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EARLY ORGAN-SPECIFIC ENDOTHELIAL ACTIVATION DURING HEMORRHAGIC SHOCK AND RESUSCITATION

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ABSTRACT—Multiple organ dysfunction syndrome (MODS) is a complication of hemorrhagic shock (HS) and related to high morbidity and mortality. Interaction of activated neutrophils and endothelial cells is considered to play a prominent role in the pathophysiology of MODS. Insight in the nature and molecular basis of endothelial cell activation during HS can assist in identifying new rational targets for early therapeutic intervention. In this study, we examined the kinetics and organ specificity of endothelial cell activation in a mouse model of HS. Anesthetized male mice were subjected to controlled hemorrhage to a MAP of 30 mmHg. Mice were killed after 15, 30, 60, or 90 min of HS. After 90 min of hemorrhagic shock, a group of mice was resuscitated with 6% hydroxyethyl starch 130/0.4. Untreated mice and sham shock mice that underwent instrumentation and 90 min of anesthesia without shock served as controls. Gene expression levels of inflammatory endothelial cell activation (P-selectin, E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1) and hypoxia-responsive genes (vascular endothelial growth factor and hypoxia-inducible factor 1α) were quantified in kidney, liver, lung, brain, and heart tissue by quantitative reverse-transcription–polymerase chain reaction. Furthermore, we examined a selection of these genes with regard to protein expression and localization using immunohistochemical analysis. Induction of inflammatory genes occurred early during HS and already before resuscitation. Expression of adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial genes CD31 and VE-cadherin, which function in endothelial cell homeostasis and integrity, were not affected during the shock phase except for VE-cadherin in the liver, which showed increased mRNA levels. The rapid inflammatory activation was not paralleled by induction of hypoxia-responsive genes. This study demonstrated the occurrence of early and organ-specific endothelial cell activation during hemorrhagic shock, as presented by induced expression of inflammatory genes. This implies that early therapeutic intervention at the microvascular level may be a rational strategy to attenuate MODS.

KEYWORDS—Hemorrhagic shock, inflammatory gene expression, endothelial cells, hypoxia

INTRODUCTION

The development of multiple organ dysfunction syndrome (MODS) after hemorrhagic shock (HS) is a problem in the care of patients who suffer major bleeding. MODS contributes significantly to morbidity and mortality (1). Advances in medical care of HS patients, including the introduction of resuscitation fluids, trauma centers, and intensive care units, have resulted in a significant decrease in early deaths caused by HS. Concomitant with recovering from a previously fatal condition, the systemic inflammatory response initiated by HS became manifest (1). The inflammatory response is nowadays considered the leading cause for the development of MODS.

Although the precise mechanisms and pathways leading to organ injury after HS are still unknown, the neutrophil is thought to be a principal cellular mediator of tissue damage. Migration of neutrophils into tissue during HS leads to significant organ damage through release of proteases and oxygen-derived radicals (2). The interaction between neutrophils and endothelium is instrumental in the migration of neutrophils into different tissues (3). This migration is regulated by adhesion molecules on both leukocytes and endothelium, the latter including P-selectin, E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1).

Most studies concerning MODS after HS have focused on the postresuscitation period in which the shock is compensated by volume infusion. A few studies showed the occurrence of early cytokine production and activation of intracellular signaling pathways that are well-established triggers of endothelial cell activation (4–7). Increased expression of P-selectin in liver and lung after 90 min of HS induction implicates early endothelial responses (8), which might be induced by hypoxia (6, 9). Based on these studies, we hypothesized that HS rapidly activates endothelial cells toward a proinflammatory status. Early endothelial activation can imply options for an early therapeutic window. Considering the organ-specific involvement in HS-associated MODS, this activation likely presents differently in different organs. It is imperative to unravel the kinetics and pathophysiological mechanisms of endothelial activation to develop specific therapeutic strategies that can either prevent or attenuate the effects of HS on organ function and related morbidity and mortality.

The aim of this study was to investigate the kinetics, profile, and organ specificity of endothelial cell activation during the earliest phase of HS. We used a mouse model of controlled arterial pressure of 30 mmHg during a period of 90 min, after which designated groups of mice were resuscitated. We examined the expression levels of a series

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of adhesion molecules (P-selectin, E-selectin, VCAM-1, and ICAM-1) known to contribute to leukocyte recruitment, hypoxia-related genes hypoxia-inducible factor (HIF) 1α and vascular endothelial growth factor (VEGF) A, and the proinflammatory cytokine TNF-α by quantitative reverse-transcription–polymerase chain reaction (RT-PCR). Furthermore, immunohistochemistry was performed to detect endothelial adhesion molecule and HIF-1α proteins. Besides information on the extent of activation, this approach provided details regarding the microvascular location of endothelial activation within the organs.

MATERIALS AND METHODS

Animals

Eight- to 12-week-old C57BL/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, the Netherlands). Mice were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-h light/dark cycle. All procedures performed were approved by the local committee for care and use of laboratory animals and were performed according to strict governmental and international guidelines on animal experimentation.

HS model

Mice were anesthetized with isoflurane (inspiratory, 1.4%), N_2O (66%), and O_2 (33%). Throughout the experiment, mice were breathing spontaneously. The animals were kept on a temperature-controlled surgical pad (37–38°C). The left femoral artery was cannulated with polyethylene tubing with an internal diameter of 0.28 mm and an external diameter of 0.61 mm for monitoring MAP, blood withdrawal, and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg within 15 to 30 min. Blood was collected in a heparinized 1-mL syringe to prevent clotting. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain MAP at 30 mmHg during this period. Mice were killed 15, 30, 60, or 90 min after MAP of 30 mmHg was achieved. In Europe, colloidal infusion fluids are frequently used in the resuscitation of shock victims. We therefore resuscitated, after 90 min of HS, additional groups of mice with 6% hydroxyethyl starch 130/0.4 (Voluven; Fresenius-Kabi, Bad Homburg, Germany), two times the volume of the blood withdrawn. 1, 4, or 24 h after volume resuscitation, these mice where killed. Control mice were left untreated and received anesthesia only before they were killed. Sham shock mice underwent instrumentation and were kept under anesthesia for 90 min; however, no blood was withdrawn. The HS 90-min shock and HS sham shock group consisted of five animals, whereas the other groups consisted of three animals per group. After the experimental procedure, the brain, heart, lungs, liver, and kidney were harvested, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

RNA isolation and quantitative RT-PCR

RNA was extracted from 20 (liver, brain, and kidney) or 30 (heart and lung) 5-μm-thick cryosections and isolated using the RNeasy Mini Kit (Qiagen, Westburg b.v., Leusden, The Netherlands), according to the manufacturer's instructions. Integrity of RNA was determined by gel electrophoreses. RNA yield (OD260) and purity (OD260/OD280) were measured by an ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE).

