CHAPTER 3

HEMORRHAGIC SHOCK INDUCED PRO-INFLAMMATORY ENDOTHELIAL CELL ACTIVATION IN LUNG AND KIDNEY IN MICE IS NOT INFLUENCED BY MECHANICAL VENTILATION AND IS NOT HYPOXIA MEDIATED

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Abstract

Introduction: The interaction between neutrophils and activated endothelium is essential for the migration of neutrophils into tissues, and for the development of multiple organ dysfunction in patients suffering from hemorrhagic shock (HS). HS leads to early and organ specific pro-inflammatory microvascular endothelial activation. Mechanical Ventilation (MV) is frequently employed in patients with HS. Intubation and MV does protect organs from hypoxia and hypercapnia, on the other hand MV may initiate an inflammatory reaction and induce inflammation of the lung and distant organs. Our aim was to investigate the consequences of mechanical ventilation of mice subjected to HS on microvascular endothelial activation in the lung and kidney.

Methods: Anesthetized wild type C57Bl/6 male mice were subjected to controlled hemorrhage. Mice were killed after 90 minutes of HS. After 90 minutes of HS, a group of mice was resuscitated and sacrificed 24 hours after shock induction. To examine the effects of mechanical ventilation, subgroups of mice were mechanical ventilated during the HS insult. To study the effect of acute hypoxia mice were housed in cages with 6, 10 and 21 % oxygen during 2 hours and harvested. Untreated mice served as controls. Gene expression levels of endothelial cell activation, (E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1), inflammation (TNF-α, IL-6 and MCP-1) and hypoxia-responsive genes (vascular endothelial growth factor and hypoxia-inducible factor 1α) were quantified in kidney and lung, by quantitative reverse-transcription polymerase chain reaction. A selection of these genes was examined with regard to protein expression levels and localization using immunohistochemical analysis. Soluble pro-inflammatory cytokine (TNF-α, IL-6, and CXCL-1) levels in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA).

Results: 90 minutes after shock induction a vascular bed specific, heterogeneous pro-inflammatory endothelial activation represented by E-selectin, VCAM-1 and ICAM-1 expression was seen in kidney and lung. No differences in adhesion molecules between the spontaneous breathing and mechanically ventilated mice were found. Also no differences in endothelial pro-inflammatory activation cytokines and hypoxia influenced genes were found for TNF-α, IL-6, MCP-1 and HIF-1α at 90 minutes after shock induction between the spontaneous breathing and mechanically ventilated mice. During HS,
HIF-1α mRNA was not induced in the kidney, while in the lung HS led to HIF-1α mRNA upregulation, with no differences between HS alone and HS combined with mechanical ventilation. To determine the contribution of tissue hypoxia due to decreased oxygen delivery to the pro-inflammatory endothelial activation observed in kidney and lung, we subsequently studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only. 2 hours of 6% hypoxia induced upregulation of proinflammatory cytokines IL-6, and MCP-1 mRNA in the lung but not in the kidney, while TNF-α mRNA was unchanged. Two hours of 6% oxygen did not however induce the expression of E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice.

Conclusions: Hemorrhagic shock leads to an early and reversible pro-inflammatory endothelial activation in kidney and lung. This proinflammatory cytokine response during HS can induce an endothelial pro-inflammatory activation. HS induced endothelial activation is not augmented nor prevented by mechanical ventilation during the shock phase. Hypoxia alone does not lead to endothelial activation.
INTRODUCTION

