Angiopoietin-1 inhibits intrinsic apoptotic signaling and vascular hyperpermeability following hemorrhagic shock

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ABSTRACT

Studies from our laboratory demonstrated the involvement of intrinsic apoptotic signaling in hyperpermeability following hemorrhagic shock (HS). Angiopoietin-1 (Ang-1), a potent inhibitor of hyperpermeability has recently been shown to inhibit apoptosis. The purpose of our study was to determine the effectiveness of Ang-1 in attenuating HS-induced hyperpermeability and its relationship to apoptotic signaling. Hemorrhagic shock was induced in rats by withdrawing blood to reduce the MAP to 40 mmHg for 1 hour followed by reperfusion. Mesenteric post-capillary venules were examined for changes in hyperpermeability utilizing intravital microscopy. Mitochondrial release of smac and cytochrome c were determined using western blot and ELISA respectively. Caspase-3 activity was determined by fluorometric assay. Parallel studies were performed in rat lung microvascular endothelial cell (RLMEC) monolayer utilizing HS serum and the pro-apoptotic BAK (BH3) peptide as inducers of hyperpermeability. In rats, Ang-1 (200 ng/ml) attenuated HS-induced hyperpermeability, versus HS group ($p < 0.05$). Ang-1 prevented HS-induced collapse of mitochondrial transmembrane potential ($\Delta\Psi_m$), smac and cytochrome c release, and caspase-3 activity ($p < 0.05$). In RLMEC monolayer, HS serum and BAK (BH3) peptide both induced hyperpermeability that was inhibited by Ang-1 ($p < 0.05$). Angiopoietin-1 attenuated HS and BAK (BH3) peptide induced-collapse of $\Delta\Psi_m$, smac release, cytochrome c release, activation of caspase-3 and vascular hyperpermeability. In vivo BAK (BH3) induced vascular hyperpermeability that was attenuated by Ang-1 ($p < 0.05$). These findings suggest that Ang-1’s role in maintaining microvascular endothelial barrier integrity involves the intrinsic apoptotic signaling cascade.

Key words: Angiopoietin, ischemia-reperfusion injury, microvascular hyperpermeability, smac, apoptotic signaling
INTRODUCTION

Hemorrhagic shock is associated with disruption of the endothelial cell barrier, which leads to vascular hyperpermeability (Baluk et al., 1997; McDonald, 1998; Childs et al., 2005a, 2005b). Hemorrhagic shock following trauma has been shown to activate apoptotic signaling cascade (Davidson et al., 2004; Childs et al., 2007; Murao et al., 2003). Activation of the ‘intrinsic’ mitochondrial pathway of apoptosis has been implicated in ischemia-reperfusion injury induced cell death (Davidson et al., 2004; Honda et al., 2005). The ‘intrinsic’ apoptotic pathway is mediated through the mitochondrial release of cytochrome c, smac (second mitochondrial-derived activator of caspases) and AIF (apoptosis inducing factor). Translocation of smac and cytochrome c from mitochondria to the cytosol through mitochondrial transition pores, are important routes of caspase activation. These mitochondrial factors are regulated by proapoptotic and antiapoptotic Bcl-2 family proteins, such as Bax/Bak and Bcl-2/xL (Kim et al., 2005). Our laboratory demonstrated an increase in pro-apoptotic BAK expression, mitochondrial release of cytochrome c and activation of caspase-3 following hemorrhagic shock (Childs et al., 2007).

Angiopoietin-1 (Ang-1), an endothelial growth factor is a potent inhibitor of vascular permeability (Gamble et al., 2000; Thurston et al., 1999; 2000; Joussen, 2001; Jho et al., 2005). Ang-1 was the first Tie-2 receptor ligand to be identified and was shown to stimulate tyrosine phosphorylation of Tie-2 receptors. Ang-1 has been shown to attenuate human umbilical cord endothelial cell apoptosis by inhibition of smac release from mitochondria (Harfouche et al., 2002).
Smac has been shown to facilitate caspase activation by its ability to bind and inhibit the function of the IAPs (Inhibitor of apoptosis proteins) (Chai et al., 2000; Cregan et al., 2004). Recent studies show that endothelial cell apoptosis can be regulated by inhibition of smac release from mitochondria (Harfouche et al., 2002). The release of smac and cytochrome c from the mitochondria results in the activation of caspase-3. Caspase-3 activation has been shown to result in the cleavage of a variety of cell adherens proteins including beta catenin (Cervello et al., 2004; Gregorc et al., 2005). Beta-catenin functions as a regulator of VE cadherin-mediated cell-cell adhesion in endothelial cells and its cleavage may result in microvascular hyperpermeability. Thus, alterations in mitochondrial membrane integrity and the subsequent mitochondrial release of proapoptotic factors such as smac and cytochrome c may have important implications in modulating vascular permeability. While recent studies show evidence that the mitochondrial release of smac is associated with endothelial cell apoptosis, its relationship to vascular hyperpermeability has not been demonstrated.

We hypothesized that Ang-1 would prevent vascular hyperpermeability following hemorrhagic shock by regulating the “intrinsic” apoptotic signaling cascade. The purpose of this study was to determine the effect of Ang-1 on vascular hyperpermeability, mitochondrial release of smac and cytochrome c, and caspase-3 activity, following hemorrhagic shock in vivo with parallel in vitro studies using microvascular endothelial cells.

MATERIALS AND METHODS
Animals.

Male Sprague-Dawley rats (275-325 g) were obtained from Charles River Laboratories (Wilmington, MA). The rats were housed at our institutional animal facility, at 25° ± 2°C and the humidity was maintained at 55%. They were maintained on a 12:12 hour dark/light cycle, with free access to food and water. All surgical procedures and experimental protocol described herein were conducted at Texas A&M University Health Science Center College of Medicine and Scott and White Hospital, after approval by the Institutional Animal Care and Use Committee. The animal facility is approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the National Institutes of Health guidelines.

Chemicals and solutions.

