Chapter 4

Histone deacetylase inhibition and IkappaB kinase/NF-kappaB blockade ameliorate microvascular pro-inflammatory responses associated with hemorrhagic shock/resuscitation in mice

Ranran Li; Adnan Aslan; Rui Yan; Rianne M Jongman; Jill Moser; Peter J. Zwiers; Henk E Moorlag; Jan G Zijlstra; Grietje Molema; Matijs van Meurs.

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1Department of Pathology and Medical Biology, Medical Biology Section, 2Department of Critical Care, 3Department of Anesthesiology, University Medical Center Groningen, University of Groningen; Groningen, the Netherlands.
Chapter 4

Abstract

Objective: To investigate the consequences of histone deacetylase inhibition by HDAC inhibitor valproic acid (VPA) respectively of IkappaB kinase/NF-kappaB (IKK/NF-κB) signaling blockade by IKK inhibitor BAY11-7082 on (microvascular) endothelial cell behavior in vitro as well as in mice subjected to hemorrhagic shock (HS)/resuscitation in vivo.

Design: Prospective, randomized laboratory investigation using an established mouse model of hemorrhagic shock.

Setting: Research laboratory at university teaching hospital.

Subjects: Endothelial cells and C57BL/6 male mice.

Interventions: Endothelial cells were incubated with tumor necrosis factor-α in the absence or presence of valproic acid or BAY11-7082 in vitro. Mice were subjected to hemorrhagic shock by blood withdrawn until the mean arterial pressure of 30 mm Hg and maintained at this pressure for 90 minutes. At 90 minutes, subgroups of mice were resuscitated with 4% human albumin in the absence or presence of vehicle, valproic acid (300 μg/g body weight) or BAY11-7082 (400 μg per mouse). Mice were killed 1 hour and 4 hours after resuscitation.

Measurements and Main Results: VPA and BAY11-7082 selectively diminished TNFα-induced endothelial pro-inflammatory activation in vitro. In vivo, both systemic and local inflammatory responses were significantly induced by HS/resuscitation. The decreased histone acetylation in kidney after HS/resuscitation was restored by VPA treatment. In glomerular endothelial cells, the nuclear translocation of NF-κB, which was induced by HS/resuscitation, was eliminated by BAY11-7082 treatment while enhanced in the presence of VPA. Both VPA and BAY11-7082 significantly attenuated the HS/resuscitation-induced protein expression of endothelial cell adhesion molecules E-selectin and vascular cell adhesion molecule-1 in the microvasculature of kidney and liver, although mRNA expression levels of these molecules analyzed in whole organ lysates of kidney, lungs, and liver were not extensively affected. The reduced protein expression of adhesion molecules was paralleled by diminished the adhesion/transmigration of polymorphonuclear leukocytes in kidney and liver after HS/resuscitation.

Conclusion: Suppression of HDAC activity and blockade of IKK/NF-κB signaling during resuscitation ameliorate microvascular endothelial pro-inflammatory responses in organs in mice after HS.
Drug interventions in hemorrhagic shock

Introduction

Hemorrhagic shock (HS), a life-threatening organ hypoperfusion caused by rapid and substantial blood loss, is a medical emergency frequently encountered by anesthesiologists and intensivists (1). Severe HS is associated with multiple organ dysfunction syndromes (MODS) and death in trauma, surgical, and medical patients. Fluid resuscitation to restore tissue perfusion is the first therapeutic intervention in HS (2), although it remains controversial because of the increased blood loss and mortality due to aggressive restoration of the intravascular volume and increased blood pressure (3, 4). Nowadays, treatment of HS combines early control of bleeding, correction of coagulopathy, maintenance of critical tissue perfusion, and management of the systemic inflammatory response syndrome (SIRS) (1). No drugs interfering with the inflammatory responses have so far proven clinical benefit. Therefore, the search for effective drugs that are able to counteract systemic inflammatory response-related MODS in HS/resuscitation continues.

Multiple mechanisms are involved in the pathogenesis of MODS after HS, including the production of pro-inflammatory cytokines and the disturbance of the (micro)circulation (5, 6). Microvascular endothelial cells actively engage in the development of MODS, orchestrating their interaction with leukocytes via induced expression of, among others, the adhesion molecules E-selectin, VCAM-1, and ICAM-1 on their membrane (7). This endothelial cell-leukocyte interaction is crucial for the recruitment and transmigration of leukocytes into underlying tissues, leading to organ injury via the release of proteases and oxygen-derived radicals (8). Furthermore, microvascular endothelial cells regulate vascular leakage and the development of tissue edema which also contribute to the development of MODS after HS. We previously showed that HS results in an early and organ specific pro-inflammatory activation of microvascular endothelial cells independent of tissue hypoxia (5, 9).

A better understanding of the molecular effects of drug intervention on specific cell types within the complex organism is important for the development of therapeutic strategies (10). In the present study, we therefore focused on the microvascular endothelial inflammatory responses in HS/resuscitation to drug interventions aimed at two mechanisms, i.e., histone (de)acetylation and IKK/NF-κB signaling.
Chapter 4

Histone (de)acetylation is a posttranslational protein modification that regulates the structure and function of chromatin, and thereby modulates the expression of genes (11). Histone acetylation is controlled by the enzymes histone acetyl transferase (HAT) and histone deacetylase (HDAC). HS/resuscitation disrupts cellular acetylation homeostasis through increasing HDAC activity, leading to histone hypoacetylation and the alteration of gene expression (12). Modulation of protein acetylation in hemorrhagic and septic shock is reviewed elsewhere (13). Valproic acid, an HDAC inhibitor, increases survival in HS models (14-16). Furthermore, the addition of HDAC inhibitors to resuscitation fluid reversed shock induced changes in histone acetylation status (12). However, the precise effects of HDAC inhibition on pro-inflammatory responses of microvascular endothelial cells in different organs during HS and resuscitation are not known.

