Vasculotide, an Angiopoietin-1 Mimetic, Restores Microcirculatory Perfusion and Microvascular Leakage and Decreases Fluid Resuscitation Requirements in Hemorrhagic Shock

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ABSTRACT

Background: Microcirculatory dysfunction is associated with multiple organ failure and unfavorable patient outcome. We investigated whether therapeutically targeting the endothelial angiopoietin/Tie2 system preserves microvascular integrity during hemorrhagic shock.

Methods: Rats were treated with the angiopoietin-1 mimetic vasculotide and subjected to hemorrhagic shock and fluid resuscitation. Microcirculatory perfusion and leakage were assessed with intravital microscopy (n = 7 per group) and Evans blue dye extravasation (n = 8 per group), respectively. The angiopoietin/Tie2 system was studied at protein and RNA level in plasma, kidneys, and lungs.

Results: Hemorrhagic shock significantly reduced continuously perfused capillaries (7 ± 2 *vs.* 11 ± 2) and increased nonperfused vessels (9 ± 3 *vs.* 5 ± 2) during hemorrhagic shock, which could not be restored by fluid resuscitation. Hemorrhagic shock increased circulating angiopoietin-2 and soluble Tie2 significantly, which associated with microcirculatory perfusion disturbances. Hemorrhagic shock significantly decreased *Tie2* gene expression in kidneys and lungs and induced microvascular leakage 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). Vasculotide had no effect on hemodynamics and microcirculatory perfusion during hemorrhagic shock but restored microcirculatory perfusion during fluid resuscitation. Interestingly, vasculotide attenuated microvascular leakage in lungs (10.1 ± 3.3 μg/g) and significantly reduced the required amount of volume supplementation (1.3 ± 1.4 *vs.* 2.8 ± 1.5 ml). Furthermore, vasculotide posttreatment was also able to restore microcirculatory perfusion during fluid resuscitation.

Conclusions: Targeting Tie2 restored microvascular leakage and microcirculatory perfusion and reduced fluid resuscitation requirements in an experimental model of hemorrhagic shock. Therefore, the angiopoietin/Tie2 system seems to be a promising target in restoring microvascular integrity and may reduce organ failure during hemorrhagic shock. **(ANESTHESIOLOGY 2018; 128:361-74)**

HEMORRHAGIC 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 patients[3] 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]

What We Already Know about This Topic

- Hemorrhagic shock can cause microcirculatory injury and organ failure; although standard resuscitation targets cardiac output and overall perfusion, there is no treatment to protect or treat the microcirculation.

What This Article Tells Us That Is New

- Hemorrhagic shock in rats activated the angiopoietin/Tie2 system and was associated with vascular leakage and fewer perfused capillaries. These effects were attenuated by pretreatment and posttreatment with a new angiopoietin-1 mimetic, vasculotide.

This article is featured in "This Month in Anesthesiology," page 1A. Corresponding article on page 252. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication January 31, 2017. Accepted for publication August 21, 2017. From the Departments of Anesthesiology (M.T., A.L.I.v.L., C.B., C.E.v.d.B.), Physiology (M.T., A.L.I.v.L., C.E.v.d.B.), and Trauma Surgery (L.M.G.G.), VU University Medical Center, Amsterdam, The Netherlands; Departments of Pathology and Medical Biology (M.v.M.) and Critical Care (M.v.M.), University Medical Center Groningen, Groningen, The Netherlands; and Vasomune Therapeutics, Toronto, Ontario, Canada (P.V.S., V.H.).

 Anesthesiology 2018; 128:361-74

<zdoi;10.1097/ALN.0000000000001907>

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 H_2O, 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% O_2/60% N_2). 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 previously[19,20] 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 shock. 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 (Fresenius Kabi, The Netherlands; 1 times volume of withdrawn blood) and shed blood (preserved in a syringe with 200 μl of heparin (LEO Pharma, The Netherlands) until baseline levels of MAP were regained and rats were monitored for an additional hour. Rats in the sham group underwent a similar surgical preparation procedure as described above and were monitored for two consecutive hours. At baseline, a

