EMORRHAGIC shock is a life-threatening condition often followed by multiple organ failure and associated with unfavorable patient outcome.1 Microcirculatory perfusion is essential for the delivery of oxygen and nutrients to the organs, and disturbances of microcirculatory perfusion seem to have a predictive value in the development of multiple organ failure.2,3 Despite stabilization of the macrocirculation, microcirculatory hypoperfusion persists for days in hemorrhagic shock patients3 and seems to be a key mediator in the development of multiple organ failure and unfavorable patient outcome.2,3

Current treatment of hemorrhagic shock combines early control of bleeding, maintenance of critical tissue perfusion, correction of coagulopathy, and management of the systemic inflammatory response syndrome.4,5 Resuscitation with fluids and/or blood products is the first step to correct hypoperfusion and prevent subsequent organ failure.5,6 However, crystalloids have poor plasma-expanding capacities, of which...
only 20% of the given volume remains in the intravascular space due to microvascular leakage.7–10 A reduction in microvascular leakage and preservation of microcirculatory perfusion may improve outcome after hemorrhagic shock, but therapeutic targets are currently lacking.

One of the molecular systems involved in the maintenance of microvascular integrity is the angiopoietin/Tie2 system.11 Tie2 is a vascular tyrosine kinase receptor that has specificity for angiopoietin-1 and angiopoietin-2 binding.11 Angiopoietin-1 promotes endothelial barrier function and prevents damage to endothelial cells, whereas angiopoietin-2 competitively binds to and inhibits Tie2 phosphorylation, thereby preventing protective downstream signaling.11 A previous animal study suggested a role for angiopoietin-1 in maintaining microvascular endothelial barrier integrity during hemorrhagic shock.10 In trauma patients, circulating angiopoietin-2 levels are associated with endothelial activation, systemic hypoperfusion, injury severity, and worse clinical outcome.12 Moreover, in the mouse renal vasculature, Tie2 gene and protein expressions were reduced during hemorrhagic shock,13 and recently it was confirmed that microvascular leakage is indeed induced by direct suppression of Tie2 in mice.14 Taken together, these results suggest that the angiopoietin/Tie2 system plays an important role in the protection of microvascular integrity and microcirculatory perfusion during hemorrhagic shock. Interestingly, a recently developed drug, Vasculotide, acts as an angiopoietin-1 mimetic and has been shown to ameliorate microvascular leakage in experimental models of sepsis and acute kidney injury.15–17 We hypothesized that therapeutically targeting the endothelial angiopoietin/Tie2 system with the angiopoietin-1 mimetic Vasculotide decreased microvascular leakage and improved microcirculatory perfusion in a rat model of hemorrhagic shock.

Materials and Methods

Study Approval
All of the procedures were approved by the institutional animal care and use committee of VU University (Amsterdam, The Netherlands; animal welfare No. ANES 13-03) and conducted following the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and the Animal Research: Reporting of In Vivo Experiments guidelines on animal research.18

Animals and Experimental Setup
Male Wistar rats (Charles River Laboratories, Belgium) were housed in a temperature-controlled room (12/12 h light dark cycle, 20° to 23°C, 40 to 60% humidity) with food and water ad libitum. The experimental setup is visualized in figure 1. Rats were allocated to undergo hemorrhagic shock (shock; Vasculotide n = 7, phosphate-buffered saline [PBS] n = 8), hemorrhagic shock with fluid resuscitation (resuscitation; Vasculotide n = 7, PBS n = 7), or sham protocol (sham; Vasculotide n = 7, PBS n = 7) for microcirculatory perfusion measurements and protein and RNA analyses. Rats were treated with Vasculotide (Vasomune Therapeutics, Canada) or PBS (Sigma-Aldrich, The Netherlands) as control using block randomization. For microvascular leakage measurements, another eight rats per intervention were included. An extra group of rats (n = 10) was added to show the clinical applicability of Vasculotide posttreatment after hemorrhagic shock on microcirculatory perfusion.

