The microvascular endothelial cell in shock
van Meurs, Matijs

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2011

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
CHAPTER 6

SHOCK INDUCED STRESS INDUCES LOSS OF MICROVASCULAR ENDOTHELIAL Tie2 IN THE KIDNEY WHICH IS NOT ASSOCIATED WITH REDUCED GLOMERULAR BARRIER FUNCTION

Matijs van Meurs*
Neng Fisheri Kurniati*
Francis M. Wulfert
Sigridur A. Ásgeirsdóttir
Inge A. de Graaf
Simon C. Satchell
Peter W. Mathieson
Rianne M. Jongman
Philipp Kümpers
Jan G. Zijlstra
Peter Heeringa
Grietje Molema

Am J Physiol Renal Physiol. 2009
Aug;297(2):F272-81
(* authors contributed equally)
ABSTRACT

Both hemorrhagic shock and endotoxaemia induce a pronounced vascular activation in the kidney which coincides with albuminuria and glomerular barrier dysfunction. We hypothesized that changes in Tie2, a vascular restricted receptor tyrosine kinase shown to control microvascular integrity and endothelial inflammation, underlie this loss of glomerular barrier function. In healthy murine and human kidney, Tie2 is heterogeneously expressed in all microvascular beds, although to different extent. In mice subjected to hemorrhagic and septic shock, Tie2 mRNA and protein were rapidly, and temporarily, lost from the renal microvasculature, and normalized within 24 hours after initiation of the shock insult. The loss of Tie2 protein could not be attributed to shedding as both in mice and healthy volunteers subjected to endotoxaemia, sTie2 levels in the systemic circulation did not change. In an attempt to identify the molecular control of Tie2, we activated glomerular endothelial cell cultures and human kidney slices in vitro with LPS or TNF-α, but did not observe a change in Tie2 mRNA levels. In parallel to the loss of Tie2 in vivo, an overt influx of neutrophils in the glomerular compartment which coincided with proteinuria, was seen. As neutrophil – endothelial cell interactions may play a role in endothelial adaptation to shock, and these effects can not be mimicked in vitro, we depleted neutrophils prior to shock induction. While this neutrophil depletion abolished proteinuria, Tie2 was not rescued, implying that Tie2 may not be a major factor controlling maintenance of the glomerular filtration barrier in this model.
INTRODUCTION

Acute kidney injury (AKI) after shock states is an often lethal complication of hemorrhagic and septic shock. Aggressive management of shock with supportive therapy has not substantially lowered the more than 50% 60-day mortality of AKI patients treated in Intensive Care units. AKI is characterized by a sudden loss of the ability of the kidneys to excrete wastes, maintain fluid balance, and conserve electrolytes and by the occurrence of proteinuria.

A number of potential mechanisms have been described to underlie the occurrence of proteinuria in AKI, including loss of microvascular integrity. One of the molecular systems controlling microvascular integrity is the Angiopoietin/Tie2 system. Tie2 is a 140 kD tyrosine kinase receptor with immunoglobulin and epidermal growth factor homology, that has specificity for Angiopoietin (Ang)-1 and Ang-2 binding. Ang-1 induced Tie2 signaling is considered essential for endothelial integrity and provides quiescent endothelial status with anti-inflammatory properties. In contrast, competition of Ang-1/Tie2 binding by Ang-2 induces inhibition of Tie2 signal transduction and is associated with inflammatory and vascular leakage disorders, similar to a diminished Ang-1/Tie2 signaling due to other causes. Both hemorrhagic shock and endotoxaemia induce a pronounced vascular activation in the kidney which coincides with vascular leakage and glomerular barrier dysfunction. An increase in Ang-2 has until now been assigned as being the dynamic factor of the system, which upon endothelial release from Weibel Palade bodies competes with Ang-1 for binding to Tie2, and thereby creates a condition of endothelial destabilization. Ang-2 overexpression in podocytes led to increased proteinuria in adult mice, while in a diabetic mouse model the administration of Ang-1 exerted protective effects with diminished proteinuria. Also in human proteinuric diseases like systemic lupus erythematosus, Ang-2 serum levels correlated positively with proteinuria. Although not considered actively regulated, preliminary observations in our critical illness models showed differences in Tie2 mRNA expression during shock onset. We therefore hypothesized, that a change in Tie2 expression may be one of the molecular responses of the Angiopoietin/Tie2 system that underlies maladaptive behavior in shock, including loss of microvascular integrity in the kidney.