Besides by posttranslational modification, endothelial inflammatory activation is regulated by IKK/NF-κB intracellular signaling (17, 18). NF-κB is a transcription factor that controls the expression of several pro-inflammatory mediators and plays a pivotal role in the onset of inflammation. NF-κB is normally complexed with its inhibitory protein IκB in the cytoplasm. Upon activation, IκB is rapidly phosphorylated by IKK leading to the dissociation from NF-κB, which is followed by the degradation of IκB and subsequent nuclear translocation of NF-κB and transcription of target genes (19). NF-κB signaling is strongly activated during HS and resuscitation (20, 21), which makes it a potential therapeutic target for suppressing inflammation and tissue damage.

Based on the knowledge available at present as summarized in Figure 1A, we hypothesized that the pro-inflammatory activation of microvascular endothelial cells in organs will be counteracted by pharmacological intervention of histone (de)acetylation and IKK/NF-κB signaling in the resuscitation phase following HS. We first examined the effects of HDAC inhibitor valproic acid and IKK inhibitor BAY11-7082 on TNFα-induced endothelial activation in vitro. Thereafter, using a mouse model of pressure-controlled HS, we investigated the consequences of HDAC activity inhibition by HDAC inhibitor valproic acid respectively blockade of IKK/NF-κB signaling by IKK inhibitor BAY11-7082 on microvascular endothelial cell behavior in kidney, lungs, and liver during HS/resuscitation (22, 23).
Materials and Methods

Animals

Eight- to twelve-week-old C57BL/6 male mice (20-30g) were obtained from Harlan (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-hour light/dark cycle. All procedures performed were approved by the local committee for care and use of laboratory animals and were performed according to strict governmental and international guidelines on animal experimentation.

Mouse Hemorrhagic shock (HS) /Resuscitation Injury Model

The HS/resuscitation injury model has been previously described (5). In brief, after induction of anesthesia, animals were placed on a temperature-controlled surgical pad (37-38°C). HS was achieved by blood withdrawal from the left femoral artery using a roller pump (Ismatec, Geldermalsen, the Netherlands) until a reduction of the MAP to 30mmHg was reached. Blood was collected in a heparinized 1mL syringe. Additional blood withdrawal or restitution of small volumes of blood was performed to maintain MAP at 30mmHg during the shock period. After 90 min of shock, a subset of mice was resuscitated with 4% human albumin in saline (Sanquin, Amsterdam, the Netherlands) using two times the volume of withdrawn blood. Mice were allowed to wake up for 1 hour or 4 hours after volume resuscitation was achieved. During sacrifice, animals were anesthetized with isoflurane, subsequently blood was drawn via cardiac puncture and thereafter, the kidneys, lungs, and liver were harvested, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Each group in this study consisted of 8 animals. The experimental setup is illustrated schematically in Figure 1B.

Pharmacological interventions during resuscitation

4% human albumin in saline (AL) was used as resuscitation fluid. Control mice were left untreated and received isoflurane anesthesia only during termination. HDAC inhibitor VPA (300µg/g body weight, which was selected based on previous publications (14, 24-27) and which is higher than the doses used in humans for treatment of epilepsy, yet in the same range as the daily dose used for treatment of cancer (28, 29)) and IKK inhibitor BAY11-7082 (400µg per mouse) (30)were used
Drug interventions in hemorrhagic shock

Figure 1-Schematic representation of molecular responses in endothelial cells involved in the pathogenesis of hemorrhagic shock and the setup of the hemorrhagic shock/resuscitation mouse model.

(A) Local blood flow is disturbed upon the induction of hemorrhagic shock. As a consequence, the disturbance of flow sensed by endothelial cells leads to the loss of KLF2 through mechanotransduction. Concomitantly, endothelial cells are exposed to pro-inflammatory cytokines (e.g., TNFα) present in the circulation. This can cause activation of NF-κB signaling, leading to the transcription of the adhesion molecules E-selectin, VCAM-1, ICAM-1, and the pro-inflammatory cytokines IL-1β, MCP1, IL-6 and IL-8. In addition, upon pro-inflammatory activation, Ang2 is released from Weibel Palade Bodies. After its release, Ang2 binds to its receptor Tie2 on the endothelial cell membrane, thereby reducing Tie2 phosphorylation, which leads to vascular instability. The hemorrhagic shock insult can furthermore disrupt the homeostasis of histone acetylation via an increase in histone deacetylase activity.

In this study, we examined the in vitro and in vivo effects of HDAC inhibitor valproic acid and IKK inhibitor BAY11-7082 (red boxes) to assess their effects on organ specific microvascular endothelial inflammation during hemorrhagic shock/resuscitation.

Abbreviations: TNFa, tumor necrosis factor alpha; IL-1β, Interleukin-1 beta; MCP1, monocyte chemotactic protein 1; IL-6, interleukin 6; IL-8, interleukin 8; CD31, cluster of differentiation 31 (also known as Platelet endothelial cell adhesion molecule-1); VE-cad, vascular endothelial-cadherin. KLF2, Kruppel-like factor-2; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; NF-kB, nuclear factor-kappa B; Ang2, Angiopoietin-2; Tie2, receptor tyrosine kinase; HDAC, histone deacetylase; HAT, histone acetyl transferase.

(B) After the induction of anesthesia, blood withdrawal was started to induce hemorrhagic shock. After 90min of shock, mice were either sacrificed (†) or resuscitated with 4% human albumin (AL) as resuscitation fluid, where appropriate containing vehicle (Dimethyl sulfoxide, DMSO), valproic acid (VPA), or BAY11-7082, after which mice were allowed to wake up for 1h or 4h before termination. Control mice were terminated (†) at the start of the experiment.
as experimental drugs. VPA was dissolved in sterile saline (50mg/ml) and BAY11-7082 was reconstituted in dimethyl sulfoxide (DMSO, 40mg/ml). Shortly before resuscitation, both drugs were further diluted in resuscitation fluid to achieve the final doses. DMSO in 4% AL at a final concentration equivalent to the concentration in the BAY11-7082-contained solution was prepared as DMSO vehicle control. This same final concentration of DMSO was created in VPA-contained resuscitation fluid.

