

# Hypoxia serves a key function in the upregulated expression of vascular adhesion protein-1 *in vitro* and in a rat model of hemorrhagic shock

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**Abstract.** Hemorrhagic shock following major trauma results in mortality, but the function of vascular adhesion protein-1 (VAP-1), implicated in intracranial hemorrhage, remains unknown. This study aimed to determine whether expression of the *AOC3* gene and its encoded protein, VAP-1, is altered by hypoxia. Rat hepatic sinusoidal endothelial cells (RHSECs) and rat intestinal microvascular endothelial cells (RIMECs) were transduced with a viral vector carrying *AOC3*, and *AOC3* mRNA expression levels were measured by reverse transcription-quantitative polymerase chain reaction. VAP-1 protein expression levels were measured by western blot analysis and compared between normoxic and hypoxic conditions. Following this, *AOC3* mRNA and VAP-1 protein expression levels in hepatic and intestinal tissues were assessed in a rat model of hemorrhagic shock with or without fluid resuscitation; and serum semicarbazide-sensitive amine oxidase (SSAO) activity was measured by fluorometric assays. The effects of 2-bromoethylamine (2-BEA) on *AOC3*/VAP-1 levels and 24 h survival were investigated. *AOC3* mRNA and VAP-1 protein levels were increased in RHSECs and RIMECs by hypoxia, and in hepatic and intestinal tissues from rats following hemorrhagic shock. Hypoxia increased serum SSAO activity in these animals. 2-BEA reduced *AOC3* mRNA and VAP-1 protein levels in hepatic and intestinal tissues from

rats following hemorrhagic shock, and appeared to improve survival in animals not receiving resuscitation following hemorrhagic shock. In conclusion, hemorrhagic shock upregulates *AOC3*/VAP-1 expressions, and this potentially occurs via hypoxia. Therefore, inhibition of VAP-1 may be beneficial in the setting of hemorrhagic shock. Further studies are required to confirm these findings and to establish whether VAP-1 may be a valid target for the development of novel therapies for hemorrhagic shock.

## Introduction

Trauma is a leading cause of death in people aged 5-44 years (1), and >50% of early deaths following trauma are due to major hemorrhage (2). The loss of blood during hemorrhagic shock results in hemodynamic instability, coagulopathy, reduced tissue perfusion and oxygen delivery, and therefore cellular hypoxia (3,4). In turn, this results in organ failure, including the liver (5) and intestine (6). Management of hemorrhagic shock includes interventions to control the bleeding, coagulation support, administration of vasoactive drugs and resuscitation with fluids to maintain tissue oxygenation (3,4,7). However, the mortality rate following major hemorrhage remains high, emphasizing the need for novel treatments. Elucidation of the cellular alterations induced by hypoxia and major hemorrhage in animal models may help identify novel therapeutic targets to minimize the detrimental effects of hemorrhagic shock and therefore improve survival.

Vascular adhesion protein-1 (VAP-1), a copper-containing amine oxidase composed of 763 amino acids, is a member of the semicarbazide-sensitive amine oxidase (SSAO) family (8). These proteins are primarily expressed in the vascular endothelial cells, adipose tissue and smooth muscle cells in humans and a variety of mammals (9,10). VAP-1 is an ectoenzyme on the vascular endothelial cell surface, but is additionally expressed in the plasma as soluble VAP-1 (sVAP-1) (11). In the plasma, sVAP-1 catalyzes the transformation of aromatic and aliphatic primary amines in the blood into corresponding aldehydes, and produces H<sub>2</sub>O<sub>2</sub> and

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NH<sub>3</sub> (12-14). VAP-1 is also a homing-associated molecule, and may mediate adhesion and effusion between leukocytes and endothelial cells by antigen-antibody binding (15-20) or enzyme activity (18,19,21,22). These dual functions of VAP-1 allow leukocytes to patrol throughout the body (23). Therefore, previous studies characterizing VAP-1 have focused mainly on inflammation.

It has been reported that plasma sVAP-1 concentrations are significantly increased in chronic liver disease, chronic kidney disease, septic shock and certain other diseases (24-26). In disease models of peritonitis, hepatitis, colitis, skin inflammation, stroke, sepsis, ischemia-reperfusion injury and allograft rejection, anti-VAP-1 monoclonal antibodies (mAbs) and VAP-1 enzyme inhibitors have been demonstrated to relieve inflammation by preventing leukocyte migration (17,27-32). As VAP-1 mediates leukocyte migration and adherence to vascular endothelium, it has additionally been implicated in cardiovascular and cerebrovascular diseases, including the dissemination of tumor cells (33-35) and ischemic stroke (36,37). Therefore, VAP-1 may prove to be a prognostic biomarker or potential drug target (23).

However, little is known about VAP-1 regulatory mechanisms despite increasing evidence suggesting that VAP-1 is closely associated with the occurrence and development of multiple diseases. Indeed, VAP-1 expression has been well described in inflammatory reactions, and inflammation is considered a major factor that increases sVAP-1 concentration in the plasma. However, no specific pro-inflammatory factors have been identified that directly induce VAP-1 expression or stimulate signal transduction pathways that regulate VAP-1 expression (9,38,39). Nonetheless, the inflammatory cascade markedly alters the local microenvironment of endothelial cells, and this may alter their gene expression to allow them to adapt to the altered environment (40,41).

In previous animal experiments, it has been demonstrated that the gene for VAP-1 (*AOC3*) was differentially expressed in hepatic and intestinal tissues between rats surviving severe hemorrhagic shock and those that died within an hour following induction of shock (42). As hypoxia is one of the major physiological insults associated with hemorrhagic shock, this raises the possibility that cellular hypoxia may be an important factor that regulates VAP-1 expression in the liver and intestine. Therefore, the present study was designed to assess the importance of severe shock and cellular hypoxia in the regulation of VAP-1 expression in the liver and intestine. The data suggested that hypoxia causes upregulation of VAP-1 expression.

## Materials and methods

**Cell culture.** Rat hepatic sinusoidal endothelial cells (RHSECs) and rat intestinal microvascular endothelial cells (RIMECs; Wuhan PriCells Biomedical Technology Co., Ltd., Wuhan, China) were cultured in primary endothelial cell basic culture medium (Wuhan PriCells Biomedical Technology Co., Ltd.) supplemented with 1% special additives SUP-0002 (Wuhan PriCells Biomedical Technology Co., Ltd.), 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 1% penicillin (Shanghai Hualan Chemical Technology Co., Ltd., Shanghai, China) and streptomycin (North China Pharmaceutical Group Corp., Shijiazhuang,

China), at 37°C in a humid environment containing 5% CO<sub>2</sub>. As VAP-1/SSAO expression is progressively lost with passage of cells (43,44), only cells of the first generation were used in the present study. HEK 293 cells were purchased from ATCC (Manassas, VA, USA) and cultured in ATCC complete growth medium, which included ATCC-formulated Eagle's Minimum Essential Medium (ATCC) and 10% FBS. Cells were maintained in a humidified environment at 37.0°C with 5% CO<sub>2</sub>.

