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Abrupt reflow enhances cytokine induced pro-inflammatory activation of endothelial cells during simulated shock and resuscitation

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Abstract

Circulatory shock and resuscitation is associated with systemic hemodynamic changes, which may contribute to the development of multiple organ dysfunction syndrome (MODS). In this study, we used an in vitro flow system to simulate the consecutive changes in blood flow as occur during hemorrhagic shock and resuscitation in vivo. We examined the kinetic responses of different endothelial genes in HUVEC pre-conditioned to 20 dyne/cm² unidirectional laminar shear stress (LSS) for 48 hours to flow cessation and abrupt reflow, respectively, as well as the effect of flow cessation and reflow on tumor necrosis factor alpha (TNFα) induced endothelial pro-inflammatory activation. Endothelial CD31 and VE-cadherin were not affected by the changes in flow in the absence or presence of TNFα. The mRNA levels of pro-inflammatory molecules E-selectin, VCAM-1 (vascular cell adhesion molecule-1), and IL-8 (Interleukin-8) were significantly induced by flow cessation respectively acute reflow, while ICAM-1 (intercellular adhesion molecule-1) was downregulated upon flow cessation and induced by subsequent acute reflow. Flow cessation also affected the Ang/Tie2 (Angiopoietin/Tie2 receptor tyrosine kinase) system by downregulating Tie2 and inducing its endothelial ligand Ang2, an effect that was further extended upon acute reflow. Furthermore, the induction of pro-inflammatory adhesion molecules by TNFα under flow cessation was significantly enhanced upon subsequent acute reflow. This study demonstrated that flow alterations per se during shock and resuscitation contribute to endothelial activation and that these alterations interact with pro-inflammatory factors co-existing in vivo such as TNFα. The abrupt reflow related enhancement of cytokine-induced endothelial pro-inflammatory activation supports the concept that sudden regain of flow during resuscitation has an aggravating effect on endothelial activation, which may play a significant role in vascular dysfunction and consequent organ injury. This study implies that the improvement of resuscitation strategies and the pharmacological interference with pro-inflammatory signaling cascades at the right time of resuscitation of shock patients may be beneficial to regain and/or maintain organ function in patients after circulatory shock.
Introduction

Vascular endothelial cells are constantly exposed to blood flow under normal physiological conditions. Shear stress produced by the blood flow modulates the structure and function of endothelial cells (1). The occurrence of blood flow disturbances in the vascular system is a hallmark of circulatory shock, which is defined as reduced organ perfusion or (micro)circulatory arrest, and contributes to patient morbidity and mortality (2). Systemic inflammatory response is conceived as the leading cause of the development of multiple organ dysfunction syndrome (MODS) following shock. In hemorrhagic shock mice, endothelial cells show early pro-inflammatory activation and vascular destabilization, a phenotype which is augmented in the early stage of resuscitation after hemorrhagic shock (3). Activated endothelial cells express adhesion molecules and chemokines which mediate interactions between leukocytes and endothelial cells as well as subsequent leukocyte migration into tissues that plays a critical role in organ injury (4).

Events proposed to be involved in endothelial activation and organ injury during hemorrhagic shock and resuscitation encompass leukocyte activation (5), the release of cytokine by innate immune cells (6), and hemodynamic alterations of microcirculatory blood flow (2). Elucidating endothelial responses to these different variables may shed light on their relative contributions to pro-inflammatory activation and vascular destabilization. They furthermore can support rational design of therapeutic strategies for maintaining proper organ function during microcirculatory arrest in shock and resuscitation as well as the period thereafter.