One microgram of total RNA was subsequently used for the synthesis of first-strand cDNA with SuperScript III RNase minus reverse transcriptase (Invitrogen, Breda, The Netherlands) in a 20-μL final volume containing 250 ng of random hexamers (Promega Benelux, Leiden, The Netherlands) and 40 units of RNase OUT inhibitor (Invitrogen). After the RT reaction, 1 μL cDNA was used for each PCR reaction. Intron-overspanning primers and minor groove binder probes used for quantitative RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJsel, The Netherlands), primers included housekeeping gene β-2 microglobulin (assay ID Mm00437762_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915_g1), endothelial cell marker CD31 (platelet endothelial cell adhesion molecule 1, Mm00476702_m1), VE-cadherin (Mm00486938_m1), VEGF-a (Mm00437304_m1), HIF-1α (Mm00468869_m1), and P-selectin (Mm00441295_m1). TNF-α (Mm00443258_m1) primers and probes were purchased as predeveloped assay reagent for gene expression (Applied Biosystems). Primers and probes for E-selectin (forward primer, 5′-CAACGTCTAGGTTCAAAACAATCAG-3′; probe, 5′-CACAAATGCAATCGTGGGA-3′; reverse primer, 5′-TTAAGCAGGCAAGAGGAACCA-3′), ICAM-1 (forward primer, 5′-ATGGGAATGTCACCAGGAATG-3′; probe, 5′-CAGTACTGTACCACTCTC-3′; reverse primer, 5′-GCACCAGAATGATTATAAGTCCAGTTATT-3′), and VCAM-1 (forward primer, 5′-TGAAGTTGGCTCACAATTAAGAAGTT-3′; probe, 5′-AACACTTGATGTAAAAGGA-3′; reverse primer, 5′-TGCGCAGTAGTAGAGTGCAAGGA-3′) were purchased as Assay-by-Design from Applied Biosystems using the same quality criteria as for Assay-on-Demand.

The final concentrations of primers and minor groove binder probes in TaqMan PCR MasterMix (Applied Biosystems, Foster City, Calif) for each gene were 900 and 250 nM, respectively. Water was used as a negative control to exclude unspecific signals arising from impurities and consistently showed no amplification signals.

TaqMan real-time RT-PCR was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. Triplicate real-time RT-PCR analyses were executed for each sample, and the obtained threshold cycle values (C_t) were averaged. According to the comparative C_t method described in the ABI manual (http://www.appliedbiosystems.com), gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the $\triangle C_t$ value. The average $\triangle C_t$ value obtained from control, a nontreated mouse, was then subtracted from the average $\triangle C_t$ value of each sample subjected to the experimental conditions described, yielding the $\triangle\triangle C_t$ value. The gene expression level, normalized to the housekeeping gene and relative to the control, was calculated as $2^{-\triangle\triangle C_t}$.

Localization of adhesion molecule expression, leukocyte infiltration, and HIF-1α using immunohistochemistry

Localization of P-selectin, E-selectin, VCAM-1, and ICAM-1 expression was determined in the kidney, liver, heart, lung, and brain by immunohistochemistry. Leukocyte recruitment was analyzed by immunohistochemical staining with an anti-CD45 pan leukocyte marker antibody. Frozen organs were cryostat-cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 min. After drying, sections were incubated for 45 min at room temperature with primary rat antimouse antibodies

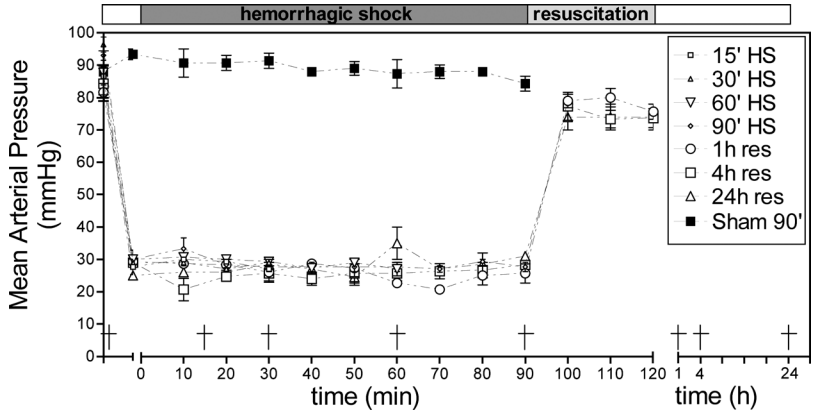

FIG. 1. **Study design and measured mean arterial pressure during experiment.** Hemorrhage was induced by blood withdrawal as described in "Materials and Methods". Mice were sacrificed (†) at start of experiment, or after 15, 30, 60 or 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a group of mice was resuscitated. Sham shock mice (■) underwent instrumentation and 90 min of anesthesia. Blood pressure is expressed as mean ± SEM, (n = 5 in the 90' HS group and 90' sham shock, other groups n = 3).