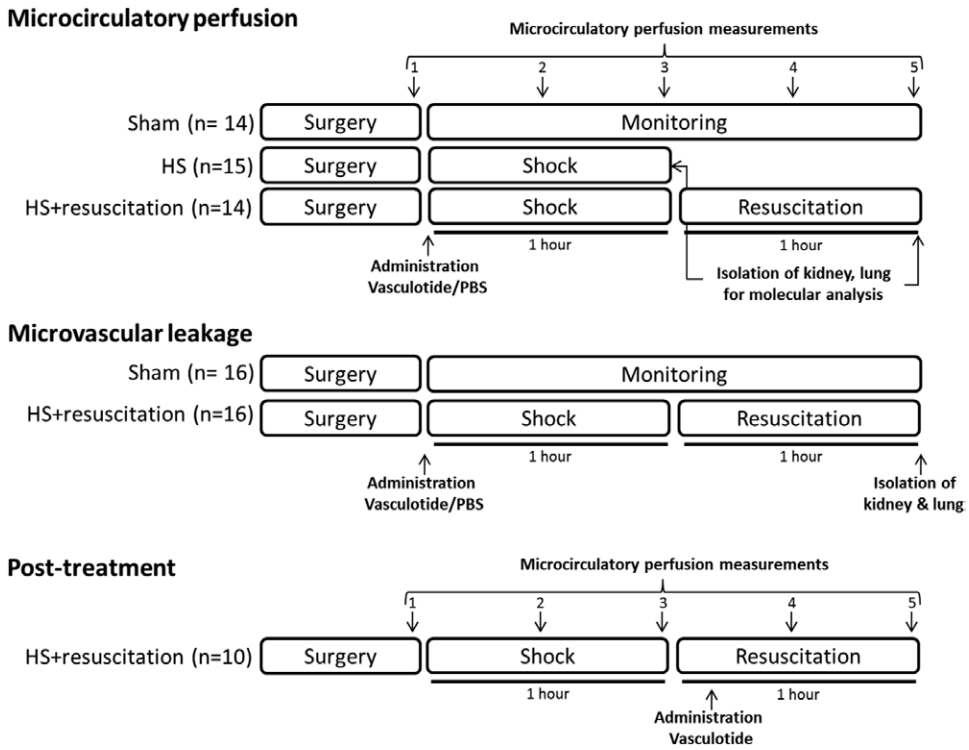

Fig. 1. Schematic overview of experimental protocol. Hemorrhagic shock was induced by pressure-controlled blood withdrawal, and mean arterial pressure (MAP) was maintained for 1 h at 30 mmHg (Shock). The groups were divided in sham surgery (Sham), hemorrhagic shock (HS), and hemorrhagic shock followed by fluid resuscitation (HS+resuscitation). Vasculotide or phosphate-buffered saline (PBS) was administered after the baseline measurements or after resuscitation with Ringer's lactate solution in the vasculotide posttreatment group (*1*). 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).

fixed-dose of 200 ng vasculotide in 100 µl PBS or 100 µl PBS alone as control was administered intravenously after baseline microcirculatory perfusion measurements or after resuscitation with Ringer's lactate in the vasculotide posttreatment group. The administrated dose of vasculotide was determined previously in pilot studies designed to explore optimal dosing. After euthanasia, kidneys and lungs were harvested, snap frozen in liquid nitrogen, and stored at –80°C for additional RNA and protein analysis.

Microcirculatory Perfusion Measurements and Analyses

Microcirculatory perfusion measurements were performed as described previously[20] and are more extensively described in appendix 1. Measurements were performed directly after the surgical preparation (1: baseline), 30 min after start of shock induction when a MAP of 30 mmHg was reached (2: shock induction), 1 h after start of shock induction (3: shock), 30 min after start of fluid resuscitation when baseline MAP was reached (4: resuscitation), and 1 h after start of fluid resuscitation (5: resuscitation; fig. 1). An intravital microscopy video of the cremaster during hemorrhagic shock and fluid resuscitation is shown in the Supplemental Digital Content (http://links.lww.com/ALN/B545).

Microvascular Leakage Assessment

Microvascular leakage was determined by Evans blue dye extravasation as described previously[21,22] 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 centrifuged twice to obtain platelet-free plasma and stored at –80°C. Levels of circulating angiopoietin-1, angiopoietin-2, soluble Tie2, vascular cell adhesion molecule-1 (VCAM-1), and interleukin (IL)-6 were measured with enzyme-linked immunosorbent assay (ELISA; Cloud-Clone Corporation, USA) in accordance with the manufacturer.

RNA Analyses

RNA was extracted from kidneys and lungs as described previously[23] and more extensively in appendix 1. Messenger RNA (mRNA) abundance of angiopoietin-1, angiopoietin-2, Tie2, intracellular adhesion molecule-1, VCAM-1, tumor necrosis factor (TNF) α, IL-6, Ras homolog gene family, member A (RhoA), vascular endothelial cadherin (VE cadherin), and vascular endothelial growth factor (VEGF) α (Applied Biosystems, USA) were measured, normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT) abundance, and expressed relative to sham.

Protein Analyses

Protein expression of angiopoietin-1 and angiopoietin-2 (Abcam, USA) in kidney and liver was analyzed and normalized to glyceraldehyde 3-phosphate dehydrogenase as described previously[23] and more detailed in appendix 1.

Primary Kidney Glomerular Endothelial Cells

Rat primary kidney glomerular endothelial cells were stimulated with 10 and 100 ng/ml vasculotide for 15 min, and tyrosine-phosphorylated Tie2 and total Tie2 were measured with ELISA, as described in appendix 1. Human angiopoietin-1 was used as positive control.

Statistical Analysis

All of the data are expressed as mean ± SD and analyzed two-tailed using GraphPad Prism 6.0 (GraphPad Software, USA).

At least a 30% reduction in microcirculatory perfusion with an SD of 1.7 and a 100% increase in microvascular leakage with an SD of 5.5 after hemorrhagic shock were expected. With a significance level (α) of 0.05 and β of 0.9, group sizes of seven for microcirculatory perfusion measurements and eight for microvascular leakage measurements were calculated. The studies were not conducted contemporaneously, because an extra experimental group (posttreatment with vasculotide) was added after the primary analyses per reviewer request, and there is no correction for interim analysis.

Time-dependent differences in the characteristics of the hemorrhagic shock model and in microcirculatory perfusion between groups were analyzed using a two-way ANOVA with repeated measurements, followed by Bonferroni *post hoc* analyses when appropriate. Evans blue dye extravasation differences and *in vitro* stimulation experiments were analyzed with one-way ANOVA with Bonferroni *post hoc* analyses. The associations between circulating levels of angiopoietin-1, angiopoietin-2, soluble Tie2, and the amount of continuously perfused and non-perfused vessels were analyzed using a Pearson correlation coefficient. *P* values less than 0.05 were considered statistically significant.