To test this hypothesis, we studied the spatiotemporal changes in Tie2 mRNA
and protein expression in the renal microvasculature of mice during endotoxic and hemorrhagic shock as models of AKI, and investigated the relation between Tie2 changes and proteinuria as a measure glomerular barrier dysfunction. The initial observations justified a further study into the role of neutrophils in the changes in Tie2 expression. For this, we depleted the neutrophils by antibody treatment prior to shock induction, and investigated its consequences for Tie2 expression and proteinuria. The observations were extended to humans, by studying a human volunteer endotoxaemia model and human kidney slices exposed to sepsis mediators.

**MATERIALS AND METHODS**

**Animals**

Eight- to 12-week-old C57Bl/6 male mice (20-30g) 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-h light/dark cycle. All procedures were approved by the local committee for care and use of laboratory animals and were performed according to governmental and international guidelines on animal experimentation.

**Mouse shock models**

The mouse hemorrhagic shock (HS) model has been extensively documented elsewhere\textsuperscript{15}. 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 MAP at 30 mmHg during this period. The mice were resuscitated after 90 minutes of HS with 6% hydroxyethyl starch 130/0.4 (Voluven®; Fresenius-Kabi, Bad Homburg, Germany) at two times the volume of blood withdrawn. After 4, 8, or 24 hours post volume resuscitation, blood was withdrawn via aortic puncture under isoflurane anesthesia, and the kidneys were excised, snap-frozen in metal cups on liquid nitrogen, and stored at -80°C until analysis.
For the induction of endotoxaemia, mice were intraperitoneally (i.p.) injected with LPS (Escherichia coli, serotype 026:B6; Sigma, St. Louis, MO) at 5 mg/kg (15,000 EU/g) body weight. 4, 8 and 24 hours later, blood was drawn and organs harvested as described above. Control mice were left untreated and were sacrificed under isoflurane anesthesia, after which blood was withdrawn and kidneys were harvested and handled as described above.

In indicated experiments, mice were housed in a metabolic cage for 24 hours at 7 days before the experimental procedure, to obtain a control urine sample. Metabolic cages were used to obtain urine samples from mice in healthy and diseased conditions. Control albumin/creatinin ratios were assessed by housing mice in metabolic cages for 24 hours 7 days prior to the insult and from 0 until 4 hours, 0 until 8 hours, and 8 until 24 hours after LPS induced shock. A subgroup of LPS treated mice was i.p. injected with 0.5 mg anti-NIMP antibody to selectively deplete the neutrophils prior to shock induction\textsuperscript{21}. One day after this procedure mice were i.p. injected with LPS at a similar dose as described above. These mice were housed in metabolic cages for urine collection immediately after LPS administration and sacrificed 8 hours later under isoflurane anesthesia, blood was withdrawn via an aortic puncture, and the kidneys were harvested, snap-frozen in metal cups on liquid nitrogen, and stored at -80°C until analysis.

**Human endotoxaemia**

For the human endotoxaemia model, human volunteers who participated in a drug intervention study were injected with a dose of 4 ng/kg body weight (10,000 endotoxin units/µg) LPS (Escherichia coli, batch EC-6, US Pharmacopeia, Twinbrook Parkway, Rockville, MD, USA). The local Investigations Review Board approved the study. Written informed consent was obtained from all subjects before enrolment in the study. Data from this study have been reported extensively elsewhere\textsuperscript{22}. From this cohort, plasma stored at -80°C was analyzed for soluble Tie2.

**In vitro cell culture and organ slice incubation**

Conditionally immortalized Human Glomerular Endothelial cells (ciGEnC)\textsuperscript{23} were cultured in EBM medium in 12-well culture dishes at a density of 100,000 cells/well for 24h at 33°C, followed by 5 days at 37°C under 5% CO\textsubscript{2}/95% O\textsubscript{2} before introducing them in
an experiment. The ciGEnC culture medium consisted of EBM-2 medium supplemented with 5% fetal calf serum (FCS) and EGM-2 MV singleQuots (Lonza Group Ltd, Basel, Switzerland). In the experiments described here ciGEnC were used up to passage 40.

Confluent ciGEnC were activated for 4 hours with 0.1, 1 and 10 ng/ml TNF-α (Boehringer, Ingelheim, Germany) and 1, 50 and 1,000 ng/ml LPS. After incubation cells were microscopically analyzed with regard to their morphology and consistently were found to be adherent and viable.