**Construction of adenovirus vectors and transduction of adenovirus.** The adenovirus vector pAd-IRES-EGFP was constructed using the Invitrogen BP Recombination and LP Recombination systems to obtain the adenovirus vector pAd-AOC3-IRES-EGFP, which was used for overexpression of rat *AOC3* (Thermo Fisher Scientific, Inc.; *AOC3* GeneID: NM\_031582.2). The vector did not contain the endogenous rat *AOC3* promoter. The pAd-AOC3-IRES-EGFP adenovirus vector was used to transfect HEK 293 cells for virus amplification. Cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

RHSECs and RIMECs were divided into three experimental groups: Control, which received no adenoviral transduction; pAd-IRES-EGFP, transduced with an empty adenovirus vector (pAd-IRES-EGFP); and pAd-AOC3-IRES-EGFP, transduced with an adenovirus vector for *AOC3* overexpression (pAd-AOC3-IRES-EGFP). In the two transduction groups, cells in the logarithmic growth phase were plated on 6-well cell culture plates at a density of 5×10<sup>4</sup> cells/well and cultured in complete medium (primary endothelial cell basic culture medium, supplemented as above) for 24 h. The medium was replaced with fresh medium and the cells were transduced (multiplicity of infection [MOI]=10) with the adenovirus vector for 24 h, and subsequently cultured under either normoxic (95% air, 5% CO<sub>2</sub>) or hypoxic (95% N<sub>2</sub>, 5% CO<sub>2</sub>) conditions for 24 h. *AOC3* mRNA expression and VAP-1 protein expression were subsequently determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, respectively.

**Animals and grouping.** Healthy specific pathogen-free (SPF) male Sprague-Dawley rats (n=60; weight, 200-220 g; age, 8-9 weeks), were purchased from Sino-British Sippr/BK Lab Animal Ltd. (Shanghai, China) and housed for >1 week in a SPF animal room at 20-25°C and in a 12-h light/dark cycle, to allow for adaptation prior to experiments. Rats were fed until they reached a desired weight (300-350 g), after which surgery was performed.

The experiment was repeated 5 times; for each experiment 12 rats were randomly divided into six groups (n=2 per group), and each group therefore contained a total of 10 rats following all 5 repeats. The six groups were as follows: Sham (sham operation); hemorrhagic shock (HS; hemorrhagic shock with no resuscitation); HS/R group (hemorrhagic shock/resuscitation following hemorrhagic shock); sham+2-bromoethylamine (2-BEA; treatment with the SSAO enzyme inhibitor 2-BEA followed by sham surgery); HS+2-BEA (treatment with 2-BEA, followed by hemorrhagic shock with no resuscitation); and HS/R+2-BEA (treatment with 2-BEA, followed by hemorrhagic shock and resuscitation).

The present study was approved by the Institutional Animal Care and Use Committee of the Second Military Medical University (SMMU; Shanghai, China) of the People's Liberation Army (protocol number 12106). The surgical procedures were conducted at the Experimental Animal Center, and other work at the Laboratory of the College of Pharmacy, SMMU (Shanghai, China).

**Treatment with 2-BEA prior to surgery.** Starting 4 weeks prior to surgery, rats in the sham+2-BEA, HS+2-BEA and HS/R+2-BEA groups received intraperitoneal injections of the reversible competitive SSAO enzyme inhibitor, 2-BEA (20 mg/kg daily; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) (45,46). Rats in the sham, HS and HS/R groups received daily intraperitoneal injections of the same volume of deionized water. The body weight of the rats was measured daily, and the drug dosage was adjusted according to the body weight.

**Rat model of hemorrhagic shock and resuscitation.** Surgery was performed 4 weeks following treatment with 2-BEA or vehicle (water). Rats were fasted for 12 h prior to surgery, but had free access to water. The rat model of hemorrhagic shock was established using a modification of Wigger's method (42). Each rat was anesthetized by intraperitoneal injection of a 3% solution of sodium pentobarbital (30 mg/kg; Sigma-Aldrich; Merck KGaA). Rats in the HS/R group were injected with sodium pentobarbital (20 mg/kg) again prior to resuscitation, and all rats were sacrificed under anesthetic, following completion of the experimental procedure.

The left carotid artery was dissected and connected to a polygraph (MPA-2000; Shanghai Alcott Biotech Co., Ltd., Shanghai, China) to allow monitoring of blood pressure. In addition, the right femoral artery and vein were dissected and intubated, and the femoral artery was connected to a micro-injector (ALC-IP900; Shanghai Alcott Biotech Co., Ltd.) for drawing blood. The tube in the femoral vein was temporarily locked, and was available for transfusion of blood following shock. Rats in the sham group received only femoral artery and vein intubation; no blood was drawn or transfused.

Once a stable state had been reached 10 min following intubation, blood was drawn from the right femoral artery of rats in the HS group at 2 ml/kg/min, until the mean arterial pressure (MAP) reached  $\leq 30$  mmHg. Repeat bloodletting was performed when the MAP reached  $\geq 40$  mmHg, and multiple drawings of blood were performed to maintain the MAP at 30-40 mmHg for 1 h, with no infusions given. The total volume of blood withdrawn was measured and recorded. Rats in the HS/R group were treated similarly to animals in the HS group. However, once a low MAP had been maintained for 1 h, a transfusion was given consisting of whole blood (half the original blood loss volume) together with lactated Ringer's solution (volume, 2X the original blood-loss volume; Baxter, Deerfield, MA, USA). Following this, rat status and MAP changes were observed for 1 h.

Following completion of the above procedures, a laparotomy was performed on sterile drapes. The hepatic left lateral lobe was cut, and 3 ml of blood was quickly drawn from the femoral artery for measurements of the partial pressures of  $O_2$  and  $CO_2$  (i-STAT 300 blood gas analyzer; Abbott

Laboratories, Chicago, IL, USA). Subsequently, the animals were sacrificed through further bleeding. Mortality was confirmed by monitoring the blood pressure and heart rate, via the left carotid artery. Specimens of the liver were taken and sliced into small sections ( $\sim 0.5 \times 0.5$  cm). Specimens of the small intestine (including the mesentery) were obtained, cut along the longitudinal axis, and then cut into small sections ( $\sim 1 \times 1$  cm). All specimens were rinsed three times in phosphate-buffered saline (PBS) and stored in liquid nitrogen. Serum samples were prepared by centrifugation of the blood ( $4^\circ C$ ,  $3,200 \times g$ , 10 min), and stored in liquid nitrogen.

The experiment was repeated to determine the 24 h survival rate with another set of healthy SPF male Sprague-Dawley rats ( $n=120$ ; weight, 200-220 g; age, 8-9 weeks), purchased from Sino-British Sippr/BK Lab Animal Ltd. and housed as aforementioned. Rats were fed until they reached a desired weight (300-350 g), and were divided into the following four groups ( $n=30$ /group): HS, HS/R, HS+2-BEA, HS/R+2-BEA. Rats underwent the same surgical procedures as aforementioned, whereby the rats were anesthetized and monitored during the intubation/shock/recovery process. When these processes were completed, rats that survived the procedure were extubated, placed into cages, and monitored up to 24 h.