Results

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.

Macrohemodynamics 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. 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

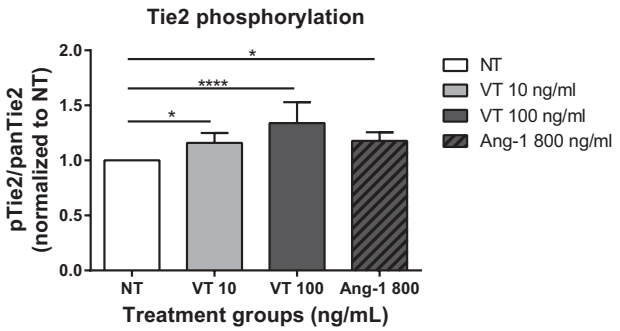

Fig. 2. Vasculotide phosphorylates the Tie2 receptor in rat glomerular endothelial cells. Rat primary kidney glomerular endothelial cells were stimulated with 10 and 100 ng/ml vasculotide (VT) and as positive control 800 ng/ml human angiopoietin-1 (Ang-1) and normalized for the nontreated (NT) group. Data represent mean ± SD, n = 7 independent experiments. One-way ANOVA with Bonferroni *post hoc* analyses, **P* < 0.05, ***P* < 0.01.

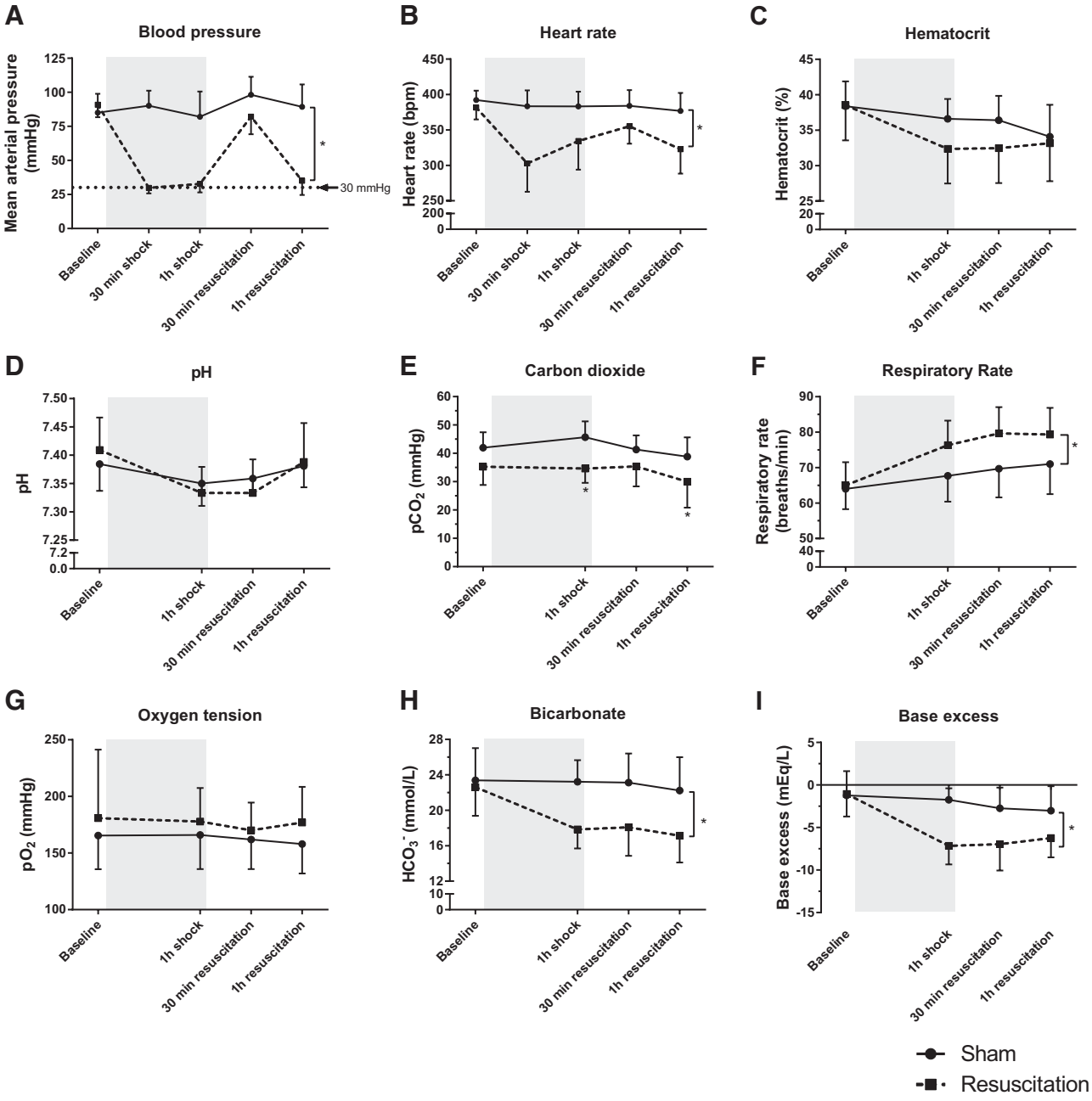

Fig. 3. Hemodynamics and blood gas analyses. Mean arterial blood pressure (*A*), heart rate (*B*), hematocrit (*C*), pH (*D*), partial pressure of carbon dioxide (pCO_2; *E*), respiratory rate (*F*), pO_2 (*G*), HCO_3^- (*H*), and base excess (*I*) in rats during hemorrhagic shock and fluid resuscitation (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.