For kidney slice incubations, human kidney tissue was obtained as tumor free surgical waste from patients subjected to kidney carcinoma surgery. The three patients were all male, age between 60 and 66 years, with normal kidney function. Tissue was prepared for precision cut tissue slices within 15 minutes. Tissue cylinders were prepared with an 8 mm diameter motor-driven coring tool, and further processed into 250 µm thick slices with a mechanical slicer as described earlier24. Slices were incubated individually in 12-well culture plates (Costar 3512; Corning Glassworks, Corning, NY) in 1.3 ml of Williams Medium E with Glutamax-I, supplemented with D-glucose (25 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). For activation, 10 ng/ml LPS was added to the medium at the start of the incubation period. The culture plates were placed at 37°C and slices were incubated under humidified carbogen on an orbital shaker (45 rpm). The condition of precision-cut slices was evaluated at different incubation time points by microscopic examination of hematoxylin and eosin stained cryosections. Intracellular ATP levels were measured in slice homogenates with ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics Nederland, Almere, The Netherlands) to judge the overall metabolic condition of the tissue. Immunohistochemical staining of Tie2 was performed on 5 µm cryosections and gene expression analysis was performed with RNA isolated from frozen slices as described below.

Laser microdissection of renal microvasculature

From mice kidneys, 5 µm cryosections mounted on 1.35 µm polyethylene-naphtalene membranes attached to normal 1 mm slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany) were fixed in acetone and stained with Mayer’s hematoxylin, washed with diethyl pyrocarbonate treated water, and air-dried. ECs from small arterioles (6 x 10^5 µm^2) and postcapillary venules (1.3 x 10^6 µm^2), as well as glomeruli (3 x 10^6 µm^2), were

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

RNA was extracted from 20 x 5 µm cryosections from mouse kidney, 250 µm human kidney slices and cells, 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 (OD260) and purity (OD260/OD280) 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, USA) used in the PCR reaction included housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm99999915_g1 for mouse and assay Hs99999905_m1 for human), Tie2 (assay ID Mm00443242_m1 for mouse and assay Hs00176096_m1 for human), E-selectin (assay ID Hs00174057_m1 for human), VEGF-A (assay ID Mm00437304_m1 for mouse), and VEGFR-2 (assay ID Mm00440099_m1 for mouse). Duplicate real time RT-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 average, relative mRNA level was calculated by $2^{-ΔCT}$.

**Localization of proteins by immunohistochemistry**

Localization of Tie2, CD31, E-selectin and neutrophils was determined using immunohistochemistry. Frozen kidneys 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 in the presence of 5% fetal calf serum (table 6.1). After washing, endogenous peroxidase was blocked by incubation with 0.1% H$_2$O$_2$ in phosphate-buffered saline (PBS) for 20 minutes. This was followed by incubation for 30 minutes at room temperature with horseradish peroxidase-conjugated secondary antibodies (table 6.1). Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected
### Table 6.1. Antibodies and their controls used for immunohistochemistry and leukocyte depletion.

<table>
<thead>
<tr>
<th>Ab name / epitope</th>
<th>Provider</th>
<th>Isotype</th>
<th>Dilution used</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Abs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Tie2</td>
<td>Tek4</td>
<td>eBioscience</td>
<td>IgG1</td>
<td>1:50</td>
</tr>
<tr>
<td>Hu Tie2</td>
<td>sc324</td>
<td>Santa Cruz</td>
<td>IgG1</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse CD31</td>
<td>PECAM-1</td>
<td>BD Pharmingen</td>
<td>IgG2a</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse E-selectin</td>
<td>MES-1</td>
<td>Dr. D. Brown, United Kingdom</td>
<td>IgG2a</td>
<td>1:10</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Ly6G</td>
<td>BD Pharmingen</td>
<td>IgG2a</td>
<td>1:10</td>
</tr>
<tr>
<td>Control IgG for rat Ab</td>
<td></td>
<td>Antigenix America</td>
<td>IgG1, IgG2a, IgG2b</td>
<td>1:50</td>
</tr>
<tr>
<td>Neutrophil</td>
<td></td>
<td>HBt</td>
<td>IgG2b</td>
<td>n.a.</td>
</tr>
<tr>
<td>Control IgG</td>
<td></td>
<td>Sigma</td>
<td>IgG</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>Secondary Abs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti rat-HRP</td>
<td>P0459</td>
<td>Dako</td>
<td>1:40</td>
<td>IHC</td>
</tr>
<tr>
<td>Envision kit, rabbit polymer</td>
<td>K4009</td>
<td>Dako</td>
<td>IHC</td>
<td></td>
</tr>
<tr>
<td>Goat anti rabbit-HRP</td>
<td>4050-05</td>
<td>Southern Biotech</td>
<td>1:50</td>
<td>IHC</td>
</tr>
<tr>
<td>Rabbit anti rat (preadsorbed)</td>
<td></td>
<td>Vector</td>
<td>1:300</td>
<td>IHC</td>
</tr>
</tbody>
</table>
with 3-amino-9-ethylcarbazole (Sigma-Aldrich Chemie, St. Louis, MO, USA), and sections were counterstained with Mayer hematoxylin (Klinipath, Duiven, The Netherlands). No immunostaining was observed with isotype-matched controls (table 6.1), demonstrating specificity of staining with the antigen specific antibodies.