**RT-qPCR.** Cells (RHSECs or RIMECs) were seeded into 6-well cell culture plates and were seeded at  $5 \times 10^4$  cells/well, and transduced as aforementioned. Total RNA was isolated from the cells, using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Samples of hepatic or intestinal tissue (100 mg; which had been stored in liquid nitrogen) were transferred into a clean 10 ml glass tube containing 1 ml TRIzol and homogenized. The above samples were transferred into 1.5 ml tubes and centrifuged at  $12,000 \times g$  at  $4^\circ C$  for 5 min. RNA purity was determined by spectroscopy [ratio of optical density at 260 nm to that at 280 nm ( $OD_{260}/OD_{280}$ )=1.8-2.0] and agarose gel electrophoresis (1.0% agarose, 1 ng total RNA/lane). Total RNA (500 ng) was reverse transcribed into cDNA using Reverse Transcriptase M-MLV. (Takara Bio, Inc., Otsu, Japan). qPCR (TP800 Thermal Cycler Dice Real Time System; Takara Bio, Inc.) was performed with 2  $\mu l$  cDNA, 0.4  $\mu l$  each of forward and reverse primer, and Takara SYBR Premix Ex Taq (DRR041A; Takara Bio, Inc.) in a final reaction volume of 20  $\mu l$ . The sequence-specific primers for *AOC3* and  $\beta$ -actin (TaKaRa Bio Inc.) were as follows: Forward, 5'-CCT AAGGCCAACCGTGAAAAGATG-3' and reverse, 5'-GTC CCGGCCAGCCAGGTCCAG-3' for *AOC3*; forward, 5'-GCT CCGGCGACACCACTCAG-3' and reverse, 5'-CGCCAGCAC CGAAGAAGAAAG-3' for  $\beta$ -actin.

The cycling conditions were:  $50^\circ C$  for 2 min, initial denaturation at  $95^\circ C$  for 10 min followed by 40 cycles of denaturation at  $90^\circ C$  for 15 sec, and annealing at  $60^\circ C$  for 1 min. Quantitation cycle (Cq) values of the target gene *AOC3* were normalized to those of  $\beta$ -actin, and relative mRNA expression levels were calculated using the  $2^{-\Delta\Delta Cq}$  method (47). The experiment was repeated three times.

**Western blotting.** Cells were lysed with radioimmunoprecipitation assay buffer (Invitrogen, Thermo Fisher Scientific, Inc.) and centrifuged at  $18,500 \times g$  for 15 min at  $4^\circ C$ . Proteins were extracted from hepatic and intestinal tissues using Tissue

Protein Extraction Reagent (T-PER; Pierce, Rockford, IL, USA). Frozen tissue (~100 mg) was added to a mixture of 1 ml T-PER reagent and protease inhibitor cocktail (Abcam, Cambridge, UK), homogenized, and centrifuged at 4°C for 5 min at 10,000 × g. Total protein content was determined with a Bicinchoninic Acid protein assay kit (Pierce), and equal amounts of proteins (100 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The non-specific sites on each blot were blocked with 5% milk powder diluted in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST) for 1 h at room temperature. Following washing with TBST, membranes were incubated overnight at 4°C in the presence of primary antibodies against glyceraldehyde 3-phosphate dehydrogenase (rabbit mAb; 1:1,000; #2118; Cell Signaling Technology, Inc., Danvers, MA, USA) and VAP-1 (mouse mAb; 1:1,000; ab81718; Abcam). Blots were subsequently incubated at room temperature for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit immunoglobulin (Ig)-G (#7074; 1:3,000) or anti-mouse IgG (#7076; 1:6000); Cell Signaling Technology, Inc.), and developed using enhanced chemiluminescence western blotting substrate (Pierce). Band intensity was quantified using TotalLab Quant v12.0 software (TotalLab Ltd., Newcastle upon Tyne, UK). Experiments were repeated twice.

**Determination of serum SSAO activity.** The Fluorescent SSAO Detection kit (Cell Technology, Inc., Mountain View, CA, USA) was used to determine SSAO activity in various specimens of serum, in accordance with the manufacturer's protocol. Briefly, serum samples were diluted in 1X reaction buffer, and a monoamine oxidase-B inhibitor was added 30 min prior to the assay, which was performed in the dark in 96-well plates. Subsequently, 100 µl sample or positive control were loaded in each well, followed by the addition of 100 µl reaction cocktail. The mixture was incubated at 37°C for 1-3 h, and the plates were read using a DU-730 ultraviolet fluorescence spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA) with excitation at 530 nm and emission at 590 nm. Serum SSAO activity was calculated according to a standard curve and expressed in mU/ml. One unit represents the amount of enzyme required to oxidize 1 µmol of SSAO substrate (benzylamine) in 1 min at 25°C.

**Immunohistochemistry.** Immunohistochemistry experiments (labeled streptavidin-biotin method) were performed using a Streptavidin-Peroxidase Immunohistochemical Detection kit (SP-9000; ZSGB-BIO, Beijing, China). In brief, formalin-fixed, paraffin-embedded sections (thickness: 4 µm) were dewaxed with fresh xylene and subjected to gradient hydration (100% ethanol, 95% ethanol, 60% ethanol, purified water). Following elimination of endogenous peroxidase activity (incubation with 3% H<sub>2</sub>O<sub>2</sub> for 15 min at room temperature), microwave antigen retrieval and incubation with normal goat serum (Abcam) for 15 min at 37°C. The sections were then incubated overnight at 4°C with a primary antibody against VAP-1 (rabbit polyclonal; 1:200; ab187202; Abcam). Following 3 washes with PBS, the specimens were incubated with a biotin-conjugated goat anti-rabbit (1:1,000; ab6720; Abcam) secondary antibody

for 15 min at 37°C, followed by streptavidin-peroxidase for 15 min at 37°C. The slides were stained with 3,3-diaminobenzidine for 10 min at room temperature, and re-stained with hematoxylin. The specimens were subsequently dehydrated in a graded series of ethanol (30, 70 and 100%), sealed with neutral resin, and observed under a light microscope (Olympus Corporation, Tokyo, Japan). A total of 10 fields were assessed (magnification, ×100) in three independent experiments.

**Statistical analysis.** Statistical analysis was performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard deviation derived from at least three independent experiments. Statistical significance was evaluated by one-way analysis of variance followed by the least significant difference post-hoc test, or the independent samples t-test. *P* < 0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of hypoxia on AOC3 mRNA expression, VAP-1 protein expression, and SSAO activity in RHSECs and RIMECs.** As tissue hypoxia is a major physiologic insult arising from hemorrhagic shock, *in vitro* experiments were performed to investigate the effects of hypoxia on the mRNA expression of AOC3 and protein expression of VAP-1 in RHSECs and RIMECs. Fluorescence microscopy revealed the successful transduction of the AD-AOC3-IRES-EGFP adenovirus vector into RHSECs (Fig. 1A) and RIMECs (Fig. 1B).

