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Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo

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The mouse model of liver ischemia and reperfusion injury has proven to be valuable for our understanding of the role that reactive oxygen and nitrogen metabolites play in postischemic tissue injury. This methods paper provides a detailed protocol for inducing partial liver ischemia followed by reperfusion. Liver ischemia is induced in anesthetized mice by cross-clamping the hepatic artery and portal vein for varying lengths of time, resulting in deprivation of blood flow to approximately 70% of the liver. Restoration of blood flow to the ischemic lobes enhances superoxide production concomitant with a rapid and marked decrease in the bioavailability of nitric oxide, resulting in alterations in the redox state of the liver in favor of a more oxidative environment. This hepatocellular oxidative stress induces the activation of oxidant-sensitive transcription factors followed by the upregulation of proinflammatory cytokines and mediators that ultimately lead to liver injury. This model can be induced in any strain or sex of mouse and requires 1–2 months of practice to become proficient in the surgery and animal manipulation. The roles of various reactive metabolites of oxygen and nitrogen may be evaluated using genetically engineered mice as well as selective molecular, cellular, and/or pharmacological agents.

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Few aspects of vascular pathobiology have garnered more attention over the past 3 decades than has ischemia and reperfusion injury. Indeed, it has been almost 30 years since Granger, Rutili, and McCord first reported that much of the postischemic tissue damage observed in a feline model of intestinal ischemia and reperfusion (I/R) was mediated directly or indirectly by the superoxide anion radical [1]. Since that publication, a voluminous literature has accumulated implicating both reactive oxygen and nitrogen species as important mediators and modulators of postischemic tissue injury in a number of different organ systems, including the gut, liver, heart, kidney, brain, and lung, to mention just a few [2]. One organ system that has produced some of the most detailed information regarding the mechanisms by which reactive metabolites of oxygen and nitrogen mediate or modulate tissue damage after I/R is the liver. It is well appreciated by the clinical community that liver I/R injury is a major complication associated with liver transplantation and resectional surgery as well as hemorrhagic or endotoxin shock and thermal injury. A large body of experimental data using rat or mouse models of I/R suggests that postischemic tissue injury is initiated by rapid alterations in the generation and/or steady-state levels of reactive oxygen and nitrogen species [3–5]. One of the earliest events associated with reperfusion of ischemic tissue is a remarkable dysfunction of sinusoidal endothelial cells (SECs) characterized by profound decreases in the steady-state levels of endothelial cell nitric oxide synthase (eNOS)-derived nitric oxide (NO) [6]. This decline in NO bioavailability occurs within the first few minutes of reperfusion and seems to be caused by decreased synthesis of NO, enhanced inactivation of NO by the overproduction of certain reactive oxygen species (ROS), or both. Coincident with the fall in NO production is an equally rapid increase in the production of ROS such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) [5,7–11]. Hepatocyte-associated xanthine oxidase, mitochondria, and Kupffer cell- and/or sinusoidal endothelial cell-associated NADPH oxidase have all been implicated as important generators of ROS in the acute or early phase of I/R-induced ischemic liver [5,7–12]. Because NO is well known to interact with and decompose certain ROS such as O₂⁻, hydroxyl radical (•OH), and ferryl hemoproteins, I/R-induced overproduction of ROS coupled to decreased steady-state levels of NO results in a rapid alteration in the redox state of the different cells within the liver in favor of a more oxidative environment [13–15].

Early studies suggested that postischemic liver injury was mediated directly by the overproduction of injurious oxidizing agents
derived from oxygen, i.e., ROS. However, more recent data suggest that oxidative stress may injure the tissue indirectly by activating redox-sensitive transcription factors such as NF-κB and activating protein-1, thereby upregulating a variety of potentially injurious cytokines, chemokines, and proinflammatory mediators [5,10,16,17]. Inherent in this hypothesis is the prediction that exogenous antioxidants or NO may attenuate postischemic tissue injury via their ability to prevent or attenuate transcription factor activation and the consequent expression of the various injurious mediators. Indeed, a number of studies, using pharmacologic interventions or genetic approaches, have demonstrated protective effects with administration of certain nonenzymatic or enzymatic antioxidants before the induction of liver ischemia [9,10,18–20]. Furthermore, the mouse model of liver I/R has been especially informative in demonstrating that endogenous or exogenous NO protects the liver from postischemic injury by normalizing the redox potential of the liver, attenuating the transcriptional activation of a number of injurious and proinflammatory genes, limiting hypoxic insult, and/or interfering with platelet–leukocyte–endothelial cell interactions [18,21–24].