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.

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

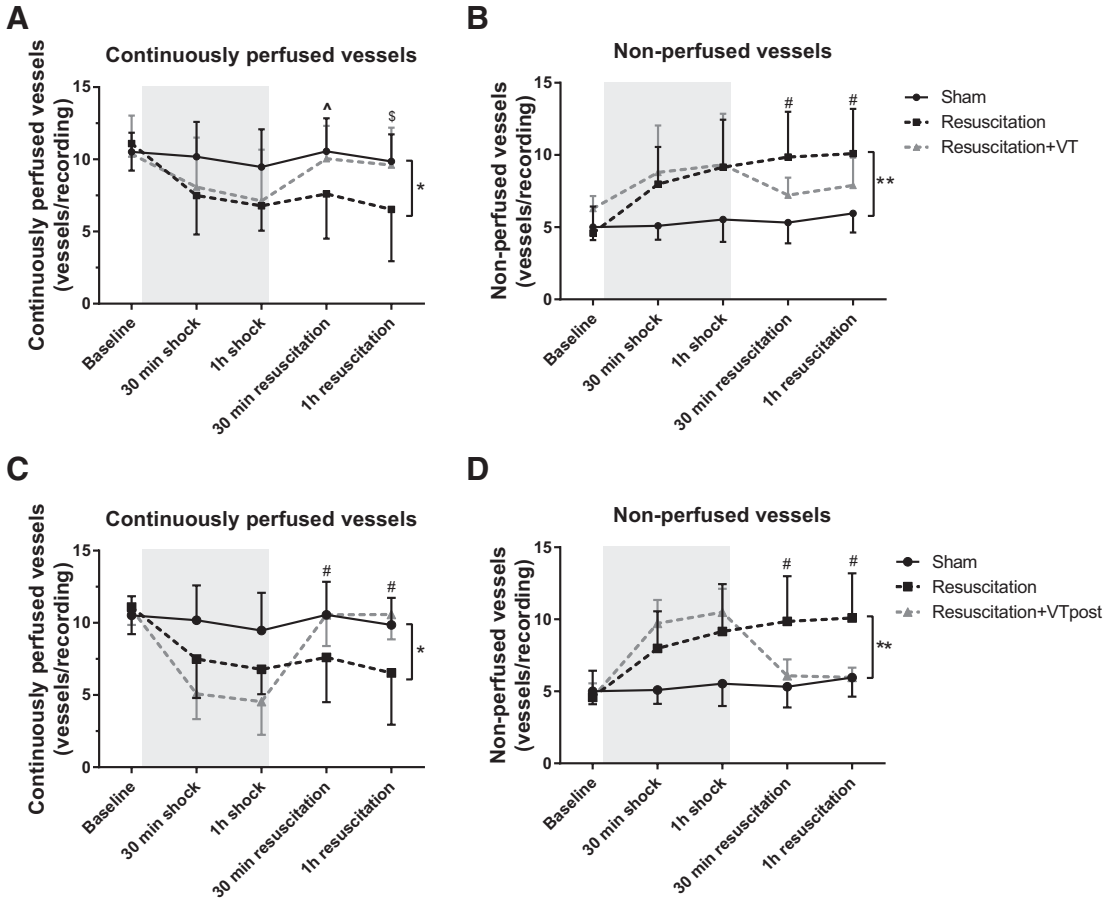

Fig. 4. Microcirculatory perfusion during hemorrhagic shock and resuscitation. Continuously perfused vessels (*A* and *C*) and nonperfused vessels (*B* and *D*) in rat cremaster muscle during hemorrhagic shock and fluid resuscitation (resuscitation; *black dotted line*), sham rats (*black line*), and 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.

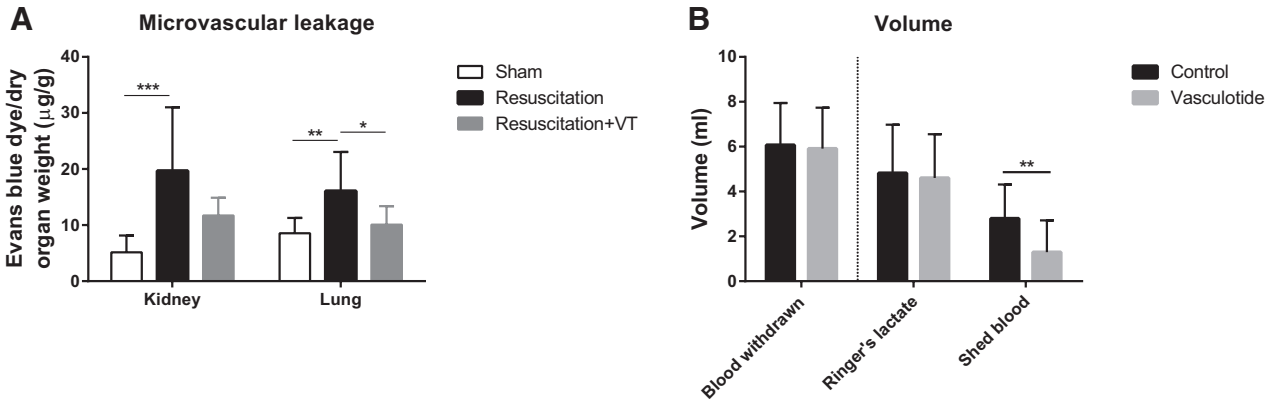

Fig. 5. Microvascular leakage and fluid resuscitation requirements. Evans blue dye extravasation in kidneys and lungs after hemorrhagic shock and fluid resuscitation (Resuscitation) with vasculotide treatment (Resuscitation+VT; *A*). Absolute amount of blood withdrawn and Ringer's lactate solution and 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.