After 90min of HS, a subset of mice was randomly allocated into the following groups: 90min HS (HS without fluid resuscitation), AL (HS resuscitated with 4% human albumin), vehicle (HS resuscitated with 4% AL containing vehicle DMSO), VPA (HS resuscitated with 4% AL containing VPA), BAY (HS resuscitated with 4% AL containing BAY11-7082). Mice were sacrificed 1 hour and 4 hours after resuscitation.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Breda, The Netherlands). Cells were cultured in EBM-2 medium supplemented with EGM-2 MV SingleQuot Kit Supplements & Growth Factors (Cat. No. CC-3202, Lonza, the Netherlands). Culture plates (Costar, Corning, New York) were incubated with EGM-2MV medium for 30min, thereafter cells were seeded and grown until confluent before the experiments. In all experiments, cells between passages 5 to 7 were used. All cell cultures were maintained by the Endothelial Cell Facility of the UMCG.

Gene expression analysis by real time RT-PCR

Total RNA was isolated from HUVEC respectively tissue cryosections of mouse kidney, lungs, and liver using the RNeasy Mini plus Kit (Qiagen, Westburg, 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 by NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). cDNA synthesis and real-time PCR were performed as described previously(9, 31). The Assay-on-Demand primers purchased from Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands) for quantitative PCR included the housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, assay ID
Drug interventions in hemorrhagic shock

Mm99999915_g1), CD31 (Platelet endothelial cell adhesion molecule, PECAM-1), assay ID Mm00476702_m1), VE-Cad (VE-Cadherin, assay ID Mm00486938_m1), KLF2 (Kruppel-like factor-2, assay ID Mm00500486_g1), Tie2 (receptor tyrosine kinase, assay ID Mm00443242_m1), Ang2 (Angiopoietin-2, assay ID Mm00545822_m1), E-selectin (assay ID Mm00441278_m1), VCAM-1 (vascular cell adhesion molecule-1, assay ID Mm00449197_m1), ICAM-1 (intracellular adhesion molecule-1, assay ID Mm00516023_m1), MCP-1 (monocyte chemotactic protein-1, assay ID Mm00441242_m1), IL-6 (Interleukin-6, assay ID Mm00446190_m1), IL-8 (Interleukin-8, assay ID Mm00433859_m1), TNF-α (tumor necrosis factor α, assay ID Mm00443258_m1), IL-1β (Interleukin-1β, assay ID Mm00434228_m1), NGAL (Neutrophil gelatinase-associated lipocalin, assay ID Mm01324470_m1), and MPO (myeloperoxidase, assay ID Mm00447886_m1). Quantitative PCR was performed in a ViiATM 7 real-time PCR System (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Gene expression levels were normalized to the expression of the housekeeping gene GAPDH. The mRNA levels relative to GAPDH were calculated by 2^ΔCT values and averaged per group. The fold change of gene expression levels relative to the control groups was calculated by 2^ΔΔCT.

Cytokine quantification by Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNFα, IL-6, and NGAL in plasma were measured by ELISA (TNFα and IL-6: Biolegend, CA, USA; NGAL: R&D Systems, Minneapolis, Minnesota, USA).

Immunofluorescence staining of NF-κB subunit p65 localization in HUVEC

HUVEC were cultured on sterile glass coverslips (Menzel-Gläser, Braunschweig, Germany) in 6-well plates. Cells were pretreated with valproic acid (5mM, sodium salt, VPA; Sigma-Aldrich, St. Louis, MO, USA) or BAY11-7082 (10µM, (E)-3-{(4-methylphenylsulfonfonyl)-2-propenenitrile; Enzo Life Sciences, Lausen, Switzerland) for 30min before being challenged with TNFα (10ng/ml, Boehringer Ingelheim, Germany) for indicated time periods. Cells were next fixed with 1% formaldehyde in PBS for 20min. Subsequently, cells were permeabilized by 5min incubation with 0.25% Triton X-100 in PBS, then blocked with
PBS/3% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min at room temperature and incubated with rabbit anti-p65 antibody (cat. no. D14E12, Cell Signaling Technology, Inc., Leiden, The Netherlands) diluted 1:200 in PBS/0.5% BSA/0.05% Tween 20 (Sigma-Aldrich). Next, coverslips were washed with PBS and incubated for 1 h with Alexa Fluor®-conjugated donkey anti-rabbit secondary antibody (cat. no. A-31572, Molecular Probe, Leiden, The Netherlands) diluted 1:100 in PBS/0.5% BSA/0.05% Tween 20. Thereafter, coverslips were washed with PBS and mounted using Aqua Poly/Mount medium (Polysciences, Warrington, PA, USA) containing DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; Molecular Probe), air dried for 24 h, and stored in the dark at 4°C. Fluorescence images were taken with a Leica DM/RXA fluorescence microscope using Quantimet HR600 image analysis software (Leica, Wetzlar, Germany).

**Protein Expression Analysis by Western Blot**

Western blot analysis was performed with mouse kidney tissues to determine the protein expression levels of IκBα and acetylated histone H3. Protein extracts (75 µg protein/lane) were separated by sodium dodecyl sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) on 10% (IκBα) and 12% (acetylated H3) polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Utrecht, the Netherlands). After 1 h blocking with 5% skimmed milk (Campina, Friesland, The Netherlands), membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study were: IκBα (cat. no. #06-494, Millipore, Temecula, CA, USA) diluted 1:500 in 5% skimmed milk, acetylated H3 (cat. no. #06-599, Millipore) diluted 1:2,000 in 5% skimmed milk, and actin (cat no. #MAB1501, Millipore) diluted 1:200,000 in 5% BSA. The primary antibodies were detected by horseradish peroxidase-coupled secondary antibodies (diluted 1:5,000 in 5% skimmed milk, Southern Biotech, Birmingham, Alabama, USA) at room temperature for 1 hour, and visualized by horseradish peroxidase substrate (Millipore), and examined by Geldoc (Bio-Rad). Blots were analyzed using Image Lab software (Bio-Rad).