The test solute used for the permeability measurements was fluorescein isothiocyanate-bovine albumin (FITC-albumin; Sigma, St. Louis, MO). The test solution was prepared by dissolving the FITC-albumin (50 mg/kg) in saline. Mitochondrial transmembrane potential was determined by using JC-1 (5,5’,6,6’ tetrachoro-1,1’,3,3’ tetraethylbenzimidazolyl carbocyanine iodide) (Cell Technology Inc. Mountain View, CA). JC-1 reagent was prepared by reconstituting the lyophilized reagent with 500µl of DMSO to obtain a 100X stock solution. Immediately prior to the experiments the 100X stock solution was diluted 1:100 in 1X assay buffer. Previous studies to determine the effects of DMSO on permeability have been published from our laboratory (Childs et al.2002; 2005a). For in vivo studies, BAK (BH3) peptide (R&D Systems, Minneapolis, MN), 80µl of a 1µg/µl stock, was mixed with TransIT (Mirus Bio-Corporation, Madison, WI) for a final concentration of 5µg/ml and 10µl/ml (TransIT) of the rat’s blood volume. BAK (BH3) peptide at a concentration of 5µg/ml of culture media was used to induce hyperpermeability in rat lung
microvascular endothelial cell (RLMEC) monolayers. The dose/concentration of Ang-1 (gift from Regeneron Pharmaceuticals Inc., Rensselaer, NY or from R&D Systems, Minneapolis, MN; 40 ng/ml for cell culture studies and 200 ng/ml for in vivo studies) and the duration of treatment (10 minutes before shock) was set based on previously published information on the effect of Ang-1 on Tie-2 receptor tyrosine phosphorylation (Harfouche et al., 2002). Rabbit polyclonal antibody for beta catenin and FITC conjugated anti-rabbit secondary antibody were obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, CA). Annexin-V FITC staining kit was obtained from Invitrogen (Carlsbad, California).

**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 as described above. Prior to each experiment the rats were fasted for 18 hours and given water ad libitum. The animals were anesthetized by a single intramuscular injection of 50% urethane (1.5g/kg). Polyethylene tubing (PE-50, 0.58 mm ID) was placed in the right internal jugular vein to give fluid intravenously (normal saline) at 2ml/hr by continuous infusion pump (Harvard Apparatus, South Natick, MA) and in the right carotid artery for blood withdrawal. The mean arterial pressure (MAP) was monitored continuously using a PE-50 cannula placed in the left femoral artery connected to a blood pressure analyzer (Dig-Med, BPA 400A, Micromed, Louisville, KY). A midline laparotomy incision was performed to expose a section of mesentery from the proximal ileum, for exteriorization. The rats were placed in a lateral decubitus position on a temperature controlled Plexiglas platform mounted to an intravital upright microscope (Nikon E600, Tokyo, Japan). The mesentery was maintained at 37 °C. The mesentery was superfused with normal saline at 2 ml/min and covered with plastic wrap to reduce evaporation.
Venules with diameters of 20 to 35 µm were selected for study with a Nikon 20x objective, 0.45 to 2.16 mm working distance (Nikon Instruments, Inc., 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 using MetaMorph 4.5/4.6 (Universal Imaging Corp., Downingtown, PA).

In vivo Animal experiments

Effect of angiopoietin-1 on vascular hyperpermeability.

The rats were allowed to recover from surgical manipulation for 30 minutes before the start of all experiments. This was followed by the recording of baseline parameters: 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, the mean values were used). The rats were divided into a sham (control) group, a hemorrhagic shock group, a hemorrhagic shock group pre-treated with angiopoietin-1 (Ang-1; 200 ng/ml) and an Ang-1 alone treated group. Each experimental group consisted of 5 rats. The experimental group of animals then underwent 60 minutes of hemorrhagic shock. To produce hemorrhagic shock, 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 shock requires approximately 40-50 % 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 at or above 90 mmHg. Parameters were recorded postshock at 10 minutes intervals for 60 minutes. Minimal
exposures, less than 15 seconds’ recordings, were performed to minimize quenching of the fluorescent indicator.

The extravasation of FITC-albumin was measured by determining the changes in integrated optical intensity by image analysis: \( \Delta I = 1 - \frac{(I_i - I_o)}{I_i} \), where \( \Delta I \) is the change in light intensity; \( I_i \) is the light intensity inside the vessel, and \( I_o \) is the light intensity outside the vessel. Each experimental frame was digitized into a 512 X 512 charged- coupled device (CCD) that yielded 16 bits of data/pixel. Gray scale 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 labeled albumin (FITC) represented relative change in permeability. Areas in the small bowel mesentery, postcapillary venules, and the adjacent extravascular space, were selected for study. 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.

**Effect of angiopoietin-1 on mitochondrial smac protein release**

The rats were divided into a sham (control) group, a hemorrhagic shock group and a hemorrhagic shock group pre-treated intravenously with Ang-1 (200 ng/ml). Hemorrhagic shock was induced as described above. The rat mesenteric tissues collected from various experimental groups were homogenized in lysis buffer containing protease inhibitors. Tissue homogenates were centrifuged (12,000 \( \times \) g for 30 min at 4°C) to remove insoluble tissue material. The supernatant was collected
and subjected to protein assay (BCA method, Pierce). Samples (50 µg protein) were heated at 95 °C for 5 min and subjected to SDS-PAGE electrophoresis and electrotransferred to nitrocellulose membranes. The membranes were then blocked for 1 hour at room temperature with TBST containing 5 % milk powder, then incubated overnight at 4 °C with primary antibody for smac (goat polyclonal, 1:200, Santa Cruz) and subsequently with horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz) for 1 hour. For control, equal amount (50 µg protein) of the same tissue lysates were loaded separately and immunobloting was performed as described above. Anti-beta actin (mouse monoclonal, Sigma, St. Louis) was used as the primary antibody. Pre-stained molecular weight markers (Invitrogen) were used to determine the molecular weight of the proteins. The membranes were later subjected to a chemiluminescence reagent (Pierce) and exposed to an X-ray film. Data analyses were performed using ‘image J’ software and expressed as a percentage of sham group.

**Effect of angiopoietin-1 on mitochondrial cytochrome c release**

The rats were divided into a sham (control) group, a hemorrhagic shock group, a hemorrhagic shock group pre-treated with angiopoietin-1 (Ang-1; 200 ng/ml) and an Ang-1 alone treated group. Hemorrhagic shock was induced as described above. Each experimental group consisted of 5 rats each. Hemorrhagic shock was induced in rats as described above and compared to sham operated animals. The cytosolic cytochrome c levels were estimated using a cytochrome c ELISA kit (MBL, Woburn, MA). Briefly, 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 Apoptinin, 10 µM Pepstatin, 10 µM Leupeptin, 1mM PMSF). The tissue homogenates were centrifuged (10,000 X 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, transferred to microwell strips coated with anti-cytochrome c antibody and incubated for 60 minutes at room temperature. The well contents were discarded and the wells were washed using a wash solution. The samples were then treated with a peroxidase substrate reagent and incubated for 15 minutes 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 minutes. 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.

Effect of angiopoietin-1 on Caspase-3 activity

The rats were divided into a sham (control) group, a hemorrhagic shock group, a hemorrhagic shock group pre-treated with angiopoietin-1 (Ang-1; 200 ng/ml) and an Ang-1 alone treated group. Hemorrhagic shock was induced as described above. Each experimental group consisted of 5 rats. Caspase-3 activity was determined using a caspase-3 activity assay kit (Calbiochem, La Jolla, CA). Briefly, the mesenteric vasculature was collected and homogenized in the sample buffer provided in the assay kit. The tissue lysates were treated with the substrate conjugate and incubated for 2 hours at 37 °C. The DEVD substrate provided in the assay kit was already labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin. The resulting fluorescence was measured in a fluorescent plate reader capable of measuring excitation 400 nm, and emission at 505 nm.