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 affected 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 patients[27] and also seem to have predictive value during hemorrhagic shock in the development of multiple organ failure.[3]

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

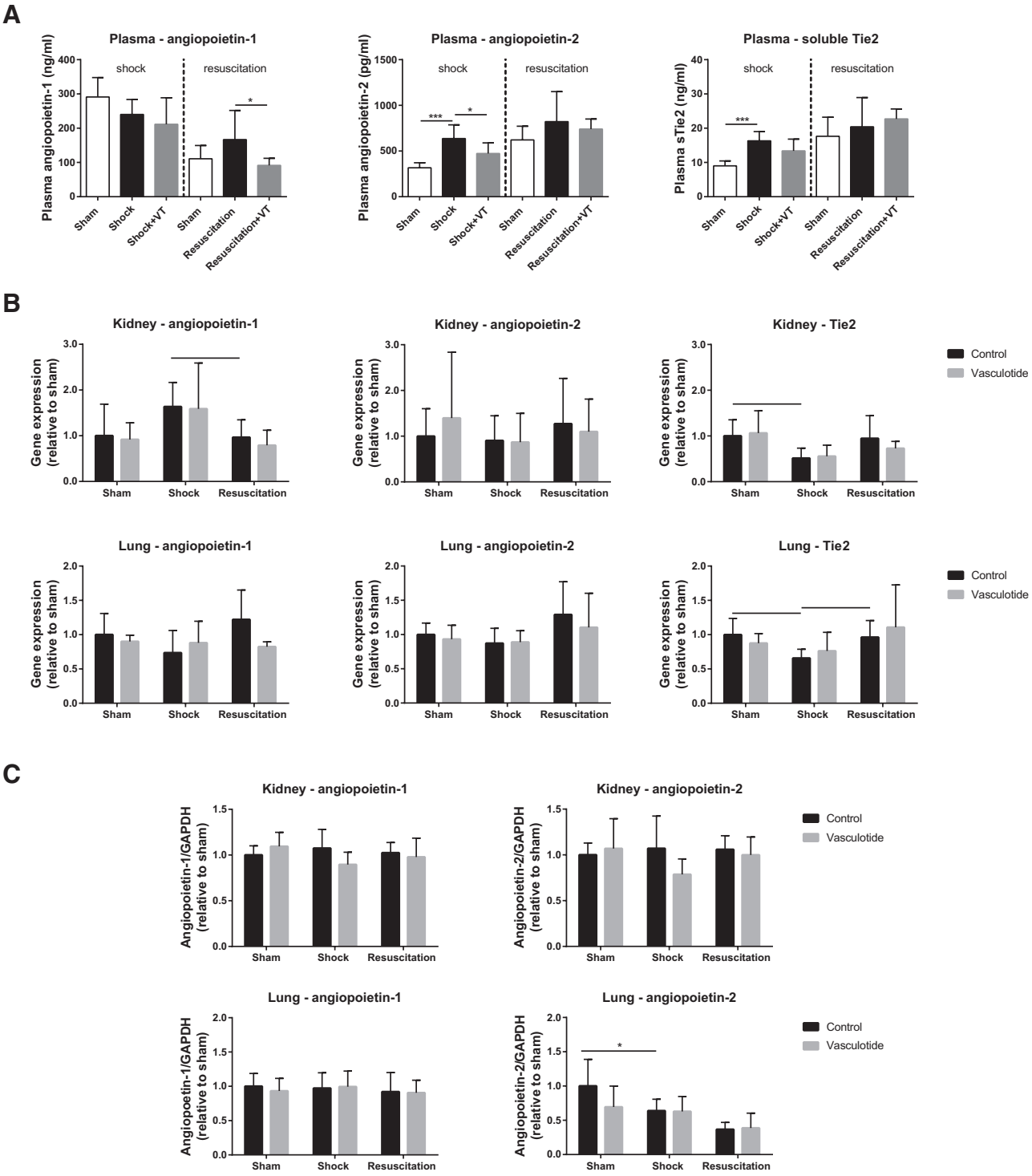

Fig. 6. Alterations in the angiopoietin/Tie2 system. Circulating levels of angiopoietin-1, angiopoietin-2, and soluble Tie2 in plasma (*A*). Gene expression of angiopoietin-1, angiopoetin-2, and Tie2 in kidneys and lungs (*B*). Protein expression of angiopoietin-1 and angiopoietin-2 in kidneys and lungs (*C*) from rats after 1 h of hemorrhagic shock (shock) and 1 h after fluid resuscitation (resuscitation). Data represent mean ± SD, n = 7. One-way ANOVA with Bonferroni *post hoc* analyses, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. VT = vasculotide.