Anesthesia and Surgical Preparation
For surgery, rats (377 ± 12 g) were anesthetized with 4% isoflurane (Ivax Farma, The Netherlands) in a plastic box filled with 100% oxygen. After endotracheal intubation with a 16-gauge catheter (Venflon Pro, Becton Dickinson, Sweden), lungs were mechanically ventilated (UMV-03, UNO Roestvaststaal BV, The Netherlands) with a positive end-expiratory pressure of 1 to 2 cm H2O, a respiratory rate of approximately 65 breaths/min, a tidal volume of approximately 10 ml/kg, and 1.5 to 2.0% isoflurane in oxygen-enriched air (40% O2/60% N2). Respiratory rate was adjusted to maintain pH and partial pressure of carbon dioxide within physiologic limits. The body temperature was continuously measured and maintained stable between 36.5° and 37.5°C using a temperature controller (TC-1000 Rat, CWE Inc., USA). A 22-gauge catheter (Venflon Pro, Becton Dickinson) was placed in the caudal (tail) artery for continuous measurements of arterial blood pressure. The left cremaster muscle was isolated under warm saline superfusion, spread out on a heated platform (34°C), and covered with gas-impermeable plastic film (Saran wrap; S. C. Johnson, USA), as described previously19,20 for microcirculatory perfusion measurements. The right femoral artery was cannulated with a 20-gauge catheter (Arterial Cannula, Becton Dickinson) for blood withdrawal, blood gas analyses (ABL80, Radiometer, Denmark), and hematocrit measurements. The right jugular vein was catheterized with a 22-gauge catheter (Venflon Pro, Becton Dickinson) for administration of the drug and infusion of Ringer’s lactate solution and blood. All of the catheter insertions were preceded by local application of 1% lidocaine. Fentanyl (1.25 to 2.50 µg) was administered as additional analgesia every 20 to 30 min throughout the experiment. Arterial blood pressure, electrocardiogram, and heart rate were continuously recorded using PowerLab software (PowerLab 8/35, Chart 8.0; ADInstruments Pty Ltd., Australia).

Hemorrhagic Shock
Hemorrhagic shock was induced by withdrawing blood from the right femoral artery until a mean arterial pressure (MAP) of 30 mmHg was reached and maintained for 1 h either by withdrawal of blood or reinfusion of heparinized shed blood. Shock was confirmed by the development of metabolic acidosis. Figure 1 illustrates the experimental time line and predefined time points. The shock group was euthanized 1 h after induction of hemorrhagic
The resuscitation group received the same protocol as described above, however, after 1 h of hemorrhagic shock, rats were resuscitated with Ringer’s lactate solution in the Vasculotide posttreatment group. Microcirculatory perfusion measurements were performed directly after the surgical preparation (1: baseline), 30 min after shock induction (2), 1 h after shock induction (3), 30 min after start of resuscitation when baseline MAP was restored (4), and 1 h after start of resuscitation (5). Plasma was collected at baseline (1), 1 h after shock induction (3), and 1 h after fluid resuscitation (5). Rats were sacrificed and kidneys and lungs were isolated at the end of each experiment for additional molecular analysis and Evans blue dye extravasation assessment (time point 5 for sham and HS+resuscitation groups and time point 3 for the HS group).

**Microvascular Leakage Assessment**

Microvascular leakage was determined by Evans blue dye extravasation as described previously and more detailed in appendix 1. Briefly, 1 h after shock induction, Evans blue dye was administered as part of fluid resuscitation. Extracted Evans blue dye from kidney and lung tissue was quantified by spectrophotometry.

**Plasma Analyses**

Arterial blood was withdrawn in EDTA tubes at three different time points, including baseline (1), 1 h after shock induction (3), and 1 h after resuscitation (5; fig. 1). Blood was
Targeting Tie2 during Hemorrhagic Shock

Vasculotide Phosphorylates Tie2 in Primary Rat Kidney Glomerular Endothelial Cells

Stimulation of rat kidney glomerular endothelial cells with Vasculotide phosphorylated the Tie2 receptor significantly in a dose-dependent manner compared with untreated endothelial cells (fig. 2). Human angiopoietin-1, which was used as a positive control, also caused a significant increase in phosphorylation of Tie2.