In vitro experiments

Effect of angiopoietin-1 on shock serum induced monolayer hyperpermeability in RLMEC
Rat lung microvascular endothelial cells (RLMEC; VEC technologies Inc., Rensselaer, NY) were maintained on fibronectin-coated dishes in complete MCDB-3 media supplemented with 10% fetal bovine serum. The cells were later grown on Costar Transwell membranes for 48 hours. The cells were treated with serum obtained from sham, shock T₀ (60 minutes shock period followed by 0 minute resuscitation) and shock T₆₀ (60 minutes shock period followed by 60 minutes resuscitation) rats, for 30 minutes. Hemorrhagic shock was induced as described above. Ang-1 (40 ng/ml of phenol red free cell culture medium) was exposed to the cells 15 minutes prior to the exposure to serum. Untreated (basal) and Ang-1 alone treated groups served as controls. FITC-albumin (5 mg/ml final concentration) was added to the luminal chamber and left for 30 minutes. The samples (100 µl) collected from the abluminal (lower) chambers were analyzed for FITC fluorescent intensity using a fluorometric plate reader at excitation 494 nM and 520 nM. The data were calculated as percentage of the control (basal) values.

**Effect of angiopoietin-1 on BAK (BH3)- induced monolayer hyperpermeability in RLMEC**

RLMEC were grown on Costar Transwell membranes as described above. The cells were transfected with BAK (BH3) peptide (5µg/ml final concentration). BAK (BH3) peptide (5 µg/ml) was exposed to the transfection medium (TransIT-LT1 polyamine) for 15 minutes and the transfection was performed for 1 hour. Ang-1 (40 ng/ml) was exposed to the cells 15 minutes prior BAK (BH3) transfection. Untreated (basal), Ang-1 alone treated and TransIT alone treated cells served as controls. FITC-albumin (5 mg/ml final concentration) was added to the luminal chamber for 30 minutes. The samples (100 µl) collected from the abluminal (lower) chambers were analyzed
for FITC fluorescent intensity using a fluorometric plate reader at excitation 494 nM and 520 nM. The data were calculated as percentage of the control (basal) values.

**Effect of angiopoietin-1 on BAK (BH3) –induced collapse of mitochondrial membrane potential in RLMEC**

To determine the mitochondrial membrane potential, RLMEC were grown on fibronectin coated glass cover slips for 24 hours and exposed to media without phenol red for 1 hour. BAK (BH3) peptide (5 µg/ml) was exposed to the transfection medium (TransIT-LT1 polyamine) for 15 minutes prior to transfection for 1 hour, as described above. Ang-1 (40 ng/ml) was exposed to the cells 15 minutes prior BAK (BH3) transfection. Untreated, Ang-1 alone treated and TransIT alone treated cells served as controls. The cells were incubated with JC-1 (5,5’,6,6’ tetrachoro-1,1’,3,3’ tetraethylbenzimidazolyl carbocyanine iodide) for 15 minutes at 37 °C, washed in PBS and observed immediately under a Leica AOBS SP2 Confocal microscope. JC-1 aggregates were viewed with a bandpass filter designed to detect Texas red (excitation 590 nm, emission 610 nm). JC-1 monomers were detected with a bandpass filter used for the detection of fluorescien (excitation 490 nm, emission 520 nm).

**Effect of angiopoietin-1 on BAK (BH3) –induced smac release in RLMEC**

To determine smac protein expression, cells were grown on fibronectin-coated dishes for 24 hours. BAK (BH3) peptide (5 µg/ml) was exposed to the transfection medium (TransIT-LT1 polyamine) for 15 minutes prior to transfection for 1 hour, as described above. Ang-1 (40 ng/ml) was exposed to the
cells 15 minutes prior BAK (BH3) transfection. Untreated cells served as controls. Cytosolic smac expression was determined using immunobloting. The cell lysates were centrifuged (12,000 × g for 30 min at 4 °C) and the supernatant was collected and subjected to protein assay (BCA method, Pierce). The protein samples were heated at 95 °C for 5 min and subjected to SDS-PAGE electrophoresis and electrotransferred to nitrocellulose membranes, as described above. The membranes were then blocked for 1 hour at room temperature with TBST containing 5 % milk powder, then incubated overnight at 4 °C with primary antibody for smac (goat polyclonal, 1:200, Santa Cruz) and subsequently with horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz) for 1 hour. The membranes were later subjected to a chemiluminescence reagent (Pierce) and exposed to an X-ray film. Mouse monoclonal antibody for beta actin (Sigma, St. Louis) was used as an internal control. Pre-stained molecular weight markers (Invitrogen) were used to determine the molecular weight of the proteins. Four replicates from each group were used for the immunoblot analysis.

**Effect of angiopoietin-1 on BAK (BH3)-induced cytochrome c release in RLMEC**

RLMEC were grown on fibronectin-coated culture dishes at 37 °C with 5% CO₂ as described above. BAK (BH3) peptide (5 µg/ml) was exposed to the transfection medium (TransIT) for 15 minutes and the transfection was performed for 1 hour. Ang-1 (40 ng/ml) was exposed to the cells 15 minutes prior to BAK (BH3) transfection. Untreated, Ang-1 alone treated and TransIT alone treated cells served as controls. The cytosolic cytochrome c levels were estimated using a cytochrome c ELISA kit. Briefly, the cells were lysed in a cold preparation buffer provided in the kit, centrifuged (10,000 X g for 60 min at 4 °C) and the supernatant (cytosolic fraction) was collected and subjected
to protein assay (BCA method, Pierce). The samples were treated with a conjugate reagent, transferred to a cytochrome c antibody coated microwell plate and incubated at room temperature for 60 minutes. The wells were washed and treated with a substrate reagent and incubated for 30 minutes followed by addition of a stop solution. The optical density was read at 450 nm in a calorimetric plate reader. 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.

*Effect of angiopoietin-1 on Caspase-3 activity in RLMEC*

BAK (BH3) peptide (5 µg/ml) was exposed to the transfection medium (TransIT) for 15 minutes and the transfection was performed for 1 hour. Ang-1 (40 ng/ml) was exposed to the cells 15 minutes prior to BAK (BH3) transfection. Untreated, Ang-1 alone treated and TransIT alone treated cells served as controls. Caspase-3 activity was determined using a caspase-3 activity assay kit (Calbiochem, La Jolla, CA) as described above. The DEVD substrate provided in the kit was labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin (AFC). The cell lysate was used for protein estimation and treated with the substrate conjugate. The resulting fluorescent intensity was measured in a fluorescent plate reader capable of measuring excitation 400 nm and emission at 505 nm.