The development of multiple organ dysfunction syndrome (MODS) is a complication in patients who suffer from major bleeding\(^1\). Advances in medical care of HS patients, including the introduction of resuscitation fluids, trauma centers, intensive care units, and mechanical ventilation have resulted in a significant decrease in early deaths caused by HS. But instead of dying, patients can develop MODS. The inflammatory response is considered the leading cause for the development of MODS. Two failing organs are the lung and the kidney. Lung failure, the so called Acute Respiratory Distress Syndrome (ARDS), and acute kidney injury (AKI) are strongly associated with patient morbidity and mortality\(^2\). To treat symptoms of ARDS and AKI, patients are treated with mechanical ventilation and renal replacement therapy. The precise mechanisms leading to MODS after HS are still largely unknown. One of the proposed mechanisms is infiltration of neutrophils into the tissues, leading to significant organ damage through release of proteases and oxygen-derived radicals. The interaction between neutrophils and endothelium is essential for the migration of neutrophils into tissues\(^3\). This migration is regulated by adhesion molecules on both leukocytes and endothelium, the latter including E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1)\(^4\). We have recently shown that HS leads to early and organ specific pro-inflammatory microvascular endothelial activation\(^5\). This organ and microvascular bed specific endothelial activation is also seen in animal models subjected to septic shock, where it coincides with increased vascular leakage\(^6; 7\). HS occurs frequently in the operating theatre as a result of difficult to control surgical bleeding. Patients with HS due to other causes including trauma or gastrointestinal bleeding require resuscitation and procedures to control the bleeding\(^8; 9\). Therefore HS patients will be frequently intubated and mechanically ventilated to allow intervention procedures and warrant a patent airway and gas exchange\(^10\). Mechanical ventilation (MV) during conditions of hemorrhagic shock can act as a double-edged sword. On the one hand intubation and mechanical ventilation may protect organs from hypoxia and hypercapnia, on the other hand it may initiate an inflammatory reaction\(^11\) and induce pro-inflammatory activation in the lung and in distant organs\(^12; 13\).
To follow up on our earlier observation of hemorrhagic shock induced microvascular endothelial priming during the shock phase\textsuperscript{6}, our aim was to investigate the beneficial, neutral or harmful consequences of mechanical ventilation in mice subjected to HS on microvascular endothelial activation in the lung and kidney. Mechanical ventilation is started to prevent hypoxia and threatened oxygen delivery. HS by definition decreases oxygen delivery\textsuperscript{14}, yet the role of decreased oxygen availability in endothelial activation is not clear. To study the effect of a decreased oxygen delivery in vivo on endothelial cell (EC) activation we also evaluated endothelial proinflammatory adhesion molecule expression in hypoxic mice.

**MATERIALS AND METHODS**

**Animals**

8- to 12-wk-old C57Bl/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water ad libitum in a temperature-controlled chamber at 24°C with a 12:12-h light-dark cycle. All procedures were approved by the local committee for care and use of laboratory animals and were performed according to national and international guidelines on animal experimentation.

**Mouse shock model**

The mouse hemorrhagic shock model has been extensively documented elsewhere\textsuperscript{15,16}. In short, mice were anesthetized with isoflurane (inspiratory, 1.4%), N\textsubscript{2}O (66%), and O\textsubscript{2} (33%). The left femoral artery was canulated for monitoring mean arterial pressure (MAP), blood withdrawal, and resuscitation. Hemorrhagic shock was achieved by blood withdrawal until a reduction of the MAP to 30 mmHg. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain a pressure constant hemorrhagic shock model with a MAP at 30 mmHg during this period. A subset of mice was resuscitated after 90 minutes of hemorrhagic shock with 6% hydroxyethyl starch 130/0.4 (Voluven\textsuperscript{®}; Fresenius-Kabi, Bad Homburg, Germany) at two times the volume of blood withdrawn and sacrificed at 24 hours post HS induction. During sacrifice, blood was withdrawn via aortic puncture under isoflurane anaesthesia, and the kidneys and...
lungs were excised, snap-frozen in metal cups on liquid nitrogen, and stored at –80°C until analysis.

**Mouse mechanical ventilation model**

The mechanical ventilation model employed has been described previously\(^ {11, 17}\). In short mice were anesthetized with isoflurane inspiratory 3.0% induction in N\(_2\)O (2 liter/min), and O\(_2\) (1 liter/min). Animals were orally intubated under direct vision with an endotracheal tube (0.82 mm ID, 1.1 mm OD, length 25 mm). After intubation anaesthesia was continued as described above. Endotracheal tube position was confirmed by bilateral chest excursions. Subsequently animals were connected to the ventilator (MiniVent\(^{®}\); Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). Tidal volume was set at 180 μl, frequency was set at 150/min. All animals received 4 cm H\(_2\)O PEEP.

**Mouse hypoxia model**

To examine the role of acute hypoxia on endothelial pro-inflammatory and hypoxia driven genes, a subset of mice was housed for 2 hours in respiratory cages to manipulate oxygen concentration. Oxygen concentration was set at 21%, 10% and 6% respectively. Hypoxia exposed mice and unexposed control mice were sacrificed under isoflurane anaesthesia, after which blood was withdrawn and kidneys and lung were harvested and handled as described above.

