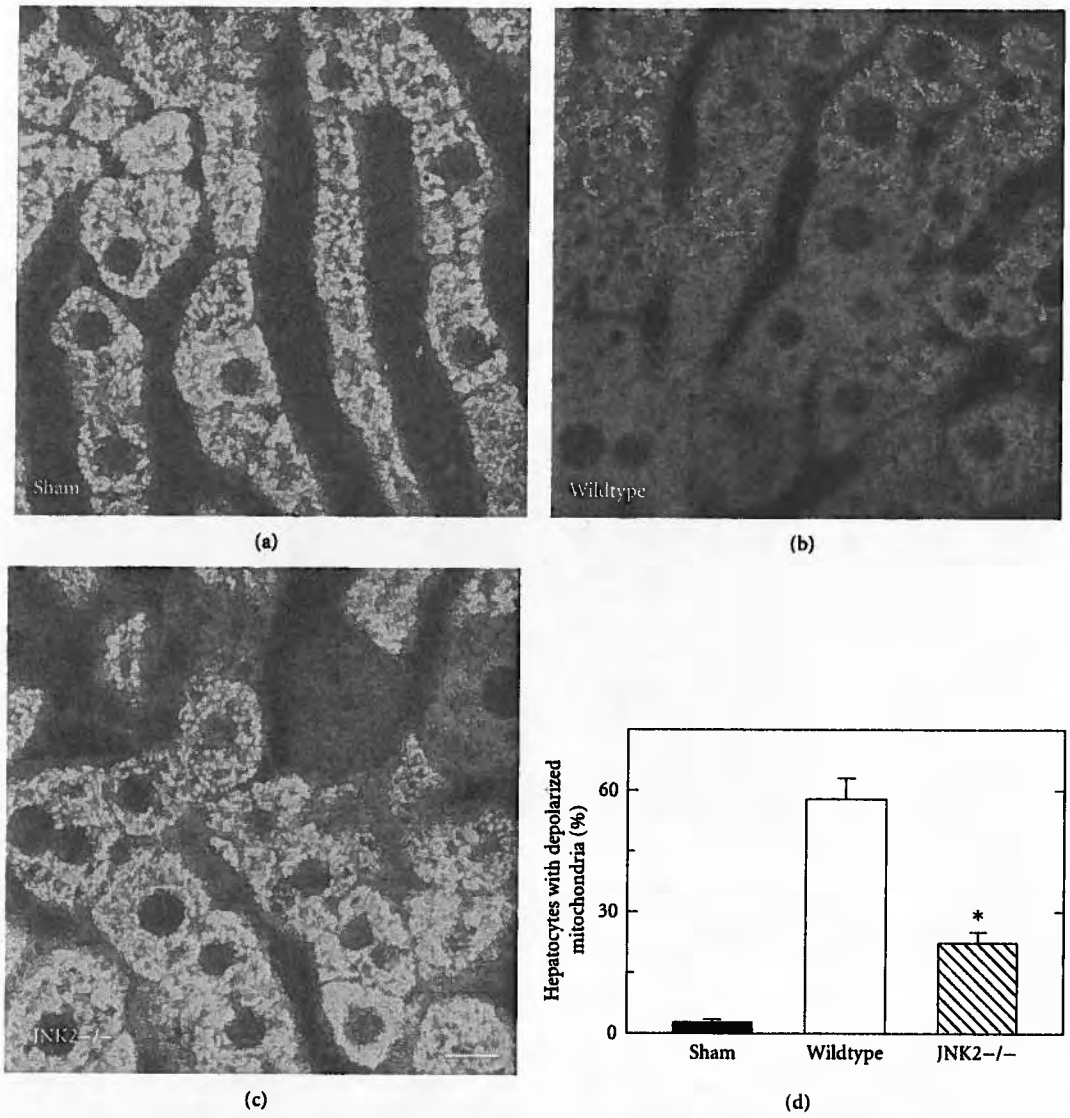

FIGURE 4: Decreased mitochondrial depolarization after hemorrhage and resuscitation of JNK2-deficient mice. Multiphoton imaging of hepatic Rh123 fluorescence was performed at 4 h after sham operation to wildtype mice (a) and H/R of wildtype- (b) and JNK2-deficient (c) mice, as described in Section 2. The percentage of hepatocytes per HPF with depolarized mitochondria is plotted in (d). Bar is 10 μm. $P < 0.05$ versus other groups; $n = 3$ per group.

immunoreactivity developed in pericentral and midzonal areas with relative sparing the periportal regions (Figure 5(b)). After H/R of JNK2-deficient mice, HNE immunoreactivity was substantially decreased and confined mostly to pericentral regions (Figure 5(c)).