TABLE 1. **Expression levels of genes investigated in this study in healthy mouse organs**

	Kidney	Liver	Heart	Lung	Brain
CD31	5.9 (5.5–6.2)	7.1 (6.8–7.4)	5.5 (5.0–5.6)	0.9 (0.5–1.6)	7.2 (7.2–7.4)
VE-Cadherin	6.0 (5.7–6.1)	6.4 (6.3–7.7)	4.4 (4.2–4.6)	−0.3 (−0.6 to 0.5)	7.1 (7.1–7.2)
P-Selectin	14.9 (14.6–15.7)	12.4 (11.6–12.4)	14.4 (12.9–14.8)	8.3 (8.2–8.8)	18.0 (17.0–18.0)
E-Selectin	14.6 (14.4–14.7)	17.0 (16.8–17.3)	14.2 (13.6–14.4)	12.3 (11.5–12.6)	18.6 (18.1–18.9)
VCAM-1	9.4 (9.3–9.5)	8.9 (8.6–9.6)	10.2 (9.3–10.2)	6.4 (5.3–6.9)	8.2 (8.1–8.5)
ICAM-1	7.6 (7.4–8.1)	7.7 (7.6–8.3)	10.4 (9.9–10.7)	2.4 (2.1–3.1)	10.7 (10.1–11.1)
HIF-lα	6.1 (5.5–6.4)	7.0 (6.6–7.3)	7.5 (6.0–8.0)	4.4 (4.4–5.0)	6.3 (6.3–6.5)
VEGF-A	4.3 (4.1–4.3)	4.5 (4.1–4.8)	5.7 (4.8–5.7)	0.8 (0.8–1.6)	6.0 (5.9–6.2)

Expression of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in control mice not subjected to any experimental procedure was determined in the lung, liver, kidney, heart, and brain using quantitative RT-PCR with GAPDH as housekeeping gene. Data represent values from three animals per group expressed as median ΔC_T (minimum-maximum).

TABLE 2. **Kinetics of EC activation before resuscitation**

		0	30 min	60 min	90 min	Sham 90 min
CD31	Lung	1.0 (0.6–1.3)	1.4 (0.2–1.9)	0.6 (0.4–2.0)	0.6 (0.1–1.7)	0.4 (0.1–1.5)
	Liver	0.8 (0.7– 1.0)	0.5 (0.4–0.8)	0.6 (0.6–0.6)	0.7 (0.4–1.1)	0.8 (0.6–1.0)
	Kidney	1.0 (0.8–1.3)	0.7 (0.4–0.8)	0.8 (0.5–1.5)	0.5 (0.5–0.7)	0.7 (0.6–0.8)
	Heart	1.1 (1.0–1.5)	1.0 (1.0–1.0)	1.3 (1.1–1.3)	1.2 (0.9–1.3)	1.5 (0.2–2.7)
	Brain	1.0 (0.9–1.0)	0.6 (0.4–0.7)	0.7 (0.6–0.7)	0.7 (0.4–1.1)	0.6 (0.6–0.9)
VE-Cadherin	Lung	1.0 (0.6–1.2)	1.2 (1.2–1.6)	0.5 (0.4–1.8)	0.7 (0.1–1.7)	0.3 (0.1–1.7)
	Liver	2.4 (1.0–2.7)	3.3 (2.5–3.4)	3.1 (3.1–3.2)	3.3 (3.0–7.8)†	4.3 (2.7–5.0)
	Kidney	1.0 (1.0–1.3)	0.9 (0.2–1.0)	1.2 (1.2–1.5)	1.2 (0.9–1.3)	0.8 (0.7–1.0)
	Heart	1.0 (0.9–1.2)	1.1 (1.0–1.1)	1.2 (1.2–1.4)	0.9 (0.9–1.2)	1.3 (0.2–2.4)
	Brain	1.0 (0.9–1.0)	0.7 (0.5–1.0)	1.0 (0.8–1.0)	0.8 (0.5–1.4)	0.9 (0.7–1.2)
P-Selectin	Lung	1.0 (0.8–1.1)	2.3 (0.2–2.0)	1.4 (1.4–7.2)	8.4 (1.1–60)*	1.5 (0.5–2.1)
	Liver	0.6 (0.6–1.0)	0.7 (0.6–1.7)	1.4 (1.0–1.5)	10 (1.8–32)	3.1 (0.4–7.4)
	Kidney	1.0 (0.6–1.2)	2.1 (0.9–4.2)	5.2 (4.7–12)	25 (3.2–112)	4.7 (0.6–7.4)
	Heart	0.4 (0.3–1.0)	0.6 (0.6–3.0)	1.3 (0.5–1.6)	15 (6.4–70)	3.7 (0.5–11)
	Brain	0.5 (0.5–1.0)	1.7 (1.0–2.5)	7.4 (6.5–8.7)	66 (28–397)	26 (1.3–63)
E-Selectin	Lung	1.0 (0.8–1.6)	1.5 (0.4–127)	7.6 (5.6–26)	54.5 (9.0–301)*†	2.5 (2.2–11)
	Liver	1.2 (1.0–1.4)	9.3 (3.4–778)	135 (106–176)	666 (472–2,289)†	637 (1.2–1,261)
	Kidney	1.0 (1.0–1.2)	7.4 (2.6–63)	39 (18–90)	133 (18–313)*†	30 (1.6–64)
	Heart	1.1 (1.0–1.7)	2.9 (2.3–25)	4.7 (3.5–8.8)	52 (16–92)	3.5 (1.8–52)
	Brain	1.0 (0.8–1.4)	9.8 (1.4–25)	34 (21–36)	124 (47–477)*†	66 (3.9–156)
VCAM-1	Lung	1.0 (0.7–2.1)	2.8 (0.8–15)	3.6 (2.0–6.2)	6.4 (1.6–29)*†	2.0 (0.9–3.0)
	Liver	0.8 (0.5–1.0)	2.5 (1.2–3.4)	3.4 (2.7–3.4)	4.9 (4.3–9.4)†	10.8 (1.9–13)
	Kidney	1.0 (0.9–1.1)	1.3 (1.2–3.5)	3.1 (2.5–3.8)	6.0 (1.4–15)*†	3.8 (0.7–5.3)
	Heart	1.0 (1.0–1.9)	1.5 (1.3–8.7)	3.4 (2.0–4.9)	12.5 (5.4–17)	3.6 (0.6–15)
	Brain	1.0 (0.8–1.1)	0.6 (0.6–0.8)	0.8 (0.6–1.1)	1.8 (1.4–2.1)*†	1.0 (0.8–1.1)
ICAM-1	Lung	1.0 (0.6–1.2)	1.9 (0.5–5.1)	1.0 (0.7–3.0)	3.1 (0.5–19)*	0.6 (0.3–1.9)
	Liver	1.0 (0.6–1.0)	0.7 (0.7–6.9)	3.9 (3.8–5.9)	24 (17–41)†	37 (0.6–43)
	Kidney	1.0 (0.7–1.1)	1.4 (1.2–4.4)	5.5 (3.1–6.7)	12 (3.7–24)*†	3.3 (0.8–4.4)
	Heart	1.2 (1.0–1.8)	3.9 (2.7–30)	12 (6.2–14)	82 (25–195)	6.2 (3.5–125)
	Brain	1.0 (0.7–1.5)	1.8 (0.7–2.8)	2.6 (1.7–4.1)	12 (6.6–33)*†	5.1 (1.6–7.9)