of fluid into the extravascular space. In accordance with previous studies that show hemorrhagic shock-induced microvascular leakage in rat intestine,[9] rat lungs,[28] and mouse cremaster muscle,[29] 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.[11,30]

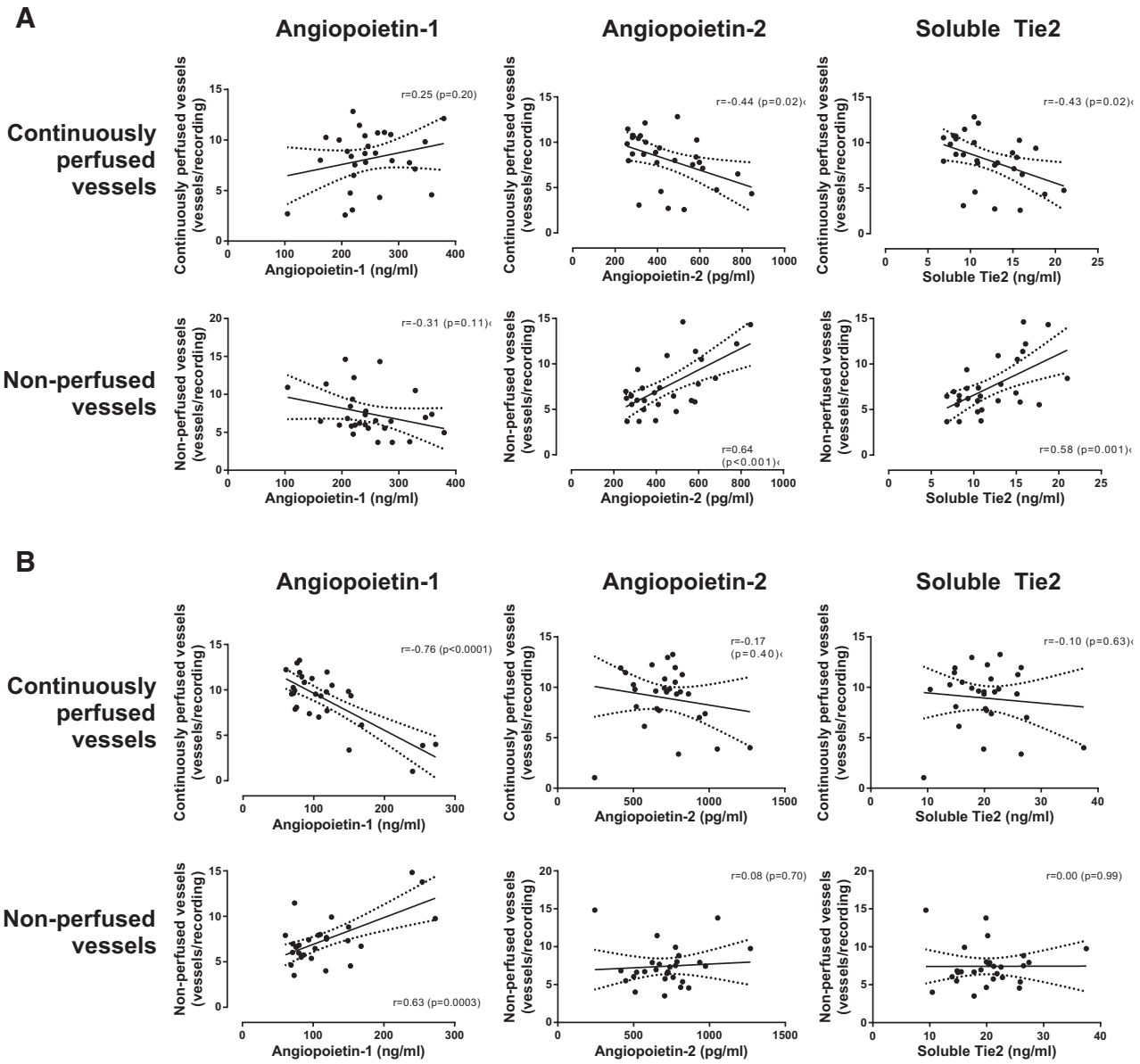

Fig. 7. Association between circulating angiopoietin/Tie2 and microcirculatory perfusion. Association between circulating levels of angiopoietin-1, angiopoietin-2, and soluble Tie2 with the amount of continuously perfused and nonperfused vessels in rats 1 h after induction of hemorrhagic shock (*A*) and 1 h after fluid resuscitation (*B*). Data were presented with a linear regression with 95% CI and tested with a Pearson correlation test.

Alterations of the angiopoietin/Tie2 system during experimental hemorrhagic shock are described in mesenteric arteries[31] and renal vasculature.[13] 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.[12] Moreover, excess circulating angiopoietin-2 is associated with pulmonary vascular leak.[32] 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.[33] Soluble Tie2 might bind to circulating angiopoietin-1 resulting in increased circulating angiopoietin-2, 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.[29] 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[34] and in kidneys of healthy mice.[15] In septic mice, vasculotide decreased microvascular leakage,[15] and, more recently, direct suppression of Tie2 in mice resulted in microvascular leakage,[14] 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 during fluid resuscitation and resulted in improved microcirculatory perfusion. These results suggest a possible clinical application for vasculotide, and future research needs to be performed to show the long-term effects of vasculotide on organ complications.

In conclusion, the present study showed that severe hemorrhagic shock resulted in microcirculatory perfusion disturbances 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 reduced 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.