4. Discussion

4.1. Decreased Liver Injury after Hemorrhagic Shock and Resuscitation of JNK2-Deficient Mice. Systemic inflammatory response syndrome (SIRS) and MODS following H/R are major problems after multiple trauma [3, 4]. H/R also causes hepatic necrosis and apoptosis [15, 23, 39]. The goal of this study was to evaluate the impact of JNK2 on hepatic injury and mitochondrial dysfunction after H/R. Our findings show a specific role for JNK2 in liver injury after H/R, since JNK2-deficient mice had decreased hepatic injury and mitochondrial dysfunction after H/R in comparison to wildtype mice (Figures 1–4).

4.2. Reperfusion Injury after Hemorrhagic Shock and Resuscitation Induces Necrosis and Apoptosis through JNK2 Signaling. JNK becomes activated in various models of liver injury, and pharmacological inhibition of JNK decreases liver injury [14, 15, 22–24, 40–42]. In particular, JNK inhibition with the peptide inhibitor, DJNKI-1, decreases hepatic damage and inflammation after H/R [23]. However, JNK inhibitors are nonspecific with regards to the two isoforms of JNK, JNK1

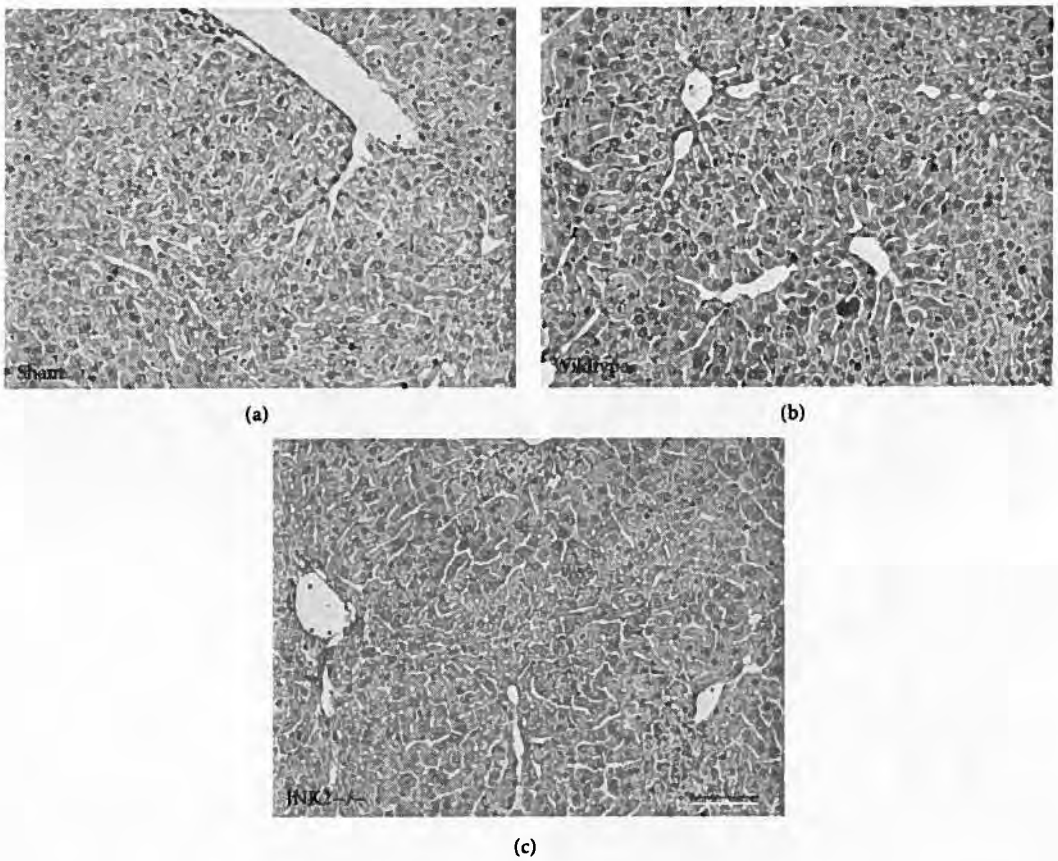

FIGURE 5: Decreased 4-hydroxynonenal immunostaining after hemorrhage and resuscitation of JNK2-deficient mice. ROS generation was assessed by 4-hydroxynonenal immunocytochemistry livers at 6 h after sham operation of wildtype mice (a) and after H/R to wildtype- (b) and JNK2-deficient (c) mice, as described in Section 2. Bar is 50 μm. $n = 5$ per group.

and JNK2, that are expressed in liver. Previous studies show that injury after orthotopic mouse liver transplantation and warm hepatic I/R decreases in JNK2-deficient livers compared to wildtype [29, 30]. In H/R, the specific roles of JNK isoforms are unknown. Therefore, we investigated the role of JNK2 by comparing JNK2-deficient mice and wildtype mice.

JNK2 deficiency decreased both necrosis and apoptosis in liver after H/R. Necrosis assessed by ALT and histology and apoptosis assessed by caspase 3 activity were decreased by 60% or more in JNK2-deficient mice compared to wildtype (Figures 1 and 2). Nonetheless, necrosis was the predominant mode of cell death after H/R. These results are in agreement with earlier results after liver transplantation and warm I/R [29, 30].