Expression levels of P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in the lung, liver, kidney, heart, and brain analyzed by quantitative RT-PCR using GAPDH as housekeeping gene. RNA levels were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Data are expressed as median (minimum-maximum) (n = 5 in the 90-min HS group and 90-min sham shock; other groups, n = 3).
*$P < 0.05$, 90 min of HS compared with 90 min of sham shock (sham, 90 min).
†$P < 0.05$, 90 min of HS compared with control, as described in "Materials and methods."

anti–E-selectin (MES-1; kindly provided by Dr. D. Brown, Celletech Group, Slough, UK), anti-CD31 (clone MEC13.3; Pharmingen BD Biosciences, Alphen aan den Rijn, The Netherlands), anti-CD45 leukocyte common antigen (Pharmingen BD Biosciences), anti–VCAM-1 (clone M/K-1.9; ATCC, Manassas Va), and anti–ICAM-1 (clone YN1/1.7; ATCC) in the presence of 5% fetal calf serum. After washing, endogenous peroxidase was blocked by incubation with 0.1% H_2O_2 in phosphate-buffered saline (PBS) for 20 min. This was followed by incubation of 30 min at room temperature with horseradish peroxidase–conjugated secondary antibodies (rabbit anti-rat–immunoglobulin G; DAKO, Glostrup, Denmark). Conjugates were diluted 1:50 in PBS supplemented with 2% normal mouse serum. Sections with isotype-matched controls, E-selectin, and VCAM-1–specific antibodies were further incubated for 30 min at room temperature with horseradish peroxidase–conjugated goat antirabbit antibody (Southern Biotech Association, Birmingham, Ala) diluted 1:100 in PBS. Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma-Aldrich Chemie, St. Louis, Mis), and sections were counterstained with Mayer hematoxylin (Klinipath, Duiven, The Netherlands). No immunostaining was observed with isotype-matched controls, demonstrating specificity of staining with the antigen-specific antibodies.

For HIF-1α staining, 5-μm acetone-fixed cryosections were incubated for 45 min at room temperature with primary HIF-1α goat polyclonal antibody (HIF-1α [Y-15]: sc-12542, Santa Cruz Biotechnology, Santa Cruz, Calif) in the presence of 5% fetal calf serum in PBS. Endogenous biotin was blocked by Biotin Blocking System (DAKO) according to manufacturer's protocol, and peroxidase activity was blocked by incubation with 0.1% H_2O_2 in PBS for 10 min. Subsequently, sections were incubated at room temperature for 45 min with rabbit antigoat antibody (DAKO; dilution 1:100 in PBS) in the presence of 2% normal mouse serum. Further staining procedure was performed as described above. Negative control samples were incubated with PBS instead of the primary antibody.

Statistical analysis

Statistical significance of differences was studied by means of the ANOVA with post hoc least significant difference. Differences were considered to be significant when $P < 0.05$.

RESULTS

Hemodynamic changes induced by HS

Figure 1 illustrates the time course of MAP during the experiment. All groups of mice exhibited initial MAP values

TABLE 3. **Kinetics of EC activation after resuscitation**

		90 min HS	1 h	4 h	24 h
CD31	Lung	0.6 (0.1–1.7)	0.1 (0.1–0.4)	0.5 (0.1–0.9)	0.8 (0.4–1.7)
	Liver	0.7 (0.4–1.1)	0.5 (0.5–0.8)	0.5 (0.4–0.5)	0.9 (0.6–1.0)
	Kidney	0.5 (0.5–0.7)	0.4 (0.4–0.4)	0.6 (0.3–1.0)	1.3 (0.8–2.7)
	Heart	1.2 (0.9–1.3)	1.0 (0.1–1.3)	0.7 (0.5–0.8)	2.0 (1.5–2.3)
	Brain	0.7 (0.4–1.1)	0.7 (0.6–0.7)	1.1 (0.5–1.2)	2.1 (1.1–2.9)
VE-Cadherin	Lung	0.7 (0.1–1.7)	0.2 (0.2–1.7)	0.7 (0.2–1.1)	0.7 (0.2–1.0)
	Liver	3.3 (3.0–7.8)	7.6 (4.9–8.1)*	6.6 (4.8–9.3)	3.0 (2.4–3.8)
	Kidney	1.2 (0.9–1.3)	1.6 (1.6–1.9)*	2.6 (2.4–2.9)	1.7 (0.8–2.1)
	Heart	0.9 (0.9–1.2)	0.8 (0.1–1.1)	0.9 (0.5–1.3)	1.5 (1.2–1.8)
	Brain	0.8 (0.5–1.4)	1.7 (1.1–1.7)	1.7 (0.9–3.2)	2.1 (1.1–2.4)
P-selectin	Lung	8.4 (1.1–60)	25 (18–65)	6.8 (5.3–28)	2.0 (1.9–6.3)
	Liver	10 (1.8–32)	118 (74–174)*	62 (6.0–75)	0.8 (0.5–1.2)
	Kidney	25 (3.2–113)	264 (255–311)*	94 (33–214)	9.3 (2.2–11)
	Heart	15 (6.4–70)	222 (33–287)*	119 (4.3–184)	2.1 (1.2–2.7)
	Brain	66 (28–398)	482 (253–9O1)*	26 (7.5–138)	13 (5.8–173)
E-selectin	Lung	55 (9.0–301)	103 (82–234)	3.9 (3.0–9.2)	6.2 (2.7–17)
	Liver	666 (473–2,290)	3,042 (2,196–3,059)*	230 (24–379)	6.3 (1.7–7.4)
	Kidney	133 (18–313)	252 (236–465)*	17 (9.5–46)	1.5 (0.6–1.7)
	Heart	52 (15.8–92)	492 (111–795)*	98 (1.5–301)	5.1 (1.6–7.5)
	Brain	124 (47–474)	287 (245–419)	30 (2.3–36)	40 (8.5–151)
VCAM-1	Lung	6.4 (1.6–29)	2.7 (2.6–7.3)	2.1 (2.0–6.4)	1.8 (1.6–2.7)
	Liver	4.9 (4.3–9.4)	12 (12–16)*	4.6 (2.5–7.8)	0.9 (0.9–1.0)
	Kidney	6.0 (1.4–15)	23 (21–27)*	6.4 (3.3–15)	1.5 (0.6–1.8)
	Heart	13 (5.4–17)	37 (2.5–46)*	11 (0.8–2)	1.9 (1.5–2.2)
	Brain	1.8 (1.4–2.1)	2.8 (2.2–3.3)*	0.8 (0.5–1.1)	1.4 (0.9–1.4)
ICAM-1	Lung	3.1 (0.5–19)	3.3 (2.9–7.8)	1.2 (0.9–5.8)	1.2 (0.9–2.8)
	Liver	24 (17–41)	33 (26–41)	9.7 (3.7–14)	1.3 (1.0–1.4)
	Kidney	12 (3.7–24)	25 (22–26)*	16 (4.2–20)	1.6 (0.7–2.6)
	Heart	82 (25–1 95)	356 (45–365)*	129 (4.4–236)	7.2 (2.2–9.1)
	Brain	12 (6.6–33)	27 (24–29)*	2.1 (1.4–11)	4.2 (1.4–6.6)