Acknowledgments

The authors thank Nick J. Koning, M.Sc., and Tom Grandjean, B.Sc. (Department of Anesthesiology, VU University Medical Center, Amsterdam, The Netherlands), for their help with the set-up and execution of the animal experiments, as well as Dan J. Dumont, M.D., Ph.D. (Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada), for his advice on the molecular analysis of the angiopoietin/Tie2 system.

Research Support

Supported by a Young Investigator Grant from the Dutch Society of Anesthesiology (NVA; Utrecht, The Netherlands; to M.v.M.) and a Research Project Grant 2016 from the European Society of Anesthesiology (Brussels, Belgium; to C.E.v.d.B.).

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 (AxiotechVario 100HD, Zeiss, Germany) connected to a digital camera (scA640, Basler, Germany) with a final magnification of 640×, as described previously.[20] 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.[20] 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 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 di-methyl-formamide removal, organs were dried at 90°C for 24 h. Extracted Evans blue dye and hemoglobin were quantified by spectrophotometry at a wavelength of 610 nm (excitation) and 740 nm (emission), and the Evans blue dye/dry organ weight ratio was calculated.

RNA Analyses

Total RNA was extracted from 10 to 30 mg frozen kidney and lung tissue and isolated using the RNeasy 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) using oligo-dT priming.

mRNA abundance was measured using a CFX96 Touch real-time polymerase chain reaction detection system (Bio-Rad). The following primers were used for quantitative polymerase chain reaction: angiopoietin-1, angiopoietin-2, Tie2, ICAM-1, VCAM-1, TNF-α, IL-6, RhoA, VE cadherin, and VEGF-α (Applied Biosystems). All of the mRNA expression levels were normalized to HPRT abundance, calculated by ΔΔcT values, and expressed relative to sham.

Protein Analyses

Frozen kidney and liver tissue were homogenized to obtain cellular protein fractions for Western blot analysis as described previously.[23] Protein expression of signaling intermediates was analyzed using the following primary antibodies: angiopoietin-1 (ab183701, monoclonal, rabbit, Abcam) and angiopoietin-2 (ab155106, monoclonal, rabbit, Abcam). Signals were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; No. 2118, polyclonal, rabbit, Cell Signaling Technology, USA). Immunoblots were quantified by densitometric analysis of films (ImageQuant TL, v8.1, GE Healthcare, USA).

Primary Kidney Glomerular Endothelial Cells

Rat primary kidney glomerular endothelial cells (Cell Biologics, USA) were grown in complete rat endothelial cell medium /w kit (Cell Biologics) in 20% O_2, 5% CO_2, and 37°C humidified chamber and split 1:3 once reaching 80 to 90% confluence. To examine the activation of Tie2 by vasculotide in rat kidney glomerular endothelial cells, 95 to 100% confluent cells were stimulated with vasculotide 10 and 100 ng/ml in serum-containing medium for 15 min. Human angiopoietin-1 (R&D Systems, USA) was used as a positive control.

Cell lysates were collected using lysis buffer recommended in the human phospho-Tie-2 ELISA kit (R&D Systems), tyrosine-phosphorylated Tie2 and total Tie2 were measured using ELISA (human phospho-Tie2 and total-Tie2 ELISA kits, R&D Systems) with substitution of capture antibody validated for rat Tie2. Mouse anti-Tie2 antibody (BD Biosciences, USA) and rabbit TEK polyclonal antibody (MyBioSource) were used as capture antibody in pTie2 and total Tie2 ELISA, respectively.

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

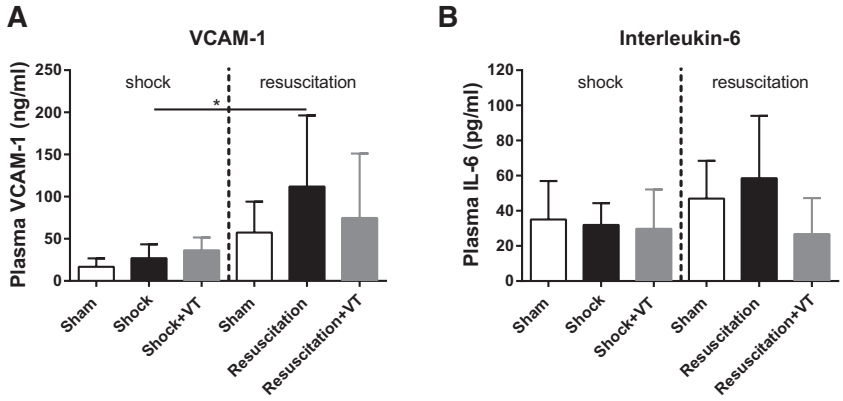

Fig. A2. Circulating levels of VCAM-1 (*A*) and IL-6 (*B*) in plasma withdrawn from rats after 1 h of shock (shock) and 1 h after fluid resuscitation (resuscitation). Data represent mean ± SD, n = 7. One-way ANOVA with Bonferroni *post hoc* analyses, $^*P < 0.05$. VT = vasculotide.