In the present study, our interest was to investigate the role in endothelial activation of flow cessation and its later recovery as appear in hemorrhagic shock and subsequent resuscitation. As it is difficult, if not impossible, to study in vivo flow alterations separately from consequential cytokine production and leukocyte activation and tissue hypoxia, we chose an in vitro approach to dissect flow changes from the other co-existing factors. The aim of this study was to better understand the role of shear stress and shear stress changes per se in endothelial activation, and to evaluate their effects on concomitant cytokine challenge. For this purpose, we used endothelial cells that
were pre-adapted to 48 hour laminar shear stress (LSS) to represent the condition of continuous blood flow in vivo until hemorrhagic shock and resuscitation happen. Endothelial pro-inflammatory activation and vascular integrity related molecules were studied during these flow changes (Figure 1A). They include Tie2, the expression of which was lost in hemorrhagic shock/resuscitation (7), and Angiopoietin-2 (Ang2), the vasculature destabilizing ligand of Tie2 signaling, which is known to be released by Weibel-Palade bodies upon stimulation and has a role in ischemia induced pro-inflammatory activation (8). We furthermore examined the responses of VE-cadherin and CD31 (or PECAM1) that function among others in endothelial permeability control (9, 10). Lastly we included cellular adhesion molecules E-selectin, VCAM-1, and ICAM-1 as well as chemokine IL-8 that were previously found to be regulated in vivo by installment of shock and following resuscitation (3). Transcription factor KLF2 was analyzed as a control gene to assess cell responsiveness to flow changes.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from the Endothelial Cell Facility of the UMCG. HUVEC were isolated from at least two umbilical cords and cultured on 1% gelatin coated plates or microchambers with medium (RPMI 1640, Lonza, BE) supplemented with endothelial growth factors, 20% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 5 U/ml heparin, 50 μg/ml endothelial cell growth factor, and antibiotics (100 IE/ml penicillin and 50 μg/ml streptomycin). HUVEC of passage 2 to 4 were used in this study.

Flow experiments

HUVEC were detached with trypsin/EDTA and seeded in 1% gelatin-coated microchambers (µ-Slide I 0.4 Luer, sterile, ibidi, Martinsried, Germany). 60,000 cells/cm² were used to obtain confluent monolayers overnight. Cells were subjected to 20 dyne/cm² LSS produced by flowing medium for 48 hours, where appropriate cells were subjected to flow cessation for indicated periods of time followed by reflow with 20 dyne/cm² LSS for different time periods as indicated. For gene expression analysis, cells were lysed with RLT buffer containing 10%
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Figure 1. Schematic representation of selected molecules investigated in endothelial cells and experimental setup of in vitro flow studies performed.

(A) E-selectin, VCAM-1 and ICAM-1 are cellular adhesion molecules that mediate leukocyte-endothelial interactions in inflammation. IL-8 is a chemokine that is released upon activation to regulate leukocyte attraction. Ang2, released by Weibel-Palade bodies upon pro-inflammatory activation, binds to receptor Tie2, thereby destabilizing the vasculature. Ang2/Tie2 as well as CD31 and VE-cadherin play important roles in maintaining vascular integrity. KLF2 is a transcription factor that shows high sensitivity to shear stress exposure and is analyzed in this study to molecularly validate flow alterations.

(B) Scheme of the experimental design: human umbilical vein endothelial cells (HUVEC) were seeded in ibidi microchambers and cultured overnight in static condition before exposure to unidirectional laminar shear stress (LSS). After 48 hours of 20 dyne/cm² LSS adaptation, flow was stopped for indicated time periods to mimic the marked loss of shear associated with hemorrhagic shock in vivo. To mimic the subsequent resuscitation following hemorrhagic shock, laminar flow at 20 dyne/cm² was restarted for indicated time periods. At time points indicated in certain experiments, cells were further exposed to TNFα stimulation, which is prominently produced in vivo during shock events.
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β-Mercaptoethanol (see below). For flow cytometric assays, cells were first detached with EDTA/trypsin from the slides before further processing (see below).

The experimental setup is illustrated schematically in Figure 1B.