RT-qPCR revealed that, for RHSECs and RIMECs cultured under normoxic conditions, AOC3 mRNA expression was low in blank controls and cells transduced with AD-IRES-EGFP (RHSECs, Fig. 1C; RIMECs, Fig. 1D). However, RHSECs transduced with AD-AOC3-IRES-EGFP had significantly increased AOC3 mRNA expression levels than blank controls (~4.3-fold increase; *P* < 0.01; *n* = 3) and cells transduced with AD-IRES-EGFP (~4.2-fold increase; *P* < 0.001; *n* = 3; Fig. 1C). Similarly, RIMECs transduced with AD-AOC3-IRES-EGFP had significantly increased AOC3 mRNA expression than blank controls (~12.5-fold increase; *P* < 0.01; *n* = 3) and cells transduced with AD-IRES-EGFP (~12.5-fold increase; *P* < 0.01; *n* = 3; Fig. 1D). In RHSECs and RIMECs, hypoxia (culture for 24 h in 0% O<sub>2</sub>) had little or no effect on blank controls and cells transduced with AD-IRES-EGFP (Fig. 1C and D). In contrast, hypoxia caused a significant upregulation of AOC3 mRNA expression in RHSECs (~12.1-fold increase; *P* < 0.01; *n* = 3) and RIMECs (~3.8-fold increase; *P* < 0.01; *n* = 3) transduced with AD-AOC3-IRES-EGFP (Fig. 1C and D, respectively).

Western blotting revealed that under normoxic conditions, VAP-1 protein expression in RHSECs and RIMECs did not differ significantly between blank controls and cells transduced with AD-IRES-EGFP (Fig. 1E and F, respectively). RHSECs transduced with AD-AOC3-IRES-EGFP had significantly increased VAP-1 protein expression compared with blank controls (~3.3-fold increase; *P* < 0.001; *n* = 3) and cells transduced with AD-IRES-EGFP (~3.4-fold increase; *P* < 0.001; *n* = 3; Fig. 1E). RIMECs transduced with AD-AOC3-IRES-EGFP additionally had significantly increased VAP-1 protein expression compared with blank controls (~2.5-fold increase;

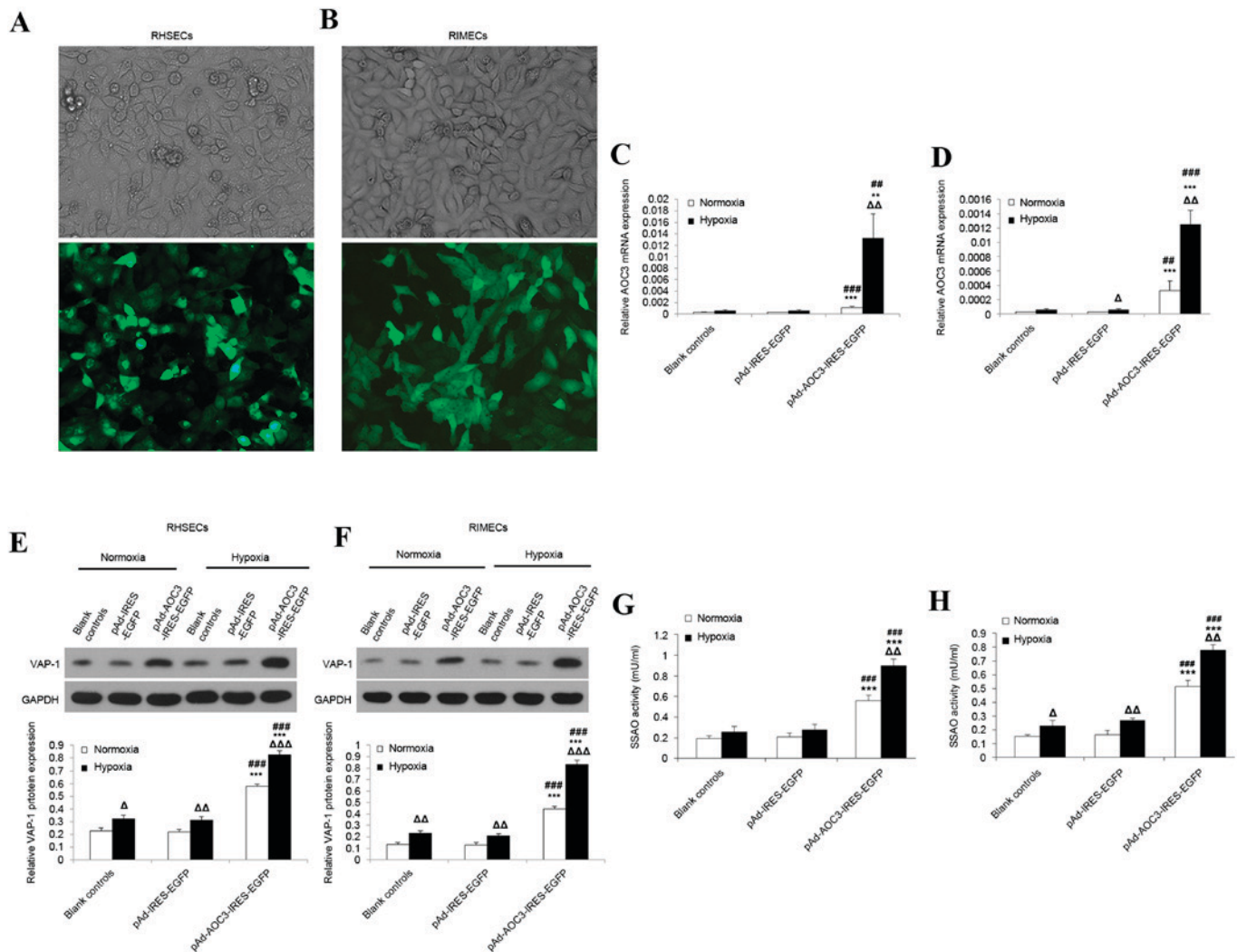


Figure 1. Effects of hypoxia on VAP-1 protein expression and *AOC3* mRNA expression in RHSECs and RIMECs. RHSECs and RIMECs were transduced with pAd-AOC3-IRES-EGFP or pAd-IRES-EGFP adenovirus vectors for 72 h; blank controls were not exposed to any adenovirus vector. Light and fluorescence microscopy images confirmed the successful transduction of (A) RHSECs and (B) RIMECs with pAd-AOC3-IRES-EGFP (magnification, x200). *AOC3* mRNA expression in (C) RHSECs and (D) RIMECs under normoxic and hypoxic conditions, determined by reverse transcription-quantitative polymerase chain reaction.  $\beta$ -actin served as an internal control. VAP-1 protein expression in (E) RHSECs and (F) RIMECs under normoxic and hypoxic conditions, determined by western blotting. GAPDH was used as an internal control. Serum SSAO activity in (G) RHSECs and (H) RIMECs was determined using a fluorometric assay. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. blank controls; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. pAd-IRES-EGFP;  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$  and  $\Delta\Delta\Delta P < 0.001$  hypoxia vs. normoxia within the same group. VAP-1, vascular adhesion protein-1; *AOC3*, VAP-1 gene; RHSECs, rat hepatic sinusoidal endothelial cells; RIMECs, rat intestinal microvascular endothelial cells; SSAO, semicarbazide-sensitive amine oxidase; pAd-IRES-EGFP, adenovirus control vector; pAd-AOC3-IRES-EGFP, adenovirus vector for *AOC3* overexpression.