Western blot analysis of acetylated H3 was also performed with HUVEC protein lysates with 30 µg protein/lane loading. The primary antibody for acetylated H3 was diluted 1:5,000. Detection was performed as described above.
Immunofluorescence double staining for NF-κB subunit p65 and CD31 in mouse kidney

Immunofluorescence staining was performed on 5µm thick cryosections of mouse kidney. Tissue cryosections were fixed in acetone for 10min. Endogenous biotin was blocked by a Biotin Blocking System (DAKO, Glostrup, Denmark). p65 was detected with rabbit anti-p65 antibody (1:200, cat. no. D14E12, Cell Signaling), and then incubated with goat anti-rabbit biotin secondary antibody (IgG (H+L)-BIOT, Southern Biotech) in the presence of 2% normal mouse serum (Sanquin), followed by the incubation with Alexa Fluor® 555-conjugated streptavidin (1:100, Molecular Probes). To detect CD31, sections were incubated with rat anti-mouse CD31 primary antibody (1:200, cat. no. #550274, BD Pharmingen, San Diego, CA, USA), followed by Alexa Fluor® 488-conjugated goat anti-rat secondary antibody (1:100, Molecular Probes) plus 2% normal mouse serum. All incubation steps were carried out in the presence of 5% fetal calf serum (FCS, Sigma-Aldrich). After proper washing, sections were then incubated with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol for 30min. Sections were mounted and examined as described above.

Immunohistochemical detection of endothelial cell adhesion molecules and leukocyte infiltration in mouse kidney and liver

The expression and localization of adhesion molecules E-selectin, VCAM-1, and ICAM-1, as well as CD45+ polymorphonuclear leukocytes in kidney and liver were determined by immunohistochemistry. Frozen organs were cryostat-cut at 5µm, mounted onto glasses, and fixed in acetone for 10min. Endogenous peroxidase was blocked by 10min incubation with Peroxidase Block (EnVision + System-HRP (AEC), DAKO, Carpentaria, CA, USA). For specific protein detection, sections were incubated for 60min at room temperature with primary rat anti mouse antibodies (10 µg/ml) recognizing E-selectin (MES-1 10 µg/ml, kindly provided by Derek Brown, Ph.D., UCB Celltech, Brussels, Belgium), VCAM-1 (1: 50, clone M/K-1.9, ATCC, Manassas VA, USA), ICAM-1 (1: 100, Southern Biotech), and CD45 (1: 100, cat. no. #550539, BD Pharmingen) diluted in PBS/5% FCS. This was followed by 30min incubation with unconjugated rabbit anti-rat IgG antibody (mouse adsorbed, Vector Laboratories, Burlingame, CA, USA) diluted 1: 300 in PBS/5% FCS supplemented with 2% normal mouse serum at room temperature.
temperature. Sections were further incubated for 30min at room temperature with anti-rabbit labeled polymer HRP antibody from the EnVision kit. Between incubations with different antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC) from the EnVision kit and sections were counterstained with Mayer’s hematoxylin (Merck, Darmstadt, Germany).

**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 Bonferoni correction for selected pairs. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered to be significant when P<0.05.

**Results**

Hemorrhagic shock/resuscitation induce systemic and local inflammatory responses and organ damage in kidneys, lungs, and liver

First, the inflammatory activation and organ damage arising during HS and subsequent resuscitation were studied. The plasma levels of pro-inflammatory cytokines TNFα and IL-6 were both significantly increased after 90min of HS (77 fold respectively 45 fold). 1h after resuscitation, the TNFα level was decreased while IL-6 remained high (Fig. 2, A, B). 4h after resuscitation, both TNFα and IL-6 were back to control levels. This systemic pro-inflammatory activation was paralleled
Drug interventions in hemorrhagic shock

**D**

**Kidney**

- Fold change of mRNA level (vs. control)
- Genes: MCP1, IL-1β, TNFα, IL-6, IL-8, E-selectin, VCAM-1, ICAM-1

**E**

**Lungs**

- Fold change of mRNA level (vs. control)
- Genes: MCP1, IL-1β, TNFα, IL-6, IL-8, E-selectin, VCAM-1, ICAM-1

**F**

**Liver**

- Fold change of mRNA level (vs. control)
- Genes: MCP1, IL-1β, TNFα, IL-6, IL-8, E-selectin, VCAM-1, ICAM-1
by an increase in mRNA levels of the pro-inflammatory cytokines MCP1, TNFα, IL-1β, IL-6, and IL-8, as well as of the endothelial adhesion molecules E-selectin, VCAM-1, and ICAM-1 in kidney, lungs, and liver both after 90min of HS and at 1h post-resuscitation (Fig. 2, D, E, and F). The expression of MPO in lungs was significantly induced after 90min HS, and was next diminished after resuscitation.

Furthermore, in response to HS/resuscitation, Tie2 expression in kidney, lungs, and liver was strongly reduced in a time-dependent manner, while the expression of Ang2, the ligand of Tie2, was induced by resuscitation in kidney and liver but decreased in lungs. CD31 expression was downregulated in kidney after 90min of HS and decreased both in kidney and lungs 4h after resuscitation. Resuscitation significantly increased VE-cadherin expression in kidney. In addition, KLF2 was significantly downregulated in both kidneys and lungs, which was gradually restored after resuscitation. NGAL, a biomarker of kidney injury, was slightly increased by HS, and this increase was markedly enhanced after resuscitation (25 fold at 1h and 59 fold at 4h). The level of circulating NGAL in blood corroborated its gene expression profile (Fig.2, C). These observations indicate that both systemic inflammation and local endothelial inflammatory responses as well as kidney organ damage occurred during HS/resuscitation.

**In vitro effects of valproic acid and BAY11-7082 on TNFα-induced pro-inflammatory activation of endothelial cells**

To study the effects of inhibiting HDAC activity or blocking IKK/NF-κB signaling on endothelial pro-inflammatory activation in vitro, we
Drug interventions in hemorrhagic shock

**A** E-selectin

**B** VCAM-1

**C** ICAM-1

**D** MCP1

**E** IL-6

**F** TNFα (h)

**G** DAPI, p65, Merged (Magnification)
p65 as revealed by CD31/p65 double staining (Fig.4, C). In addition, VPA administration caused an increase of p65 nuclear translocation in the glomeruli of the kidney compared to vehicle control, which corroborates the *in vitro* observation in HUVEC (Fig. 3, G). These results indicate that treating the mice with VPA and BAY11-7082 during the resuscitation phase pharmacologically affected the increase of histone acetylation respectively attenuated NF-κB nuclear translocation in glomerular endothelial cells.