Macrophemodynamics during Hemorrhagic Shock and Fluid Resuscitation

MAP and heart rate decreased significantly after induction of hemorrhagic shock compared with the sham group, in which MAP and heart rate remained stable throughout the experiment (fig. 3, A and B). MAP regained baseline levels during fluid resuscitation, accompanied by an increase in heart rate. Hematocrit levels were unaltered in the resuscitation group compared with the sham group (fig. 3C), whereas bicarbonate and base excess levels were significantly decreased, indicating hemorrhagic shock-induced metabolic acidosis (fig. 3, D–I).

In rats, no effect of Vasculotide was observed on MAP, heart rate, hematocrit, pH, and partial pressure of carbon dioxide during hemorrhagic shock and fluid resuscitation compared with the PBS group. However, Vasculotide tended to diminish metabolic acidosis 1 h after resuscitation compared with controls, which was reflected by higher bicarbonate levels (20.0 ± 2.5 vs. 17.0 ± 3.0 mM; *P < 0.05) but not by higher base excess levels (–4.0 ± 2.4 vs. –6.3 ± 2.3 mEq/L; P = 0.11).

Hemorrhagic Shock Disturbed Microcirculatory Perfusion

One hour of hemorrhagic shock reduced the amount of continuously perfused vessels (7 ± 2 vs. 11 ± 2 per recording; fig. 4A) and increased the amount of nonperfused vessels compared with sham rats (9 ± 3 vs. 5 ± 2 per recording; fig. 4B), without differences in intermittently perfused vessels (fig. 4B).

Results

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vessels. Microcirculatory perfusion was not restored 1 h after resuscitation (fig. 4, A and B).

Treatment with Vasculotide did not affect microcirculatory perfusion during hemorrhagic shock. However, during fluid resuscitation the amount of continuously perfused vessels and nonperfused vessels returned back to baseline levels in Vasculotide-treated animals but remained deranged in the PBS-treated animals (fig. 4, A and B). Posttreatment with Vasculotide after hemorrhagic shock also restored microcirculatory perfusion 1 h after fluid resuscitation (fig. 4, C and D).

**Vasculotide Reduced Microvascular Leakage**

Hemorrhagic shock with fluid resuscitation increased Evans blue dye extravasation in kidneys (19.7 ± 11.3 vs. 5.2 ± 3.0 µg/g) and lungs (16.1 ± 7.0 vs. 8.6 ± 2.7 µg/g; fig. 5A), suggesting microvascular leakage. Treatment with Vasculotide significantly reduced extravasation of Evans blue dye after hemorrhagic shock in lungs (10.1 ± 3.3 µg/g; fig. 5A) but not in kidneys.

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**Fig. 3.** Hemodynamics and blood gas analyses. Mean arterial blood pressure (A), heart rate (B), hematocrit (C), pH (D), partial pressure of carbon dioxide (pCO₂; E), respiratory rate (F), pO₂ (G), HCO₃⁻ (H), and base excess (I) in rats during hemorrhagic shock and fluid resuscitation (resuscitation; dotted line) and sham rats (bold line). Data represent mean ± SD, n = 15. Two-way ANOVA with repeated measurements and Bonferroni post hoc analyses, *P < 0.05.
Fig. 4. Microcirculatory perfusion during hemorrhagic shock and resuscitation. Continuously perfused vessels (A and C) and nonperfused vessels (B and D) in rat cremaster muscle during hemorrhagic shock and fluid resuscitation (resuscitation; black dotted line), sham rats (black line), and hemorrhagic shock and fluid resuscitation pretreated with Vasculotide (resuscitation+VT; grey dotted line, A and B) or posttreated with Vasculotide (resuscitation+VTpost; grey dotted line, C and D). Data represent mean ± SD, n = 7 to 10. Two-way ANOVA with repeated measurements and Bonferroni post hoc analyses. *P < 0.05 and **P < 0.01 sham vs. resuscitation, #P < 0.05, ^P = 0.10, $P = 0.07 resuscitation+VTpost vs. resuscitation.