$P < 0.001$ ;  $n = 3$ ) and cells transduced with AD-IRES-EGFP (~2.6-fold increase;  $P < 0.001$ ;  $n = 3$ ; Fig. 1F). In RHSECs and RIMECs, hypoxia for 24 h resulted in a moderate (1.4-1.7-fold) but significant enhancement of VAP-1 protein expression in blank controls ( $P < 0.05$  and  $P < 0.001$ , respectively;  $n = 3$ ; Fig. 1E and F) and cells transduced with AD-IRES-EGFP ( $P < 0.001$  and  $P < 0.001$ ;  $n = 3$ ; Fig. 1E and F). Hypoxia also caused an upregulation of VAP-1 protein expression in RHSECs (~1.4-fold increase;  $P < 0.001$ ;  $n = 3$ ; Fig. 1E) and RIMECs (~1.9-fold increase;  $P < 0.001$ ;  $n = 3$ ; Fig. 1F) transduced with AD-AOC3-IRES-EGFP.

Under normoxic conditions, SSAO activity in RHSECs and RIMECs did not differ significantly between blank controls and cells transduced with AD-IRES-EGFP (Fig. 1G and H, respectively). RHSECs transduced with

AD-AOC3-IRES-EGFP had significantly higher SSAO activity than blank controls (~2.9-fold increase;  $P < 0.001$ ;  $n = 3$ ) and cells transduced with AD-IRES-EGFP (~2.7-fold increase;  $P < 0.001$ ;  $n = 3$ ; Fig. 1G). RIMECs transduced with AD-AOC3-IRES-EGFP also had significantly higher SSAO activity than blank controls (~3.4-fold increase;  $P < 0.001$ ;  $n = 3$ ) and cells transduced with AD-IRES-EGFP (~3.2-fold increase;  $P < 0.001$ ;  $n = 3$ ; Fig. 1H). Hypoxia caused an upregulation of SSAO activity in RHSECs (~1.6-fold increase;  $P = 0.002$ ;  $n = 3$ ) and RIMECs (~1.5-fold increase;  $P = 0.002$ ;  $n = 3$ ) transduced with AD-AOC3-IRES-EGFP (Fig. 1G and H, respectively).

*PaO<sub>2</sub>* and *PaCO<sub>2</sub>* in rats following surgically induced hemorrhagic shock or sham surgery. In view of the effects of hypoxia in RHSECs and RIMECs described above, an additional series

of experiments was performed to determine whether changes in *AOC3* mRNA expression and VAP-1 protein expression were observed *in vivo* in a rat model of surgically induced hemorrhagic shock. Normal values for  $\text{PaO}_2$  and  $\text{PaCO}_2$  were measured in the sham group ( $98 \pm 9$  and  $42 \pm 4$  mmHg, respectively;  $n=10$ ; Fig. 2), whereas those in the HS group were markedly reduced ( $43 \pm 6$  and  $32 \pm 7$  mmHg, respectively;  $n=10$ ; Fig. 2), consistent with hemorrhage-induced hypoxia and a compensatory hypocapnia secondary to hypoxic stimulation of ventilation. The  $\text{PaO}_2$  and  $\text{PaCO}_2$  values in the HS/R group were intermediate between those of the HS and sham groups ( $79 \pm 13$  and  $37 \pm 5$  mmHg, respectively,  $n=10$ ; Fig. 2), indicating a partial reversion of hypoxia by the administration of blood and fluids. The administration of 2-BEA had no significant effect on  $\text{PaO}_2$  and  $\text{PaCO}_2$  in all three groups ( $P>0.05$ ; Fig. 2).

***AOC3* mRNA expression and VAP-1 protein expression in rat hepatic and intestinal tissues following surgically-induced hemorrhagic shock or sham surgery.** The expression of *AOC3* mRNA in hepatic tissue was significantly increased in the HS (~4.0-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3A) and HS/R (~3.2-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3A) groups compared with the sham group. Notably, mRNA expression of *AOC3* was significantly reduced in the HS/R group than in the HS group ( $P<0.05$ ; Fig. 3A). VAP-1 protein expression was additionally significantly increased in the HS (~2.2-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3B) and HS/R (~1.8-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3B) groups compared with the sham group. However, although the level of VAP-1 expression reduced in the HS/R group compared with the HS group, the difference was not statistically significant ( $P=0.073$ ; Fig. 3B).

Similar results were obtained in intestinal tissue. *AOC3* mRNA expression was significantly increased in the HS (~3.1-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3C) and HS/R (~2.3-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3C) groups compared with the sham group, and significantly lower in the HS/R group than in the HS group ( $P<0.01$ ; Fig. 3C). VAP-1 protein expression was additionally significantly increased in the HS (~2.4-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3D) and HS/R (~2.0-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 3D) groups compared with the sham group, but did not differ significantly between the HS/R and HS groups ( $P=0.089$ ; Fig. 3D).

**Effect of the SSAO enzyme inhibitor, 2-BEA, on *AOC3* mRNA expression and VAP-1 protein expression in rat hepatic and intestinal tissues.** For hepatic and intestinal tissues, there were no significant differences in *AOC3* mRNA expression or VAP-1 protein expression between the sham and sham-2-BEA groups (Fig. 3). However, in hepatic tissue, *AOC3* mRNA expression was reduced ~1.9-fold in the HS-2BEA group compared with the HS group ( $P<0.001$ ;  $n=10$ ; Fig. 3A), and ~2.0-fold in the HS/R-2-BEA group compared with the HS/R group ( $P<0.001$ ;  $n=10$ ; Fig. 3A). Consistent with the changes in mRNA expression, VAP-1 protein expression in hepatic tissue was decreased ~1.3-fold in the HS-2BEA group compared with the HS group ( $P<0.05$ ;  $n=10$ ; Fig. 3B), and ~1.5-fold in the HS/R-2BEA group compared with the HS/R group ( $P<0.01$ ;  $n=10$ ; Fig. 3B). Similar findings were obtained in intestinal tissue. *AOC3* mRNA expression in intestinal tissue was decreased ~1.9-fold in the HS-2BEA group compared with

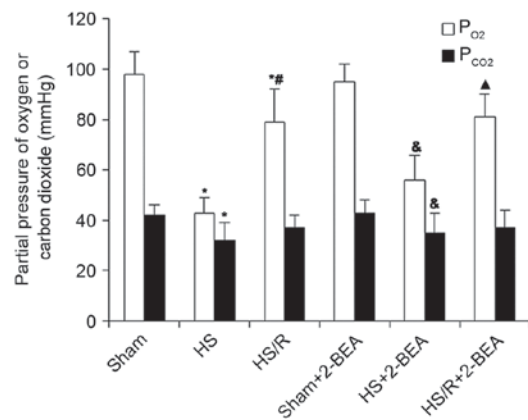


Figure 2. Arterial blood  $\text{PaO}_2$  and  $\text{PaCO}_2$  in rats. Data are presented as the mean  $\pm$  standard deviation ( $n=10$  per group). \* $P<0.05$  vs. sham group; # $P<0.05$  vs. HS group; & $P<0.05$  vs. sham-2-BEA group; ^ $P<0.05$  vs. HS-2-BEA group.  $\text{PaO}_2$ , partial pressure of oxygen;  $\text{PaCO}_2$ , partial pressure of  $\text{CO}_2$ ; sham, sham operation; HS, surgically-induced hemorrhagic shock with no resuscitation; 2-BEA, 2-bromoethylamine; HS/R, surgically-induced hemorrhagic shock with resuscitation.

the HS group ( $P<0.001$ ;  $n=10$ ; Fig. 3C), and ~1.9-fold in the HS/R-2BEA group compared with the HS/R group ( $P<0.001$ ;  $n=10$ ; Fig. 3C). VAP-1 protein expression in intestinal tissue was reduced ~1.4-fold in the HS-2BEA group compared with the HS group ( $P<0.05$ ;  $n=10$ ; Fig. 3D), and ~1.5-fold in the HS/R-2BEA group compared with the HS/R group ( $P<0.05$ ;  $n=10$ ; Fig. 3D).

