Apoptotic signaling induces hyperpermeability following hemorrhagic shock

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HEMORRHAGIC SHOCK (HS) has been implicated in the pathogenesis of multiple-organ failure and accounts for 30% of the deaths associated with traumatic injury (26). One of the primary clinical manifestations of HS is disruption of the endothelial cell barrier, which leads to microvascular hyperpermeability (3, 8, 37). HS following trauma has been shown by several investigators (10, 35, 40, 58) to activate mediators of apoptosis. Recent studies (19, 34) have demonstrated the involvement of endothelial cell apoptosis in vascular permeability.

Cell-cell detachment is one of the hallmarks of endothelial cell apoptosis. Apoptosis requires induction and activation of a variety of cellular pathways that culminate in morphological changes in cell shape, detachment of cells from the matrix, and cell-cell detachment (17, 18, 30, 45). As the primary component of adherens junction in exchange vessels, the transmembrane adhesion protein vascular endothelial cadherin (VE-cadherin) connects adjacent endothelial cells through calcium-dependent homophilic binding of its extracellular domain, whereas its intracellular domain interacts with the actin cytoskeleton via a family of catenins, including α-catenin, β-catenin, γ-catenin, and p120 (4, 11, 24). The interaction of the catenins with VE-cadherin and the cytoskeleton has been shown to be the most important regulator of junctional strength and paracellular permeability (12, 60). Dissociation of this complex is associated with endothelial barrier dysfunction (20, 23).

Apoptosis is executed by caspases, which can be activated by two main pathways (47, 53). The nonmitochondrial or “extrinsic” pathway is initiated by binding the extracellular ligand (CD95, TNF-α, and TNF-related apoptosis-inducing ligand TRAIL) (2, 29). The second or “intrinsic” pathway is mediated via activation of the mitochondria (18, 30, 32, 44). Mitochondrial regulation of apoptosis is mediated through the release of cytochrome c, apoptosis-inducing factor, and second mitochondrial-derived activator of caspases, all of which are regulated by members of the Bcl-2 family of proteins (14, 51). The Bcl family consists of both antiapoptotic (Bcl-2, Bcl-xL) and proapoptotic (BAK, BAX) factors (28, 31, 41). The antiapoptotic members of this family prevent apoptosis by sequestering proforms of death-driving cysteine proteases or by preventing the release of the aforementioned mitochondrial apoptogenic factors. In contrast, the proapoptotic members of this family, such as BAK, trigger the release of mitochondrial apoptogenic factors into the cytoplasm by acting on the mitochondrial permeability transition (PT) pore, thereby leading to caspase activation. Translocation of cytochrome c from mitochondria to the cytosol through mitochondrial transition pores is an important route of caspase activation. Cytochrome c triggers the release of apoptosome assembly from apoptotic protease-activating factor-1, ATP, and procaspase-9, which activates caspase-3 and caspase-7, leading to cellular alterations (28). Thesalutationsinmitochondrialmembraneintegrityviaproapoptotic factors and the subsequent mitochondrial release of cytochrome c have very important roles in the apoptotic signaling cascade.

Previous work from our laboratory (9) implicated the mitochondria of endothelial cells as a major producer of reactive oxygen species (ROS) following HS. We reported a marked increase in endothelial ROS following HS associated with an increase in leukocyte adherence and microvascular hyperpermeability. By using an in vivo intravital microscopy model, ROS were visualized with dihydrorhodamine 123. This is a hydroperoxide-sensitive fluorescent probe that is trapped within viable cells in a nonfluorescent form and is then converted into the mitochondrion-selective form rhodamine 123 (15, 32). This work demonstrated mitochondrial ROS generation following acute HS. This is particularly important because ROS activation of the mitochondria has been shown to result in the release of apoptogenic factors such as cytochrome c (46).

We postulated that activation of the intrinsic mitochondrial-signaling pathway would result in the disruption of the endo-

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thelial cell membrane cell-cell adherence and thus cause hyperpermeability. To test this, we developed a novel in vivo protein-transfection technique to introduce the intrinsic pathway proapoptotic factor BAK into exchange vessels. In addition, we monitored mitochondrial membrane potential in vivo by using 5,5′,6′-tetrachloro-1,1′,3,3′-tetracycthylbenezimidazolylicarbocyanine iodide (JC-1; Cell Technology, Mountain View, CA) and investigated the release of cytochrome c and caspase-3 activation. Our data show that HS increases microvascular permeability via the intrinsic mitochondrial apoptotic-signaling cascade.

MATERIALS AND METHODS

Animals. The surgical procedures and experimental protocol were conducted at Texas A&M University Health Science Center/Scott and White Hospital, after approval by the Institutional Animal Care and Use Committee. The facility is approved by the American Association for Accreditation of Laboratory Animal Care in accordance with National Institutes of Health guidelines.