Appendix 3. Gene Expression Profile in Kidneys and Lungs

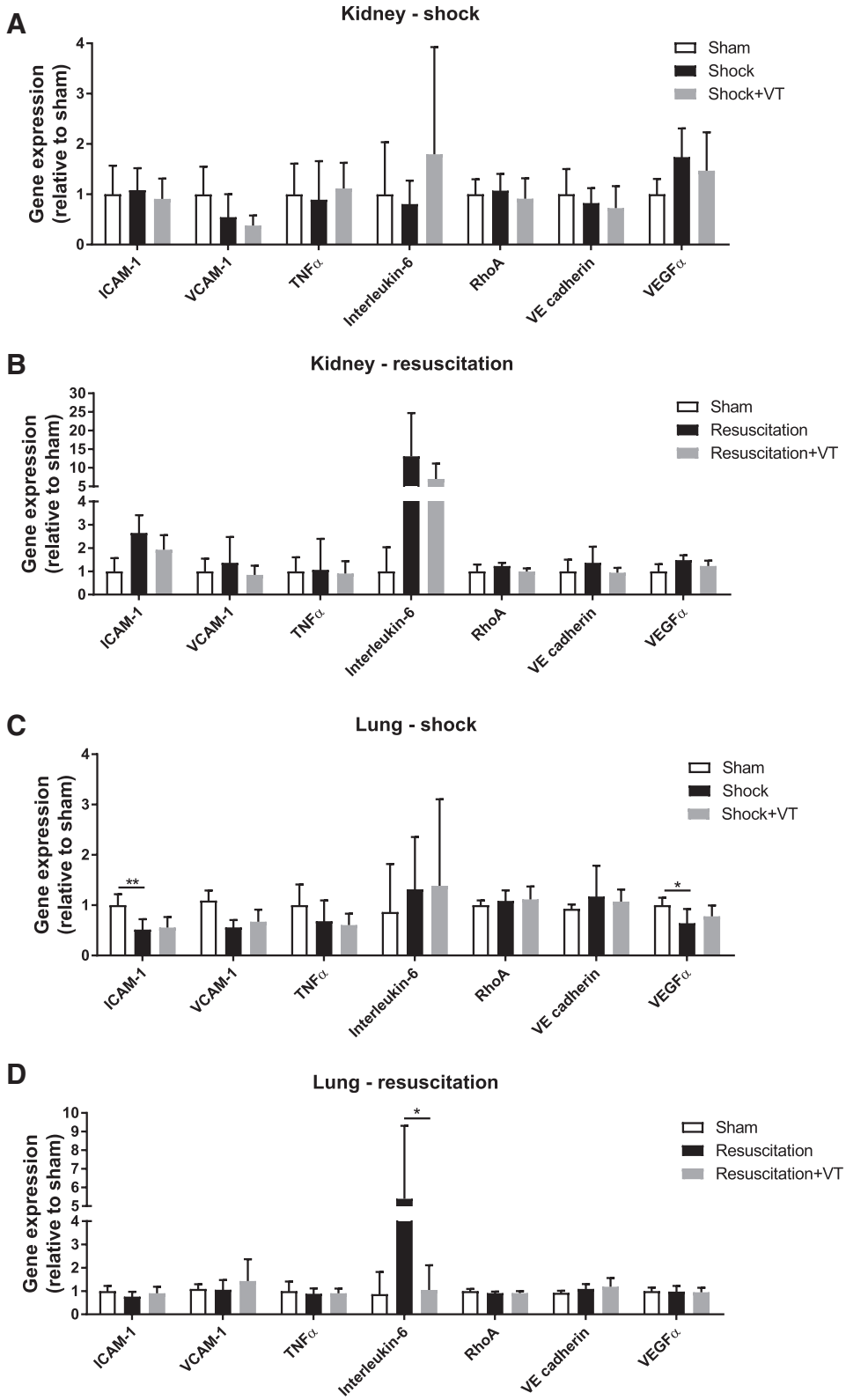

Fig. A3. Gene expression of ICAM-1, VCAM-1, TNF-α, IL-6, RhoA, VE cadherin, and VEGF-α in kidneys (*A* and *B*) and lungs (*C* and *D*) from rats after 1 h of hemorrhagic shock (shock; *A* and *C*) and 1 h after fluid resuscitation (resuscitation; *B* and *D*). Data represent mean ± SD, n = 7. One-way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

Appendix 4. Western Blots of Angiopoietin-1 and Angiopoietin-2

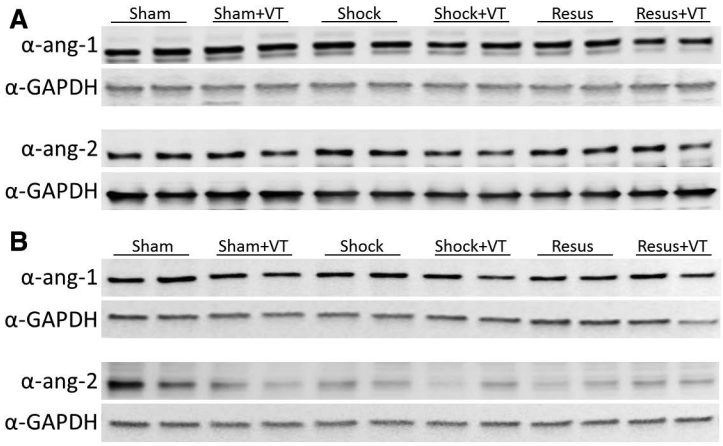

Fig. A4. Representative examples of Western blots of angiopoietin-1 (ang-1), angiopoietin-2 (ang-2), and household protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the kidneys (*A*) and lungs (*B*).

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