RNA isolation and quantitative RT-PCR

Total RNA from cultured cells was isolated with the RNeasy Mini plus Kit, (Qiagen, Leusden, The Netherlands) according to the manufacturer’s instructions. Integrity of RNA was determined by gel electrophoresis, while RNA concentration (OD260) and purity (OD260/OD280) were measured using an ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). One microgram of total RNA was subsequently used for the synthesis of cDNA with SuperScript III RNase reverse transcriptase (Invitrogen, Breda, The Netherlands) in 20μl final volume containing 250ng of random hexamers (Promega, Leiden, The Netherlands) and 40 units of RNase OUT inhibitor (Invitrogen). 1μl cDNA was used for each PCR reaction. The Assay-on-Demand primers purchased from Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands) for quantitative PCR included housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, assay ID Hs99999905_m1), CD31 (Platelet endothelial cell adhesion molecule, PECAM-1, assay ID Hs00169777_m1), VE-Cad (VE-cadherin, assay ID Hs00174344_m1), KLF2 (Kruppel-like factor-2, assay ID Hs00360439_g1), Tie2 (assay ID Hs00176096_m1), Ang2 (Angiopoietin-2, assay ID Hs00169867_m1), E-selectin (assay ID Hs00174057_m1), VCAM-1 (vascular cell adhesion molecule-1, assay ID Hs00365486_m1), ICAM-1 (intracellular adhesion molecule-1, assay ID Hs00164932_m1), IL-8 (Interleukin-8, assay ID Hs00174103_m1). Quantitative PCR was performed in a ViiATM 7 real-time PCR System (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Amplification was performed using the following cycling conditions: 15min 95˚C and 40 two-step cycles of 15sec at 95˚C and 60sec at 60˚C.

Duplicate real time PCR analyses were executed for each sample, and the obtained threshold cycle values (CT) were averaged. According to the comparative CT method described in the ABI manual, gene expression was normalized to the expression of the housekeeping gene, yielding the DCT value. The average mRNA level relative to GAPDH was calculated by $2^{-\Delta CT}$.
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Data shows mean ± SD of one experiment with three replicates (n=3), which is representative of three independent experiments.

**Flow cytometric analysis**

After incubation at different conditions, HUVEC were detached with Trypsin/EDTA, and subsequently resuspended in 5% FCS solution to neutralize Trypsin/EDTA. After washing with PBS /5% FCS, HUVEC were incubated with monoclonal antibodies against E-selectin, VCAM-1 and ICAM-1 (hybridoma supernatants, kindly provided by Dr. M. Gimbrone, Boston, MA, USA), or with monoclonal antibody against CD31 (dilution 1:25; DakoCytomation, the Netherlands) for 45min on ice followed by 45min incubation with FITC labeled rabbit anti-mouse antibodies (dilution 1:50; Jackson Immunoresearch, Suffolk, UK) on ice. Cells were fixed with 0.5% paraformaldehyde in PBS and the fluorescence was detected by flow cytometry using the FACSCalibur (Becton Dickinson, the Netherlands). Nonspecific binding was assessed by staining with irrelevant isotype-matched monoclonal antibody and fluorescence intensity was measured in arbitrary units corrected by isotype control.

Data shows mean ± SD of one experiment with three replicates (n=3), which is representative of three independent experiments.

**Quantification of IL-8 protein levels by ELISA**

To quantify the concentration of IL-8 protein in the medium under different conditions, medium supernatants were collected and protein expression of IL-8 was quantified using human IL-8 ELISA max standard sets (Biolegend, UK), according to the manufacturer’s instructions. In graphs, IL-8 levels were expressed as pg per ml per cm² total cell surface to normalize for cell surface differences in different experimental conditions.

**Statistical analysis**

Statistical significance of differences was studied by means of the Student’s T-test or a one way analysis of variance (ANOVA) followed by a Bonferoni correction for selected pairs of columns. All statistical analyses were performed using GraphPad Prism software (GraphPad Prism Software Inc., San Diego, California, USA). Differences were considered to be significant when P<0.05.
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Results

The expression of endothelial genes is affected by prolonged laminar shear stress

Since LSS exposure for 1 to 2 days is essential for endothelial cells in vitro to develop maximal responses to the changes of flow in a more physiological way (11), endothelial cells in this study were allowed to be adapted to 48h of LSS before they were subsequently subjected to flow alterations (Figure 1B).