Chemicals and solutions. The test solute for the permeability measurements was FITC-albumin (Sigma, St. Louis, MO). The test solution was prepared by dissolving the FITC-albumin in 50 mg/kg saline. JC-1 reagent was prepared by reconstituting the lyophilized reagent with 500 μL DMSO to obtain a 100× stock solution. Immediately prior to the experiments, the 100× solution was diluted 1:100 in 1× assay buffer. BAK (BH3) peptide, BAK L-to-A mutant peptide (R&D Systems, Minneapolis, MN), and BAK (BH3)-fluorescein peptide (custom synthesized by Sigma-Genosys, Woodlands, TX; 80 μl of a 1-μg/μl stock), were each mixed with TransIT (Mirus Bio, Madison, WI) for a final concentration of 5 μg/ml BAK, BAK L-to-A mutant, or BAK-fluorescein and 10 μl/ml TransIT of the rat’s blood volume. The caspase-3 inhibitor Z-DEVD-FMK (R&D Systems) was dissolved by 1 mg in 75 μL DMSO to obtain a 20 mM stock solution. The stock solution was further diluted to 1 mM in PBS and was given to rats at a final concentration of 1 μM of blood volume. BAK and von Willebrand factor polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon (Temecula, CA), respectively.

Animal surgery and intravital microscopy. Male Sprague-Dawley rats (275–325 g) obtained from Charles River Laboratories (Wilmington, MA) were housed in the institutional animal facility. The animals were maintained on a 12:12-h light-dark cycle with free access to food and water. The room temperature and humidity were maintained at 25 ± 2°C and 55%, respectively. Prior to each experiment, the rats were fasted for 18 h and were given water ad libitum. The animals were anesthetized by a single intramuscular injection of 50% urethane (1.5 g/kg). Polyethylene tubing (PE-50, 0.58 mm ID) was placed in the right femoral artery connected to a blood-pressure analyzer (Dig-Med BPA 400A). The rats were allowed to recover from surgical manipulation for 30 min before the start of all experiments. This was followed by the recording of baseline parameters for MAP, red cell centerline velocity, and vessel diameter. During this period, the animals were dosed with FITC-albumin (50 mg/kg) and baseline-integrated optical intensities were obtained intra- and extravascularly (two sites, same computed areas; mean values were used). The experimental group of animals then underwent 60 min of HS. To produce HS, the MAP was decreased to 40 mmHg by withdrawing blood from the right carotid artery into a syringe containing 100 units of heparin. To obtain this level of hemorrhage requires ~50–60% of the animal’s blood volume (level IV shock). After the shock period, the shed blood plus two times the volume of normal saline was reinfused to maintain a MAP ≥90 mmHg. Parameters were recorded after shock at 10-min intervals for 60 min. Minimal exposures, <15-s recordings, were performed to minimize quenching of the fluorescent indicator.

In vivo HS and vascular permeability. The experimental groups for HS-induced permeability consisted of sham-operated controls (n = 5) and HS for 60 min (n = 5). The animals were anesthetized by a single intramuscular injection of 50% urethane (1.5 g/kg). The right internal jugular vein and the right carotid arteries were exposed and cannulated using polyethylene tubing (PE-50, 0.58 mm ID) to give fluids intravenously and for the withdrawal of blood, respectively. The MAP was monitored continuously by using a PE-50 cannula placed in the left femoral artery connected to a blood-pressure analyzer (Dig-Med BPA 400A). The rats were placed on a temperature-controlled Plexiglas platform mounted to an intravital upright microscope (Nikon E600; Nikon, Tokyo, Japan). The mesentery was draped over a Plexiglas stage and was maintained at 37°C. The mesentery was superfused with normal saline at 2 ml/min and was covered with plastic wrap to reduce evaporation. Venules with diameters of 20–35 μm were selected for study with a Nikon ×20 objective, 0.45–2.16 mm working distance (Nikon Instruments, Natick, MA). Images were obtained with a photometric cascade camera (Roper Scientific, Tucson, AZ). A video time and date generator (WJ-810; Panasonic, Secaucus, NJ) provided on-screen time, date, and stopwatch functions. The images were projected onto a computer monitor (Trinitron 20-inch monitor; Sony, New York, NY) and were captured digitally on computer disc. Data were analyzed by using MetaMorph 4.5/4.6 (Universal Imaging, Downingtown, PA).