4.3. JNK2 Deficiency Attenuates Formation of Reactive Oxygen Species after Hemorrhage and Resuscitation. Reactive oxygen species (ROS) mediate, at least in part, liver injury after H/R, warm I/R, and storage/reperfusion injury occurring in liver transplantation. A consequence of ROS formation is peroxidation of polyunsaturated fatty acids, such as linoleic and arachidonic acids, which leads to 4-HNE generation and formation of 4-HNE-protein adducts. In the present study, hepatic 4-HNE immunostaining was marked after H/R to wildtype mice but substantially diminished in JNK2-deficient mice (Figure 5). This indicates that JNK2 signaling has a role in promoting ROS generation after H/R. Such ROS can directly damage proteins, lipids, and DNA, as well as to help induce the MPT.

4.4. JNK2 Signaling after H/R Induces Mitochondrial Depolarization and Promotes Liver Injury. To test the hypothesis that the JNK2 isoform specifically promotes mitochondrial dysfunction after H/R, we used intravital multiphoton microscopy of Rh123 to assess mitochondrial polarization. This technique allows direct assessment of mitochondrial polarization in livers of living animals. Four hours after H/R of wildtype livers, mitochondrial depolarization occurred in more than 50% of hepatocytes. Mitochondrial depolarization occurred prior to cell death, since after 4 h few cells labeled with propidium iodide, a marker of nonviable cells (data not shown), as described previously [29]. After H/R of JNK2-deficient mice, mitochondrial depolarization was markedly decreased in comparison to wildtype mice (Figure 4). Minocycline and N-methyl-4-isoleucine cyclosporin are specific inhibitors of the MPT that prevent mitochondrial depolarization after I/R and orthotopic rat liver transplantation with no direct effect

on mitochondrial respiration and oxidative phosphorylation [29]. Thus, mitochondrial depolarization visualized by intravital multiphoton microscopy, which was attenuated in JNK2-deficient mice, most likely represents onset of the MPT. Several studies indicate involvement of the MPT in acetaminophen hepatotoxicity [12, 20]. In acetaminophen hepatotoxicity, activated JNK translocates to mitochondria to induce MPT onset, which can be prevented by JNK inhibitors [20]. Thus, protection against mitochondrial depolarization in JNK2-deficient livers after H/R implies that JNK2 is directly involved in promoting the MPT in wildtype livers after H/R stress.

4.5. Other Mechanisms Promoting JNK2-Dependent Toxicity. H/R is also associated with a proinflammatory milieu in the gut lumen that promotes loss of barrier function [31]. Moreover, JNK2 mediates osmotic stress-induced tight junction disruption in the intestinal epithelium [36], although JNK1 is reported to mediate apical junction disassembly triggered by calcium depletion [37]. Impaired intestinal barrier function promoted by JNK during H/R may therefore also lead to portal vein endotoxemia, activation of TLR4 with phosphorylation of MAPKs, and increased production of inflammatory cytokines and ROS by hepatic Kupffer cells [34, 35, 43, 44]. Future studies will be needed to characterize how JNK2-dependent actions inside and outside hepatocytes contribute causally to liver injury, mitochondrial dysfunction, and development of MODS/SIRS after H/R.

4.6. Therapeutic Implications. An important implication of the present findings is that JNK2 represents a unique therapeutic target for treatment and prevention of hepatic injury and possibly SIRS and MODS after H/R. D-JNKI-1 and other existing JNK inhibitors are nonspecific and inhibit all JNK isoforms: JNK1, JNK2, and JNK3 [45]. JNK2 in our model of H/R plays a detrimental role, but JNK1 and/or JNK3 may have beneficial effects in liver and other tissues, especially since JNK1/JNK2 double knockout mice are not viable [46]. Thus, a specific JNK2 inhibitor might provide greater and more specific benefit after H/R and decrease the potential of toxicity by JNK1 and/or JNK3 inhibition, but such an inhibitor still awaits development.

List of Abbreviations

4-HNE: 4-hydroxynonenal
ALT: Alanine aminotransferase
H/R: Hemorrhage and resuscitation
H&E: Hematoxylin and eosin
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF: High-power field
MODS: Multiple organ dysfunction syndrome
MPT: Mitochondrial permeability transition
Rh123: Rhodamine 123
ROS: Reactive oxygen species
SIRS: Systemic inflammatory response syndrome.

Acknowledgments

This work was supported, in part, by Grants DK37034 and DK073336 from the National Institutes of Health and Grant W81XWH-09-1-0484 from the Department of Defense. Imaging facilities for this research were supported, in part, by Cancer Center Support Grant P30 CA138313 to the Hollings Cancer Center, Medical University of South Carolina. Portions of this work were presented at the International Shock Congress, Cologne, Germany, June 28–July 2, 2008 and at the Annual Meeting of the American Association for the Study of Liver Diseases, San Francisco, CA, USA, October 31–November 4, 2008.

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