Fig. 5. Microvascular leakage and fluid resuscitation requirements. Evans blue dye extravasation in kidneys and lungs after hemorrhagic shock and fluid resuscitation (Resuscitation) with Vasculotide treatment (Resuscitation+VT; A). Absolute amount of blood withdrawn and Ringer’s lactate solution and heparinized shed blood required for fluid resuscitation after hemorrhagic shock (B). Data represent mean ± SD. A, n = 8, one-way ANOVA with Bonferroni post hoc analyses; B, n = 15, Student t test. *P < 0.05, **P < 0.01, ***P < 0.001.
Vasculotide Treatment Reduced Resuscitation Requirements

Similar volumes of blood were withdrawn to induce hemorrhagic shock in rats treated with Vasculotide or control (fig. 5B). During resuscitation, rats received 1 times the withdrawn volume of blood in Ringer’s lactate solution, followed by heparinized shed blood until baseline MAP was reached. The volume of Ringer’s lactate solution infused was equal between the rats treated with Vasculotide and control but slightly lower compared with volume of blood withdrawn due to the fact that some rats reached baseline MAP before total volume of Ringer’s lactate solution was infused (fig. 5B). Interestingly, rats treated with Vasculotide required significantly less heparinized shed blood to reach baseline MAP compared with the untreated hemorrhagic shock rats (1.3 ± 1.4 vs. 2.8 ± 1.5 ml; fig. 5B).

Hemorrhagic Shock Increased Circulating Angiopoietin-2 Levels

Hemorrhagic shock significantly increased circulating levels of angiopoietin-2 and soluble Tie2, whereas angiopoietin-1, VCAM-1, and IL-6 levels remained unaltered compared with sham rats (fig. 6A and appendix 2). Fluid resuscitation did not restore plasma angiopoietin-2 and soluble Tie2 levels but significantly increased VCAM-1. Treatment with Vasculotide prevented the increase in circulating angiopoietin-2 after hemorrhagic shock compared with untreated rats and decreased circulating angiopoietin-1 during the resuscitation phase compared with untreated rats (fig. 6A).

Hemorrhagic Shock Deranged Angiopoietin/Tie2 and Inflammation-related Gene Expression

Gene expression was studied in two severely injured vital organs during hemorrhagic shock.

In kidneys, hemorrhagic shock decreased Tie2 gene expression compared with sham without affecting angiopoietin-1, angiopoietin-2, TNF-α, IL-6, intracellular adhesion molecule (ICAM)-1, VCAM-1, RhoA, VE cadherin, and VEGF-α gene expression (fig. 6B and appendix 3). Fluid resuscitation decreased angiopoietin-1 gene expression (fig. 6B) and significantly increased ICAM-1 gene expression. Vasculotide treatment did not have any effect on gene expression during hemorrhagic shock or after fluid resuscitation (appendix 3).

In lungs, hemorrhagic shock significantly decreased Tie2, ICAM-1, and VEGF-α, but did not affect angiopoietin-1, angiopoietin-2, VCAM-1, TNF-α, IL-6, RhoA, and VE cadherin gene expression compared with sham (fig. 6B and appendix 3). Fluid resuscitation significantly restored Tie2 and VEGF-α gene expression compared with hemorrhagic shock rats. Treatment with Vasculotide did not affect gene expression during hemorrhagic shock but significantly decreased IL-6 gene expression during fluid resuscitation (appendix 3).