**Gene expression analysis by quantitative RT-PCR**

RNA was extracted from 20 x 5-μm cryosections from kidney and mouse lung, and isolated using the RNeasy Mini Plus kit (Qiagen, Leusden, The Netherlands) according to the manufacturer’s instructions. Integrity of RNA was determined by gel electrophoresis. RNA yield and purity were measured by an ND-1,000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The Assay-on-Demand primers (ABI Systems, Foster City, CA) used in the PCR included the housekeeping gene GAPDH (assay ID Mm99999915_g1), E-selectin (assay ID Mm00441278_m1), ICAM-1 (assay ID Mm00516023_m1), VCAM-1 (assay ID Mm00449197_m1), TNF-α (assay ID
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Mm00443258_m1), IL-6 (assay ID Mm00446190_m1), MCP-1 (assay ID Mm00441242_m1), HIF-1α (assay ID Mm00468869_m1), VEGF-A (assay ID Mm00437304_m). 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 ΔCT value. The relative mRNA level was calculated by $2^{ΔCT}$ and per group averaged.

Cytokine analysis

TNF-α, interleukin (IL)-6, and CXCL-1 levels in plasma were analyzed by enzyme-linked-immunosorbent assay (ELISA) (TNF-α, IL-6, CytoSet, BioSource, CA; CXCL-1: ELISA kit, R&D Systems, Minneapolis, MN). Lower detection limits: 32 pg/ml for tumour necrosis factor-α; 160 pg/ml for IL-6; 160 pg/ml for for CXCL-1.

Localization of adhesion molecule expression using immunohistochemistry

Localization of CD31, E-selectin, VCAM-1, and ICAM-1 expression was determined in kidney by immunohistochemistry. Snap frozen organs were cryostat cut at 5 µm, mounted onto glass slides, and fixed with acetone for 10 minutes. After drying, sections were incubated for 45 minutes at room temperature with primary rat anti-mouse antibodies recognizing CD31 (clone MEC13.3; Pharmingen BD Biosciences, Alphen aan de Rijn, The Netherlands), E-selectin (MES-1, kindly provided by Dr. D. Brown, UCB Celltech, Brussels Belgium), and ICAM-1 (clone YN1/1.7; ATCC) in the presence of 5% fetal calf serum. After washing, endogenous peroxidase was blocked by incubation with 0.1% H$_2$O$_2$ in PBS for 20 minutes. This was followed by incubation for 30 minutes at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (rabbit anti rat-Ig, DAKO, Glostrup, Denmark). Conjugates were diluted 1:50 in PBS supplemented with 2% normal mouse serum. Sections with isotype matched controls and E-selectin antibodies were further incubated for 30 min at room temperature with HRP-conjugated goat anti-rabbit antibody (Southern Biotech Association, Birmingham, Alabama, USA) diluted 1:100 in PBS. Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich Chemie, St.Louis, Missouri, USA) and sections were counterstained with Mayer’s
hematoxylin (Klinipath, Duiven, The Netherlands).

Statistical analysis

Statistical significance of differences was studied by means of Student’s t-test or ANOVA with post hoc comparison using Dunnet correction. First 90 minutes time points and 24 hours time points HS were compared with HS combined with mechanical ventilation. When there were no differences, HS and MV at the same time points were pooled, for increased statistical power. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered to be significant when \( p < 0.05 \).

RESULTS

Mice under anesthesia were intubated and mechanically ventilated or allowed to breathe spontaneously while blood was withdrawn to reach a MAP of 30 mmHg. No differences in blood pressure were observed between the groups, neither during the

Figure 3.1. Study design and measured mean arterial pressure in mice during hemorrhagic shock in the presence or absence of mechanical ventilation.

Hemorrhage was induced by blood withdrawal as described in ‘Materials and Methods’. Groups of mice were sacrificed (†) at the start of the experiment (control), or after 90 minutes of HS. Following 90 minutes of hemorrhagic shock, a subgroup of mice was resuscitated and sacrificed 24 hours after shock induction. Spontaneous breathing mice (□) were compared with mechanically ventilated mice (▲). Data are expressed as mean ± SD, \( n>5 \) per group.
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Figure 3.2. Kinetics of mRNA changes of endothelial cell adhesion molecules during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.