Measurement of vascular permeability. The extravasation of FITC-albumin was measured by determining the changes in integrated optical intensity by image analysis. The FITC-labeled albumin represented relative change in permeability. Areas in the small bowel mesentery, postcapillary venules, and adjacent extravascular space were selected for study. The following formula was used for calculating change in integrated optical intensity: ΔI = 1 – (I − I0)/I0, where ΔI is the change in light intensity, I0 is the light intensity inside the vessel, and I is the light intensity outside the vessel. Each experimental frame was digitized into a 512 × 512-pixel charged-coupled device camera that yielded 16 bits of data per pixel. Grayscale values were measured in the postcapillary venules and in the extravascular space around the venule’s per-unit area throughout the experiment and at selected times using the MetaMorph image-analysis systems. The images were standardized to images taken at the beginning of each experiment within the same animal and at selected timed intervals between different animals. This method of standardization was selected to minimize the bias incurred with changes in room lighting and hematocrit concentrations. This method of measuring vascular permeability by using image analysis was validated by Bekker et al. (5).

Experimental protocols. The rats were allowed to recover from surgical manipulation for 30 min before the start of all experiments. This was followed by the recording of baseline parameters for MAP, red cell centerline velocity, and vessel diameter. During this period, the animals were dosed with FITC-albumin (50 mg/kg) and baseline-integrated optical intensities were obtained intra- and extravascularly (two sites, same computed areas; mean values were used). The experimental group of animals then underwent 60 min of HS. To produce HS, the MAP was decreased to 40 mmHg by withdrawing blood from the right carotid artery into a syringe containing 100 units of heparin. To obtain this level of hemorrhage requires ~50–60% of the animal’s blood volume (level IV shock). After the shock period, the shed blood plus two times the volume of normal saline was reinfused to maintain a MAP ≥90 mmHg. Parameters were recorded after shock at 10-min intervals for 60 min. Minimal exposures, <15-s recordings, were performed to minimize quenching of the fluorescent indicator.

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pixels per area. The area was predetermined in all groups and was consistent.

**HS and BAK protein expression.** HS was induced in rats as described in *Experimental protocols* and was compared with sham-operated animals. The mesenteric microvasculature was dissected from the rats at T0 (n = 4) and at T60 (n = 4), where T0 was the mesenteric microvasculature collected after 60 min of HS and 0 min of resuscitation and T60 was mesenteric microvasculature collected after 60 min of HS and 60 min of resuscitation. The microvasculature was weighed and homogenized in lysis buffer containing protease inhibitors (Pierce, Rockford, IL). Microvascular tissue homogenates were centrifuged (12,000 g for 20 min at 4°C), and the supernatant was collected and subjected to protein estimation by the bicinchoninic acid method (Pierce). The samples (15 μg protein) were heated at 95°C for 5 min, subjected to SDS-PAGE electrophoresis, and elektrotransferred to nitrocellulose membranes. The membranes were then blocked for 1 h at room temperature with Tris-buffered saline supplemented with 0.1% Tween 20 and 5% powdered milk and were incubated overnight at 4°C with primary antibody for BAK (rabbit polyclonal; Santa Cruz Biotechnology) and subsequently with horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz) for 1 h. The protein bands were visualized by using a Western blot protein detection kit (Pierce).

**HS and cytosolic cytochrome c levels.** HS was induced in rats as described in *Experimental protocols*, and rats were compared with sham-operated animals. Briefly, the rat mesenteric microvasculature was collected from sham-operated (n = 5), T0 (n = 5), and T60 (n = 5) animals. The cytosolic cytochrome c levels were estimated by using a cytochrome c ELISA kit (MBL, Woburn, MA). The mesenteric vessels were dissected from the rat, weighed, and homogenized in a cold preparation buffer (10 mM Tris-HCl, pH = 7.5, 0.3 M sucrose, 10 μM aprotinin, 10 μM pepstatin, 10 μM leupeptin, and 1 mM PMSF). The tissue homogenates were centrifuged (10,000 g for 60 min at 4°C), and the supernatant (cytosol fraction) was collected and subjected to protein estimation. The samples were then treated with a conjugate reagent (horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody), transferred to MicroWell strips coated with anti-cytochrome c antibody, and incubated for 60 min at room temperature. The well contents were discarded, and the wells were washed with a wash solution. The samples were then treated with a peroxidase substrate reagent and were incubated for 15 min at room temperature. Following the addition of a stop solution (0.5 M H2SO4), the optical density of each well was measured at 450 nm within 30 min. A serial dilution of cytochrome c calibrator was subjected to the assay along with the samples, and the values were plotted. The concentration of cytochrome c was calibrated from the standard curve.

**HS and caspase-3 activity.** HS was induced in rats as described above, and HS rats were compared with sham-operated animals. Briefly, the rat mesenteric microvasculature was collected from sham-operated (n = 5), T0 (n = 5), and T60 (n = 5) animals. Caspase-3 activity was determined by using a caspase-3 activity assay kit (Calbiochem, La Jolla, CA). Active caspase-3 cleaves after aspartate residues in a particular peptide sequence (DEVD). The DEVD substrate was labeled with the fluorescent molecule 7-amino-4-trifluoromethyl coumarin. The mesenteric microvessels harvested from the animals were homogenized in caspase-3 sample lysis buffer provided in the kit. The homogenates were centrifuged at 500 g, and the resulting supernatant was used for protein estimation and caspase-3 assays. The lysates were treated with the substrate conjugate, and the resulting fluorescence was measured in a fluorescent plate reader at 400 nm excitation and 505 nm emission.