**Quantification of Tie2 protein levels by ELISA**

To quantify the amount of Tie2 protein in the renal tissues of mice, 15 x 10 µm kidney slices were homogenized in 50mM Tris-HCl buffer (pH 7.5), containing 150mM NaCl and protein inhibitor cocktail (Sigma-Aldrich Chemie) and centrifuged at 13.000g for 15 minutes. Total protein was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, USA), before quantification of Tie2 by ELISA (mouse Tie2 MTE200, R&D Systems Inc. Minneapolis, USA) according to the manufacturer’s instructions. Tie2 levels were normalised to total protein concentrations in the tissue homogenate and expressed as pg Tie2 per μg total protein.

The level of soluble Tie2 in the plasma was analysed using a commercially available Tie2 ELISA (human DTE200 and mouse MTE200; R&D Systems) according to the manufacturer’s instructions. The DTE200 ELISA kit was previously used to measure changes in soluble Tie2 in different patients groups\(^{25, 26}\). During this investigation, we validated the MTE200 ELISA for suitability to measure soluble Tie2 using commercially available soluble mouse Tie2 (R&D systems; 762-T2).

**Kidney function measured by albumin:creatinin ratio**

To assess glomerular barrier function, the micro-albumin and creatinine levels were measured in mouse urine using a commercial available kit (Exocell Inc., Philadelphia, USA) according to the manufacturer’s instructions.

**Statistical analysis**

Statistical significance of differences was studied by means of the a Student’s t-test or ANOVA with post hoc comparison using Bonferroni correction. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad Prism software (GraphPad Prism Software Inc., San Diego, California, USA). Differences were considered to be significant when \( p < 0.05 \).
RESULTS

In healthy mouse kidney, Tie2 is expressed in all vascular beds to different extents

To examine the expression pattern of Tie2 in healthy mouse kidney, we immunohistochemically stained tissue for Tie2 protein (figure 6.1A). Tie2 is located in all microvascular beds, with a clear differential level of expression between the microvascular segments that can be histologically discriminated. Pronounced expression of Tie2 was observed in arterioles, glomeruli, and peritubular endothelium, while the expression was lower in the endothelium of the postcapillary venules. These vascular bed specific differences were corroborated by Tie2 mRNA levels in microvascular segments microdissected from mouse kidneys prior to gene expression analysis. Most Tie2 mRNA was localized in glomeruli while the least was seen in venules (figure 6.1B).

Tie2 expression is diminished in the kidney in different shock states

After initiation of hemorrhagic shock and LPS induced shock, Tie2 was rapidly lost, both at the mRNA and protein level. 24 hours after the shock insult the mRNA in hemorrhagic shock had normalized, while an increase in mRNA was seen in the LPS treated groups (figure 6.2A). Of note is the fact that 24 hours after the shock insult, levels of Tie2 protein in both groups of shock subjected mice had normalized. The decrease in mRNA content was accompanied by a reduction in Tie2 protein levels in kidney homogenates in both

Figure 6.1. Tie2 is expressed in different microvascular beds in healthy mouse kidney. (A) Protein expression detected by immunohistochemical staining. Arrows point at different microvascular beds: arteriole (a), glomerulus (g), peritubular vasculature (pt), and venule (v). (B) Expression of Tie2 mRNA levels by quantitative RT-PCR (relative gene expression adjusted to GAPDH) assessed in three microvascular beds laser microdissected from kidney. Mean values ± SD of 3 mice per group, * p <0.05.
models, with the most prominent reduction visible in the LPS model (figure 6.2B). Immunohistochemical detection of Tie2 revealed that the protein was lost from all vascular beds, i.e., the arteriolar, glomerular, peritubular and venular vasculature (figure 6.2C).