**Histological observations of VAP-1 protein expression.** Representative immunohistologically stained sections comparing VAP-1 protein expression between the groups are presented in Fig. 4. VAP-1 was mainly distributed in the vascular endothelial cells, primarily in tissues rich in small blood vessels including the liver blood sinus and intestinal mucosal vascular endothelium. The results were similar to those obtained via western blotting.

**Serum SSAO activity in rats following surgically-induced hemorrhagic shock or sham surgery, and the effects of 2-BEA.** Further experiments were undertaken to establish whether alterations in *AOC3* mRNA expression and VAP-1 protein expression described above were reflected by alterations in serum SSAO activity in rats. Serum SSAO activity was significantly increased in the HS (~2.7-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 5) and HS/R (~2.2-fold increase;  $P<0.001$ ;  $n=10$ ; Fig. 5) groups than in the sham group, and significantly reduced in the HS/R group than in the HS group ( $P<0.01$ ; Fig. 5). Compared with the corresponding groups not administered with an SSAO inhibitor, 2-BEA was associated with significant reductions in serum SSAO activity in the sham-2-BEA (~1.6-fold reduction;  $P<0.05$ ;  $n=10$ ), HS-2-BEA (~2.6-fold reduction;  $P<0.001$ ;  $n=10$ ) and HS/R-2-BEA (~2.2-fold reduction;  $P<0.001$ ;  $n=10$ ) groups (Fig. 5).

**Effect of the SSAO inhibitor, 2-BEA, on the 24 h survival rate of rats following surgically-induced hemorrhagic shock or sham surgery.** The 24 h survival rate was 100% (30/30 animals) in the sham and sham-2-BEA groups. In contrast, the

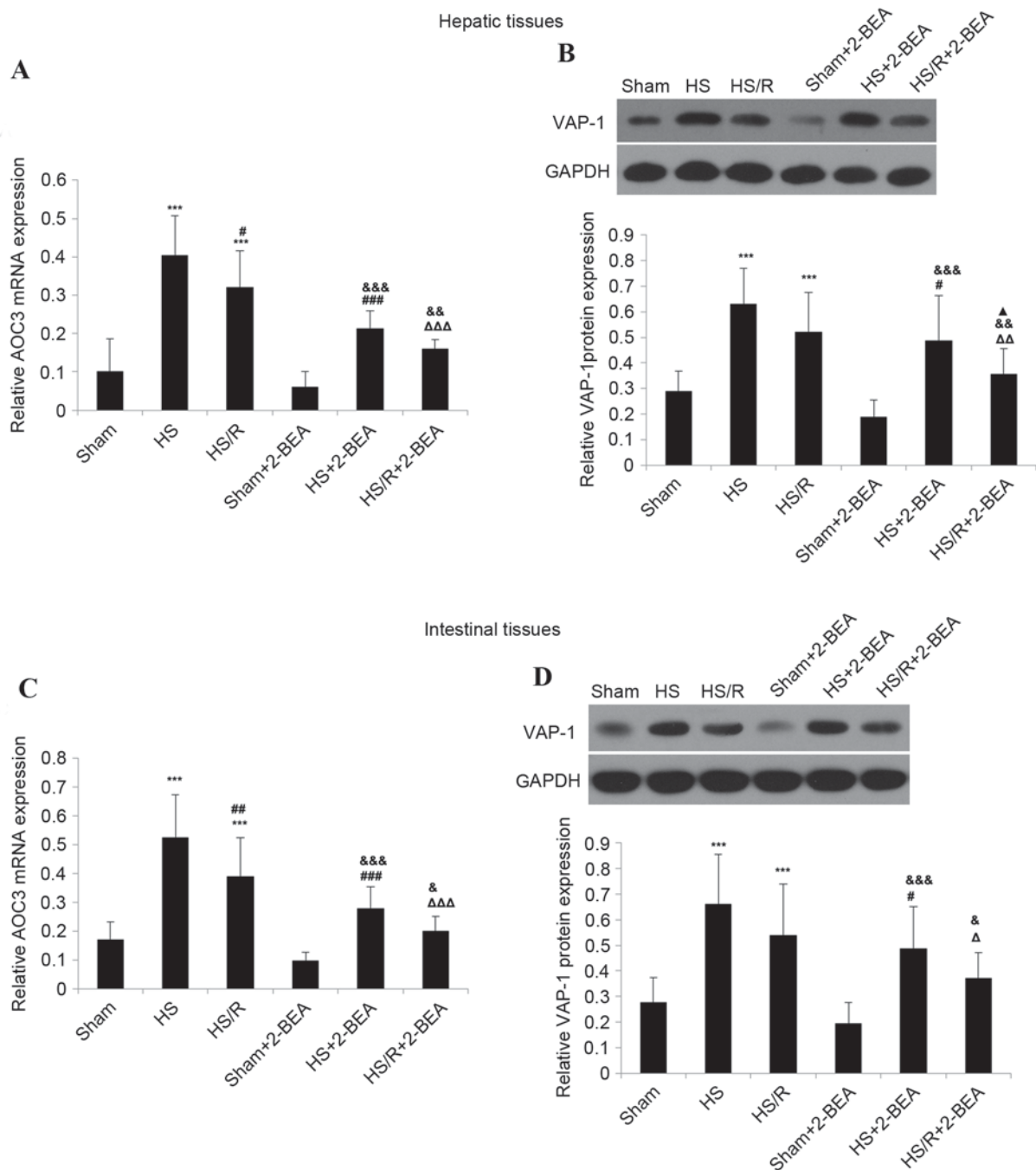


Figure 3. VAP-1 protein expression and *AOC3* mRNA expression in hepatic and intestinal tissues from rats of the various experimental groups, and the effects of 2-BEA. (A) *AOC3* mRNA expression and (B) VAP-1 protein expression in rat hepatic tissue. (C) *AOC3* mRNA expression and (D) VAP-1 protein expression in rat intestinal tissue.  $\beta$ -actin and GAPDH were used as internal controls for reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Data are presented as the mean  $\pm$  standard deviation (n=10 per group). \*\*\*P<0.001 vs. sham group; #P<0.05, ##P<0.01 and ###P<0.001 vs. HS group;  $\Delta$ P<0.05,  $\Delta\Delta$ P<0.01 and  $\Delta\Delta\Delta$ P<0.001 vs. HS/R group; &P<0.05, &&P<0.01 and &&&P<0.001 vs. sham+2-BEA group;  $\Delta$ P<0.05 vs. HS+2-BEA group. VAP-1, vascular adhesion protein-1; *AOC3*, VAP-1 gene; 2-BEA, 2-bromoethylamine; sham, sham operation; HS, surgically-induced hemorrhagic shock with no resuscitation; HS/R, surgically-induced hemorrhagic shock with resuscitation.