**HS and mitochondrial transmembrane potential.** HS (n = 5) was induced in rats as described in *Experimental protocols*, and rats were compared with sham-operated animals (n = 5). The animals were placed on a temperature-controlled Plexiglas platform mounted to an intravital upright microscope (Nikon E600). HS was induced by withdrawing blood to reduce the MAP to 40 mmHg for 60 min and resuscitating to 90 mmHg with the shed blood and normal saline solution. The rat mesenteric vasculature was superfused with JC-1 reagent (1:100) to measure changes in mitochondrial membrane potential by using intravital microscopy. In healthy cells, the cationic fluorescent indicator JC-1 fluoresces the mitochondria red (Cy3 filter, emission wavelength 590 nM) and cytoplasm fluoresces green (FITC filter, emission wavelength 530 nM). The negative charge exchanged by the mitochondrial membrane potential allows the lipophilic dye bearing a delocalized positive charge to enter the mitochondrial matrix, where it accumulates. When the critical concentration is exceeded, J-aggregates form that fluoresce red. In cells undergoing apoptosis, the mitochondrial membrane potential collapses and the JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytoplasm in a green-fluorescent monomeric form. The images were captured and were analyzed for changes in fluorescent intensity over time compared with baseline values.

**In vivo BAK peptide transfection.** The rats were subjected to surgical procedures as described in *Animal surgery and intravital microscopy* and were allowed to recover for 30 min. The animals were given 5 μg/ml BAK (BH3) peptide and 10 μl/ml TransIT (n = 5), 10 μl/ml TransIT alone (n = 5), or 5 μg/ml BAK L-to-A mutant and 10 μl/ml TransIT as a negative control. TransIT concentration was determined from previously published data in intact microvessels (54) as 10 μl/ml. In our animals the dose was 10 μl/ml of the rat’s blood volume. The BAK concentration was determined to be 5 μg/ml of blood volume following a dose-response experiment in our laboratory (Childs et al., unpublished data). The cocktail was prepared by mixing 200 μl of serum-free medium with 160 μl of TransIT and letting it sit at room temperature for 15 min. BAK or BAK L-to-A mutant peptide (80 μl of a 1 μg/μl stock) was mixed with the prepared TransIT solution and was allowed to sit at room temperature for an additional 15 min. The cocktail was injected intravenously into the internal jugular vein cannula at the beginning of each experiment. After 60 min of peptide delivery, the animals were injected intravenously with FITC-albumin (50 mg/kg) to quantify changes in albumin flux, as described in *In vivo HS and vascular permeability*. BAK peptide transfection and vascular tissue cytochrome c levels. In separate experiments, animals were sham-operated or were given 5 μg/ml BAK peptide and 10 μl/ml TransIT (n = 5) or 5 μg/ml BAK L-to-A mutant peptide and 10 μl/ml TransIT (n = 5). BAK or BAK L-to-A mutant was prepared for delivery as described above. Animals from the different groups were killed, and the mesenteric microvascular tissue was harvested at T0 and T60, where T0 was at 0 min following a 1-h transfection period and T60 was at 60 min following a 1-h transfection period. The tissues were processed for cytoplasmic cytochrome c levels by using an ELISA kit as described in *HS and cytosolic cytochrome c levels*. The optical density of samples was measured at 450 nM excitation by using a microplate reader.

**BAK peptide transfection and caspase-3 activity.** In separate experiments, rats were sham-operated (n = 5) and were given 5 μg/ml BAK peptide and 10 μl/ml TransIT or 5 μg/ml BAK L-to-A mutant and 10 μl/ml TransIT. BAK or BAK L-to-A mutant was prepared for delivery as described in *In vivo BAK peptide transfection*. The mesenteric microvascular tissue was collected at T0 and T60, where T0 was at 0 min following a 1-h transfection period and T60 was at 60 min following a 1-h transfection period. The tissues were processed for caspase-3 activity by using a caspase-3 activity assay kit as described in *HS and caspase-3 activity*. Following protein extraction, the samples were measured in a fluorescent plate reader at 400 nm excitation and 505 nm emission.
baseline values as described in HS and mitochondrial transmembrane potential.