Gene expression levels of E-selectin (a, b), VCAM-1 (c, d), and ICAM-1 (e, f) in kidney (a, c, e) and lung (b, d, f) were analysed by quantitative RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SEM, n>5, * p< 0.05.

shock phase nor during the post shock phase (figure 3.1). The initial blood pressure after induction in the spontaneous breathing group (mean 82 mmHg, SD 16.2) was statistically not significantly different than the blood pressure in the mechanical ventilation group (mean 71 mmHg, SD 13.8). Mice in all the groups remained normoxic and did not became hypercapnic (data not shown).

Ninety minutes after shock induction, an endothelial pro-inflammatory activation was observed reflected by increased E-selectin, VCAM-1, ICAM-1 mRNA expression levels (figure 3.2). After 24 hours the pro-inflammatory endothelial activation genes in both the mechanical ventilation and the spontaneously breathing group were back to baseline.
level (figure 3.2). No differences between the spontaneous breathing and mechanically ventilated mice were found for E-selectin, VCAM-1, and ICAM-1 at 90 minutes or 24 hours after shock induction. The lung and the kidney have different endothelial response patterns during HS. VCAM-1 mRNA levels were upregulated in the lung after shock (figure 3.2d) but unchanged in the kidney (figure 3.2c). In the kidney and the lung, both mRNA of E-selectin and ICAM-1 were upregulated after 90 minutes of HS (figure 3.2a, 3.2b, 3.2e and 3.2f).

To further extend our knowledge on microvascular bed specific differences of endothelial activation in HS, we examined these endothelial pro-inflammatory activation
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Endothelial pro-inflammatory activation can be caused by pro-inflammatory cytokines and hypoxia. First, we investigated the mRNA expression levels in lungs and kidneys of the pro-inflammatory cytokines TNF-α, IL-6 and MCP-1 in time. No differences between the spontaneous breathing and mechanically ventilated HS mice were found for TNF-α, IL-6, and MCP-1 90 minutes after shock induction (figure 3.4a-f). In the kidney and lung, 90 minutes HS induced a small increase in mRNA for TNF-α (figure 3.4a, 3.4b) and IL-6 (figure 3.4c and 3.4d), while MCP-1 mRNA levels were unchanged. (figure 3.4e, 3.4f). To investigate whether the pro-inflammatory cytokines TNF-α, CXCL-1 and IL-6 were produced in remote organs we measured soluble cytokine proteins in plasma. While TNF-α protein in plasma was not changed at 90 minutes (figure 3.5a), levels of the pro-inflammatory cytokines CXCL-1 and IL-6 were significantly increased after 90 minutes (figure 3.5b, 3.5c). Mechanical ventilation during 90 minutes of hemorrhagic shock did not affect these HS induced changes in IL-6 and CXCL-1 in the systemic circulation (figure 3.5). At 24 hours after the shock period, all pro-inflammatory cytokines in the plasma were back to baseline (figure 3.5).

Hypoxia can induce HIF-1α both via transcriptional control and via posttranslational processes affecting the protein level, with an increase in VEGF-A as one of the downstream consequences. We therefore investigated whether shock induced changes in cellular oxygen levels influenced HIF-1α and VEGF-A, in our model. During the shock
Figure 3.4. Kinetics of expression of pro-inflammatory and hypoxia related genes in kidney and lung during hemorrhagic shock in the presence or absence of mechanical ventilation.

Gene expression levels of the pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 and the hypoxia related molecules HIF-1α and VEGF-A in kidney and lung analysed by quantitative RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SEM, n>5. p < 0.05 values are marked with *.
period, HIF-1α mRNA was not induced in the kidney, while in the lung it led to HIF-1α mRNA upregulation, with no differences between HS alone and HS combined with mechanical ventilation (figure 3.4g and 3.4h). VEGF-A mRNA showed a small but significant upregulation in the 90 minutes with MV compared to HS alone, both in lung and kidney compared to the spontaneous breathing mice (figure 3.4i and 3.4j).

The upregulation of HIF-1α in the lung and the upregulation of VEGF-A in the mechanical ventilation group implies that a hypoxic condition may have occurred during the shock.