24 h survival rate was only 40% (12/30) in the HS group, but was 83.3% (25/30) in the HS/R group. Furthermore, the 24 h survival rate was 56.7% (17/30) in the HS+2-BEA group and 86.6% (26/30) in the HS/R+2-BEA group (Table I).

## Discussion

The main findings of the present study were that *AOC3* mRNA expression and VAP-1 protein expression were upregulated by

hypoxia in RHSECs and RIMECs cultured *in vitro*, and in hepatic and intestinal tissue obtained from an *in vivo* rat model of hemorrhagic shock. Furthermore, hypoxia additionally enhanced serum SSAO activity in rats subjected to experimental hemorrhagic shock. In addition, the SSAO inhibitor, 2-BEA, reduced *AOC3* mRNA expression and VAP-1 protein expression in hepatic and intestinal tissues from rats subjected to experimental hemorrhagic shock, and may improve 24 h survival in rats that did not receive resuscitation following

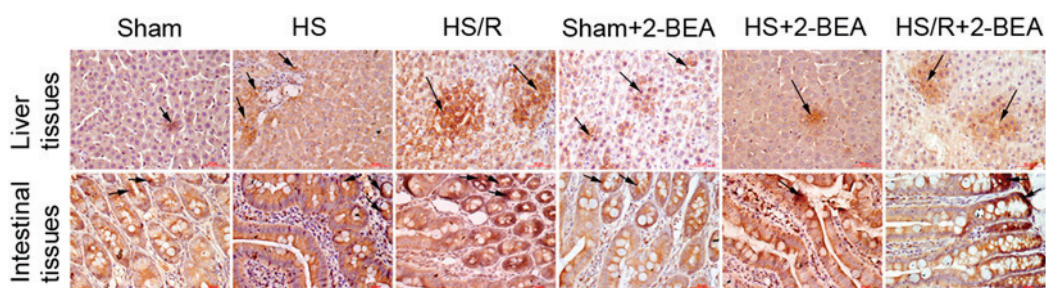


Figure 4. Representative immunohistochemistry images depicting the expression of VAP-1 protein in hepatic and intestinal tissues from rats of the various experimental groups, and the effects of 2-BEA (magnification, x400). VAP-1 was mainly distributed in the vascular endothelial cells, primarily in tissues rich in small blood vessels including the liver blood sinus and intestinal mucosal vascular endothelium. Black arrows indicate the localization of VAP-1. Brown staining represents VAP-1 and purple staining represents hematoxylin. VAP-1, vascular adhesion protein-1; 2-BEA, 2-bromoethylamine; sham, sham operation; HS, surgically-induced hemorrhagic shock with no resuscitation; HS/R, surgically-induced hemorrhagic shock with resuscitation.

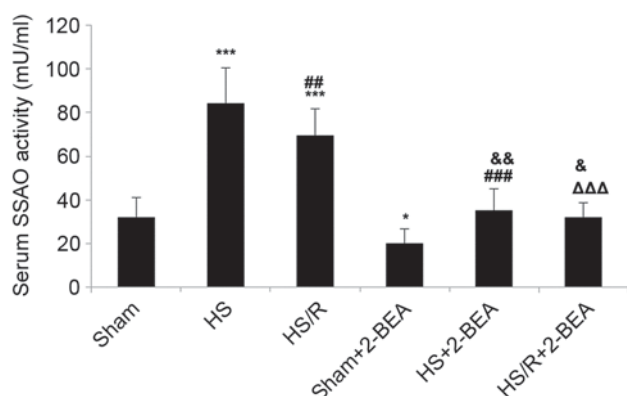


Figure 5. Serum SSAO activity in rats of the various experimental groups, and the effects of 2-BEA. Serum SSAO activity was determined using a fluorometric assay. Data are presented as the mean  $\pm$  standard deviation (n=10 per group). \* $P<0.05$  and \*\*\* $P<0.001$  vs. sham group; ## $P<0.01$  and ### $P<0.001$  vs. HS group; ΔΔΔ $P<0.001$  vs. HS/R group; & $P<0.05$  and && $P<0.01$  vs. sham+2-BEA group. SSAO, semicarbazide-sensitive amine oxidase; 2-BEA, 2-bromoethylamine; sham, sham operation; HS, surgically-induced hemorrhagic shock with no resuscitation; HS/R, surgically-induced hemorrhagic shock with resuscitation.

hemorrhagic shock. Taken together, these observations suggest that hypoxia-induced upregulation of VAP-1 may be one of the mechanisms that contributes to the detrimental physiological effects of hemorrhagic shock.

VAP-1 exists in two distinct forms, as a transmembrane protein in endothelial cells and as a serum protein (9-11). For this reason, VAP-1 protein expression was studied in solid organs containing blood vessels (liver and intestine) and in the serum. The liver and small intestine (including mesentery) were chosen as parenchymal organs that are known to be susceptible to damage following hemorrhagic shock (5,6). The liver, which collects venous blood from abdominal organs, contains abundant vascular endothelial tissue in the hepatic sinusoids (11,24), while high endothelial venules in the mesentery of the small intestine are regarded as having high levels of VAP-1 (9,45,46). In addition, hepatic and intestinal tissues in the abdominal cavity are easily collected, adding to their suitability for the *in vivo* study of VAP-1 expression.

The *in vitro* experiments were performed using RHSECs and RIMECS transduced with the *AOC3* gene, since the expression of *AOC3*/VAP-1 by these cells in culture is normally very

low (18,43). The use of a recombinant adenovirus expressing VAP-1 permitted the study of the effects of hypoxia on VAP-1 expression. These *in vitro* studies revealed that culture of RHSECs or RIMECS under hypoxic conditions resulted in a significant upregulation of *AOC3* mRNA expression and VAP-1 protein expression. This suggested that hypoxia directly induces an increase in VAP-1 levels via enhanced transcription of the *AOC3* gene. These data were supported by those obtained from the *in vivo* rat model of hemorrhagic shock: Maintenance of a low MAP (30-40 mmHg) for 1 h resulted in an upregulation of *AOC3* mRNA expression and VAP-1 protein expression in hepatic and intestinal tissue, and this enhanced expression was inhibited by prompt resuscitation with blood and fluids. These findings indicated that regulation of VAP-1 expression occurred rapidly over a short timescale, and were consistent with a previous study, which reported that hepatic and intestinal *AOC3* expression differed between rats surviving hemorrhagic shock and those that died within 1 h (42). Notably, a previous investigation in endothelial cells expressing VAP-1 demonstrated that VAP-1 increased the susceptibility of these cells to deprivation of oxygen and glucose, and enhanced tissue damage (48). As VAP-1 also mediates leukocyte recruitment via its SSAO activity, the enhanced VAP-1 expression following hemorrhagic shock/hypoxia observed in the present study may increase the susceptibility of the liver and intestines, and potentially other organs, to damage and necrosis.