Expression levels of CD31, VE-cadherin, P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA in the lung, liver, kidney, heart, and brain analyzed by quantitative RT-PCR using GAPDH as housekeeping gene. The RNA levels of adhesion molecules were normalized to their respective levels in RNA isolates of healthy organs obtained from untreated control mice. Data are expressed as median (minimum-maximum) (n = 5 in the 90-min HS group; other groups, n = 3). Hemorrhagic shock (90 min) was compared with 1 h postresuscitation group (1 h). $P < 0.05$ values are marked with * as described in "Materials and methods."

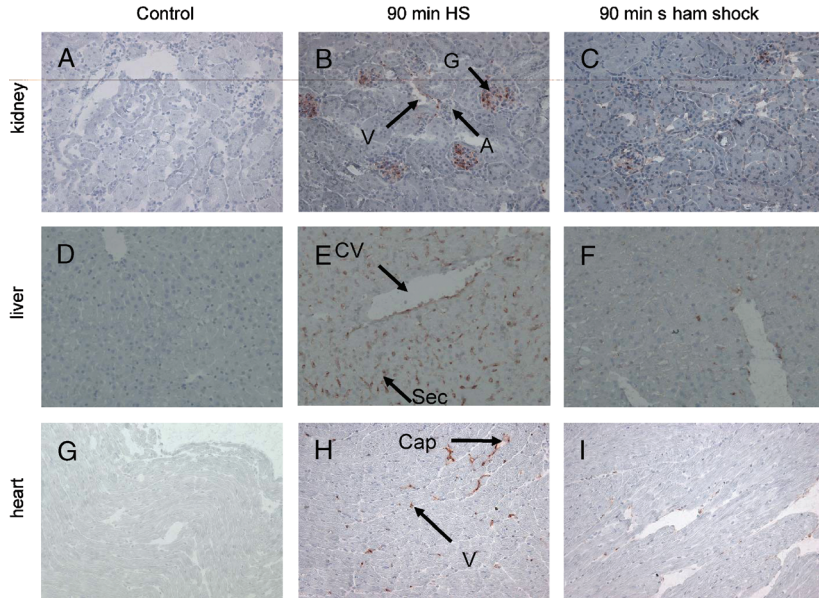

FIG. 2. **Localization of E-selectin expression in kidney, liver and heart during the early phase of HS.** Immunohistochemical detection of E-selectin in healthy mouse tissue (A, D, G), after 90 minutes of HS (B, E, H), and after 90 min of sham shock (C, F, I). Staining was performed respectively on kidney (A–C), liver (D–F), and heart (G–I). Original magnification 200×. E-selectin is stained red, with increased staining in blood vessels after 90 minutes of shock. Arrows indicate: G = Glomerulus, V = venule, A = arteriole, CV= Liver central vene, Sec = Liver sinusoidal endothelium, Cap = capillary.

in the range of 75 to 100 mmHg. In sham shock mice, the MAP remained stable during 90 min between 80 and 100 mmHg. In the HS group, the MAP was maintained between 20 and 40 mmHg. At the end of resuscitation, all groups experienced a comparable increase in MAP, which nevertheless did not reach the baseline values observed before HS induction. All mice survived the procedure until the end of the experiments.