*Tie2 is not shed in LPS mediated shock*

The cause of Tie2 protein downregulation in LPS shock can be either internalization and degradation or shedding of the membrane associated protein. To determine the potential occurrence of Tie2 shedding during and after the shock period, we measured

![Figure 6.2. Spatiotemporal changes in renal Tie2 mRNA and protein expression in mice subjected to LPS induced shock and hemorrhagic shock followed by resuscitation.](image)

*Figure 6.2. Spatiotemporal changes in renal Tie2 mRNA and protein expression in mice subjected to LPS induced shock and hemorrhagic shock followed by resuscitation.*

In the endotoxemia model, LPS was administered at a dose of 0.5 mg/kg mice, while in the hemorrhagic shock model mice were subjected to blood withdrawal to a mean arterial pressure of 30 mmHg for 90 minutes, after which they were resuscitated with Voluven® as described in Materials and Methods. (A) mRNA levels shown are relative to GAPDH as housekeeping gene and determined by quantitative RT-PCR as described in Materials and Methods. Mean values ± SD of at least 5 mice per group, * p<0.05. (B) Protein levels were measured in kidneys homogenates by ELISA as described in Materials and Methods. Mean values ± SD of at least 8 mice per group, * p <0.05. (C) Representative light microscopy pictures of the microvascular localization of Tie2 protein after LPS or hemorrhagic shock respectively and resuscitation 8 hours and 24 hours after induction of shock, assessed by immunohistochemistry. Original magnification 200x, insert 400x * p <0.05.
soluble Tie2 in the systemic circulation after LPS induced shock. In mice, no increased shedding occurred during the first 24 hours after LPS administration (figure 6.3A). Similar to the mouse model, no shedding of Tie2 into the plasma could be observed in human endotoxaemia (figure 6.3B). These mouse and human data suggest that the diminished Tie2 protein expression observed in the kidney is not due to systemic protein shedding.

**Endothelial cell loss of Tie2 cannot be induced in in vitro and ex vivo conditions**

In order to determine the molecular mechanism underlying shock induced loss of Tie2 from endothelial cells, we incubated glomerular endothelial cells with LPS and with TNF-α, which is one of the rapid responder cytokines in vivo after LPS administration (figure 6.4A and 6.4B). Neither low, nor relatively high concentrations of LPS or TNF-α, changed the mRNA levels of Tie2 in vitro. The strong induction of E-selectin mRNA expression under these pro-inflammatory conditions ruled out an overall non-responsiveness of the cells toward LPS and TNF-α.

As glomeruli contain mesangial cells and podocytes next to endothelial cells, theoretically these non-endothelial cells could have contributed to the observed Tie2 decrease in vivo. Compared to glomerular endothelium, however, their Tie2 expression level was more than 100 – 1,000 fold lower, and no effect of both short term and long term LPS exposure on Tie2 mRNA levels could be detected (supplemental figure 6A).

To determine whether a possible interplay between cells determined the main

![Figure 6.3. Soluble Tie2 levels in plasma of mice and humans administration did not change after LPS administration.](image)

Quantitation of soluble Tie2 protein levels in plasma from LPS challenged mice (A; n=4) and LPS challenged humans (B; n=6) was performed using ELISA. Results are expressed as the mean ± SD, *p <0.05.
cause of Tie2 gene and protein expression loss, we incubated 250 μm precision cut human kidney slices in the absence and presence of LPS. In human kidneys, Tie2 was expressed in all microvascular beds in a pattern similar to that in mice (figure 6.5A). Upon ex vivo incubation of the slices in normal medium for 8 hours, Tie2 mRNA levels significantly dropped compared to levels in control kidney snap frozen directly prior to slice production. These lower mRNA levels were still well above the detection limit of Figure 6.4. In vitro, LPS nor TNF-α stimulation of glomerular endothelial cells did not affect Tie2 gene expression, while E-selectin was strongly induced. Conditionally immortalized glomerular endothelial cell (ciGEnC) were exposed for 4 hours to LPS (A) or TNF-α (B), at increasing concentrations. White bars represent Tie2 mRNA levels, black bars E-selectin mRNA levels. Values are mean of 3 ± SD, * p<0.05.

Supplemental figure 6A. In vitro, cells from glomerular origin express Tie2 to a different extent which is not affected by exposure to LPS. Mesangial cells, podocytes and endothelial cells derived from human glomeruli were analysed for Tie2 mRNA expression levels in the absence and presence of 1 μg/ml LPS. mRNA was harvested and analysed by real-time RT-PCR as described in Materials and Methods and relative to GAPDH as house keeping gene. Striped bars represent mesangial cells, black bars represent podocytes, white bars represent glomerular endothelial cells. Values are mean of 3 ± SD.
the analytical procedure. Loss of Tie2 was not accompanied by a concurrent drop in ATP content of the slices (ATP data not shown). Incubation of the slices with LPS for 8 hours had, however no extra effect on the Tie2 mRNA levels (figure 6.5B).