Because one cannot completely recapitulate the complexity of the physiologic setting in vitro, it is becoming increasingly important for investigators who wish to study the normo- and/or pathobiology of reactive oxygen and nitrogen metabolism to work in a physiologically relevant environment. Thus, the objective of this methods paper is to provide a detailed protocol describing the mouse model of I/R. It is our hope that this model will be useful for investigators interested in exploring the many different molecular, cellular, and physiological mechanisms associated with vascular pathobiology, free radical metabolism, and inflammation in vivo.

Materials

Animals

Eight- to 12-week-old wild-type or genetically engineered mice of the same sex are used. Important: Although the strain of the mouse is not critical for this model, gender differences may exist in response to liver ischemia and reperfusion injury. Therefore, it is recommended that at least seven mice of the same sex and strain be used for each experimental group. All animals are maintained on a standard laboratory diet with free access to food and water until the time of the experiment. All experimental procedures must comply with the Guide for the Care and Use of Laboratory Animals (revised 1996), approved by the Council of The American Physiological Society, and with federal and state regulations.

Reagents

The following is a list of the reagents used:

- Ketamine (Ketaset Ill 100 mg/ml; Fort Dodge Animal Health, Fort Dodge, IA, USA; Cat. No. 44016)
- Xylazine (Xylazine-20 injection 20 mg/ml; The Butler Co., Columbus, OH, USA; Cat. No. WAB10945)
- 0.9% Sodium chloride
- Heparin sodium (10,000 units/ml; Baxter, Deerfield, IL, USA; Cat. No. 462-274-01)
- Sterile alcohol prep pad (Fisher, Pittsburgh, PA, USA; Cat. No. 06-669-62)
- Povidone–iodine swab stick (Professional Disposables, Orangeburg, NY, USA; Cat. No. S41350)
- 10% Phosphate-buffered formalin
- Hematoxylin
- Eosin
- Trizol (Invitrogen, Carlsbad, CA, USA; Cat. No. 15596-018)
- DNase (Invitrogen)
- MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA)
- Mouse IL-1α/IL-1F1 ELISA Kit (Quantikine, Minneapolis, MN, USA; Cat. No. MLA00)
- Mouse TNF-α/TNFSF1A ELISA Kit (Quantikine; Cat. No. MTA00).

Surgical instruments

The instruments needed are as follows:

- Tissue scissors (blunt, 11.5 cm; Fine Science Tools, Foster City, CA, USA; Cat. No. 14072-10)
- Hemostats (Micro-Mosquito, straight, 12 cm; Fine Science Tools; Cat. No. 13010-12)
- Atraumatic clip (Micro Serrefine, jaw length 6 mm, jaw width 1 mm, total length 17 mm, jaw pressure 110 g; Fine Science Tools; Cat. No. 18055-05)
- Microclip applicator with lock (total length 15 cm; Fine Science Tools; Cat. No. 18056-14).

Equipment

The following equipment is used:

- 25-gauge needle
- 1-ml syringe
- Gauze sponges (Fisher; Cat. No. 22-362-178)
- Cotton-tipped swabs (Fisher; Cat. No. 23-400-100)
- Suture with needle (4-0 silk, C-3, 45 cm; Ethicon, Somerville, NJ, USA; Cat. No. 736G)
- Serum separator tube (Becton–Dickson, Franklin Lakes, NJ, USA; Cat. No. 365956)
- JB4 plastic mounting medium (Polysciences, Warrington, PA, USA)
- Heat lamp and temperature controller
- Electric razor
- Bio–Rad iCycler (Bio-Rad, Hercules, CA, USA).