**Systemic and local effects of valproic acid and BAY11-7082 treatment in mice subjected to hemorrhagic shock/resuscitation**

The next step was to determine which molecular consequences were associated with these inhibitory effects of the drugs. The administration of VPA during resuscitation to HS mice increased the circulating level of TNFα but did not affect IL-6, while BAY11-7082 significantly downregulated the plasma level of both TNFα and IL-6 compared to vehicle control (Fig.5, A and B). The systemic level of NGAL was not affected by either VPA or BAY11-7082 (Fig.5, C). At whole organ mRNA level, VPA and BAY11-7082 treatment did not show extensive effects on endothelial activation in either kidney, lungs or liver although a reduction was seen in the expression of some pro-inflammatory molecules, i.e., of...
first treated HUVEC with HDAC inhibitor VPA respectively IKK inhibitor BAY11-7082 for 30min before challenging the cells with TNFα. VPA selectively inhibited the expression of endothelial adhesion molecule VCAM-1 and pro-inflammatory cytokines MCP1 and IL-6 induced by TNFα (Fig. 3, A-E). BAY11-7082 significantly reduced the upregulation of endothelial adhesion molecules E-selectin, VCAM-1, and ICAM-1 as well as of the pro-inflammatory cytokine MCP1 (Fig. 3, A-E). In contrast, NF-κB inhibition led to a significant upregulation of the pro-inflammatory cytokine IL-6 (Fig. 3, E).

Acetylation of histone H3 in endothelial cells subjected to TNFα was decreased compared to unstimulated control in a time-dependent manner, and this decrease was effectively inhibited by VPA pretreatment (Fig. 3, F). TNFα stimulation-related nuclear translocation of NF-κB subunit p65 was effectively eliminated by BAY11-7082 pretreatment. It is of note that VPA pre-incubation led to increased p65 nuclear accumulation (Fig. 3, G). These data demonstrated that both VPA and BAY11-7082 ameliorated endothelial inflammatory responses in vitro, albeit to a different extent.

**Effects of valproic acid and BAY11-7082 treatment on histone acetylation status and activation of IKK/NF-κB signaling after hemorrhagic shock/resuscitation**

Acute kidney injury is often the first complication of HS/resuscitation and it is an important factor associated with mortality in critically ill patients (32). Therefore, here we focused on the molecular changes occurring in the kidney during HS/resuscitation.

To determine the effects of HDAC inhibitor VPA on histone acetylation patterns during HS/resuscitation, the acetylation level of histone H3 in kidney was assessed by western blot. Mice subjected to 90min HS and resuscitation showed a decrease in acetylation of histone H3 compared to control mice, while the addition of VPA in resuscitation fluid markedly restored histone acetylation (Fig. 4, A).

Similarly, the effects of BAY11-7082 on NF-κB signaling was examined. HS and resuscitation lowered the expression of IkBα in kidney compared to healthy control. IKK inhibitor BAY11-7082 inhibited the degradation of IkBα both at 1h and 4h after resuscitation (Fig. 4, B). In glomerular endothelial cells, the blockade of the NF-κB signaling cascade by BAY11-7082 was confirmed by the markedly reduced nuclear translocation of
Chapter 4

Figure 4-Effects of valproic acid and BAY11-7082 treatment on histone acetylation status and activation of IkappaB kinase/NF-kappaB signaling during hemorrhagic shock/resuscitation.

Representative images of western blot for the expression of acetylated histone H3 respectively IκBα. Western blot analysis of (A) the acetylation of histone H3 and (B) IκBα in the kidney of control (CT) mice, and mice subjected to 90min hemorrhagic shock (90min HS) followed by resuscitation in the absence or presence of vehicle, VPA, or BAY11-7082. Actin was used as loading control. (C) Immunofluorescence staining for cell nucleus (DAPI blue), endothelial marker CD31 (green), and NF-xB subunit p65 (red) in glomerulus of control kidney and kidney of mice subjected to hemorrhagic shock and resuscitation in the presence of vehicle, VPA, respectively BAY11-7082. Original magnification 400x.
Drug interventions in hemorrhagic shock

A

B

C

D

1h after reperfusion

mRNA level relative to GAPDH

E-selectin

ICAM-1

NF200

ICAM-1

IL-1β

TNFα

IL-6

L-4

L-8
Figure 5—Systemic and local effects of valproic acid and BAY11-7082 treatment in mice subjected to hemorrhagic shock/resuscitation.
Concentrations of pro-inflammatory cytokines TNFα (A) and IL-6 (B) as well as kidney damage biomarker NGAL (C) in plasma after hemorrhagic shock/resuscitation and the effects of pharmacological intervention thereon were quantified by ELISA. Expression of endothelial adhesion molecules and pro-inflammatory cytokines (D), vascular integrity related molecules (Tie2, CD31, VE-cadherin, Ang2), flow responsive molecule (KLF2) (E), and organ damage related markers (NGAL and MPO) (F) in kidney (Ki), lungs (Lu), and liver (Li), was determined by real time RT-PCR using GAPDH as housekeeping gene. Data are expressed as mean ± SD of 8 mice per group. *, P<0.05, VPA vs. vehicle; #, P<0.05, BAY11-7082 vs. vehicle.
Drug interventions in hemorrhagic shock

E-selectin, ICAM-1, TNFα, and IL-8 in kidney, and of ICAM-1, MCP1 and IL-8 in liver by BAY11-7082, as well as the reduction of MCP1 and IL-6 in lungs and liver by VPA treatment (Fig. 5, D, E and F).