HS and caspase-3 inhibition. HS was induced in rats (n = 5) as described in Experimental protocols, and rats were compared with sham-operated (n = 5) and caspase-3 inhibitor Z-DEVD-FMK-treated (n = 5) animals. The animals were given Z-DEVD-FMK intravenously into the internal jugular vein 10 min before the beginning of the shock period at a final concentration of 10 μM calculated based on each animal’s blood volume. HS was induced by withdrawing blood to reduce the MAP to 40 mmHg for 60 min. Following the shock period, the animals were resuscitated to 90 mmHg MAP with the shed blood and normal saline solution. The animals were also injected intravenously with FITC-albumin (50 mg/kg) to quantitate changes in albumin flux, as described in In vivo HS and vascular permeability.

In vivo BAK-fluorescein peptide transfection and von Willebrand factor immunostaining. This experiment was designed to demonstrate that the peptide-transfection approach used in the present study would effectively deliver BAK (BH3) peptide to vascular endothelial cells. Rats were anesthetized as described above, and the superior mesenteric artery was exposed surgically and cannulated with a polyethylene catheter. BAK-fluorescein peptide (5 μg/ml of blood volume) was given through the catheter, and the superior mesenteric vein was clamped. After 30 min of BAK-fluorescein treatment, the superior mesenteric vein was unclamped and the superior mesenteric artery was flushed repeatedly with saline. The mesenteric tissue was collected, rinsed in saline, mounted immediately in optimum cutting temperature-freezing medium, and sectioned at 10–15 μM in a cryostat microtome at −20°C. The tissue sections were fixed in 4% paraformaldehyde for 10 min and were processed for immunofluorescence staining by using a polyclonal antibody for von Willebrand factor, which is an endothelial cell-specific marker. Briefly, the tissue sections were washed in PBS, blocked with normal goat serum for 30 min, and exposed to the primary antibody for 1 h at room temperature. Following this, the tissue sections were washed and exposed to a secondary antibody conjugated to Texas red for 45 min. The tissue sections were further washed in PBS, mounted using Vectashield antifade reagent (Vector Laboratories, Burlingame, CA), and observed by using fluorescein (green) and Texas red (red) filters at ×60 under an Olympus-Fluo View confocal microscope (Olympus, Center Valley, PA).

In vivo BAK-fluorescein transfection and intravital microscopy. The animals were anesthetized by a single intramuscular injection of 50% urethane (1.5 g/kg), and the right internal jugular vein and the right carotid arteries were exposed and cannulated with polyethylene tubing (PE-50, 0.58 mm ID) as described in In vivo HS and vascular permeability. The MAP was monitored continuously by using a PE-50 tubing (PE-50, 0.58 mm ID) as described in

HS induces hyperpermeability. Figure 1A, left, is a composite image of a rat mesenteric postcapillary venule prior to the shock period demonstrating minimal extravasation of FITC-albumin into the extravascular space. Figure 1A, middle, was taken 60 min into the shock period (0 min of resuscitation; T0) and demonstrates marginal extravasation of FITC albumin compared with Fig. 1A, right (60 min shock and 60 min of resuscitation, T60), showing in a marked increase in FITC-albumin extravasation. Figure 1B shows mean changes in microvascular permeability after 60 min of HS. Albumin extravasation became significant between 10 and 20 min into resuscitation (*P < 0.05). In data from our laboratory that are not shown, FITC-albumin extravasation continued to increase after shock, with a plateau between 60 and 90 min into resuscitation.

HS induces apoptotic signaling. Mesenteric vascular tissue was harvested from rats following 60 min of HS at T0 and at T60 and was subjected to immunoblot analysis. The mesenteric vascular tissue includes a mixture of arterioles and venules with a few associated mesenteric support-structure cells. However, care was taken to delicately dissect the vascular structure from the surrounding support structure. The tissue was scanned for both pro- and antiapoptotic factors, including Bad, Bcl-xL, Bcl-2, and Mcl-1, with minimal increase (data not shown). However, there was a marked elevation in the proapoptotic factor BAK (Fig. 2) at both T0 and T60. BAK, a multidomain peptide that contains the essential BH3 proapoptotic region, induces mitochondrial membrane potential loss, swelling, and the release of cytochrome c into the cytoplasm. Cytochrome c release from the mitochondria has been reported to be the key event in apoptosis induced by various stimuli. Mesenteric vascular tissue levels of cytoplasmic cytochrome c were also measured following HS at T0 and at T60 (Fig. 3). Cytoplasmic cytochrome c was elevated at T0 and T60, which indicates loss of mitochondrial transmembrane potential and the release of cytochrome c. Once released, cytochrome c promotes the activation of procaspase-9 directly within the apoptosome complex, eventually leading to the activation of the effector caspase-3. Mesenteric vascular tissue following HS also demonstrated an elevation of active caspase-3 at T0 and T60 (Fig. 4).