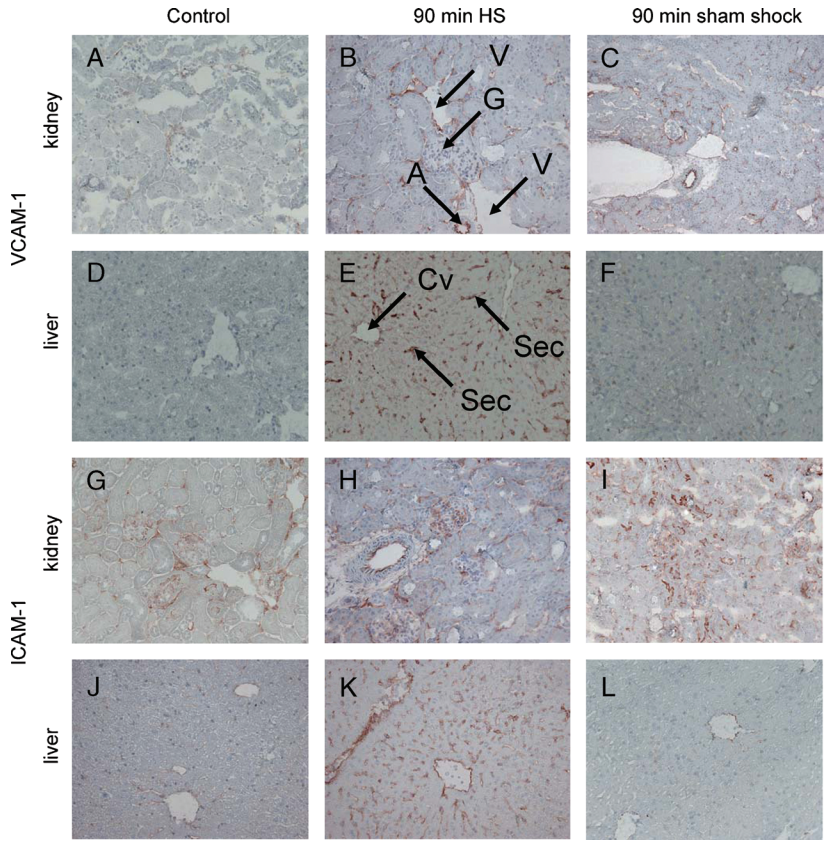

FIG. 3. **Localization of VCAM-1 and ICAM-1 expression in kidney and liver during the early phase of HS.** Immunohistochemical detection of VCAM-1 and ICAM-1 in healthy mouse tissue (A, D, G, J), after 90 minutes of HS (B, E, H, K), and after 90 min of sham shock (C, F, I, L). Staining was performed respectively on kidney (A–C, G–I) and liver (D–F, J–K). Original magnification 200×. VCAM-1 and ICAM-1 and CD31 are stained red, with increased staining in blood vessels after 90 minutes of shock.

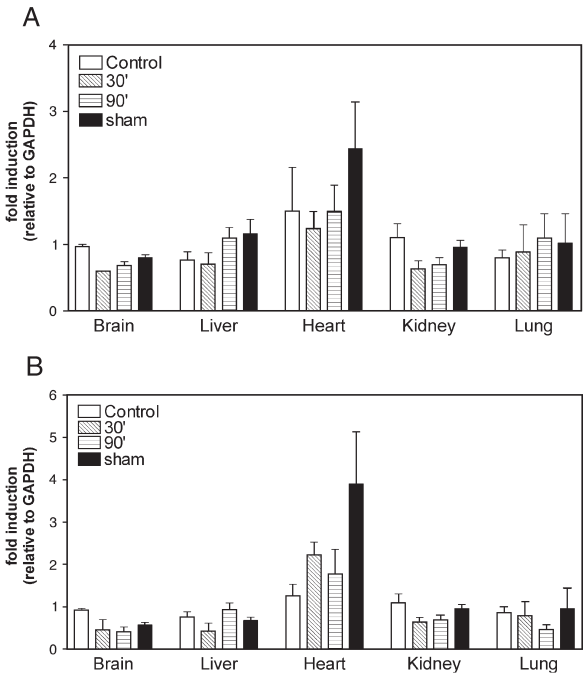

FIG. 4. **Effect of hemorrhagic shock on hypoxia related markers.** Gene expression of HIF-1α (A) and VEGF-A (B) was determined in lung, liver, kidney, heart, and brain by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold induction of hypoxia related genes in comparison to the level of activity that was present in RNA isolates of normal healthy tissue. Data presented are the mean ± SEM of each group (n = 5 in the 90' HS group and 90' sham shock, other groups n = 3). 90 minutes of HS was compared with 90 minutes of sham shock (sham 90'), no significant differences were found.

Baseline gene expression levels in different organs

Quantitative RT-PCR was used to quantitatively measure the basal and HS-affected mRNA levels of adhesion molecules and the hypoxia-related genes *HIF-1α* and *VEGF-A* in the different organs. In control mice (Table 1), the levels of mRNA of the genes under study differed between genes within one organ and between organs. CD31 and VE-cadherin expression levels within one organ were within the same magnitude. The low $\triangle C_t$ values of endothelial marker genes *CD31* and *VE-cadherin* in the lung (representing high mRNA levels) most likely reflects the high degree of vascularization in this organ compared with the other organs. Although the $\triangle C_t$ values do not directly represent protein levels, these data do imply that organ-specific vascular heterogeneity in microvascular endothelial cell status exists.

Kinetics of expression of endothelial adhesion molecules and hypoxia-related genes during the shock phase

The kinetics of endothelial cell activation during onset and maintenance of HS were studied by quantitative analysis of P-selectin, E-selectin, VCAM-1, and ICAM-1 mRNA levels (Table 2). P-Selectin, E-selectin, and ICAM-1 were strongly up-regulated at 90 min after initiation of HS compared with sham shock. Furthermore, E-selectin expression in the kidney and the lungs especially showed a trend toward early induction. Although the early increase was statistically not significant compared with the activation induced by 90 min of sham shock, it did show statistical significance when compared with control mice. Neither CD31 nor VE-cadherin expression changed

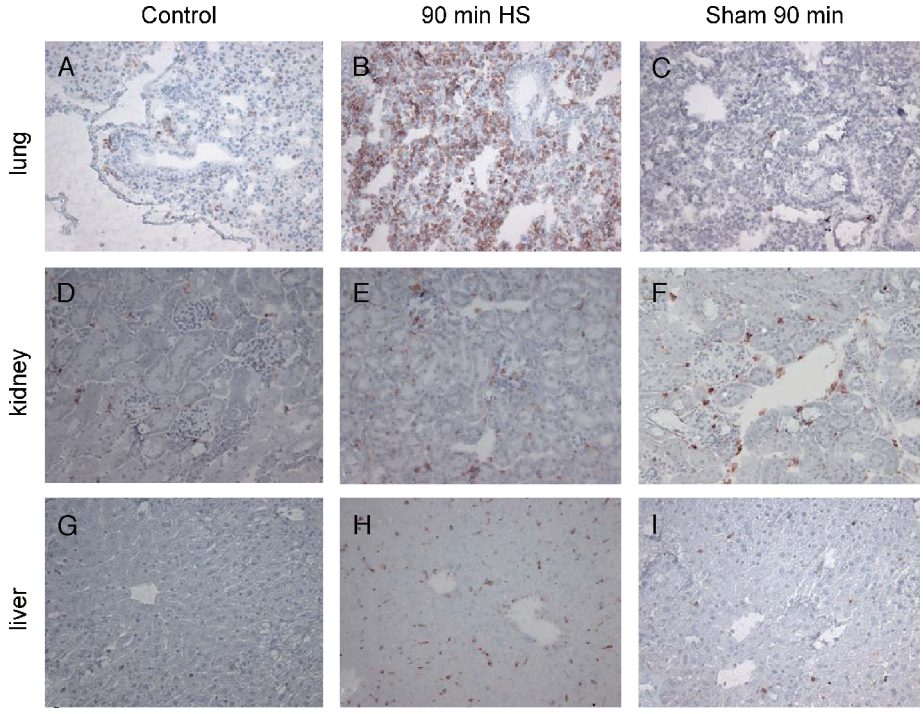

FIG. 5. **Influx of leukocytes in the different organs during HS.** Immunohistochemical staining with an anti- CD45 pan leukocyte antibody showed influx of leukocytes in lungs (A–C), kidney (D–F), and heart (G–I). Staining was performed respectively on healthy mouse tissue (control) (A, D, G) and 90 min HS (B, F, H), and 90 min sham shock (C, E, I). Original magnification 200×. Leukocytes are stained red, with increased influx of CD45 positive cells is seen at 90 minutes of shock.