Methods

Surgery

1. Fasted (16–18 h) mice are anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection using a 25-gauge needle into the lower right quadrant of the mouse.

2. Once the animal is anesthetized, it is immobilized by taping the animal’s legs and arms to a clean flat surface (e.g., washable plastic) with the abdomen facing up. The abdomen is then shaved up to the xiphoid process and is cleaned by swabbing the skin with a 70% ethanol solution followed by a commercially available betadine preparation to prevent the introduction of bacteria during the surgical procedure.

3. A small pair of scissors is then used to open the abdomen at the midline to expose the abdominal contents. This incision should be approximately 3 cm, beginning at the midadomen and ending at the xiphoid process. Important: Be careful not to extend the incision beyond the xiphoid process as there a number of large vessels within the skin and muscle layers, which can bleed extensively. Attach hemostats to either side of the incision and xiphoid process and pull the abdominal cavity open (Fig. 1). Hemostats can be placed on stacks of gauze to raise the abdominal walls and allow for easier visualization.

4. Moistened cotton swabs, carefully externalize the intestines as gently as possible from the cavity and place them on premoistened (sterile 0.9% saline) gauze to expose the portal vein and associated structures. Cover
the intestine with the second piece of gauze to prevent drying. Carefully separate the quadrate lobe from its attachment to the left lateral lobe with scissors to better reveal the portal triad (portal vein, hepatic artery, and bile duct) (Fig. 1). It may be necessary to first lift the median and left lateral lobes against the diaphragm to identify the connection between the left lateral and the quadrate lobe.

5. Now place an atraumatic clip across the portal vein, hepatic artery, and bile duct just above the branching to the right lateral lobe (Fig. 1). The median and left lateral lobes (approximately 70% of the liver) should quickly show significant blanching, i.e., they should quickly change from their normal reddish brown color to a pale brown or cream color. **Important:** If the lobes do not show significant blanching (Fig. 1), remove the clip and reapply. Depth of clamp placement can be critical, as the hepatic artery (which you cannot see) can be significantly deeper than the portal vein and require deeper clamp placement for occlusion. **Important:** The clamps are very fragile and can become stretched with repeated use, decreasing their clamping force over time, resulting in variable degrees of ischemia. Extreme care is therefore necessary when applying and removing clamps, and specific clamp holder should always be used.

6. After clamp placement, replace the intestines into the abdominal cavity carefully, add 500 µl of 10 U/ml heparinized saline directly into the peritoneal cavity via syringe, and cover the incision with well-moistened gauze. Place the animal under a heat lamp to maintain body temperature at 37°C and monitor closely during the ischemic period, making sure the covering gauze remains moist with saline. During this time, additional anesthesia may be required by placing a small volume (50 µl) of anesthetic into the open abdomen.

7. After the desired period of ischemia, remove the clamp carefully and administer 500 µl of sterile saline to the peritoneal cavity to replenish any fluid loss during surgery. **Important:** Verify reperfusion visually, as the blanched color of the ischemic lobes should begin to show restoration of the normal reddish-brown color within a few seconds of clamp removal. No reflow can occur, especially after extended periods of ischemia, potentially causing significant variations in results.

8. Close the abdomen by suturing the muscle layer and then the skin with 4-0 silk sutures and allow the animal to recover for the required reperfusion period.

9. Immediately after the reperfusion period, blood may be collected from the anesthetized mouse as described below for serum preparation.

10. When blood samples have been obtained the mouse is euthanized by cutting the diaphragm.

11. Representative samples of the posts ischemic liver (and/or bypass lobes) are immediately frozen in liquid nitrogen and stored at -80°C.