We first investigated the response of endothelial cells to prolonged LSS exposure at 20 dyne/cm$^2$ for 48 hours. As shown in Figure 2, HUVEC cultured under LSS showed a significantly increased mRNA level of KLF2 compared to static control, which is in agreement with other studies (11, 12). At the same time, Tie2 was induced more than 3.5 fold by prolonged LSS while Ang2 was downregulated by more than 90% of initial expression in static conditions. Flow exposure suppressed the expression of adhesion molecules E-selectin and VCAM-1 as well as chemokine IL-8. In contrast, ICAM-1 expression was induced. The

Figure 2. The expression of endothelial genes is affected by prolonged laminar shear stress. HUVEC were kept under static condition or exposed to laminar shear stress at 20 dyne/cm$^2$ for 48 hours before gene expression analysis. mRNA levels of genes were determined by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold changes compared to static control. Data are expressed as mean ± SD, n=3. *, P<0.05, LSS exposure vs. static control.
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expression of CD31 and VE-cadherin in endothelial cells was unchanged during long-term flow exposure. Similar results were found at 24 hours though the changes were less extensive (data not shown).

**Flow cessation after laminar shear stress pre-adaptation leads to a pro-inflammatory response of endothelial cells**

To study the effect of loss of flow to represent the shock period in extensive hemorrhage, after initial 48h exposure of HUVEC to 20 dyne/cm² LSS flow was ceased completely for different time periods (Figure 3A). The gene expression results in Figure 3B showed that the expression of flow sensor KLF2 was rapidly abolished by flow cessation. In contrast, Tie2 expression was gradually downregulated after an initial slight increase, while the loss of flow significantly upregulated Ang2 expression starting at a later time point, i.e., at 8h, and continuing up to 24h. E-selectin, VCAM-1 as well as IL-8 were induced by the loss of flow compared with LSS control, with an apparent peak expression of all three genes at 8h. After 24h loss of shear stress, VCAM-1 expression was still as high as its 8h expression level, while the expression of E-selectin and IL-8 had decreased but were still significantly higher than their levels under LSS conditions. The expression of ICAM-1, which was the only cellular adhesion molecule that was upregulated under LSS condition, was decreased upon loss of flow. Neither CD31 nor VE-cadherin expression was markedly affected by flow cessation in vitro.

At the protein level, the concentration of IL-8 in the medium of cells exposed to LSS was decreased compared to static control, while subsequent exposure to 8 hour flow cessation induced its upregulation (Figure 3C). Protein expression of ICAM-1 followed its mRNA changes,
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B

mRNA changes (relative to LSS control as 100%)

KL2

Tki2

Ang2

CO31

VE-cadherin

C

IL-8 (pg/ml/cm²)

static control  LSS  8h flow cessation

D

Mean Fluorescence Intensity (MFI)

E-selectin  VCAM-1  ICAM-1

static control  LSS  6h flow cessation

E-selectin  VCAM-1  ICAM-1

static control  LSS  24h flow cessation
Abrupt reflow enhances pro-inflammatory activation of endothelial cells. Increased protein levels were measured upon LSS exposure while they decreased upon flow cessation (Figure 3D). At the same time, the expression levels of E-selectin and VCAM-1 protein did not change compared to baseline, which might relate to the limited extent of mRNA expression induction upon flow cessation. We also examined the expression of adhesion molecules at 24 hour, showing similar results as at the 8h time point (Figure 3D). Ang2 protein that is stored in Weibel-Palade bodies may have been released into the medium upon the loss of flow, we however did not assess its concentration in the circulating medium in this study.

Although focusing on complete flow cessation, we found that reduction of shear stress from 20 dyne/cm$^2$ to 5 dyne/cm$^2$ also leads to endothelial activation. Under this condition, the pro-inflammatory molecules and Ang2 were induced, while Tie2 showed a significant downregulation (Figure 4), the latter being in agreement with a previous study (13).

Thus, endothelial cells are quickly activated by flow reduction/cessation after long term adaptation to laminar flow, though the kinetics of changes in expression of the genes involved in vascular integrity as well as pro-inflammatory activation of endothelium are variable.
Abrupt reflow associated endothelial responses after flow cessation

Fluid resuscitation to improve microvascular blood flow is an essential therapy for the treatment of any form of shock (2). Our previous in vivo study showed that endothelial pro-inflammatory status was significantly induced by fluid resuscitation after hemorrhagic shock (3). To investigate the role of shear stress changes by itself in the process of endothelial

![Graph showing endothelial responses to acute reduction of laminar shear stress to 5 dyne/cm².]