under the influence of HS except for VE-cadherin expression in the liver, which showed increased mRNA levels. Resuscitation facilitated an additional up-regulation of the inflammatory endothelial genes compared with the activation status at 90 min of HS (Table 3). It is of note that especially in the liver and the kidney, VE-cadherin mRNA levels were strongly up-regulated at 1 h after resuscitation.

To corroborate the gene expression levels, cell adhesion molecules were immunohistochemically analyzed. In the kidney, liver, and heart, E-selectin protein was strongly expressed at 90 min of shock, whereas in the sham shock group, expression was minor or absent (Fig. 2). Immunohistochemical staining did not only show differences in expression between different organs, thereby supporting the gene expression data, but also demonstrated differences in activation between the diverse organ microvascular endothelial cells. In the kidney, for example, protein expression of E-selectin was mainly found in glomeruli (Fig. 2), whereas VCAM-1 expression was seen primarily in the peritubular endothelial cells, arteries, and venules, but not in glomeruli (Fig. 3). From this, it was concluded that HS under anesthesia strongly affected the activation status of microvascular endothelial cells in all organs. Unexpectedly, however, anesthesia and instrumentation without concurrent induction of HS also affected endothelial cell activation, as observed by up-regulation of adhesion molecule mRNA and protein levels (Figs. 2, 3; Table 2).

Hypoxia-related genes *VEGF-A* and *HIF-1α* were not transcriptionally affected during the 90 min of HS (Fig. 4). Moreover, immunohistochemical staining did not reveal nuclear localization of HIF-1α in any of the organs (data not shown), implying absence of activation of the HIF-1α system during the HS phase.

Early organ activation is paralleled by leukocyte influx

Adhesion molecule expression is instrumental in facilitation of leukocyte migration into the tissues. Influx of leukocytes into tissue, as determined with pan leukocyte marker CD45, was observed in the kidney, lung, and liver at 90 min of HS (Fig. 5). In contrast, in the brain and heart, no leukocyte influx was seen after 90 min of HS. No difference in leukocyte influx could be observed compared with control mice (data not shown) 24 h after volume resuscitation.

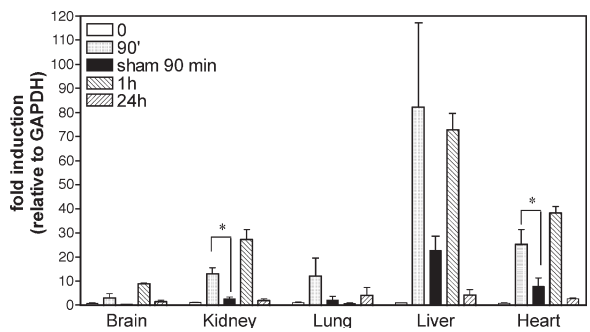

FIG. 6. **Effect of hemorrhagic shock on TNF-α in tissue.** Gene expression of TNF-α was determined in lung, liver, kidney, heart, and brain tissue homogenates by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold induction of TNF-α in comparison to the level of activity that was present in extracts of normal healthy organs. Data presented are the mean ± SEM of each group (n = 5 in the 90' HS group and 90' sham shock, other groups n = 3). 90 minutes of HS was compared with 90 minutes of sham shock (sham 90'). $P < 0.05$ values are marked with *.

HS TNF-α is produced in kidney and heart tissue

Leukocyte-endothelial interactions are often initiated by proinflammatory cytokines. These cytokines are either produced locally in the organ where the inflammatory response is taking place or systemically released, for example, by the liver upon exposure to bacterial products. TNF-α gene expression analysis showed a significant TNF production in the kidney and heart at 90 min after initiation of HS compared with sham shock mice (Fig. 6). Interestingly, in the heart, this induction of TNF-α was not paralleled by leukocyte influx (data not shown).

DISCUSSION

The cellular and organ response to HS is complex and results in profound changes in gene expression and organ function (10). The earliest effects of HS on microvascular endothelial cells and its causes remain poorly understood. In models of HS, ample data exist regarding the detrimental influx of leukocytes into organ parenchyma. Because endothelial cell activation is instrumental in leukocyte recruitment, we aimed to investigate the kinetics and organ specificity of microvascular endothelial cell activation during the early phase of HS. We showed that induction of inflammatory gene expression is an early event that occurs before resuscitation is instituted. Expression of all adhesion molecules was significantly induced in all organs, albeit to a different extent depending on the organ. Endothelial integrity genes *CD31* and *VE-cadherin* were only affected in the liver, whereas activation of the hypoxia HIF-1α pathway was absent during the shock period.