*Effect of hemorrhagic shock serum on endothelial adherens junction and Annexin-V binding*

Rat lung microvascular endothelial cells (RLMEC) were grown on fibronectin coated glass chamber slides. The cells were treated with serum obtained from sham or shock T60 (60 minutes shock period
followed by 60 minutes resuscitation) rats, for 30 minutes. A control group of cells received no
treatment. Hemorrhagic shock was induced as described above. Following the serum treatment, cells
were washed in PBS and fixed in 4% paraformaldehyde. After repeated washing steps, Triton X-100
treatment and blocking for non-specific binding, cells were incubated with a primary antibody for
beta catenin (1:100) raised in rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight.
Cells were washed in PBS and exposed to an FITC conjugated secondary antibody for 1 hour.
Following repeated washing steps the cells were mounted in an antifade mounting medium that
contained the nuclear stain DAPI (Vectashield) and observed under a fluorescent microscope with
appropriate filters for visualizing FITC and DAPI.

Annexin-V is a phospholipid binding protein with high affinity for phosphatidyl serine found in the
outer cell membrane beginning early in the process of apoptosis. During apoptosis, a redistribution
of phosphotidylerine from the cytoplasmic location to the outer leaflet takes place, making it
available for Annexin-V binding. For, Annexin-V FITC binding study, control cells and serum
treated cells were exposed to 5 µl Annexin-V FITC (Invitrogen, Carlsbad, CA) per chamber and
incubated for 15 minutes in the dark, washed in PBS followed by exposure to Annexin-V binding
buffer. The cells were observed under a fluorescent microscope for visualizing Annexin-V FITC
fluorescence.

In vivo BAK transfection

Effect of angiopoietin-1 on BAK induced vascular hyperpermeability.

BAK (BH3) peptide trasfection in rats was previously shown to induce hyperpermeability similar to
that of hemorrhagic shock in rats (Childs et al., 2007). This experiment was designed to test if Ang-1
prevents BAK (BH3)-induced vascular hyperpermeability. The rats were divided into a sham group,
a transfection reagent (TransIT) treated group, a BAK (BH3)-peptide (5µg/ml blood volume) treated group, and a BAK (BH3)-peptide (5µg/ml blood volume) group pre-treated with Ang-1 (200 ng/ml). Each experimental group consisted of 5 rats. BAK (BH3)-peptide solution was prepared by exposing the peptide to a transfection reagent (TransIT) as described previously (Childs et al., 2007). The extravasation of FITC-albumin to determine vascular hyperpermeability was measured as described above.

**Statistical Analysis**

All data are expressed as mean ± SE. Student’s t-test and analysis of variance (ANOVA) were used for determining significant differences between two mean values. Inter-group comparisons were made utilizing ANOVA followed by the Bonferroni’s post-test for multiple comparisons. In *in vivo* vascular permeability studies, each experimental value was compared to initial baseline value and expressed as percentage change. This method decreases bias between animals due to red blood cell accumulation and changes in room lighting. A *p* value of < 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

*A Angiopoietin-1 attenuates hemorrhagic shock-induced vascular hyperpermeability in vivo*

Hemorrhagic shock induced hyperpermeability in rat mesenteric post-capillary venules, which was attenuated by Ang-1 pre-treatment. Figure 1a is a composite image of a rat mesenteric
postcapillary venule. In control, minimal extravasation of FITC albumin into the extravascular space was observed. The second image corresponds to 60 minutes of hemorrhagic shock and 60 minutes of resuscitation (T_{60}). Hemorrhagic shock resulted in a marked increase in FITC albumin extravasation. The third image corresponds to Ang-1 treatment prior to 60 minutes of hemorrhagic shock and 60 minutes of resuscitation.

Figure 1b is the graphic representation of the changes in vascular permeability after 60 minutes of hemorrhagic shock period and 60 minutes of resuscitation (Shock group), a sham group that received neither shock nor Ang-1 treatment, and a shock group pre-treated with Ang-1 (Shock + Ang-1) and an Ang-1 alone treated group. The change in FITC albumin extravasation indicated the change in permeability. FITC-albumin extravasation became significant at 20 minutes into resuscitation as indicated by asterisk ($p<0.05$). Animals treated with Ang-1 (200 ng/ml) prior to shock demonstrated a significant decrease in FITC-albumin extravasation compared with shock group without Ang-1 pre-treatment ($p<0.05$). Ang-1 alone treated group showed no significant change in FITC-albumin extravasation compared with the sham group.

**Angiopoietin-1 inhibits hemorrhagic shock-induced smac release**

The release of smac from mitochondrial to the cytosol occurs following the opening of the mitochondrial transition pores. Opening of mitochondrial transition pores are directly related to the decrease in mitochondrial transmembrane potential. Release of smac from mitochondria to the cytosol inhibits the functions of IAP’s (inhibitor of apoptosis proteins) leading to the activation of caspase-3. Figure-2 shows representative immunoblot images (upper panel) and corresponding
quantitative data (lower panel) of smac protein, obtained from sham group, shock group and shock group pre-treated with Ang-1. In rat mesenteric tissue collected from sham operated animals, cytosolic smac levels were low. Hemorrhagic shock for 1 hour followed by 60 minutes of resuscitation T$_{60}$ resulted in an increase in cytosolic smac, compared to the sham operated group (185.72 ± 8.5 % of sham; $p<0.05$). Ang-1 treatment prior to hemorrhagic shock and 60 minutes of resuscitation T$_{60}$ resulted in significant decrease in smac levels, compared to hemorrhagic shock group (102.05 ± 3.7 % of sham; $p<0.05$). These results show that Ang-1 attenuated hemorrhagic shock induced release of mitochondrial smac an activator of caspase-3.

**Angiopoietin-1 prevents hemorrhagic shock-induced cytochrome c release**

The release of cytochrome c from mitochondria to the cytosol occurs following the opening of the mitochondrial transition pores. Cytochrome c release to the cytosol and subsequent activation of caspase-3 has been reported to be the key event in apoptosis induced by various stimuli. Cytosolic cytochrome c levels were elevated following hemorrhagic shock and 60 minutes of resuscitation T$_{60}$ group (43.98 ± 5.39 ng/mg protein) compared with the sham group (18.03 ± 42.5 ng/mg protein; Figure-3 A; $p<0.05$). Cytochrome c levels in hemorrhagic shock group pre-treated with Ang-1 (40ng/ml) showed a significant decrease (25.51 ± 2.7 ng/mg protein) compared to hemorrhagic shock group (Figure-3 A; $p<0.05$). Ang-1 alone treated group showed no significant change in cytochrome c levels (21.61 ± 42.5 ng/mg protein) compared with the sham group. These results show that Ang-1 attenuated HS-induced release of cytochrome c from mitochondria to the cytosol.