HS induces mitochondrial transmembrane potential loss. Under normal conditions, the inner mitochondrial membrane is nearly impermeable to maintain inner transmembrane potential. Opening of the PT pore results in immediate dissipation of the membrane potential with consequent loss of cytochrome c. We used a novel approach to monitor mitochondrial membrane potential in vivo with JC-1. In nonapoptotic cells, JC-1 exists as a monomer in the cytosol (green) and also accumulates as J-aggregates in the mitochondria, which fluoresce as red (16). On induction of apoptosis, JC-1 exists only in a monomeric form (green). Figure 5, top, is an image of a postcapillary venule from the same animal (preshock); the animal’s vessel demonstrates both green (cytosol) and red (mitochondria) fluorescence in the endothelial cells. Following shock, at the beginning of resuscitation (T0; Fig. 5, middle), there was a decrease in red (mitochondrial) fluorescence, indicating the

RESULTS

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beginning of mitochondrial transmembrane potential loss. This decrease in red fluorescence continued throughout the 60-min resuscitation period, with almost a complete absence of red at T60 (Fig. 5, bottom).

Figure 6 shows mean red Cy3 fluorescence signaling generated by JC-1 in sham-operated and shock groups. The Cy3 signals were sustained throughout the 60-min shock experiment period in sham-operated rats, indicating mitochondrial integrity. Following HS, the mitochondria began to release red Cy3 (mitochondrial) fluorescence at 5–10 min into resuscitation, showing significant difference at all time points from the sham-operated group. This is particularly important because the loss of the red Cy3 signal precedes significant hyperpermeability and correlates with our previous ROS in vivo data time points (8).

**BAK peptide induces apoptosis signaling and hyperpermeability in vivo.** To elucidate the direct effect of BAK on hyperpermeability, the BAK (BH3) peptide was transfected into rats following preparation with TransIT-LT1 (transfection agent). BAK induced a marked increase in mesenteric venular hyperpermeability; conversely, the BAK L-to-A mutant did not demonstrate a significant change in permeability (Fig. 7).

Figure 8 demonstrates the temporal relationship of BAK transfection vs. FITC albumin leak. Compared with TransIT and

![Fig. 2. A: BAK expression in rat mesenteric vasculature following HS showing increased expression at T0 and T60. B: BAK protein levels are expressed as percentage in sham-operated (control), T0, and T60 rats. *P < 0.05; n = 4.

![Fig. 3. Effect of HS on cytosolic cytochrome c levels in mesenteric vasculature. Levels were significantly increased after HS at T0 and T60. *P < 0.05; n = 5.

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BAK L-to-A mutant, BAK increased microvascular leak following transfection. Mesenteric vascular tissues were harvested from rats transfected with BAK peptide to measure cytoplasmic cytochrome c (Fig. 9) and active caspase-3 levels (Fig. 10). Both cytoplasmic cytochrome c and active caspase-3 were elevated at 60 min following transfection (T0) and 120 min following transfection (T60). In addition, mesenteric vascular tissue was harvested from rats following 60 min of HS at 0 min into resuscitation (T0) and at 60 min into resuscitation (T60) and was subjected to immunoblot analysis. The tissue was analyzed for both pro- and antiapoptotic factors, including Bad, Bcl-xL, Bcl-2, and Mcl-1, with minimal changes in expression (data not shown).

**Fig. 4.** Effect of HS on caspase-3 activity in mesenteric vasculature. Activity was significantly increased following HS at T0 and T60. *P < 0.05; n = 5.

BAK transfection induces mitochondrial transmembrane potential loss. As found in HS induces apoptotic signaling, under normal conditions the inner mitochondrial membrane maintains transmembrane potential. The initiation of an apoptotic signal results in immediate dissipation of the membrane potential with consequent loss of cytochrome c into the cytoplasm. Figure 11 demonstrates the Cy3 fluorescent signaling from animals following BAK or BAK L-to-A mutant peptide transfection. In the BAK-transfected group, the Cy3 fluorescent intensity decreased significantly over time from 10 min up to 60 min (P < 0.05) compared with the BAK L-to-A mutant-transfected group.

Caspase-3 inhibitor prevents vascular hyperpermeability in vivo. To elucidate the direct effect of caspase-3 inhibition on hyperpermeability, the caspase-3 inhibitor Z-DEVD-FMK (10

**Fig. 5.** Effect of HS on mitochondrial membrane integrity. Preshock, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluoresced the mitochondria red (Cy3) and the cytoplasm green (FITC), indicating intact mitochondria. T0 demonstrated minimal change in mitochondrial membrane potential, allowing diffusion of the Cy3 fluorescence into the cytoplasm.