Figure 3.5. Pro-inflammatory cytokines in plasma during hemorrhagic shock and resuscitation in the presence or absence of mechanical ventilation.
Levels of TNF-α, CXCL-1, and IL-6 in control (C), HS during 90 minutes, with and without mechanical ventilation and 24 after the HS insult with or without mechanical ventilation. Data are expressed as mean ± SD, n>4 for all groups. * p < 0.05 compared with control mice.
period in the absence and presence of mechanical ventilation. To determine the potential contribution of tissue hypoxia to the pro-inflammatory endothelial activation observed, we next studied endothelial pro-inflammatory activation in response to short term exposure to severe hypoxia only. To expose the mice to hypoxia during a similar period of time as the HS mice, including the time needed for instrumentation and anesthesia, mice were housed for 120 minutes in hypoxic cages with three different oxygen levels (figure 3.6). Neither 120 minutes of 6% oxygen, nor 10% oxygen (data not shown), induced the expression of the pro-inflammatory genes E-selectin, VCAM-1 and ICAM-1 in the kidneys and the lung of mice (figure 3.7). Surprisingly, this acute and severe hypoxia also did not increase the mRNA expression levels of HIF-1α and VEGF-A in lung and kidney (figure 3.7m-p). The severe hypoxia, did, however induce a pro-inflammatory response in the lung; 2 hours of 6% hypoxia led to the upregulation of pro-inflammatory cytokines IL-6 and MCP-1 mRNA, while TNF-α mRNA was unchanged (figure 3.7). In the kidney no differences in mRNA for IL-6, MCP-1 and TNF-α were observed between normal oxygen levels and severe hypoxia (figure 3.7g-k).
Figure 3.7. Kinetics of mRNA of endothelial cell adhesion molecules, pro-inflammatory cytokines and hypoxia related genes during acute hypoxia.
Gene expression levels of E-selectin (a, b), VCAM-1 (c, d), and ICAM-1 (e, f), and the pro-inflammatory cytokines TNF-α (g, h), IL-6 (i, j), and MCP-1 (k, l) and the hypoxia related molecules HIF-1α (k, l) and VEGF-A (m, n) in kidney (a, c, e, g, i, k, m, o) and lung (b, d, f, h, j, l, n, p). Gene expression was analysed by quantitative RT-PCR using GAPDH as housekeeping gene after 120 minutes of acute hypoxia, during normoxia (21%) and hypoxia (6%). Data are expressed as mean ± SEM, n>5. N.D. = not detectable.
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DISCUSSION

The interaction between neutrophils and activated endothelium is crucial for the development of multiple organ dysfunction in patients suffering from hemorrhagic shock, but the effects of MV on this endothelial activation is not known. In this study of mechanical ventilation during hemorrhagic shock in mice, we demonstrated that the pro-inflammatory endothelial cell activation in the lung and the kidney was not prevented nor augmented by mechanical ventilation. Furthermore, we found that the organ specific endothelial activation in HS is not induced by an impaired oxygen delivery but merely by a systemic pro-inflammatory response induced by the hemorrhagic shock.

To our knowledge, we are the first to report the effect of mechanical ventilation during HS and its effects on EC activation in mice. Mechanical ventilation alone is able to induce an inflammatory response in mice and man. In mice, mechanical ventilation induced TNF-α, IL-6 and IL-1β in mice lung already 60 minutes after MV initiation, whereas plasma protein levels followed some time thereafter. Moreover mechanical ventilation in mice led to endothelial activation in lung and distant organs within 120 minutes after MV initiation. The data in our studies show that, compared to the HS insult, no additional detrimental effects on endothelial inflammation are induced when mechanical ventilation is performed in haemorrhagic shock animals. This implies that the insult given is too mild or too short to add additional detrimental effects.

Our results on the early pro-inflammatory cytokine release during HS corroborate several other animal studies. For example, Liu and Dubick showed in rats with an HS to a MAP of 50 mmHg during 60 minutes that the mRNA encoding for TNF-α was up-regulated in ileum, kidney, liver, and skeletal muscle. Serum TNF-α was increased in a mouse model already 30 minutes after initiation of shock.