**Angiopoietin-1 decreases hemorrhagic shock-induced caspase-3 activation**
Caspase-3 activation occurs following cytochrome c release from mitochondria. Caspase-3 activation leads to the proteolytic cleavage of a variety of cellular substrates including endothelial cell adherens junction proteins. The caspase-3 activity assay has been used to detect caspase-3 activation in tissue samples and cells. Hemorrhagic shock induced caspase-3 activity in rat mesenteric tissue vs. sham (205 ± 12.38 %; Figure-3 B; p< 0.05). Rats pre-treated with Ang-1 followed by hemorrhagic shock showed significant decrease in caspase-3 activity compared to hemorrhagic shock group (108.63 ± 19.26 %; Figure-3 B; p< 0.05). Rats treated with Ang-1 followed by hemorrhagic shock were similar to the sham operated animals. Ang-1 alone treated group showed no significant change in caspase-3 activity (93.39 ± 17.94 %) compared with the sham control group. These results indicate that Ang-1 prevented hemorrhagic shock-induced caspase-3 activation. This might prevent caspase-3 mediated proteolytic cleavage of endothelial cell adherens proteins such as beta catenins.

**Angiopoietin-1 attenuates hemorrhagic shock serum-induced monolayer hyperpermeability in RLMEV**

Serum from sham animals did not induce RLMEC monolayer hyperpermeability (102.3 ± 6.2 %; Figure-4 A). Serum obtained from rats following 60 minutes of hemorrhagic shock and 0 minutes of resuscitation (T0 group) or 60 minutes of resuscitation (T60 group), induced hyperpermeability (172 ± 15.9 % and 165 ± 9.3 % respectively; Figure-4 A; p< 0.05). Ang-1 (40 ng/ml) pre-treatment significantly attenuated the hyperpermeability (115 ± 9.7 %; p< 0.05; Figure-4 A). Ang-1 treatment alone did not show any significant change in permeability compared to the untreated (basal) or the sham serum treated cells (112.3 ± 14 % of basal).

**Angiopoietin-1 attenuates BAK induced monolayer hyperpermeability in RLMEC**
BAK (BH3) peptide transfection significantly increased permeability versus untreated (basal) cells (226 ± 16 %; Figure-4 B; p< 0.05). Pre-treatment of Ang-1 decreased the BAK induced hyperpermeability (115 ± 19 %; p< 0.05; Figure-4 B). Ang-1 alone treatment showed no significant change in permeability compared to untreated (basal) cells (118 ± 18 %). Treatment of TransIT alone showed no significant change in permeability compared to untreated (basal) cells (data not shown).

**Angiopoietin-1 prevents BAK induced collapse of mitochondrial transmembrane potential in RLMEC**

BAK (BH3) transfected cells showed a decrease in red fluorescence (J-aggregates) compared with control cells, indicating the collapse of mitochondrial transmembrane potential (ΔΨm) (Figure-5). BAK (BH3)-transfected cells pre-treated with Ang-1, showed no visible change in red fluorescence compared to the control cells indicating intact mitochondria. In non-apoptotic cells, JCl-1 exists as a monomer in the cytosol (green) and also accumulates as J-aggregates in the mitochondria, which fluoresce as red. Upon the induction of apoptosis, and mitochondrial degradation JC-1 exists only in a monomeric form (green). Ang-1 alone or TransIT alone treated cells showed no change in mitochondrial membrane potential.

**Angiopoietin-1 prevents BAK (BH3) –induced smac release in RLMEC**

In untreated RLMEC, smac cytosolic content was very low or not detected (Figure-6 A). BAK (BH3) transfected cells showed increase in smac cytosolic content compared with the untreated cells (Figure-6 A). BAK (BH3) transfected cells pre-treated with Ang-1 demonstrated significant attenuation of smac release evident from the decrease in smac cytosolic content.
Angiopoietin-1 prevents BAK induced cytochrome c release in RLMEC

In RLMEC, the cytosolic levels of cytochrome c was significantly higher (47.1 ± 5.1 ng/mg protein following BAK (BH3)-transfection, in comparison to the control cells (28.6 ± 5.48 ng/mg protein; \( p < 0.05 \); Figure-6 B). The cytosolic cytochrome c levels were significantly attenuated in BAK (BH3)-transfected cells (30.13 ± 5.03 ng/mg protein; \( p < 0.05 \); Figure-6 B) pre-treated with Ang-1 (40ng/ml) in comparison with BAK (BH3)-transfected cells without Ang-1 treatment (26.7 ± 7.1 ng/mg protein; \( p < 0.05 \); Figure-6 B). No significant difference in cytochrome c levels were observed between TransIT alone treated cells (26.6 ± 4.5 ng/mg protein) compared with untreated control cells.

Angiopoietin-1 inhibits BAK induced caspase-3 activation in RLMEC

In BAK (BH3) transfected cells, caspase-3 activity was significantly higher in comparison to the control cells (208.3 ± 5.9 %; \( p < 0.05 \); Figure-7). The caspase-3 activity was significantly decreased (127.6 ± 4.6 %) in BAK (BH3)-transfected cells pre-treated with Ang-1 in comparison to BAK (BH3)-transfected cells without Ang-1 treatment (\( p < 0.05 \); Figure-7). BAK (BH3) transfected cells treated with Ang-1 showed no significant difference in caspase-3 activity compared to the control cells (\( p < 0.05 \); Figure-7). No significant difference in caspase-3 activity was observed between TransIT alone treated cells compared with untreated control cells.

Hemorrhagic shock serum induces disruption of endothelial cell adherens junction but does not induce cell membrane damage
Control cells showed strong and continuous beta catenin immunofluorescence at the endothelial cell-cell junctions (Figure 8) indicating intact cell barrier. Treatment of serum obtained from rats following 60 minutes of hemorrhagic shock and 60 minutes of resuscitation (T\textsubscript{60} group), induced disruption of endothelial cell junctions evidenced by irregular and scattered beta catenin fluorescence (Figure 8) indicating disruption of adherens junctions. Serum from sham animals did not induce disruption of adherens junctions.

Figure-8 B shows representative images from RLMEC following annexin-V FITC treatment. When control, sham serum treated and shock serum treated cells were exposed to Annexin-V FITC, no Annexin-V binding was observed.