At the protein level, hemorrhagic shock, fluid resuscitation, and treatment with Vasculotide did not affect angiopoietin-1 and angiopoietin-2 protein expression in kidneys (fig. 6C and appendix 4). In lungs, hemorrhagic shock significantly decreased angiopoietin-2 without affecting angiopoietin-1 protein expression compared with sham rats (fig. 6C and appendix 4). Fluid resuscitation and Vasculotide treatment did not affect angiopoietin-1 and angiopoietin-2 protein expression in lungs (fig. 6C and appendix 4).

Association between Circulating Markers and Microcirculatory Perfusion

Interestingly, circulating angiopoietin-2 and soluble Tie2 levels were negatively associated with the amount of continuously perfused vessels and positively associated with the amount of nonperfused vessels 1 h after the induction of hemorrhagic shock (fig. 7A). One hour after fluid resuscitation, circulating angiopoietin-1 levels associated negatively with the amount of continuously perfused vessels and positively with the amount of nonperfused vessels (fig. 7B).

Discussion

Hemorrhagic shock disturbs microcirculatory perfusion in the cremaster muscle of rats, which could not be restored by fluid resuscitation and was paralleled by microvascular leakage. Hemorrhagic shock-induced microcirculatory perfusion disturbances were associated with circulating angiopoietin-2 and soluble Tie2 levels, and kidneys and lungs showed a deranged angiopoietin/Tie2 system. Treatment with the angiopoietin-1 mimetic Vasculotide restored hemorrhagic shock-induced microcirculatory perfusion disturbances during fluid resuscitation, most probably through inhibition of microvascular leakage. Interestingly, restoration of microvascular integrity by Vasculotide reduced fluid resuscitation requirements. Therefore, the angiopoietin/Tie2 system seems a promising target in restoring microvascular integrity and may reduce organ failure during hemorrhagic shock.

Although several studies have described microcirculatory perfusion disturbances after hemorrhagic shock despite fluid resuscitation, this pathologic condition is limited in its study.3,24–26 The present study confirmed that hemorrhagic shock disturbed microcirculatory perfusion in the cremaster muscle, which did not restore 1 h after fluid resuscitation. Despite the fact that the cremaster muscle is a well-established and commonly used model to evaluate microcirculatory perfusion, future research is needed to evaluate the impact of hemorrhagic shock-induced microcirculatory perfusion disturbances on kidneys and lungs. However, in patients, microcirculatory perfusion is measured sublingually in daily practice, and disturbances in sublingual microcirculatory perfusion have been described as strong predictors of outcome in septic patients27 and also seem to have predictive value during hemorrhagic shock in the development of multiple organ failure.3

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The underlying mechanisms of microcirculatory perfusion disturbances during hemorrhagic shock and fluid resuscitation are yet to be elucidated. Microvascular leakage is suggested to impair microcirculatory perfusion during hemorrhagic shock and is manifested clinically by loss of fluid into the extravascular space. In accordance with previous studies that show hemorrhagic shock-induced microvascular leakage in rat intestine, rat lungs, and mouse...
cremaster muscle, the present study confirms that hemorrhagic shock also induces microvascular leakage in kidneys and lungs.

One of the molecular systems involved in microvascular leakage is the angiopoietin/Tie2 system. Alterations of the angiopoietin/Tie2 system during experimental hemorrhagic shock are described in mesenteric arteries and renal vasculature. In the present study only mild alterations in angiopoietins after hemorrhagic shock and fluid resuscitation were found. As expected, hemorrhagic shock reduced Tie2 gene expression in kidneys and lungs, but fluid resuscitation could only restore Tie2 gene expression in lungs. Future experiments should elaborate on the activation of Tie2 in kidneys and lungs. Moreover, the present study showed that hemorrhagic shock increased circulating angiopoietin-2 and soluble Tie2, which associated with microcirculatory perfusion disturbances. This is in accordance with elevated circulating angiopoietin-2 levels in trauma patients, which were associated with endothelial activation, systemic hypoperfusion, injury severity, and worse clinical outcome. Moreover, excess circulating angiopoietin-2 is associated with pulmonary vascular leak. A link between circulating soluble Tie2 levels and perfusion is not yet shown in hemorrhagic shock but is associated with pulmonary vascular leakage in septic patients. Soluble Tie2 might bind to circulating angiopoietin-1 resulting in increased circulating...
angiopeitoin-1, thereby inhibiting the angiopoietin/Tie2 system. This suggests that increased levels of soluble Tie2 might contribute to hemorrhagic shock-induced microvascular leakage and microcirculatory perfusion disturbances in hemorrhagic shock.