To reveal possible microvascular differences in endothelial pro-inflammatory activation and in response to drug interventions during HS/resuscitation, we examined the protein levels of endothelial cell adhesion molecules in kidney and liver using immunohistochemistry. In kidney, E-selectin was not present in healthy controls, while VCAM-1 showed basal expression in arteriole, peritubular capillaries, and post-capillary venules. Constitutive ICAM-1 expression was observed in all vascular beds in healthy kidney (Fig. 6, A, F, K). Upon 90 min HS, E-selectin was induced in glomerular capillaries and VCAM-1 in arterioles, peritubular capillaries, and post-capillary venules. The induction of ICAM-1 was observed in all renal vascular beds (Fig. 6, B, G, L). The upregulation of these adhesion molecules was aggravated 1 h after resuscitation, with VCAM-1 having also been induced in the glomerulus compared to its absence after 90 min HS (Fig. 6, C, H, M). The presence of VPA and BAY11-7082 during resuscitation diminished the high upregulation of E-selectin and VCAM-1 protein, while effects on ICAM-1 could not be discerned (Fig. 6, D and E, I and J, N and O).

In the liver of control mice, E-selectin was not expressed while the basal expression of VCAM-1 and ICAM-1 mainly occurred in central veins and sinusoidal endothelial cells (Fig. 7, A, F, K). The expression of all three adhesion molecules was significantly upregulated after HS, which was even further increased at 1 h after resuscitation compared to HS insult only (Fig. 7, B and C, G and H, L and M). VPA and BAY11-7082 treatment prevented the upregulation of E-selectin and VCAM-1 in the vasculature of the liver, while no effects on ICAM-1 were observed (Fig. 7, D and E, I and J, N and O).

These data indicate that HDAC inhibition and NF-κB blockade exert anti-inflammatory effects by suppressing the expression of endothelial adhesion molecule proteins in the microvasculature of kidney and liver.

The effect of VPA and BAY11-7082 treatment on polymorphonuclear leukocyte adhesion/influx in kidney and liver

The expression of adhesion molecules facilitates the recruitment and transmigration of polymorphonuclear leukocytes from blood into underlying tissues, which results in tissue damage under conditions of...
Figure 6 - Effects of valproic acid and BAY11-7082 treatment on renal microvascular bed specific localization of endothelial cell adhesion molecules induced by hemorrhagic shock/resuscitation

Immunohistochemical staining of adhesion molecules E-selectin (A-E), VCAM-1 (F-J), and ICAM-1 (K-O) in kidney from control mice, mice subjected to 90min HS only, and mice subjected to HS followed by resuscitation with 4% human albumin containing vehicle (DMSO), VPA or BAY11-7082. E-selectin, VCAM-1, and ICAM-1 stain red. Specific microvascular beds are indicated by arrows. A = arteriole; G = glomerulus; Pt = peritubular capillary; V = venule. Original magnification: 200x.
Figure 7-Effects of valproic acid and BAY11-7082 treatment on liver microvascular bed localization of endothelial cell adhesion molecules induced by hemorrhagic shock/resuscitation

Immunohistochemical staining of adhesion molecule E-selectin (A-E), VCAM-1 (F-J) and ICAM-1 (K-O) of liver from control mice, mice subjected to 90 min HS only, and mice subjected to HS followed by resuscitation with 4% human albumin containing vehicle (DMSO), VPA or BAY11-7082. E-selectin, VCAM-1, respectively ICAM-1 stain red. Specific vascular beds are indicated by arrows. CV = central vein, S = sinusoid. Original magnification: 200x.
Figure 8-Effects of valproic acid and BAY11-7082 administration on leukocyte recruitment in kidney and liver during hemorrhagic shock/resuscitation

Immunohistochemical staining of CD45 positive leukocytes in kidney (A-E) and liver (F-J). Staining was performed on organ sections of control mice, and mice subjected to 90min HS only respectively mice subjected to HS followed by resuscitation with 4% human albumin in the presence of vehicle (DMSO), VPA or BAY11-7082. CD45+ leukocytes stain red. Original magnification: 200x.
Drug interventions in hemorrhagic shock

HS (33). Upon HS and subsequent resuscitation, the number of adhering and invading CD45+ leukocytes in kidney and liver was increased. VPA and BAY11-7082 treatment during resuscitation reduced this number of adhering and invading leukocytes in both organs compared to vehicle control (Fig. 8, A-J). This finding led us to conclude that the molecular inhibitions of histone deacetylation and NF-κB activation during resuscitation after HS is an effective strategy to interfere with detrimental influx of leukocytes into the organs.

Discussion

Hemorrhagic shock (HS) is associated with a high morbidity and mortality (1). Upon HS, several endogenous compensatory mechanisms are stimulated to mitigate organ dysfunction. Insufficiency of these mechanisms combined with the reperfusion-associated damage can lead to microvascular endothelial inflammatory activation and leukocyte recruitment into tissues, which may manifest as tissue damage. Various molecular mechanisms have been proposed to be involved in HS-associated MODS, and amongst others the roles of histone (de)acetylation and NF-κB activation were highlighted (15, 34). The current work aimed to examine the pharmacological effects of interfering with HDAC activity respectively IKK/NF-κB signaling activation on HS/resuscitation-associated microvascular endothelial inflammatory activation and leukocyte recruitment into tissues. In a mouse model of HS/resuscitation, we demonstrated that the administration of either the HDAC inhibitor VPA or the IKK inhibitor BAY11-7082 in the resuscitation fluid inhibited the pro-inflammatory activation of the microvasculature in kidney, lungs, and liver. The HS/resuscitation-related induction of protein expression of endothelial cell adhesion molecules E-selectin and VCAM-1, as well as the consequent influx of polymorphonuclear leukocytes in kidney and liver were significantly reduced by treatment with either drug. In addition, BAY11-7082 reduced the systemic level of cytokines TNFα and IL-6. Taken together, our data show that either inhibition of HDAC activity or of NF-κB signaling during the resuscitation phase of HS/resuscitation has anti-inflammatory effects on endothelial cells in organs.