**Serum preparation and quantification of liver transaminase activity and cytokine levels**

1. Serum levels of alanine aminotransferase (ALT) as well as other liver-specific transaminases are quantitative indices of liver damage and can be quantified in serum obtained from mice subjected to liver I/R. At varying times after ischemia and/or reperfusion, blood is removed from the inferior vena cava after 45 or 90 min of ischemia and different times of reperfusion using a 25-gauge syringe attached to a 1-ml syringe and placed directly into the peritoneal cavity via syringe, and cover the anesthetized mouse as described below for serum preparation.

2. The samples are allowed to clot on ice for approximately 10 min, after which they are centrifuged at 4000 g for 10 min. Serum is removed and ALT is measured using a kit from Sigma Chemical Corp. (St. Louis, MO, USA). Data are presented as units per liter (U/L) at 37°C. Aliquots (50 or 100 µl) of serum may be frozen before measurement of ALT at -80°C. **Repeated freeze–thaw cycles should be avoided as ALT may become inactivated.**

3. Serum levels of TNF-α as well as other cytokines are quantified using an enzyme-linked immunosorbent assay kit according to the manufacturer’s specifications (Quantikine; R&D Systems, Abingdon, UK). Data are presented as pg/ml of serum cytokines.

**Histopathology**

1. After euthanasia, representative pieces of ischemic, I/R, or bypass lobes are quickly removed and fixed in ice-cold 10% phosphate-buffered formalin for 24 h at 4°C.
2. The tissue is then partially dehydrated with ethanol and embedded in JB4 plastic mounting medium using standard histological methods.

3. Five-micrometer sections are cut and stained with hematoxylin and eosin. After staining, the sections are scored in a blinded fashion as described below.

NF-κB activation and nuclear translocation

Nuclear extract preparation
1. Place 5 ml of cold Solution A (see below) into a prechilled Dounce homogenizer.
2. Add frozen liver (one median lobe) and homogenize with ~10 strokes.
3. Decant homogenized liver into a cold 15-ml conical centrifuge tube and centrifuge for 30 s at 2000 rpm (4°C).
4. Decant the supernatant into a new cold 15-ml conical tube.
5. Incubate for 5 min on ice.
6. Centrifuge for 5 min at 5000 rpm (4°C).
7. Decant the supernatant and keep the nuclear pellet.
8. Resuspend the nuclear pellet in 400 μl Solution B (see below) thoroughly, as it may be difficult to suspend.
9. Transfer the nuclear suspension to a cold 1.5-ml microfuge tube.
10. Extract the protein by incubating the suspension for 20 min on a rotating platform at 4°C.
11. Centrifuge the extract for 15 s at 12,000 rpm.
12. Decant the supernatant into a new cold microfuge tube.
13. Remove 50-μl aliquots and place into cold 0.5-ml microfuge tubes and flash freeze in liquid nitrogen.
14. Store extracts at ~80°C.
15. Determine protein concentration with Pierce BCA reagent.

Solution A
Solution A consists of 0.6% NP-40, 150 mM NaCl, 10 mM Hepes (pH 7.9), 1 mM EDTA, and 0.5 mM PMSF (added fresh!).

Solution B
Solution B consists of 25% glycerol, 20 mM Hepes (pH 7.9), 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT (added fresh), 0.5 mM PMSF (added fresh), and protein inhibitor cocktail (add fresh according to manufacturer).

Oligonucleotide preparation and electrophoretic mobility shift assay (EMSA)
1. The NF-κB consensus oligonucleotide 5′-AGTTGAGGG-GACTTTCCCAGGC is end labeled with [γ-32P]ATP using T4 polynucleotide kinase according to the manufacturer’s instructions (Promega). Labeled oligonucleotide (35 fmol) is incubated with 20 μg nuclear extracts for 10 min on ice in binding buffer [1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), 0.05 μg/μl poly(dI-dC)–poly(dI-dC), and 4% glycerol] in a total volume of 35 μl. A 50-fold molar excess of nonlabeled consensus or mutated NF-κB consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA, USA) is included in the respective reactions.
2. Samples are incubated for 30 min at 25°C. For supershift studies, antibody specific for either the p50 (Santa Cruz Biotechnologies) or the p65 (Rockland, Gilbertsville, PA, USA) subunit is added after the initial incubation on ice. Protein–DNA complexes are resolved on 4% nondenaturing polyacrylamide gels by electrophoresis in 0.5× Tris–borate–EDTA. Gels are dried and visualized using a PhosphorImager (Molecular Dynamics, Hercules, CA, USA). Activation of NF-κB (relative to non-TNF-treated controls) is determined by performing densitometric analysis (ImageQuant Software; Molecular Dynamics) on shifted bands from scanned autoradiograms.