Systemic inflammation is also described in the development of microvascular leakage. In the present study, inflammatory and adhesion molecules in kidneys and lungs were only affected during fluid resuscitation. However, more circulating adhesion molecules and inflammatory markers should be studied in combination with neutrophil accumulation to conclude on the contribution of systemic inflammation to the development of hemorrhagic shock-induced microvascular leakage.

To therapeutically target the angiopoietin/Tie2 system during hemorrhagic shock, rats were treated with the angiopoietin-1 mimetic Vasculotide. Vasculotide binds to and activates Tie2 in healthy endothelial cells and in kidneys of healthy mice. In septic mice, Vasculotide decreased microvascular leakage, and, more recently, direct suppression of Tie2 in mice resulted in microvascular leakage, emphasizing the importance of Tie2 in microvascular leakage. In the present study, we first showed that Vasculotide phosphorylated Tie2 in vitro in rat kidney glomerular cells in a dose-dependent manner. Accordingly, Vasculotide attenuated microvascular leakage in kidneys and lungs after hemorrhagic shock and fluid resuscitation in rats and restored microcirculatory perfusion during fluid resuscitation. In addition, Vasculotide was able to inhibit hemorrhagic shock-induced elevation of circulating angiopoietin-2, suggesting an important role for circulating angiopoietin-2 in the development of microcirculatory perfusion disturbances. Interestingly, rats undergoing hemorrhagic shock and fluid resuscitation required less blood volume after treatment with Vasculotide. These decreased volume supplementation requirements are probably the consequence of attenuation of microvascular leakage by Vasculotide and emphasize the clinical relevance of targeting the angiopoietin/Tie2 system during hemorrhagic shock. As proof of concept, rats were treated with Vasculotide before hemorrhagic shock. To confirm these findings in a clinically more relevant setting, rats were posttreated with Vasculotide after hemorrhagic shock. To confirm these findings in a clinically more relevant setting, rats were posttreated with Vasculotide after hemorrhagic shock and fluid resuscitation and resulted in improved microcirculatory perfusion. These results suggest a possible clinical application for Vasculotide after hemorrhagic shock.

In conclusion, the present study showed that severe hemorrhagic shock resulted in microcirculatory perfusion disturbance paralleled by microvascular leakage, which were not restored by fluid resuscitation. Targeting Tie2 with the angiopoietin-1 mimetic Vasculotide restored microcirculatory perfusion disturbances during fluid resuscitation, reduced microvascular leakage, and restored the required amount of volume supplementation necessary to restore systemic hemodynamics in rats. These results suggest that pharmacologic modulation of the angiopoietin/Tie2 system might be a novel therapeutic target to prevent microvascular leakage and microcirculatory perfusion disturbances in hemorrhagic shock and finally may reduce organ failure during hemorrhagic shock.

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Competing Interests

Dr. Van Slyke is listed as inventor on three patents related to Vasculotide filed by Vasomune Therapeutics (Toronto, Ontario, Canada). The other authors declare no competing interests.

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Appendix 1. Extended Materials and Methods

Microcirculatory Perfusion Measurements and Analyses

After stabilization of the exposed cremaster muscle for at least 30 min, microcirculatory perfusion measurements were performed using a 10× objective on an intravital microscope (AxioetchVario 100HD, Zeiss, Germany) connected to a digital camera (scA640, Basler, Germany) with a final magnification of 640×, as described previously. Three regions of the microvasculature in the cremaster muscle with adequate perfusion quality were selected during baseline. These predefined regions were followed throughout the experiment. Measurements were performed directly after the surgical preparation (1: baseline), 30 min after shock induction when a MAP of 30 mmHg was reached (2: shock induction), 1 h after the start of shock induction (3: shock), 30 min after start resuscitation when baseline MAP was reached (4: resuscitation), and 1 h after start resuscitation (5: resuscitation; fig. 1).