HS/resuscitation modulates the acetylation of histone and non-histone proteins, which affects cell signaling pathways and gene
transcription (13). The current study showed that the level of histone acetylation in kidney was reduced upon HS/resuscitation, which was restored by the administration of the HDAC inhibitor VPA during resuscitation. This observation is consistent with studies which showed that VPA administration effectively modulated the balance between the activity of HATs and HDACs in cardiac tissue in a rat model of HS, and induced global hyperacetylation of several histone proteins in the liver (12, 16). Besides the effects on histone proteins, HDAC inhibitors may affect other mechanisms via acetylating non-histone proteins. For example, HDAC inhibition has been shown to exert protective effects on organ injury via the upregulation of pro-survival molecule Bcl-2 through acetylation-associated nuclear translocation of β-catenin (14). Furthermore, multiple studies have demonstrated the beneficial effects of HDAC inhibition on the outcome of sepsis-associated multiple organ injury, such as enhanced recovery of acute kidney injury, inhibition of septic cell apoptosis, and protection against pulmonary inflammation (27, 35, 36). A downside of HDAC inhibition is the potential harmful consequences to the host due to the here observed enhancement of nuclear accumulation of NF-κB in endothelial cells by VPA. It is tempting to speculate that a combination of HDAC inhibition and IKK/NF-κB signaling blockade would be a potent anti-inflammatory therapeutic approach for HS patients as the latter could counteract VPA-induced NF-κB nuclear accumulation in endothelial cells while not affecting VPA’s other anti-inflammatory effects. To investigate the possibilities of this combination in an animal model, extensive dose optimization of drug combinations and the effects on survival, organ function, and microvascular endothelial behavior and the molecular causes thereof should be undertaken, which was beyond the scope of our studies. It is of note that in these studies the translation of the VPA dosing has to be taken into account, as in humans treated for advanced cancer, the maximum tolerated dose of VPA was 30mg/kg (37), while in our current mouse study the dose of VPA was 300mg/kg based on previous animal studies (27, 28).

Surprisingly, in our model the administration of VPA during resuscitation resulted in upregulation of TNFα while it did not significantly affect the level of IL-6 (Figure 5, A and B). Similar observations have been reported before, showing that VPA treatment did not have effects on inflammatory features in mice with cecal
Drug interventions in hemorrhagic shock

ligation and puncture (CLP)-induced sepsis, and even upregulated the expression of TNFα in lungs (36). The exact reason for this is not clear to us. There are a few explanations possible. First, in our study, the mice were resuscitated with two times the volume of blood loss, which may excessively dilute the systemic levels of VPA. VPA at different concentrations might have different effects, which can result in the variation in effects of VPA on different cytokines. Second, the expression of TNFα and IL-6 have different kinetics in HS, indicating that the production of IL-6 may be partially independent of those mechanisms in hemorrhage that are involved in the release of TNFα (38). Furthermore, VPA, used as an HDAC inhibitor, has broad effects via different molecular mechanisms. Thus, it may affect the production of TNFα and IL-6 through different mechanisms, contributing to the observed differences between the two cytokines. In addition, cytokines are secreted by several different cell types, and the effects of HDAC inhibition on inflammatory genes may vary between different cell types and challenges, or even between different mediators in the same cells (39, 40).

Activation of IKK/NF-κB signaling is a hallmark of inflammation and organ injury, and various strategies have been aimed towards blocking this pathway at different molecular and cellular levels. In the present study, in the kidney of mice subjected to HS/resuscitation, we observed degradation of IκB and the consequent nuclear translocation of NF-κB subunit p65 in glomerular endothelial cells (Fig 3, G), both of which were abolished by administration of IKK inhibitor BAY11-7082 during resuscitation. Our results corroborate the study by Coldewey et al. who demonstrated in experimental sepsis that delayed inhibition of IKK by the selective inhibitor IKK 16 effectively suppressed inflammation (41). In addition, Yang et al. revealed that inhibition of NF-κB DNA-binding activity using double-stranded oligodeoxynucleotides reduced tissue damage and cytokine expression in the liver following a rat polytrauma model of combined closed femur fracture, laparotomy and lipopolysaccharide injection (34). However, the IKK/NF-κB pathway has a critical role in innate and adaptive immune responses, and systemic blockade of IKK/NF-κB signaling is associated with severe side effects such as immunosuppression and cell apoptosis (42). Strategies have been developed to circumvent these severe side effects by selectively inhibiting NF-κB in endothelial cells only. This approach is protective to the host in
Chapter 4

models of sepsis and arthritis, and likely reduces the risk of side effects compared to the general blockade of NF-κB in all cells in the body (43, 44). Our group has recently shown that using endothelial-targeted immunoliposomes as the carrier, selective pharmacological inhibition of NF-κB in inflamed microvascular endothelial cells can be achieved to interfere with disease-associated endothelial activation (45).

In the present study, the nuclear accumulation of NF-κB after both TNFα activation in vitro and HS/resuscitation in vivo was enhanced by treatment with VPA. HDAC inhibitors modulate NF-κB-mediated gene transcription via the acetylation of both histone proteins and NF-κB itself (46). Although not completely understood, acetylation of NF-κB works in concert with the acetylation of histones to regulate DNA binding affinity, transcriptional activation, and the duration of action (47). The reported anti- respectively pro-inflammatory effects of HDAC inhibition are not fully understood, as the drugs are studied in various cell types and HDAC inhibitors employed lack HDAC subtype selectivity (48-50). Moreover, HDAC3 acts as a positive regulator of IL-1-induced gene expression in HEK293 (Human Embryonic Kidney 293) cells by removing inhibitory acetyl groups from NF-κB subunit p65, which suggests an inhibitory effect of selective HDAC3 inhibition on NF-κB signaling (51). In our study, NF-κB subunit p65 accumulation in the nucleus was increased by VPA treatment (Fig 3, G). However, whether this led to increased DNA binding activity of p65 in the nucleus of endothelial cells, or was dependent on the effect of certain HDAC subtypes was not part of the research question addressed here and hence not studied.