Tie2 reduction is paralleled by, but not directly related to, neutrophil influx and loss of glomerular barrier function integrity

In mice, LPS administration resulted in a rapid increase in expression of inflammatory proteins. For example, E-selectin was strongly expressed by glomerular and arteriolar endothelium, while scattered expression occurred in the peritubular microvasculature, and limited expression was observed in the postcapillary venules, which normalized within 24 hours (figure 6.6A). This inflammatory response was accompanied by a loss of glomerular barrier integrity as evidenced by the occurrence of a gradual increase in urinary albumin/creatinine ratio from 0 to 24 hours after the initiation of the insult (figure 6.6B).

By semi-quantitative analysis we showed that neutrophils represent the main responding white cell population in this model. Glomerular neutrophil influx was at a maximum at 4 hours after LPS injection (figure 6.7B). Leukocyte-endothelial cell interactions can contribute to changes in the molecular status of the endothelium, and
Figure 6.6. LPS administration to mice induced proinflammatory microvascular endothelial cell activation in parallel with proteinuria due to loss of glomerular barrier function. (A) Immunohistochemical staining of CD31 and E-selectin at two different time points after i.p. LPS administration show a minor loss of CD31 mainly from peritubular endothelial cells during the initial stage of shock, while at the same time E-selectin expression was mainly induced in arteriolar, glomerular and peritubular endothelium. Original magnification 200x. Representative sections of biopsies from 5 mice per group are shown, *p <0.05. (B) After LPS administration to mice (0.5 mg/kg) loss of glomerular barrier function became visible by an increase in urine albumin/creatinin ratio.

Figure 6.7. Kinetics of neutrophil influx in kidneys of mice subjected to LPS challenge. (A) Renal infiltrating neutrophils were detected by Ly6G immunohistochemical staining in mouse kidneys at different time points after LPS administration. Original magnification 200x, inserts show glomeruli at original magnification of 400x. (B) Quantification of the extent of neutrophil influx was assessed by counting 50 randomly chosen glomeruli per biopsy at 400x magnification *p<0.05.
represents a process that is absent in the in vitro cell culture system employed. Especially in the microvasculature, leukocyte-endothelial cell interactions can be rather extensive as the diameter of the capillaries if often as small as, or even smaller than the diameter of the white blood cells passing by. To examine the hypothesis that neutrophil-endothelial cell interactions contribute to the loss of renal microvascular Tie2, and that this loss is related to loss of glomerular endothelial integrity, we depleted neutrophils prior to LPS administration and studied its consequences for Tie2 expression and proteinuria. FACS analysis of whole blood of mice 24 hours after injection of NIMP-antibody demonstrated that the mice had become severely neutropenic, with only 2.1 +/- 1.5% of total white blood cell count being neutrophils versus 23.7 +/- 9.1% in mice treated with control IgG.

Figure 6.8. Neutrophil depletion did not affect LPS induced loss of Tie2, yet abolished loss of glomerular barrier function.
(A) Effect of LPS on Tie2 mRNA (top) and protein (bottom) expression in neutrophil depleted mice. Mice were 24 hours prior to LPS challenge treated with anti-NIMP antibody to deplete PMN, or control IgG antibody that did not affect PMN count as assessed by flowcytometry (not shown). At t=0 mice were i.p. challenged with LPS at 0.5 mg/kg and sacrificed 8 hours later. Mean of n=5 ± SD, * p <0.05 by using ANOVA with post hoc comparison of all groups by using Bonferonni correction. Mean ± SD, * p <0.05 by using ANOVA with post hoc comparison of all groups by using Bonferroni correction. (B) Neutrophil depletion by pre-treatment of mice with anti-NIMP antibody abolished LPS induced proteinuria, while pre-treatment with control antibody did not affect this pathophysiological process. All mice were housed in a metabolic cage prior to the experiment (PMN-, LPS – group), while after the PMN of IgG and LPS injection mice were housed for 8 hours in a metabolic cage before they were sacrificed. Mean and individual values are shown, * p <0.05 by using ANOVA with post hoc comparison of all groups by using Bonferroni correction.
antibody (p < 0.001). Interestingly, neutrophil depletion did not block the LPS induced Tie2 downregulation, neither at the mRNA nor at the protein level (figure 6.8A). VEGF-A has a role in the maintenance of glomerular endothelial integrity under physiological circumstances and VEGF signaling may play a protective role in pathophysiological stress. In control kidneys, mRNA encoding VEGF-A and VEGFR-2 were mainly localized in the glomeruli (supplement figure B-A), corroborating previous reports\(^\text{31}\). 8 Hours after LPS administration no differences in VEGF-A nor VEGFR-2 between the neutrophil depleted and neutrophil competent mice could be observed (supplemental figure 6B). At the same time neutrophil depletion did diminish the occurrence of proteinuria in response to LPS administration (figure 6.8B).