For perfusion analyses, each video screen was divided into three parts by two vertical lines to objectify microcirculatory perfusion. The total amount of capillaries per screen was obtained by averaging the counted capillary crossings per part of the screen. These small vessels were categorized in continuously perfused, intermittently perfused (blood flow was arrested at least once or flow was reversed), and nonperfused capillaries (vessels without erythrocytes or nonflowing erythrocytes). Analyses were performed blinded.
Microvascular Leakage Assessment
All of the surgical and experimental procedures were performed as described above except for the isolation of the cremaster muscle and microcirculatory perfusion measurements. Microvascular leakage was visualized in the Miles assay based on extravasation of albumin bound Evans blue dye as described previously.21,22 Evans blue dye binds rapidly to serum albumin (66.5 kDa), and due to the lack of cellular uptake it remains relatively constant within the hours after injection. One hour after shock induction, 1 ml of Evans blue dye (10 mg dye per milliliter 0.9% NaCl) was administered as part of the fluid resuscitation. The rats were further resuscitated with Ringer’s lactate solution and shed blood until baseline MAP was reached. One hour after resuscitation, rats were rinsed with five times 20 ml of 0.9% NaCl via the caudal artery and removed via the jugular vein. Resection samples of the kidney and lung were weighed, placed in di-methyl-formamide (Sigma-Aldrich Corporation, USA) and incubated in a water bath at 55°C for 48 h. After dimethyl-formamide removal, organs were dried at 90°C for 24 h.

RNA Analyses
Total RNA was extracted from 10 to 30 mg frozen kidney and lung tissue and isolated using the RNaseasy mini kit (Qiagen, The Netherlands), as described previously.23 The RNA concentration and purity were determined using NanoDrop 1000 (NanoDrop Technologies, USA). A total of 1 µg RNA was transcribed into complementary DNA using an iScript cDNA synthesis kit (Bio-Rad, The Netherlands), as described previously.23 The RNA concentration and purity were determined using NanoDrop 1000 (NanoDrop Technologies, USA). A total of 1 µg RNA was transcribed into complementary DNA using an iScript cDNA synthesis kit (Bio-Rad, The Netherlands), as described previously.23

mRNA abundance was measured using a CFX96 Touch real-time polymerase chain reaction detection system (Bio-Rad, The Netherlands) using oligo-dT priming. mRNA abundance was measured using a CFX96 Touch real-time polymerase chain reaction detection system (Bio-Rad, The Netherlands) using oligo-dT priming.

The following primers were used for quantitative polymerase chain reaction detection system (Bio-Rad).

Appendix 2. Plasma Levels of VCAM-1 and IL-6

A

B

Fig. A2. Circulating levels of VCAM-1 (A) and IL-6 (B) in plasma withdrawn from rats after 1 h of shock (shock) and 1 h after fluid resuscitation (resuscitation). Data represent mean ± SD, n = 7. One-way ANOVA with Bonferroni post hoc analyses, *P < 0.05. VT = Vasculotide.
Appendix 3. Gene Expression Profile in Kidneys and Lungs

Fig. A3. Gene expression of ICAM-1, VCAM-1, TNF-α, IL-6, RhoA, VE cadherin, and VEGF-α in kidneys (A and B) and lungs (C and D) from rats after 1 h of hemorrhagic shock (shock; A and C) and 1 h after fluid resuscitation (resuscitation; B and D). Data represent mean ± SD, n = 7. One-way ANOVA with Bonferroni post hoc analyses, *P < 0.05, **P < 0.01. VT = Vasculotide.
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