Our present study has several strengths and weaknesses. A strong point is the combination of studying in vitro and in vivo pharmacological effects of VPA and BAY11-7082 on endothelial pro-inflammatory behavior. The in vitro study enabled us to determine the effects of drugs on endothelial cells, which was confirmed by the in vivo study in which we examined the molecular consequences of drug intervention in the mouse model of HS/resuscitation in the complex context of the whole organism. A first limitation is that the differences between in vitro and in vivo drug effects, as well as the discrepancies between protein and mRNA expression of endothelial adhesion molecules in organs in response to drug interventions, are still not completely understood. At the mRNA level, both VPA and BAY11-7082 significantly reduced TNFα-induced upregulation of endothelial adhesion molecules and
Drug interventions in hemorrhagic shock

pro-inflammatory cytokines *in vitro*. When studied in the organs in the mouse HS model, neither drug showed strong effects on the mRNA levels of endothelial activation in kidney, lungs or liver (Fig 5, D-F). At the same time, however, the protein levels of endothelial adhesion molecules in the specific microvascular beds in the kidney and liver were extensively reduced (Fig 6). These discrepancies are possibly due to the fact that the behavior of endothelial cells within the organism are controlled by their microenvironment, including mechanical forces and interactions with leukocytes and other cell types, which cannot be easily mimicked *in vitro*. Furthermore, the link between the changes in mRNA level and protein level *in vivo* is not as clear as it is *in vitro*, due to the complexity of the organism. In addition, endothelial behavior is dependent on different microvascular beds in different organs as reflected by, e.g., the different basal mRNA expression of genes in different organs (Supplementary table 1), as well as the glomerulus-restricted upregulation of E-selectin protein in the kidney after HS (Fig 6, B and C). The microvascular bed specific behavior of endothelial cells is concealed when mRNA expression profiles are studied in whole organs and can be unmasked using laser microdissection of microvascular beds from organs prior to mRNA analysis (10, 45). Another issue is that it is difficult to directly translate our data from mice to HS patients. Translation is hindered because patients are faced with multiple comorbid diseases which are not mimicked in the healthy young male mice in our study. Moreover, the surgical rescue procedures and intensive care measures were not simulated in our animal model. In addition, while shock patients are resuscitated with fluid regimens including crystalloids, human colloids, and blood products, 4% human albumin in 0.9% NaCl was used as the fluid regimen for resuscitation in our study. We chose this colloid regimen in order to compare the results with our previous studies in which a 6% hydroxyethyl starch (HES) 130/0.4 was used as a colloidal resuscitation fluid (5, 9). Care should be taken when extrapolating data from one study to the other, as endothelial microvascular behavior might be different when using different resuscitation fluids. For example, the response of cremaster microvasculature in rats resuscitated with crystalloid and colloid infusion fluids after HS differs (52), and the choice of resuscitation fluids influences neutrophil activation and soluble plasma levels of endothelial adhesion molecules in trauma patients (53).}

Our observations however give rise to some options for translation,
and the analyses of soluble cell adhesion molecules and organ biopsies of patients who died during HS can be instrumental to validate the observations described here in patients (54, 55).

In summary, the current work demonstrated that in vivo inhibition of HDAC activity as well as IKK/NF-κB signaling activation during resuscitation resulted in an attenuation of endothelial inflammatory response induced by HS/resuscitation. The protective effects of these interventions as therapeutic strategies for the treatment of HS-associated systemic inflammation needs further study, encompassing validation in larger animal models and examination of specimens from HS patients.

Acknowledgements

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References

Drug interventions in hemorrhagic shock


Chapter 4

Drug interventions in hemorrhagic shock

49. Furumai R, Ito A, Ogawa K, et al: Histone Deacetylase Inhibitors Block Nuclear Factor-Kappab-Dependent Transcription by Interfering with Rna Polyme-


Drug interventions in hemorrhagic shock

Supplementary data

Table 1. Basal expression of genes investigated in this study in kidney, lungs, and liver of healthy mouse

<table>
<thead>
<tr>
<th>Gene</th>
<th>Kidney (x10^-4)</th>
<th>Lung (x10^-4)</th>
<th>Liver (x10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial adhesion molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>0.013 (0.008-0.020)</td>
<td>0.030 (0.010-0.041)</td>
<td>0.013 (0.001-0.055)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>0.11 (0.05-0.13)</td>
<td>2.9 (1.4-4.5)</td>
<td>0.43 (0.18-0.85)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.08 (0.06-0.10)</td>
<td>5.3 (2.9-9.0)</td>
<td>0.20 (0.05-0.13)</td>
</tr>
<tr>
<td>Vascular integrity-related molecules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>0.45 (0.35-0.53)</td>
<td>11.5 (6.3-18.5)</td>
<td>0.24 (0.16-0.41)</td>
</tr>
<tr>
<td>Ang2</td>
<td>0.04 (0.02-0.06)</td>
<td>0.18 (0.02-0.22)</td>
<td>0.004 (0.01-0.06)</td>
</tr>
<tr>
<td>CD31</td>
<td>0.54 (0.42-0.68)</td>
<td>19.6 (10.3-25.8)</td>
<td>0.29 (0.17-0.46)</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>0.72 (0.60-0.83)</td>
<td>51.1 (28.1-84.5)</td>
<td>0.82 (0.44-1.43)</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP1</td>
<td>0.002 (0.0005-0.0034)</td>
<td>0.009 (0.005-0.016)</td>
<td>0.025 (0.006-0.119)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.004 (0.003-0.006)</td>
<td>0.17 (0.03-0.38)</td>
<td>0.057 (0.009-0.12)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.0001 (0.00008-0.0003)</td>
<td>0.003 (0.0002-0.0006)</td>
<td>0.007 (0.0002-0.006)</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.004 (0.002-0.006)</td>
<td>0.10 (0.02-0.16)</td>
<td>0.020 (0.004-0.074)</td>
</tr>
<tr>
<td>Flow sensitive marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>0.3 (0.2-0.4)</td>
<td>10.5 (4.3-15.5)</td>
<td>0.31 (0.25-0.38)</td>
</tr>
</tbody>
</table>

mRNA expression levels of genes in healthy control mice was analyzed in kidney, lungs and liver using real time RT-PCR. GAPDH was taken as housekeeping gene. Data are expressed as the mean of relative mRNA level vs. GAPDH (minimum-maximum) from 8 mice per group.