Supplemental figure 6B. VEGF-A and VEGFR2 are expressed in different microvascular beds in healthy mouse kidney. Their expression levels were not affected by neutrophil depletion.

(A) Expression of VEGF-A and VEGFR-2 mRNA levels analysed by quantitative RT-PCR (relative gene expression adjusted to GAPDH) assessed in three renal microvascular beds obtained by laser microdissection. Mean values ± SD of 3 mice per group, * p<0.05.

(B) Effect of LPS on VEGF-A and VEGFR-2 mRNA expression in PMN depleted versus neutrophil competent mice. Mice were 24 hours prior to LPS challenge treated with anti-NIMP antibody to deplete neutrophils, or control IgG antibody that did not affect PMN count as assessed by flow cytometry (not shown). At t=0 mice were i.p. challenged with LPS at 0.5 mg/kg and sacrificed 8 hours later. Mean of n=5 ± SD.
**Discussion**

In various conditions of shock, the microvasculature of the kidney loses its integrity, leading to protein leakage and loss of kidney function. As the receptor tyrosine kinase Tie2 is implicated in the control of vascular integrity, we studied in mouse kidney the consequences of hemorrhagic shock and endotoxaemia on Tie2 expression in relation to proteinuria. In this study, we demonstrated for the first time that both Tie2 mRNA and protein were rapidly, and temporarily lost from the renal microvasculature in reaction to shock conditions. At the same time, the microvasculature was strongly activated leading to recruitment of neutrophils into the glomerular compartment and concurrent proteinuria. Neutrophil depletion resulted in reduction of proteinuria, which was however not accompanied by Tie2 mRNA or protein rescue, implying that Tie2 may not be a major factor controlling in the maintenance of the glomerular filtration barrier in acute shock.

Tie2 protein loss can be explained by shock induced Tie2 degradation. Bogdanovic et al elegantly showed in HUVEC cultures that in response to Ang-1, Tie2 is rapidly internalized and degraded, while Ang-2 mildly induced Tie2 degradation\(^\text{27}\). In healthy human volunteers subjected to LPS, a systemic increase in Ang-2 levels was observed with a maximum peak of 5 times control values at 4.5 hours after LPS challenge, while Ang-1 remained relatively unchanged\(^\text{32}\). Would a similar change in serum levels occur in our mouse model, one could hypothesize that in vivo a rise in Ang-2 levels may be the cause of Tie2 internalization. As at present systemic Ang-2 levels cannot be assessed in mice due to lack of proper analytical tools, the role of Ang-2 binding to Tie2 as a trigger for Tie2 protein degradation in the renal microvasculature remains speculative. The rapid and temporary loss of Tie2 mRNA can at present not be accounted for. Possibly, shear stress induced changes may acutely affect endothelial Tie2 expression, as was previously reported to be a major controlling factor for the expression of the orphan receptor Tie1\(^\text{33}\). If this would be the case, it could explain why a reduction in Tie2 mRNA levels was not be brought about in our static in vitro model systems. Preliminary studies on the effects of i.v. TNF-α administration on Tie2 in our laboratory revealed a direct or indirect role for NF-κB in the control of renal mRNA loss, as pretreatment of mice with an NF-κB inhibitor resulted in Tie2 mRNA rescue upon TNF-α challenge (unpublished data).
As in vitro studies could not mimic the in vivo observations, further studies on the molecular mechanisms underlying the current observations should be executed in vivo, and may need to make use of pharmacological tools, or endothelial cell specific knock outs to affect specific kinases. Whether a causal relation exists between Tie2 loss and changes in pO\textsubscript{2} or shear stress, immune cell expressed Tie2 – microvascular endothelial cell Angiopoietin/Tie2 interactions\textsuperscript{34}, or e.g., Interleukin-18\textsuperscript{35}, and what the functional consequences of Tie2 loss will be for the renal microvasculature, will be subject of future studies.

The fact that under acute shock conditions, the renal microvasculature temporarily loses both Tie2 protein and mRNA, but that the loss is not per se associated with major changes in glomerular barrier function, implies that other factors are likely involved in the regulation of the integrity of glomerular microvascular segments\textsuperscript{4}. In our effort to identify these factors we demonstrated that neither VEGF-A nor its receptor VEGFR-2 were differentially affected in the neutrophil competent versus the neutrophil incompetent mice.