The mechanism by which hypoxia upregulates *AOC3* mRNA and VAP-1 protein expression remains unclear. Acute hypoxia rapidly activates endothelial cells to release inflammatory mediators and growth factors, and one hypothesis is that the regulation of VAP-1 expression by hypoxia may be mediated by hypoxia-inducible factors (HIFs). HIFs are key transcription factors that alter gene expression in hypoxic environments, and activate multiple cytokines and chemokines via regulation of signal transduction pathways (49,50). However, the pathophysiologic processes underlying severe hemorrhagic shock are complicated and its consequences are reflected in multiple aspects, including alterations in the immune response, coagulation disorders, initiation of a variety of signal transduction pathways and release of numerous pro-inflammatory and anti-inflammatory cytokines. Therefore, cellular hypoxia constitutes just one step in this complex

Table I. Effect of semicarbazide-sensitive amine oxidase enzyme inhibitor 2-BEA on 24 h survival rate in a rat model of hemorrhagic shock and resuscitation.

Group (n=30/group)	24 h survival rate, n (%)
HS	12 (40.0)
HS/R	25 (83.3)
HS+2-BEA	17 (56.7)
HS/R+2-BEA	26 (86.6)

Survival rate of sham group and sham+2-BEA was 100%. Sham group, sham-operation; HS group, surgically-induced hemorrhagic shock with no resuscitation; HS/R group, surgically-induced hemorrhagic shock with resuscitation; sham+2-BEA group, sham-operation, pre-treated with the serum semicarbazide-sensitive amine oxidase enzyme inhibitor 2-bromoethylamine; HS+2-BEA group, surgically-induced hemorrhagic shock with no resuscitation, pre-treated with 2-bromoethylamine; HS/R+2-BEA group, surgically-induced hemorrhagic shock with resuscitation, pre-treated with 2-bromoethylamine.

process, and the upregulation of VAP-1 expression may be due to factors other than hypoxia. Nonetheless, the *in vitro* experiments support the idea that hypoxia is the primary factor that causes increased expression of VAP-1.

A notable finding of the *in vivo* experiments was that hemorrhagic shock was associated with an increase in the activity of serum SSAO, and that this enhancement was attenuated by prompt resuscitation. This suggested that hemorrhagic shock results in the detachment of VAP-1 from the surface of endothelial cells, raising the possibility that circulating VAP-1 may be involved in severe shock. In support of these observations, deprivation of oxygen and glucose has been demonstrated to stimulate the release of sVAP-1, via a mechanism partially mediated by matrix metalloproteinase-2 (48). In addition, an increase in plasma sVAP-1/SSAO activity following intracranial hemorrhage has been reported in humans, and serum sVAP-1/SSAO activity <2.7 pmol/min/mg is an independent predictor of neurologic improvement following hemorrhagic stroke (51).

For competitive inhibitors, increasing the dose of the drug results in the enzyme being almost completely inhibited. 2-BEA is a reversible competitive enzyme inhibitor, and enzyme function is associated with the concentration of 2-BEA. The action time of 2-BEA is short and it needs regular, continuous administration in order to maintain stable blood drug concentration and effect (52,53). In the pre-experiment, three doses were tested: 10, 20 and 30 mg/kg. There were no significant differences in SSAO activity between the 10 mg/kg and control groups. The mortality rate of rats injected with 30 mg/kg was high. Therefore, the 20 mg/kg dose was selected. As expected, the administration of the SSAO inhibitor, 2-BEA, resulted in a decrease in serum SSAO activity in rats, irrespective of whether they were subjected to hemorrhagic shock (with or without resuscitation). However, a notable finding in animals that had received surgically-induced hemorrhagic shock was that 2-BEA additionally reduced the expression of *AOC3* mRNA and

VAP-1 protein in intestinal and liver tissues, while no such effect of 2-BEA was observed in the sham group. This implied that, at least under the conditions of hemorrhagic shock, a degree of positive feedback exists whereby VAP-1 is able to upregulate the expression of its gene. The potential mechanisms underlying this effect remain unknown. However, previous studies have demonstrated that VAP-1 not only serves a pro-inflammatory function through its SSAO activity, but rapidly regulates the expression of inflammatory factors including E-selectin, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein-1 and C-X-C motif chemokine ligand 8, via activation of signaling pathways including nuclear factor- $\kappa$ B, phosphoinositide 3-kinase and mitogen-activated protein kinases (54,55). Nevertheless, there is a possibility that chronic treatment with 2-BEA may alter other molecular targets or molecular pathways responsible for the decreased *AOC3* and VAP-1 expression. Further studies are warranted to elucidate the mechanisms by which the SSAO activity of VAP-1 leads to upregulation of *AOC3* mRNA and VAP-1 protein expression during hemorrhagic shock.

Another notable finding was that 2-BEA improved 24 h survival in rats subjected to hemorrhagic shock without resuscitation. This finding is consistent with numerous studies demonstrating a benefit of SSAO inhibition in intracranial hemorrhage/ischemia. In a rat model of subarachnoid hemorrhage, SSAO inhibition has been reported to decrease leukocyte trafficking and improve neurologic outcome (56), and prevent dysfunction of arteriolar dilation, potentially via suppression of neutrophil recruitment (57). Similarly, SSAO inhibition has been demonstrated to reduce neutrophil extravasation and infarct volume in a rat middle cerebral artery occlusion model (58). In addition, inhibitors of SSAO have been demonstrated to reduce neurobehavioral deficits and brain edema following intracerebral hemorrhage, potentially via a mechanism involving reduced neutrophil infiltration, microglia/macrophage activation, and expression of ICAM-1, monocyte chemoattractant protein-1 and tumor necrosis factor- $\alpha$  (59). Previous clinical data have revealed increased VAP-1 expression in the inflammatory response of local vasculature (9,11,18). Furthermore, anti-VAP-1 mAbs and SSAO inhibitors have been demonstrated to partially restrain endovascular extravasation of lymphocytes, neutrophils, and macrophages into the peritoneum, liver, pancreas and other tissues during an inflammatory response (28,29,60), and to suppress the inflammatory response in conditions including colitis, skin infection, ischemia-reperfusion injury, sepsis, pulmonary infection, arthritis and experimental allergic cerebrospinal meningitis (19,22,30,31). Thus, VAP-1 is an important factor in the inflammatory cascade (17,61), and its upregulation during hemorrhagic shock may contribute to the detrimental processes that result in tissue damage.

In conclusion, the present study provided evidence that VAP-1 expression is upregulated by hypoxia during acute hemorrhagic shock, and that inhibition of VAP-1 may be beneficial in the setting of hemorrhagic shock. Further studies are required to confirm these findings and to establish whether VAP-1 may be a valid target for the development of novel therapies for hemorrhagic shock.

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