**Angiopoietin-1 decreases BAK (BH3)-induced vascular hyperpermeability in vivo**

Figure 9 A shows representative image of rat mesenteric post-capillary venules from control, BAK (BH3) transfection and Ang-1 treatment prior to BAK (BH3) transfection. Control images show minimum FITC-albumin extravasation whereas BAK (BH3) transfection shows increased extravasation indicating hyperpermeability. Ang-1 pre-treatment shows minimum FITC-albumin extravasation indicating the protective effects of Ang-1. Figure-9 B shows the graphical representation of results from this experiment. Mesenteric post-capillary venules of rats transfected with BAK (BH3) peptide showed hyperpermeability versus sham or TransIT alone treated rats (\(p<0.05\)). Rats pre-treated with Ang-1 followed by BAK (BH3) transfection showed a significant decrease in permeability compared to BAK (BH3) peptide transfected rats without Ang-1 pre-treatment (\(p<0.05\)). Rats transfected with BAK (BH3) following Ang-1 showed no significant change in permeability versus sham group or TransIT alone treated group (\(p<0.05\)).
DISCUSSION

This study demonstrates that angiopoietin-1 (Ang-1) attenuated vascular hyperpermeability following hemorrhagic shock in a rat model *in vivo* and in rat lung microvascular endothelial cell monolayer *in vitro*. Studies conducted to delineate the mechanism of attenuation showed that the protective effect of Ang-1 is related to its inhibition on mitochondrial ‘intrinsic’ apoptotic signaling pathway. Hemorrhagic shock increased the cytosolic levels of smac and cytochrome \( c \). Both smac (second mitochondrial activator of caspases) and cytochrome \( c \) are key-components of apoptotic signaling and are located within the mitochondria. Thus, it is evident that hemorrhagic shock induced collapse of mitochondrial transmembrane potential resulted in the release of smac and cytochrome \( c \) into the cytosol. Ang-1 pre-treatment prevented the smac and cytochrome \( c \) release into the cytoplasm suggesting its protective functions regulating mitochondrial membrane potential. In parallel studies conducted utilizing rat lung microvascular endothelial cells (RLMEC), serum collected following hemorrhagic shock when tested on microvascular endothelial cells, disrupted the adherens junctions. Ang-1 attenuated monolayer hyperpermeability. The monolayer permeability was induced either by serum from hemorrhagic shock-rats or by BAK (BH3) peptide transfection. Previous studies from our laboratory have demonstrated that the pro-apoptotic factor BAK is a mediator of vascular hyperpermeability following hemorrhagic shock (Childs *et al.*, 2007). A synthetic BAK (BH3) peptide was shown to induce vascular hyperpermeability following hemorrhagic shock, where as a mutant BAK (BH3) peptide was ineffective (Childs *et al.*, 2007). In
addition to the observations in vivo, our results show that Ang-1 treatment prevented BAK (BH3)-
induced collapse of ΔΨm, release of smac and cytochrome c and also inhibited BAK (BH3)-induced
caspase-3 activation in RLMEC. These observations suggest that the treatment with Ang-1
effectively control vascular hyperpermeability in hemorrhagic shock by regulating smac and
cytochrome c mediated ‘intrinisc’ apoptotic signaling.

Hemorrhagic shock is known to induce vascular hyperpermeability (Childs et al., 1999; 2002;
2005a,b; Lu et al., 2004). We have recently shown the involvement of intrinsic apoptotic signaling in
vascular heperpermeability following hemorrhagic shock (Childs et al., 2007). In human umbilical
cord endothelial cells, induction of apoptosis by serum deprivation for 6 hours increased cytoplasmic
cytochrome c and smac protein levels (Harfouche et al., 2002). Our study provides further evidence
of the involvement of proapoptotic factors in vascular hyperpermeability and also shows how the
selective regulation of various steps in the process is achieved by using an inhibitor of vascular
permeability. Our results show that hemorrhagic shock stimulated the release of the proapoptotic
factor smac from mitochondria (evident from the increase in smac levels in the cytoplasm) in rat
mesenteric vasculature. The release of the mitochondrial protein smac has been previously shown to
induce caspase activation through its ability to bind to and inhibit the function of members of the IAP
family (Chai et al., 2000; Martins et al., 2002; Cregan et al., 2004). Thus, our observations support
the involvement of a caspase dependent apoptotic pathway in vascular permeability following
hemorrhagic shock.

Hemorrhagic shock-induced collapse of mitochondrial permeability transition may have
resulted in the mitochondrial release of smac and cytochrome c to the cytosol. Although the precise
mitochondrial mechanisms that mediate hemorrhagic shock-mediated smac release are not known,
mitochondrial ROS may have a significant role in this. ROS induced oxidative stress is one of the most important stimulants of apoptotic cell death (Kim et al., 2005). Oxidative stress is known to alter mitochondrial permeability transition (Zhao et al., 2005). Mitochondrial transition pores are major targets of reactive oxygen species and ROS have been implicated in vascular hyperpermeability following hemorrhagic shock in rats (Childs et al., 2002; 2005b; Zhao et al., 2005). Mitochondrial oxidative stress caused by ROS, after reperfusion injury leads to accumulation of cytosolic smac and release of cytochrome c (Sugawara et al., 2002; Saito et al., 2004). Hydrogen peroxide-induced apoptosis in cardiomyocytes and myogenic cells was accompanied by the release of smac from mitochondria, activation of caspase-9 and caspase-3, and DNA fragmentation (Jiang et al., 2005). It is quite possible that in hemorrhagic shock-induced vascular hyperpermeability, mitochondrial oxidative stress played an important role in the translocation of smac and cytochrome c to the cytoplasm through mitochondrial transition pores. The preservation of mitochondrial transition pores by ang-1 is thus an effective approach to control mitochondrial release of smac and cytochrome c to the cytosol and to prevent vascular hyperpermeability.

Ang-1 has anti-apoptotic effects in endothelial cells and protective function against vascular leakage (Thurston et al., 1999; Harfouche et al., 2002). The angiopoietin/Tie receptor system is known to contribute to angiogenesis and vascular remodeling by mediating interactions of endothelial cells with smooth muscle cells and pericytes (Lin et al., 2000). Ang-1 over expressed mice have leakage-resistant blood vessels and Ang-1 protected adult vasculature from leaking, counteracting the potentially lethal actions of VEGF and inflammatory agents (Thurston et al., 1999; 2000). Further studies showed that Ang-1 inhibited mitochondrial smac release and caspase-3 activity in human HUVEC subjected to apoptotic signaling (Harfouche et al., 2002). Our results that Ang-1 inhibited
shock-serum-induced as well as proapoptotic factor BAK-induced microvascular hyperpermeability suggests that Ang-1 in part exert its protective effects on hyperpermeability through its inhibitory actions on the intrinsic apoptotic signaling pathway.