Cytokine message determinations
1. Liver cytokine message expression is quantified using real-time polymerase chain reaction in a Bio-Rad iCycler. Total RNA is...
extracted from approximately 100 mg of liver tissue using the Trizol method according to the manufacturer’s instructions.

2. One microgram of total RNA is subjected to DNase treatment according to the manufacturer’s instructions. The DNase-treated RNA is then reverse transcribed using MuLV reverse transcriptase in a two-step procedure: 30 min at 42°C followed by 5 min at 95°C. Two hundred nanograms of cDNA is then amplified by real-time polymerase chain reaction with SYBR green as the fluorescent indicator (which binds to double-stranded DNA) with the designated primer pairs.

3. The resultant data are analyzed using the Bio-Rad MyIQ software package, with samples being normalized against their sham vehicle controls.

Evaluating the roles of superoxide or nitric oxide in liver I/R

1. To assess specifically the role of superoxide we have utilized a genetically engineered polycationic chimeric form of manganese SOD consisting of a fusion protein of the mature human Mn-SOD (SOD2) sequence followed by the 26 C-terminal residues of human extracellular SOD (SOD3) [18]. This chimeric SOD2/3 fusion proteins binds to the microvascular endothelium as well as the extracellular matrix [25]. Mice are treated with SOD2/3 (1000U/kg; iv) 15 min before ischemia.

2. The role of eNOS-derived NO (or any other NOS or enzyme) in I/R injury may be evaluated using the genetically engineered eNOS-deficient mouse. In addition, the NOS inhibitor L-nitroarginine methyl ester (L-NAME) (Sigma–Aldrich, St. Louis, MO, USA) may be administered at a dose of 4 mg/kg (iv) or vehicle (0.9% sodium chloride) in a volume of 100 μl 15 min before ischemia [23,24,26].

Statistical analysis

All values are presented as means ± the standard error of the mean. Data are analyzed using Student’s t test or analysis of variance and significance was set at p < 0.05.

Results and discussion

Depriving 70% of the liver of blood flow for 90 min followed by varying times of reperfusion induces dramatic and time-dependent increases in liver injury as assessed by increases in serum ALT levels (Fig. 2). Important: We have found that addition of heparin during the surgical preparation reduces animal-to-animal variation by maintaining more consistent reperfusion while limiting the “no reflow” problems associated with coagulation. Histopathological evaluation of the livers confirms liver damage, showing necrosis in the absence of significant polymorphonuclear cell (PMN) infiltration. These data are consistent with the acute phase of liver injury as described above (Fig. 3).

One of the earliest molecular events that occurs after liver I/R is the activation and nuclear translocation of the transcription factor NF-κB. It has been demonstrated by several different laboratories that I/R-induced activation of NF-κB may represent an important pathophysiological event in liver damage [5,10,16,17]. Fig. 4 demonstrates that as early as 1 h after reperfusion, a time when liver injury is minimal, the p50/p65 active heterodimeric transcription factor has translocated into the nuclei of the liver cells. Important: Because the nuclear preparation was obtained from whole liver the vast majority of the nuclei are derived from hepatocytes. This is an important consideration if one wishes to assess the role of NF-κB activation in other cell types such as SECs or Kupffer cells (KCs), both of which represent a small population of the total cells in the liver. We, as well as others, have reported that I/R-induced NF-κB activation may induce hepatocellular injury in the

Fig. 4. EMSA demonstrating activation and nuclear localization of the p50/p65 heterodimeric transcription factor NF-κB after 90 min of ischemia and varying times of reperfusion.