**Figure 4. Endothelial responses to acute reduction of laminar shear stress to 5 dyne/cm².**

HUVEC were exposed to LSS (20 dyne/cm²) for 48 hours and then shear stress was reduced to 5 dyne/cm² for 8 hours. Cells cultured in static condition were taken as control. Genes were determined by quantitative RT-PCR and GAPDH was taken as housekeeping gene. Values represent fold change of genes in comparison to the levels in static control. Bars show mean ± SD, n=3. *, P<0.05 vs. 48h 20 dyne/cm².
Abrupt reflow enhances pro-inflammatory activation during resuscitation, we therefore examined the effect of acute reflow on the endothelium (Figure 5A). Considering that prolonged no-reflow does exist due to reperfusion defects after hemorrhagic shock in the clinic (14), we continued our study with the 8h flow cessation time point.

Upon reflow, KLF2 expression was rapidly regained (Figure 5B). Interestingly, Tie2, which was downregulated during flow cessation, was decreased even further by reflow within the 4h time period studied. On the other hand, the induction of adhesion molecules by 0.5h reflow after 8h flow cessation suggests a quick pro-inflammatory effect of rapid onset of reflow. 4 hour after the start of reflow, VCAM-1, IL-8 and Ang2 were reduced significantly, while ICAM-1 remained elevated. In addition, reflow did not affect the expression of CD31 and VE-cadherin.

The pro-inflammatory response of endothelial cells to TNFα stimulation was enhanced by 0.5h reflow

Cytokine production is a prominent systemic inflammatory response during hemorrhagic shock and resuscitation. After hemorrhage, TNFα production shows an early increase at the 1 hour time point, and remains high until 5 hours (15). Furthermore, systemic TNFα levels induced during hemorrhagic shock are further increased by subsequent resuscitation (6). It is not known whether the changes of flow during circulatory shock and resuscitation will influence the pro-inflammatory consequences of endothelial cells induced by cytokine exposure. To study this, LSS adapted endothelial cells were stimulated with TNFα for the final 4 hours of flow cessation period and next subjected to 0.5h reflow exposure or maintained without reflow (Figure 6A). As shown in Figure 6B, the additional drop in KLF2 mRNA level by 4h TNFα exposure during flow cessation was rapidly increased upon 0.5h reflow to the similar level as it was under flow cessation conditions. Tie2 and Ang2, both not responsive to TNFα per se, showed a further reduction, respectively induction in their expression by the installation of abrupt reflow. At the same time, reflow increased the expression levels of pro-inflammatory molecules E-selectin, VCAM-1 and ICAM-1 as well as IL-8 beyond the levels induced by TNFα challenge during flow cessation. The protein expression data corroborated that subsequent acute reflow further upregulated the protein levels of VCAM-1 and ICAM-1 induced by TNFα exposure during flow cessation (Figure 6C). In addition, there
Figure 5. Abrupt reflow associated endothelial responses following initial flow cessation.

(A) To mimic the resuscitation procedure following hemorrhagic shock in vivo, LSS pre-adapted HUVEC were subjected to LSS (20 dyne/cm²) for 0.5, 1 and 4 hours after an 8 hour flow cessation period. (B) mRNA was harvested and genes were determined by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold changes of genes in comparison to the levels after 8h flow cessation conditions. Data are expressed as mean ± SD, n=3. *, P<0.05, 0.5, 1, 4h reflow vs. flow cessation which was set as 100%.
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is a tendency to increased IL-8 production upon reflow (Figure 6D). These results indicate that TNFα exposure of endothelial cells during flow cessation strongly activates the cells, and that the application of subsequent reflow aggravates this cytokine-induced endothelial response.