The cellular and molecular mechanisms by which Ang-1 protected mitochondrial membrane integrity are not clearly understood at this time. Ang-1 is known to attenuate apoptotic cell death via PI-3 kinase/AKT dependent pathway and inhibition of smac release from the mitochondria (Valable et al., 2003; Harfouche et al., 2002; 2003). It is possible that Ang-1, in addition to its direct protective effects on mitochondrial transition pores, targeted and inhibited pro-apoptotic proteins that induce smac release from mitochondria. Activation of the mitochondrial pathway with the activation of the effector caspase-3 plays an important role in endothelial cell apoptosis (Ackermann et al., 1999; Tran et al., 1999; Harfouche et al., 2002). Smac is released from mitochondria to the cytoplasm and is known to potentiate the functions of cytochrome c/apoptosome complex (Du et al., 2000). It has been demonstrated that death receptor-initiated mitochondrial release of cytochrome c and smac is caspase dependent (Jiang et al., 2005). Smac promotes not only the proteolytic activation of procaspase-3 but also the enzymatic activity of mature caspase-3, both of which are dependent upon its ability to interact physically with the IAPs (Harfouche et al., 2002, 2003). The IAP family inhibits apoptosis by binding the catalytic domains of caspases-3, -7, and –9 (Deveraux and Reed, 1999; Harfouche et al., 2002). Smac binds to one of the BIR domains of IAPs and block the caspase binding sites on IAPs. PR39, a proline- and arginine-rich peptide implicated in myocardial ischemia protection, inhibited hypoxia-induced apoptosis and decreased caspase-3 activity through an increase of IAP-2 expression in endothelial cells (Wu et al., 2004). Similarly, decreased expression of antiapoptotic proteins Bcl-2 and c-IAP-1 resulting in the activation of apoptotic signaling pathway,
involving release of cytochrome c and smac and activation of caspase-9 and then caspase-3 has been observed in human coronary artery endothelial cells (Chen et al., 2004). Similarly, transgenic mice overexpressing XIAP showed protection against hypoxia-ischemia (Wang et al. 2004). Our finding of the inhibition of smac release and caspase-3 activation by Ang-1 suggests its permissive role on XIAP.

Our results show that disruption of beta catenin/endothelial cell adherens junction occurs following treatment of cells with hemorrhagic shock serum. However, hemorrhagic shock serum does not induce phosphatidyl serine externalization, an indicator of cell membrane damage normally observed in apoptotic cells. This study supports our hypothesis that endothelial cell hyperpermeability occurs due to activation of caspase-3 and subsequent cleavage of beta catenin resulting in cell-cell detachment and may not be due to apoptotic cell death. Caspase-3 cleaves a variety of cell adhesion proteins. The caspase-3-dependent cleavage of beta-catenin occurs during apoptosis (Cervello et al., 2004; Gregorc, 2005; Nakamoto et al., 2005). In endothelial cells, beta-catenin functions as a regulator of cadherin-mediated cell-cell adhesion. Thus, Ang-1 mediated maintenance of mitochondrial transmembrane potential and inhibition of smac and cytochrome c release may be possible mechanisms by which it prevents caspase-3 activation and protect endothelial cell barrier integrity to prevent vascular hyperpermeability.

In this study we utilized an in vivo mesenteric post-capillary venular preparation to study changes in microvascular hyperpermeability. In addition, parallel studies were performed in rat lung microvascular endothelial cell (RLMEC) monolayers. Endothelial cells from the lung and mesenteric venules were of particular interest due to the associated morbidity observed secondary to pulmonary
edema and intra-abdominal compartment syndrome. It also allowed us to study two different vascular systems to determine if activation of the apoptotic cascade has similar effects on barrier integrity.

In conclusion, our findings suggest that hemorrhagic shock induces mitochondrial release of pro-apoptotic smac and cytochrome c. The mitochondrial release of smac and cytochrome c may subsequently activate caspase-3 leading to the disruption of cell adherens junctions and vascular hyperpermeability. Angiopoietin-1 treatment maintains mitochondrial membrane integrity, prevents mitochondrial smac and cytochrome c release, caspase-3 activation and attenuates vascular hyperpermeability. The Ang-1 mediated protective effects may be due to its inhibitory effects on caspase mediated cleavage of endothelial cell adherens junction proteins and disruption of cell-cell junctions. Angiopoietin-1 could be tested as a therapeutic protein for specific protection against hemorrhagic shock induced endothelial cell injury and vascular leakage.

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FIGURE LEGENDS

Figure-1 (A). Angiopoietin-1 (Ang-1) attenuates vascular hyperpermeability following hemorrhagic shock (T\textsubscript{60}) in rat mesenteric post-capillary venules. The images of mesenteric post-capillary venules from hemorrhagic shock for 1 hour MAP 40 mm Hg followed by 60 minutes of resuscitation (label T\textsubscript{60}), sham and Ang-1 treatment in hemorrhagic shock are shown. FITC-albumin extravasation into the extravascular space is observed following shock T\textsubscript{60} but is minimal in control and hemorrhagic shock with Ang-1 treatment. Each image represents the images obtained from one of the five rats tested for each group under two microscopic fields per animal. (B). Angiopoietin-1 (Ang-1) attenuates vascular hyperpermeability following hemorrhagic shock in rat mesenteric post-capillary venules. Permeability is expressed as change in fluorescent intensity inside the vessel compared to the intensity outside the vessel. Asterisk indicates significant difference vs. shock group and shock group pre-treated with Ang-1, sham group or Ang-1 alone group (\textit{p} < 0.05; n = 5).

Figure-2. Angiopoietin-1 (Ang-1) prevents hemorrhagic shock induced mitochondrial release of pro-apoptotic smac to the cytoplasm. (A). Typical immunoblot images of smac cytosolic content in rat mesenteric vasculature, in sham, hemorrhagic shock (T\textsubscript{60}) and hemorrhagic shock (T\textsubscript{60}) with Ang-1 pre-treatment, are given. Smac cytosolic content is increased at T\textsubscript{60} compared to sham. Ang-1 pre-treatment shows decrease in smac cytosolic content compared with hemorrhagic shock (T\textsubscript{60}). Beta
actin is used as an internal control. (B). The lower panel shows graphical representation of smac cytosolic content expressed as percentage of sham (control). Smac cytosolic content showed significant increase at T\(_{60}\) compared with sham (\(p < 0.05; n = 4\)). Ang-1 pre-treatment shows decrease in smac cytosolic content compared with hemorrhagic shock group (T\(_{60}\)) (\(*p < 0.05; n = 4\)).

**Figure-3.** (A) Angiopoeitin-1 (Ang-1) inhibits hemorrhagic shock-induced cytochrome c release in rat mesenteric vasculature. Cytosolic cytochrome c level is increased following hemorrhagic shock (Shock T\(_{60}\)) compared with sham-control (\(p < 0.05; n = 5\)). Ang-1 pre-treatment in hemorrhagic shock shows significant decrease in cytochrome c levels compared with hemorrhagic shock (T\(_{60}\)) without Ang-1 treatment (\(*p < 0.05; n = 5\)). (B) Angiopoeitin-1 (Ang-1) inhibits hemorrhagic shock-induced caspase-3 activity in rat mesenteric vasculature. Change in caspase-3 activity is expressed as percentage of the sham-control value. Caspase-3 activity is increased following hemorrhagic shock (Shock T\(_{60}\)) compared with sham-control (\(p < 0.05; n = 5\)). Ang-1 pre-treatment in hemorrhagic shock shows significant decrease in caspase-3 activity compared with hemorrhagic shock (T\(_{60}\)) without Ang-1 treatment (\(*p < 0.05; n = 5\)).