**Fig. 6.** Effect of HS on mitochondrial membrane integrity. HS collapsed the mitochondrial membrane potential. Mitochondrial depolarization allowed diffusion of the Cy3 fluorescence, as evident from the significant decrease in fluorescent intensity over time compared with the sham-operated group. *P < 0.05; n = 5.
μM was introduced into the rats 10 min prior to the shock period. HS induced a marked increase in mesenteric venular hyperpermeability. Z-DEVD-FMK significantly attenuated hyperpermeability following HS when given prior to the shock period (Fig. 12). The attenuation of HS-induced hyperpermeability was similar to that in sham-operated animals (*P < 0.05).

Fluorescein-tagged BAK colocalizes with von Willebrand factor. To establish that the BAK (BH3) peptide-transfection approach used in this study can effectively deliver BAK peptide to endothelial cells, BAK-fluorescein was given to rats and the mesenteric tissue was processed for the localization of BAK-specific fluorescence (green) and immunofluorescent visualization of endothelial cell-specific marker von Willebrand factor (red). Red fluorescence was observed in the mesenteric venule representing von Willebrand factor (Fig. 13A). Green fluorescence was observed in the same mesenteric venule, showing the presence of transfected BAK-fluorescein peptide (Fig. 13B). Note the cobblestone appearance of the endothelial surface marker von Willebrand factor vs. the uniform uptake of the BAK-fluorescein peptide in the cytoplasm. Figure 13C contains overlay of images showing that BAK-fluorescein was colocalized with von Willebrand factor, indicating that the peptide-transfection approach used can effectively deliver BAK peptide to vascular endothelial cells.

Fig. 7. Effect of BAK transfection on vascular permeability in rat mesenteric postcapillary venules. BAK peptide or BAK L-to-A mutant peptide was transfected for 60 min, and the fluorescence intensity was measured at 0 (T₀) or 60 (T₆₀) min after the 1-h transfection period. FITC-albumin extravasation into the extravascular space is observed following BAK transfection but not after BAK L-to-A mutant transfection. Scale bar = 30 μM.

Fig. 8. Effect of BAK peptide transfection on vascular permeability in rat mesenteric postcapillary venules. The fluorescent intensity of FITC-albumin from transfection of TransIT (control), transfection of BAK peptide, and transfection of BAK L-to-A mutant groups are shown. Permeability is expressed as change in fluorescent intensity inside the vessel compared with the intensity outside the vessel. Significant increase in fluorescent intensity is observed following BAK transfection compared with TransIT alone group or BAK L-to-A mutant-transfected group. *P < 0.05; n = 5.

Fig. 9. Effect of BAK and BAK L-to-A mutant transfection in vivo on cytosolic cytochrome c levels in rat mesenteric vasculature. BAK treatment, but not BAK L-to-A mutant treatment, significantly increased the cytochrome c levels. *P < 0.05; n = 5.
In addition, the BAK-fluorescein peptide was transfected into animals as the previously described untagged peptide. Figure 14 is a series of images demonstrating the uptake of the labeled peptide into the endothelial cell wall of a postcapillary mesenteric venule. Following the initial bolus injection, the images clearly demonstrated layering of the peptide into the endothelium in vivo within 5 min.

**DISCUSSION**

In this study, we tested the ability of BAK (BH3) peptide, an intrinsic mitochondrial signaling proapoptotic factor, to induce hyperpermeability following in vivo transfection. Our results showed that BAK was significantly increased following HS in the mesenteric vasculature. Similar to HS, BAK transfection resulted in an increase in cytochrome c and active caspase-3 in rats. In addition, we also used a novel technique to visualize the mitochondria of a microvascular endothelial cell in vivo by using JC-1. These experiments demonstrated mitochondrial depolarization following HS and BAK transfection. BAK transfection was also associated with an increase in hyperpermeability similar to that observed following HS. To directly examine the intrinsic apoptotic-signaling cascade as a potential contributor to hyperpermeability following HS, we blocked caspase-3 activation with Z-DEVD-FMK and demonstrated a marked attenuation of microvascular hyperpermeability. These results suggested a relationship between activation of the intrinsic apoptotic cascade and hyperpermeability following HS.

Apoptosis is known to alter cell morphology by interrupting the cell-cell and cell-matrix interaction, eventually resulting in complete removal of endothelial cells from their underlying basement membrane. Regardless of the insult, these characteristic series of morphological changes are consistently observed. This suggests the existence of a common pathway, which we now know increases the activation of a family of proteolytic enzymes known as caspses (53). HS following trauma has been shown by several investigators to activate mediators of apoptosis, including caspses (10, 35, 40, 58). We report here an increase in intrinsic apoptotic signaling factors, including BAK, cytochrome c, and caspase-3. In addition, we demonstrate loss of mitochondrial transmembrane potential in endothelial cells following HS.