Discussion

Fluctuations in blood flow during shock and resuscitation lead to flow disturbances in vessels. In parallel, endothelial pro-inflammatory activation and vascular leakage occur (3, 16). These activations are more extensive at 1 hour after resuscitation compared to the endothelial priming observed at 90 minute of hemorrhagic shock (3). In this in vitro study we addressed whether, and to what extent, the changes in shear stress resulting from the loss as well as the subsequent regaining of blood flow during hemorrhagic shock and resuscitation contribute to endothelial activation. Using an in vitro model to mimic the flow changes as occur during hemorrhagic shock and resuscitation after long term LSS exposure, we here demonstrated that flow changes per se affected the expression of pro-inflammatory adhesion molecules by the endothelial cells. Each gene studied showed its own kinetic of expression upon the challenge of flow cessation and reflow. For these
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![Bar charts showing the fold change in mRNA levels of various genes under different conditions.](image_url)
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Figure 6. TNFα induced pro-inflammatory activation of endothelial cells during flow cessation is aggravated by subsequent abrupt reflow. (A) 48 hour LSS (20 dyne/cm²) pre-conditioned HUVEC were subjected to 8 hour flow cessation with or without subsequent 0.5 hour reflow (20 dyne/cm²), and TNFα (10 ng/ml) was added for the last 4 hours under both conditions. (B) Cells were lysed for mRNA isolation and genes were analyzed by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold change of genes in comparison to the levels under 8 hour flow cessation set as 1. Results are expressed as mean ± SD, n=3. *, P<0.05, 4h TNFα under flow cessation vs. flow cessation control; #, P<0.05 4h TNFα with reflow vs. 4h TNFα without reflow. (C) The expression levels of adhesion molecules on the endothelial surface were analyzed by flow cytometry and shown as mean fluorescence intensity (MFI) arbitrary units corrected by isotype control. Data are expressed as mean ± SD, n=3. *, P<0.05, vs. flow cessation condition alone; #, P<0.05, significant difference between with or without abrupt reflow exposure. (D) The concentration of IL-8 in the medium was determined by ELISA. Data are shown as mean of three independent experiments.
pro-inflammatory molecules E-selectin, VCAM-1, ICAM-1 as well as IL-8, however, the effects of flow alteration per se were minor compared to their expression induced by TNFα challenge. Yet, the induction of pro-inflammatory adhesion molecules by TNFα stimulation during flow cessation was significantly higher due to the combined exposure of reflow, implying that both blood flow changes and pro-inflammatory cytokines may contribute to endothelial activation during clinical shock and resuscitation. In addition, we showed that at 4h after reflow Tie2 had not regained its normal expression under LSS control, and that the combined exposure of reflow and TNFα extended the downregulation of Tie2 and upregulation of Ang2 expression.

Hemorrhagic shock/resuscitation can be conceived as a systemic ischemia/reperfusion injury insult, and the extent of ischemia in tissues during hemorrhagic shock is considered a major determinant of the systemic inflammatory response (17). We demonstrated in the present study that flow changes per se, i.e., flow cessation and reflow implementation, induce pro-inflammatory activation of endothelial cells. The flow characteristics in different microvascular beds from different vital organs in health and disease are not exactly known. It has been reported that microcirculatory responses to hemorrhagic shock are dependent on the vessel type. After a hemorrhage period slow and constant blood flow existed in some preferential capillary channels while other capillaries were eliminated from the circulation, and the blood flow in arterioles ceased several times (18). This indicates that endothelial cells from different microvascular beds likely experience variable flow changes during hemorrhagic shock. Similarly, hypotension is a hallmark of septic shock, and microcirculatory alterations characterized by increased number of intermittent- or stopped-flow capillaries may play an important role in sepsis-associated organ dysfunction (19). In addition, heterogeneity of microvascular endothelial cells likely underlies high cell to cell variability in their adaptation to pathology-related microenvironmental changes, which can be organ specific and microvascular-bed specific (20).
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Figure 7. Summary of the effects of shear stress changes in the absence/presence of TNFα on endothelial gene expression in vitro.