Moreover, in the acute shock conditions in both mice and man studied here, loss of Tie2 was not associated with increased plasma levels of sTie2, while sTie2 has previously been shown to be associated with microvascular dysfunction under pathological conditions in both mice and men\textsuperscript{25; 36}. For example, sTie2 plasma levels are elevated in Crohn’s disease\textsuperscript{37}, critical limb ischemia\textsuperscript{38}, and acute myocardial infarction\textsuperscript{39}. Lowering of sTie2 is furthermore a prognostic marker in the treatment of renal carcinoma\textsuperscript{40}, a tumor type associated with elevated VEGF production which was identified as one of the triggers for Tie2 shedding from the endothelial membrane\textsuperscript{28}. Proteinuria is present early in septic patients and a prognostic factor for the development of sepsis in postoperative patients\textsuperscript{41}, and we cannot rule out that in more complex situations of shock deviant Tie2 expression is a contributing factor for proteinuria, and that sTie2 levels are subject to change.

Recently, Mofarrahi and colleagues reported on the downregulation of Tie2 protein in liver, lungs and diaphragm of LPS challenged mice\textsuperscript{42}. The kinetics of Tie2 downregulation between these organs and the kidney examined in our study, differed quite significantly. While in the liver and lung Tie2 protein levels did not normalize up to 24 hours after LPS administration, in the kidneys they do. These deviations may be explained by the fact
that effects of LPS are dosage, and LPS and mouse strain dependent. Both studies used C57Bl/6 mice, yet the strains of E. Coli were different, as was the dose (serotype O55:B5 at 20 mg/kg vs 026:B61 at 5 mg/kg respectively). The considerable heterogeneity in basic microvascular endothelial cell behavior in organ specific microenvironments may contribute to differences in molecular control of the observed changes. As Mofarrahi and colleagues did not relate Tie2 loss with vascular leakage, it remains to be established whether in other vascular segments in the body loss of Tie2 is associated with loss of vascular integrity.

Our mouse models to represent patients with critical illness have some shortcomings. The hemorrhagic shock models may have resemblance with patients with trauma hemorrhage, while LPS induced shock is certainly a laboratory model for human sepsis. Shock induced organ failure is a multi-step and time dependent process and in our models full development of the organ failure is not awaited. Also the influence of organ failure support, like mechanical ventilation which per se could induce multiple organ failure, is not studied in our animal models. Although there are more clinical relevant animal models for sepsis and trauma hemorrhage, we chose to use our models based on the fact that these highly standardized and frequently used single hit animal models are reproducible and make comparison with the published research possible. The lack of multiple insults in our models, which are seen in trauma hemorrhage and sepsis patients, might compromise translation of our findings. Yet it does not affect our findings per se that also Tie2 can be dynamically controlled.

To study Tie2 down regulation in kidneys of septic patients, kidney biopsies are required. Because of the risks of bleeding, it is unethical to do this for research purposes. We therefore tried to mimic the septic response in an ex vivo kidney slice model. Kidney slices were incubated with different sepsis mediators, yet none of them invoked a Tie2 downregulatory response more than the downregulation already induced by the 8 hours ex vivo incubation. Of note was, the fact that in all experiments, incubation of kidney slices per se in well oxygenated conditions induced Tie2 mRNA loss already within 4 hours while the ATP content of the slices were not compromised (data not shown). Likely, early in the ex-vivo experiments reactions in the kidney tissue are activated. As Tie2 is related to vascular integrity, it may be worthwhile to follow up this observation in the scope of organ preservation for transplantation purposes.
In summary, we observed a rapid and temporary, substantial loss of Tie2 mRNA and protein from the renal microvasculature in reaction to hemorrhagic shock and LPS mediated endotoxaemia without concurrent sTie2 level increase. Loss of Tie2 could not be directly related to the occurrence of proteinuria.

Acknowledgments

Martin Houwertjes is acknowledged for excellent animal experimental assistance. Martin Schipper, Ageeth Knol and Peter Zwiers are acknowledged for excellent technical assistance. Betty van der Veen is acknowledged for conducting several cell experiments. Jan de Jong and Annemarie M. Leliveld-Kors are acknowledged for the kind provision of human kidney material. Dr. Bernhard Banas from Regensburg and Dr. Moin A. Saleem from Bristol are acknowledged for their kind gifts of, respectively, the mesangial cell line and the podocyte cell line. P. Heeringa is supported by the Dutch Organization of Scientific Research (NWO VIDI 917.66.341).
REFERENCES


34. Venneri MA, De PM, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C,