Fig. 5. Liver message and serum TNF-α levels after 90 min of ischemia and varying periods of reperfusion. *p < 0.05 vs sham-operated control.

Fig. 6. Effect of a monoclonal antibody to mouse TNF-α (200 μg/mouse) on serum ALT levels after 90 min of ischemia and varying periods of reperfusion. *p < 0.05 for antibody-treated versus vehicle-treated control. Data are derived from Ref.[18].
acute, PMN-independent phase by promoting the expression of injurious cytokines and mediators. One such cytokine is TNF-α.

Fig. 5 shows the time-dependent increase in both message and serum protein levels of TNF-α after 90 min of ischemia and varying times of reperfusion. As expected, TNF-α message level increases rapidly, preceding enhanced protein expression by 1 h after reperfusion. Serum protein levels of TNF-α increase in a time-dependent fashion with maximum production at 6 h after reperfusion. Because a number of different gene products are upregulated in response to I/R, a systematic effort to identify which of the many cytokines and mediators are important in the pathophysiology of postischemic tissue damage should be undertaken. Fig. 6 demonstrates that pretreatment of mice with a single injection of a TNF-α monoclonal antibody (mAb) 15 min before ischemia attenuates I/R-induced liver injury after 90 min of ischemia and 6 h of reperfusion. It is interesting to note the pleiotropic properties of TNF-α, as immunoneutralization of TNF-α actually enhances postischemic injury at 1 h of reperfusion. The reasons for these interesting but perplexing activities of TNF-α are not known at the present time.

Numerous investigations have identified ROS as important mediators of reperfusion injury in the liver [5,7–11]. To assess specifically the role that O$_2^-$ plays in postischemic liver damage, we have tested the effectiveness of a genetically engineered fusion protein consisting of the mature human Mn-SOD (SOD2) sequence followed by the polycationic 26 C-terminal residues of human extracellular SOD (SOD3). This fusion protein (SOD2/3) is membrane impermeable but capable of binding to the surface of sinusoidal and microvascular endothelial cells (estimated half-life of 30 h) [18,25]. We have found that SOD2/SOD3 administration is very effective at attenuating I/R-induced liver injury (Fig. 7). Indeed, we have shown, using mice deficient in KCs or NADPH oxidase, that KC-derived O$_2^-$ plays a major role in the pathophysiology of postischemic liver injury [18]. The protective effect of SOD2/3 correlates well with its ability to significantly reduce serum levels of TNF-α (Fig. 8). The mechanisms by which superoxide may directly or indirectly mediate postischemic tissue are not known; however, we and others have suggested that it may act to rapidly inactivate the important cytoprotective molecule NO. Indeed, if we subject eNOS knockout mice to liver I/R we observe a dramatic exacerbation of liver injury as shown in Fig. 9 [24,26]. Very similar effects are observed when wild-type mice are pretreated with the NOS inhibitor L-NAME (data not shown) [24,26]. Important: It should be noted that all NOS experiments have to be performed using only 45 min ischemia, as longer periods increase dramatically the mortality of the mice. To test the hypothesis that superoxide indirectly mediates I/R injury by inactivating NO, eNOS-deficient mice were treated with SOD2/3 before I/R. We reasoned that SOD2/3 administration should provide no protection to the enhanced liver injury observed in eNOS-deficient mice if our hypothesis were correct. Indeed, SOD2/3 did not provide any protection to the eNOS knockout animals [23]. Taken together, these data suggest that eNOS-derived NO acts to limit postischemic tissue injury and that the overproduction of superoxide indirectly mediates this injury by rapidly inactivating the cytoprotective free radical NO.

Concluding remarks

The mouse model of liver I/R has proven invaluable to our understanding of the role that reactive oxygen and nitrogen metabolites play in modulating and mediating tissue injury in vivo. This model should be useful to investigators who are interested in exploring the various molecular, cellular, and/or physiological mechanisms associated with vascular pathobiology, free radical metabolism, and inflammation in vivo.

References