Genes associated with endothelial integrity (Tie2, Ang2) and inflammatory responses (E-Selectin, VCAM-1, ICAM-1 and IL-8) that were affected by the studied experimental conditions are shown. VE-cadherin and CD31 mRNA levels did not change under the conditions studied (data not shown in this table). Effects of TNFα exposure during flow cessation respectively flow cessation followed by reflow are given as fold changes of mRNA expression (*, fold change vs. flow cessation control). ↑↑, >10 fold induction; ↑, <10 fold induction; ↔, no change; ↓, <50% reduction; ↓↓, >50% reduction.
important follow-up study will therefore be to investigate the effects of
different shock forms like hemorrhagic shock and septic shock in animal
models and to study in detail the different microvascular segments
within organs using e.g., quantification of gene expression in laser micro
dissected microvascular segments, and intravital microscopy to study
blood flow specificities in different segments (21, 22).

Several other in vitro studies have reported on the relation between
shear stress and endothelial activation, with the majority focusing on
effects of shear stress loss. Krizanac-Bengez et al found that under
normoxic and normoglycemic conditions, loss of shear stress was able to
independently induce leukocyte-mediated pro-inflammatory activation
in brain microvascular endothelial cells, leading to failure of blood-brain
barrier function (23). Fisher and colleagues reported that flow-adapted
bovine pulmonary artery endothelial cells (BPAEC at 1 dyne/cm² for
48h) generated reactive oxygen species (ROS) upon 1h flow cessation
(mimicking ischemia), which resulted in the activation of NF-κB and AP-1
(24). They furthermore showed that in flow-adapted BPAEC (5 dyne/
cm², 24h) the sudden removal of shear stress led to ROS-dependent
phosphorylation of ERK1/2 as well as Ca²⁺-dependent NO generation
within 60 seconds (25). NF-κB, AP-1 and ERK1/2 are also involved in
the induction of pro-inflammatory activation of endothelial cells (26,
27), and NF-κB shows time-dependent activation during hemorrhagic
shock and resuscitation in mice (28). Therefore, it can be speculated
that in our study the activation of these kinases/transcription factors is
associated with the endothelial activation observed. Since endothelial
cells responded to flow changes with a similar gene expression profile
as that observed in cytokine stimulation, we measured TNFα production
in this in vitro system and found that its mRNA level was undetectable
(data not shown). The exact molecular control of endothelial activation
during flow cessation as well as reflow exposure are as of yet unknown
and will be investigated in future studies using pharmacological
inhibition of these different pathways.

KLF2 plays an important role in maintaining a quiescent endothelial
phenotype. The current study showed that the upregulation of KLF2
by long-term shear stress adaptation was rapidly abolished by flow
cessation. Interestingly, the ability of 0.5h reflow to restore KLF2
expression was inhibited by TNFα challenge (see Figure, Supplemental
Digital Content 1, which demonstrates the expression of KLF2 upon 0.5h
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TNFα exposure under flow cessation or reflow conditions), implying a diminished adaptive capacity of endothelial cells to cytokine stimulation under abrupt reflow conditions. Gracia-Sancho et al have shown that endothelial cells with reduced KLF2 expression upon flow cessation under cold storage condition (4 degree) show higher responsiveness to cytokine IL-1β (29). Possibly, the TNFα-related inhibition of KLF2 re-expression upon reflow in our study contributes to the relatively higher responsiveness of endothelial activation under reflow conditions. Clinically this may be relevant to sepsis where flow changes and cytokines are both abundantly present. It is worthwhile to follow up on this observation and to investigate the in vivo kinetics of KLF2 expression during HS and resuscitation, and to verify whether a diminished KLF2 re-expression by TNFα is involved in the underlying molecular mechanisms of endothelial activation during resuscitation.

There is accumulating evidence emphasizing the critical role of the Ang/Tie2 system in vascular dysfunction during critical illness. We previously showed in vivo that Tie2 expression was reduced at 4 h after HS and resuscitation, and normalized at 24 h (7). In the present study we showed that the removal of shear stress in vitro caused loss of Tie2, and that the subsequent acute re-administration of flow did not recover its expression. At the same time, Ang2 was induced while the re-expression of KLF2 was abolished by TNFα challenge, showing an inverse relationship between KLF2 and Ang2 that was previously described in a study with KLF2 overexpressing endothelial cells (30). The decrease of Tie2 as well as the induction of Ang2 due to flow alterations may thus contribute to microvascular dysfunction, including increased vascular leakage and extended pro-inflammatory activation as observed in vivo.