**Figure-4** (A). Angiopoeitin-1 (Ang-1) attenuates hemorrhagic shock-serum induced hyperpermeability in rat lung microvascular endothelial cell monolayers. Change in permeability is expressed as percentage of the basal fluorescence. Shock serum (T\(_{0}\) and T\(_{60}\)) induced hyperpermeability in the monolayer compared to sham serum (\(p < 0.05; n = 5\)). Ang-1 (40 ng/ml) pre-treatment in shock-serum (T\(_{0}\)) treated cells showed decrease in FITC-albumin fluorescence compared with untreated cells (\(*p < 0.05; n = 5\)). (B). Angiopoeitin-1 (Ang-1) attenuates pro-apoptotic BAK (BH3) peptide induced hyperpermeability in rat lung microvascular endothelial cell
monolayers. BAK (BH3) peptide transfection induced monolayer hyperpermeability \((p < 0.05; n = 5)\). Ang-1 (40 ng/ml) attenuated BAK (BH3) (5µg/ml) induced hyperpermeability as evident from the decrease in FITC-albumin fluorescence in this group compared with BAK (BH3) group \(* p < 0.05; n = 5\). Change in permeability is expressed as percentage of the basal fluorescence.

**Figure-5.** Angiopoietin-1 (Ang-1) protects mitochondrial membrane integrity in rat lung microvascular endothelial cells (RLMEC). Confocal microscopy images of the mitochondrial membrane potential indicator JC-1 in its monomeric (green) and dimeric (red) forms are shown. BAK (BH3) transfection leads to the collapse of mitochondrial membrane potential showing predominantly monomeric forms. Ang-1 (40 ng/ml) treatment prevents the collapse of mitochondrial membrane potential evidenced by the restoration of dimeric form-red fluorescence. Ang-1 alone or TransIT treatment shows no change in mitochondrial membrane potential compared with the untreated control cells.

**Figure-6.** Angiopoietin-1 (Ang-1) prevents pro-apoptotic smac and cytochrome \(c\) release to the cytoplasm in rat lung microvascular endothelial cells (RLMEC). (A). Typical immunoblot image of smac cytosolic content in RLMEC, in control, BAK (BH3) transfected and Ang-1 treatment followed by BAK (BH3) transfection, are given. The smac cytosolic content is increased following BAK (BH3) transfection compared with control. Ang-1 treatment shows decrease in smac content compared with BAK (BH3) transfected cells. Beta actin is shown as an internal control. (B). Cytosolic cytochrome \(c\) levels are increased following BAK (BH3) transfection compared with control \((p < 0.05; n = 5)\). Ang-1 pre-treatment in BAK (BH3) transfected cells shows significant
decrease in cytochrome c levels compared with BAK (BH3) transfection without Ang-1 pre-treatment (*p < 0.05; n = 5).

**Figure-7.** Angiopoeitin-1 (Ang-1) inhibits pro-apoptotic BAK (BH3) peptide induced caspase-3 activity, in rat lung microvascular endothelial cells (RLMEC). BAK (BH3) transfection significantly increased caspase-3 activity compared with control cells (p < 0.05; n = 5). Ang-1 (40 ng/ml) inhibited BAK (BH3) (5µg/ml) induced increase in caspase-3 activity. Data expressed as percentage of basal (control) values. Asterisk indicates significant difference vs. BAK (BH3) transfected cells (p < 0.05; n = 5).

**Figure-8.** (A) Hemorrhagic shock serum disrupts endothelial cell adherens junction. Immunofluorescence images of endothelial cell adherens junction protein beta catenin in rat lung microvascular endothelial cells (RLMEC) are shown. Control cells and sham serum treated cells show intact junctions evidenced by the strong and continuous presence of beta catenin at the junctions. Shock serum treated cells show disruption of the junctions evidenced by irregular and scattered beta catenin fluorescence. (B). Hemorrhagic shock serum does not induce cell death. Control, sham serum treated or shock serum treated cells show no Annexin-V FITC binding.

**Figure-9.** Angiopoeitin-1 (Ang-1) attenuates pro-apoptotic BAK (BH3)-induced vascular hyperpermeability in rat mesentric post-capillary venules in vivo. (A). Images of FITC-albumin extravasation in mesenteric post-capillary venules of control, BAK (BH3) or BAK (BH3) transfection following Ang-1 treatment. Control or BAK (BH3) trasfection following Ang-1 treatment shows minimal extravasation of FITC-albumin. BAK (BH3) trasfection (BAK T_{60}) shows increased
extravasation of FITC-albumin. Each image represents the images obtained from one of the five rats tested for each group under two microscopic fields per animal. (B). Graphic representation of FITC-albumin extravasation from rat mesenteric post-capillary venules following BAK (BH3) transfection. Permeability is expressed as change in fluorescent intensity inside the vessel compared to the intensity outside the vessel. BAK (BH3) transfection shows significant increase in hyperpermeability compared with sham or TransIT group \( p < 0.05; n = 5 \). Ang-1 pre-treatment decreases BAK (BH3)-induced hyperpermeability. Asterisk indicates significant difference vs. BAK (BH3) transfection and BAK (BH3) transfection following Ang-1 treatment \( p < 0.05; n = 5 \).
Control                       Shock T60                 Ang-1 + Shock T60

A

Control       Shock 30 min       Shock 60 min

B

Ang-1 given  Start Resuscitation

ΔI (Fluorescent Intensity)

0 1 2 3 4

Time (min)
A

Cytochrome c (ng/mg protein)

Control      Shock T_{60}      Ang-1 + Shock T_{60}      Ang-1

B

Caspase-3 Activity (% Control)

Control      Shock T_{60}      Ang-1 + Shock T_{60}      Ang-1
A

Smac Cytosolic Content

Control  BAK  Ang-1 + BAK

β-actin

B

Cytochrome c Cytosolic levels

Control  BAK (BH3)  Ang-1 + BAK (BH3)  Ang-1  TransIT

Cytochrome c (ng/mg protein)
**A**

Control | BAK T<sub>60</sub> | Ang-1 + BAK T<sub>60</sub>

**B**

ΔI (Fluorescent Intensity)

- **Ang-1 + BAK**
- **BAK**
- **Sham**
- **TransIT**

Control | 30 min. | 60 min. | BAK given

Time (min.)