To our knowledge, we are the first to report the earliest effects of HS on the more complex pattern of microvascular endothelial cell activation and its organ specificity. Although no data on these early endothelial responses have been reported before, Xu et al. (8) showed that in the liver and lung, P-selectin and ICAM-1 were up-regulated 3 h after resuscitation in a 90-min HS model. The early endothelial activation during HS found in our study is in accordance with reports of early activation of inflammatory signal transduction routes in HS. In a volume-controlled HS model in which 25% of blood volume was withdrawn, the proinflammatory P38 mitogen–activated protein kinase (mitogen-activated protein kinase [MAPK]) activity increased in the kidney 60 min after initiation of HS (5). McCloskey et al. observed that Jun N-terminal kinase activation is an early event in the liver. After 30 min of HS with a MAP of 25 mmHg, they observed an increase in Jun N-terminal kinase that persisted throughout the duration of the 120-min experiment (6). Although in these studies these activated kinases were not assigned to specific cell types in the organ, these signaling pathways are known to control endothelial adhesion molecules expression (11). During the progression of HS condition, the cytokine-driven MAPK and nuclear factor κB intracellular signaling pathways

are likely to become activated in the microvascular endothelium (12, 13). Subsequent signaling via leukocyte integrin-endothelial immunoglobulin superfamily members is implied to take place in microvascular segments in which cell-cell interactions are most prominent. This signaling relays via small GTPase and p38 MAPK (14, 15). In an early stage of hemorrhage, changes in shear stress may furthermore affect endothelial activation in those microvascular beds where autoregulatory arteriolar control of blood flow cannot be sufficiently controlled (16). Because most of the studies on endothelial responses to inflammatory cytokine and shear stress changes have been performed in *in vitro* culture systems, and endothelial cells throughout the vascular tree are phenotypically heterogenic, the exact nature of signaling pathways leading to organ-specific shock-related endothelial activation needs to be identified.

The expression of endothelial adhesion molecules is essential for endothelial-leukocyte interaction (17). After activation of the endothelium, E-selectin is synthesized by *de novo* protein synthesis. Its expression on the endothelial cell membrane is induced a few hours after TNF-α stimulation of endothelial cells in culture (13). We showed that E-selectin is up-regulated at mRNA and protein levels within the first 90 min after initiation of HS in the kidney, lung, and brain. Of note are the organ-specific patterns of endothelial activation, which can theoretically form the basis for an organ-specific leukocyte recruitment process. Indeed, the early endothelial up-regulation of adhesion molecules was paralleled by early leukocyte recruitment in the liver, kidney, and lung. In contrast, the microvascular endothelium in the brain and the heart responded to HS with an increase in different adhesion molecule expression, but no leukocyte recruitment was seen in these organs. A similar organ heterogeneity in leukocyte recruitment was reported by Song et al. (18), who showed an increase in lung neutrophil count after 1 h, being maximal at 4 h after HS, without any change in myocardial neutrophil counts. A more detailed study on chemokine and cytokine expression within the different organs combined with infiltrating leukocyte subset typing may shed light on this organ specificity of leukocyte recruitment.

Cellular hypoxia is considered to be an important mediator of MODS after HS (19). During hypoxic conditions, HIF-1α accumulates in the cell and forms a stable heterodimer with HIF-1β, where it translocates to the nucleus afterwards. HIF-1α can also be regulated at the transcriptional level as demonstrated in tumor models (20, 21). In a rat model of permanent focal ischemia of the brain, Bergeron et al. (22) showed that mRNA of HIF-1α was significantly increased after 7.5 h of vascular occlusion. vascular endothelial growth factor A is a downstream target gene of HIF-1α that is primarily regulated at the transcriptional level and a major controller of vascular permeability and angiogenesis. Under hypoxic conditions, up-regulation of VEGF-A by HIF-1α occurs within minutes (23). However, in our model, we did not see any significant induction of mRNA of HIF-1α and VEGF-A during the HS phase, nor did we see an increased nuclear localization of HIF-1α after 90 min of HS. This may imply that either the duration or the severity of hypoxia in the early HS phase is too short or too minor, respectively, to activate the HIF-1α system. On the other hand, Koury et al. (24) showed that in a pressure-controlled HS model in the rat, the HIF-1α level as measured by Western blotting was increased in the ileac mucosa after 90 min of HS accompanied by a MAP of 30 mmHg. Although Hierholzer et al. (25) observed an increase in HIF-1α DNA binding activity of 3.2-fold in the lung after a 40-mmHg MAP shock period of 2.5 h, no increased HIF-1α activation in livers of animals subjected to 40 mmHg MAP HS for 60 min was found (26). Our data and those reported by others therefore suggest that, although cellular hypoxia may play a role, it is not necessarily a key factor in the up-regulation of inflammatory genes in the early phase of HS. In future experiments, the possible role of hypoxia and its cellular effects, including blood gas analysis, will be performed to examine its precise role in HS.

TNF is a proinflammatory cytokine, with the primary target including vascular endothelial cells. When TNF-α is administered to humans, it produces fever, inflammation, tissue destruction, and, in some cases, shock and death (27). Effects on endothelial cells include protein-independent changes in cell shape and motility and induction of proteins that regulate other parameters of the inflammatory response such as vasoregulation, leukocyte adhesion, leukocyte activation, and coagulation (13, 28) As such, TNF-α may be implicated in organ dysfunction in HS. Liu and Dubick (29) showed in rats with an HS to a MAP of 50 mmHg during 60 min that the mRNA encoding for TNF-α was up-regulated in ileum, kidney, liver, and skeletal muscle. Serum TNF-α was increased in a mouse model already after 30 min after initiation of shock (30). Combined with our increase in mRNA TNF-α levels in the various organs reported here, these studies indicate an early TNF-α response in HS. However, a direct relation between local TNF-α production and early activation of endothelial cells with respect to leukocyte adhesion cannot be easily inferred. Additional experiments, with induction of HS in TNFR1/R2 knockout mice, need to be performed to get a more detailed view on the role of TNF on early endothelial cell activation in the HS model used.

We showed that the combination of surgical instrumentation and anesthesia by itself strongly affected endothelial cell-activation status. The administration of anesthetics may contribute to endothelial activation by exerting depressive effects on respiratory and cardiovascular functions or by direct cellular actions. The use of a high-chloride resuscitation fluid could have induced a metabolic acidosis that might have influenced the extra endothelial activation seen 1 h after resuscitation. There also may be an effect associated with the use of heparin that is administered to maintain blood flow through the catheter (31). Previous studies showing early increased proinflammatory IL-6 cytokine expression in mice that underwent a sham shock procedure (32, 33) corroborate our findings of sham shock group proinflammatory responses (34).

In summary, our study revealed an early and organ-specific endothelial cell activation pattern during HS that occurred before resuscitation and was not per se hypoxia-driven. The early endothelial activation found in this study suggests that a temporal therapeutic window exists that can be used to

attenuate endothelial cell activation at an early stage during resuscitation to prevent neutrophil sequestration in organs and subsequent MODS. To identify potential targets for early therapeutic interference, we will, in future studies, assess complex kinase activity profiles (35) at different points after HS induction and resuscitation.

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