Our current study has several limitations that should be kept in mind. First of all, it is difficult to directly translate the in vitro flow induced endothelial changes to the bed of the shock patient. Beyond the studied flow effects, other processes will likely occur simultaneously to induce endothelial activation during shock and resuscitation. Amongst others, impaired oxygen delivery induced tissue hypoxia (31), leukocyte activation (5) and cytokine release (6) are all known to be involved in endothelial activation. At the same time, exclusion of other cell types co-existing in vivo in the microenvironment of the endothelium may also make that the in vitro flow model dose not recapitulate the in vivo
situation during shock and resuscitation. Second, unidirectional laminar flow has been used throughout our present study, whereas in the shock patient the different features of shear stress in various vessel types, e.g., arterial bifurcations and stenotic vessels, can influence downstream endothelial behavior (32). Third, we have used 20 to 5 dyne/cm² and 20 to 0 dyne/cm² flow changes in our study. Although the shear stress values in various vessels has been estimated (33), the exact flow magnitudes and patterns in distinct macro- and microvascular beds under healthy condition as well as in critically ill patients are not known. Fourth, we chose specifically pre-defined time points of flow cessation and reflow to compare this in vitro study with our in vivo hemorrhagic shock (3) and LPS exposure mouse models (13). In patients, this time frame may be shorter or much longer, as microvascular flow derangements can be limited to a few seconds (preoperative hypotension after anesthesia induction), to several minutes (massive surgical bleeding), or be present for days (critically ill sepsis patients). Therefore, only carefully combining our in vitro data presented here with data from animal shock model and human organ biopsies of deceased patients (34) will help to bridge this bench-to-bedside gap.

Collectively, our study revealed that flow alterations per se, as experienced by endothelial cells during microcirculatory shock and resuscitation, act as a pro-inflammatory stimulus, and that the flow changes in combination with other pro-inflammatory factors such as TNFα that co-exist in critically ill patients likely interact with each other. The abrupt-reflow related enhancement of cytokine-induced endothelial inflammatory activation suggests that inflammation related signaling cascades at the time of resuscitation may be potential targets of pharmacological interventions to attenuate endothelial activation and the development of consequent multiple organ failure. Moreover, the changes in expression of Ang2/Tie2 by flow alterations observed in this study indicate that improving organ perfusion, a hallmark of modern critical care, is likely to positively affect microvascular function. In addition, it has been shown that phosphorylation dependent activation of Tie2 can be induced by the onset of shear stress (35). This Tie2 phosphorylation upon flow cessation and reflow will be further investigated in our in vitro flow study as well as in vivo models (hemorrhagic shock and septic shock mice, patients) in the future. Since the flow alteration-associated endothelial dysfunction may be a critical
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determinant for the development of pathological changes during shock
and resuscitation as well as for post-resuscitation organ function, future
studies will be executed to investigate which molecular pathways can be
therapeutically interfered with in the microvasculature.

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Supplementary data

Supplemental Digital Content 1. TNFα exposure diminished the expression of KLF2 restored by 0.5h reflow.

48h LSS (20 dyne/cm²) adapted HUVEC were subjected to 8 hour flow cessation with or without subsequent 0.5h reflow exposure (20 dyne/cm²). TNFα (10 ng/ml) was added for the last 0.5h under flow cessation condition or 0.5h reflow condition. Cells were lysed for mRNA isolation and genes were analyzed by quantitative RT-PCR, using GAPDH as housekeeping gene. Values represent fold change of genes in comparison to the levels under 8 hour flow cessation condition alone. Data are expressed as mean ± SD, n=3. *, P<0.05, significant induction of KLF2 by reflow compared to the level under flow cessation condition; #, P<0.05, significant downregulation of KLF2 by TNFα vs. PBS under 0.5 hour reflow condition.