The intrinsic or mitochondrial-dependent mechanism of caspase activation depends on the Bcl-2 family of proteins that regulate mitochondrial outer membrane permeabilization (17, 43). The Bcl-2 family members share one or more Bcl-2 homology (BH) domains and are divided into two main groups according to whether they promote or inhibit apoptosis. The proapoptotic family members are subdivided according to whether they contain multiple BH domains or only the required BH3 domain. BAK is a multidomain BH3 protein that has been reported to induce mitochondrial transmembrane potential loss via mitochondrial PT pores (38). However, precisely how BAK permeabilizes membranes is unknown. BAK and BAX become oligomerized in the mitochondrial outer membrane during apoptosis in endothelial cells (1, 13, 50, 59), when recombinant Bid (BH3 only) is added to isolated mitochondria (52). The oligomerization is thought to be important for the permeabilization function.

When PT is induced, the inner mitochondrial membrane potential dissipates, leading to the loss of mitochondrial func-
tions such as energy production and protein import into the organelle. In contexts in which mitochondrial outer membrane permeabilization is accompanied without PT, by the action of Bcl-2-family proteins or the outer membrane, mitochondrial respiration is affected indirectly and possibly temporally (32).

Caspases activated by the release of cytochrome c can apparently enter the mitochondria to cleave key substrates in the mitochondrial electron-transport chain, leading to a loss of respiration and increased ROS production (6, 49, 57). In these situations, intracellular ATP pools can maintain at least a small inner membrane potential for some period of time through the reverse action of ATP synthase. Under certain circumstances, caspase activation can be blocked by overexpressed inhibitors of apoptosis protein, by the loss of caspase activation, or by the loss of inhibitor of apoptosis protein degradation pathways (25, 36, 38). In caspase-inhibited cells, cytochrome c-dependent respiration can continue for some time despite the dilution of cytochrome c into cytoplasm. Reduced cytosolic concentrations of cytochrome c have been shown sufficient to support respiration (39, 56, 57). This suggests possible restoration or reversal of mitochondrial activation. However, if unabated, the cytochrome c pools become depleted, respiration declines, and eventually so do intracellular ATP concentrations. As a result, the mitochondrial transmembrane potential cannot be maintained and the mitochondria suffer a disruption of protein import and consequently a loss of other functions that depend on sustained protein import.

In addition to indicting members of the intrinsic apoptotic signaling cascade following HS, we propose a possible mechanism. Caspase-3, the key executioner of apoptosis, is either partially or totally responsible for the proteolytic cleavage of many key proteins involved in endothelial cell morphological homeostasis. One such adherens-junctional protein is β-catenin (22, 52, 55, 61). Caspase-3 is widely distributed, with high expression in endothelial cells (21, 42, 45), especially following ischemic insult (42). Caspase-3 is responsible for cleaving substrates that contain a common Asp-Xaa-Xaa-Asp (DXXD) motif (33). Caspase-3 recognizes the tetrapeptide motif and cleaves their substrate behind aspartate. Steinhausen and co-workers (52) identified caspase-3 cleavage sites of β-catenin at consensus motifs corresponding to positions Asp-764, Asp-115, Asp-83, and Asp-32. β-Catenin associates with VE-cadherin in the endoplasmic reticulum shortly after synthesis, whereas uncomplexed cadherins are rapidly degraded (24).

In Fig. 13. Confocal microscopy images show BAK-fluorescein colocalization with endothelial cell-specific marker von Willebrand factor in rat mesenteric tissue sections, demonstrating the successful transfection of BAK peptide to endothelial cells. A: red fluorescence shows von Willebrand factor observed in the selected portion of a rat mesenteric venule in cross-section. B: green fluorescence shows successful transfection of BAK-fluorescein observed in rat mesenteric venule. C: colocalization of BAK-fluorescein and von Willebrand factor showing the presence of BAK peptide in endothelial cells.

Fig. 14. Intravital microscopy images demonstrating the uptake of fluorescein-labeled BAK peptide into the endothelial cell wall of a postcapillary mesenteric venule in rat in vivo. The layering of the peptide into the endothelium is observed in a time-dependent manner.
The cytoplasmic tails of the type II cadherin, VE-cadherin, contain PEST sequences, which are motifs associated with rapid protein turnover (48). These sequences overlay or encompass the β-catenin-binding region of type II cadherins and are hypothesized to be sequestered by β-catenin, thereby protecting bound cadherins from degradation (27). Therefore, VE-cadherins that are not bound to β-catenin would be turned over rapidly, whereas cadherins bound to β-catenin would proceed to the cell surface to participate in the adherens-junction complex. We hypothesize that cleavage of β-catenin by caspase-3 exposes VE-cadherin to rapid degradation, leading to disruption of the cell-cell adherence and resulting in hyperpermeability.

Our results suggest that the intrinsic apoptotic signaling cascade contributes to the hyperpermeability observed following HS, and a balance between the pro- and antiapoptotic factors may be important in regulating the endothelial cell barrier following systemic ischemia-reperfusion injury, such as HS.

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