Cellular hypoxia is considered to be an important mediator of MODS after HS. During hypoxic conditions, HIF-1α accumulates in the cell, translocates to the nucleus and forms a stable heterodimer, after which it induces gene transcription. HIF-1α can also be regulated at the transcriptional level as demonstrated in tumour models. Vascular endothelial growth factor A is a downstream target gene of HIF-1α that is primarily regulated at the transcriptional level and a major controller of vascular permeability in shock states. Under hypoxic conditions, up-regulation of VEGF-A by HIF-1α occurs
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within minutes\textsuperscript{26}. In cellular experiments using the human umbilical vein cell line, EA.hy926, incubation for 16 hours in 1\% oxygen, induced HIF-1 nuclear translocation. This was accompanied by ICAM-1 and E-selectin mRNA upregulation compared to cells that were incubated in normoxia\textsuperscript{19}. In Human Aortic Endothelial Cells (HAEC) however incubated for 8 hours in 4\% oxygen hypoxia no upregulation of E-selectin, VCAM-1 or ICAM-1 could be observed by Illumina gene microarray analysis\textsuperscript{27}. In our model however, we did not see any significant induction of mRNA of HIF-1\textalpha\ nor VEGF-A during HS alone, while mechanical ventilation combined with HS led to a small increase in HIF-1\textalpha\ and VEGF-A mRNA. Moreover HIF-1\textalpha\ and VEGF-A were not affected during our acute hypoxia experiments in mice without shock. Our data are contrary to Koury et al.\textsuperscript{28}, who showed that in a pressure-controlled HS model in the rat, HIF-1\textalpha\ levels as measured by Western blotting were increased in the ileac mucosa after 90 minutes of HS of a MAP of 30 mmHg. Hierholzer et al.\textsuperscript{29} observed an increase in HIF-1\textalpha\ DNA binding activity of 3.2-fold in the lung after a 40 mmHg MAP shock period of 2.5 h. No increased HIF-1\textalpha\ activation in livers of animals subjected to 40 mmHg MAP HS for 60 minutes was found\textsuperscript{30}. From these studies it becomes clear that there are large organ, insult and time-frame dependent differences that regulate HIF-1\textalpha\ responsiveness in HS models. Our data and those reported by others therefore suggest that severe whole body hypoxia is not the driving factor for EC activation in HS in kidney and lung, but that large inter-organ differences might exist.

Our animal model of HS with or without MV has several limitations. We used a short shock period to mimic the short and severe non-resuscitated HS seen in the clinic, while the described pro-inflammatory effects of MV might be more pronounced after longer periods of MV\textsuperscript{11; 12}. Possibly, after the shock insult has resolved, ongoing MV may lead to additional organ damage. This clinical important question can however not be tested in this small rodent model because of instabilities of this model after prolonged MV. A larger animal model with more physiological monitoring might in the future provide more insight in the consequences of longer mechanical ventilation exposure after the shock insult on EC activation. Furthermore in our hypoxic cage experiments we did not measure blood gases nor oxygen delivery capacity. In man however, it has been shown that 12\% normobaric hypoxia is sufficient to cause hyperlactatemia as an indicator of insufficient oxygen transport\textsuperscript{31}. Therefore we hypothesised that 6\% oxygen is sufficient
to induce an impaired oxygen delivery. This oxygen concentration leads to an estimated
\( pO_2 \) of 4-5 kPa and an oxygen saturation of 50-60%. In our model the severe hypoxia
induced a proinflammatory response in the lung; 2 hours of 6% hypoxia lead to the
upregulation of IL-6 and MCP-1 mRNA. It is of note that in the hypoxic animals blood
gases were uncontrolled. It cannot be excluded that the severe hypoxic animals became
hypercapnic secondary to a decrease in ventilation, i.e. hypoxic ventilatory decline and/
or muscle fatigue during the 2 hours exposure period. Lastly the translation of our rodent
results to HS patients is difficult. In our experiments, we used young male, otherwise
healthy mice, which do not model older patients with multiple co morbidities. These
effects of aging and co-morbidity on the systemic and microvascular responsiveness to
HS and mechanical ventilation are now under investigation.

The early endothelial activation found in this study suggests that the therapeutic
window to attenuate endothelial cell activation takes place early during the shock insult.
This EC activation is induced at least in part by a pro-inflammatory response induced by
pro-inflammatory cytokines, while in the mouse whole body cellular hypoxia does not
induce endothelial adhesion molecules in the same time frame. Mechanical ventilation
perse does not add to the pro-inflammatory endothelial activation seen in lung and
kidneys in our model of HS. Further studies will investigate potential therapeutic strategies
to diminish pro-inflammatory endothelial activation in HS on endothelial dysfunction.

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