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Shock-induced stress induces loss of microvascular endothelial Tie2 in the kidney which is not associated with reduced glomerular barrier function

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van Meurs M, Kurniati NF, Wulfert FM, Asgeirsdottir SA, de Graaf IA, Satchell SC, Mathieson PW, Jongman RM, Kümpers P, Zijlstra JG, Heeringa P, Molema G. Shock-induced stress induces loss of microvascular endothelial Tie2 in the kidney which is not associated with reduced glomerular barrier function. *Am J Physiol Renal Physiol* 297: F272–F281, 2009. First published June 10, 2009; doi:10.1152/ajprenal.00137.2009.—Both hemorrhagic shock and endotoxemia 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 extents. 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 h 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 endotoxemia, 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 cannot be mimicked in vitro, we depleted neutrophils before 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.

endothelium; hemorrhagic shock; endotoxemia; neutrophil

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 >50% 60-day mortality of AKI patients treated in intensive care units (31). AKI is characterized by a sudden loss of the ability of the kidneys to excrete wastes, maintain fluid balance, and conserve electrolytes (36) and by the occurrence of proteinuria (2).

A number of potential mechanisms have been described to underlie the occurrence of proteinuria in AKI (18, 28), including loss of microvascular integrity. One of the molecular systems controlling microvascular integrity is the angiopoietin/Tie2 system (12). Tie2 is a 140-kDa tyrosine kinase receptor with immunoglobulin and epidermal growth factor homology (20) that has specificity for angiopoietin (Ang)-1 and Ang-2 binding (8, 40). Ang-1-induced Tie2 signaling is considered essential for endothelial integrity and provides quiescent endothelial status with anti-inflammatory properties (13). 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 (6, 7, 33, 34). Both hemorrhagic shock and endotoxemia induce a pronounced vascular activation in the kidney, which coincides with vascular leakage and glomerular barrier dysfunction (37, 42, 44). 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 (38). Ang-2 overexpression in podocytes led to increased proteinuria in adult mice (7), while in a diabetic mouse model the administration of Ang-1 exerted protective effects with diminished proteinuria (26). Also in human proteinuric diseases like systemic lupus erythematosus, Ang-2 serum levels correlated positively with proteinuria (21). 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 relationship between Tie2 changes and proteinuria as a measure glomerular barrier dysfunction. The initial observations justified further study into the role of neutrophils in the changes in Tie2 expression. For this, we depleted the neutrophils by antibody treatment before shock induction and investigated its conse-

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quences for Tie2 expression and proteinuria. The observations were extended to humans by studying a human volunteer endotoxemia model and human kidney slices exposed to sepsis mediators.

MATERIALS AND METHODS

Animals. Eight- to 12-wk-old C57BL/6 male mice (20–30 g) were obtained from Harlan Nederland (Horst, The Netherlands). Mice were maintained on mouse chow and tap water ad libitum in a temperature-controlled chamber at 24°C with a 12:12-h light-dark cycle. All procedures were approved by the local committee for care and use of laboratory animals and were performed according to governmental and international guidelines on animal experimentation.

Mouse shock models. The mouse hemorrhagic shock model has been extensively documented elsewhere (37). In short, mice were anesthetized with isoflurane (inspiratory, 1.4%), N_2O (66%), and O_2 (33%). The left femoral artery was cannulated 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 min of hemorrhagic shock 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 h 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 endotoxemia, mice were intraperitoneally (ip) injected with LPS (*Escherichia coli*, serotype 026:B6l; Sigma, St. Louis, MO) at 5 μg/g (15,000 endotoxin units/g) body wt 4, 8, and 24 h later, blood was drawn, and organs were harvested as described above.

Control mice were left untreated and were killed 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 h 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/creatinine ratios were assessed by housing mice in metabolic cages for 24 h 7 days before the insult and from 0 to 4 h, 0 to 8 h, and 8 to 24 h after LPS-induced shock. A subgroup of LPS-treated mice was ip injected with 0.5 mg anti-NIMP antibody to selectively deplete the neutrophils before shock induction (43). One day after this procedure, mice were ip 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 killed 8 h 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 endotoxemia. For the human endotoxemia model, human volunteers who participated in a drug intervention study were injected with a dose of 4 ng/kg body wt (10,000 endotoxin units/μg) LPS (*E. coli*, batch EC-6, US Pharmacopeia, Twinbrook Parkway, Rockville, MD). The local Investigations Review Board approved the study. Written informed consent was obtained from all subjects before enrollment in the study. Data from this study have been reported extensively elsewhere (14). 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) (35) were cultured in EBM medium in 12-well culture dishes at a density of 100,000 cells/well for 24 h at 33°C, followed by 5 days at 37°C under 5% CO_2-95% air before they were introduced in an experiment. The ciGEnC culture medium consisted of EBM-2 medium supplemented with 5% FCS and EGM-2 MV singleQuots (Lonza Group, Basel, Switzerland). In the experiments described here, ciGEnC were used up to *passage 40*.

Confluent ciGEnC were activated for 4 h with 0.1, 1, and 10 ng/ml TNF-α (Boehringer, Ingelheim, Germany) and 1, 50, and 1,000 ng/ml LPS. After incubation, the 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 yr, with normal kidney function. Tissue was prepared for precision-cut tissue slices within 15 min. 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 earlier (17). 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

Table 1. *Antibodies and their controls used for immunohistochemistry and leukocyte depletion*

	Ab Name/Epitope	Provider	Isotype	Dilution Used	Application
Primary Abs					
Mouse Tie2	Tek4	eBioscience	IgG1	1:50	IHC
Hu Tie2	sc324	Santa Cruz Biotechnology		1:50	IHC
Mouse CD31	PECAM-1	BD Pharmingen	IgG2a	1:100	IHC
Mouse E-selectin	MES-1	Dr. D. Brown, United Kingdom	IgG2a	1:10	IHC
Neutrophil	Ly6G	BD Pharmingen	IgG2a	1:50	IHC
Control IgG for rat Ab		Antigenix America	IgG1, IgG2a, IgG2b	1:50	IHC
Neutrophil	Anti-NIMP	HBt	IgG2b	NA	PMN depletion
Control IgG		Sigma	IgG	NA	Control depletion
Secondary Abs					
Rabbit anti-rat-HRP	P0459	Dako		1:40	IHC
Envision kit, rabbit polymer	K4009	Dako			IHC
Goat anti rabbit-HRP	4050-05	Southern Biotech		1:50	IHC
Rabbit anti-rat (preadsorbed)		Vector		1:300	IHC

Ab, antibody; IHC, immunohistochemistry; PMN, polymorphonuclear neutrophils; HRP, horseradish peroxidase; NA, not applicable.

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-naphthalene membranes attached to normal 1-mm slides (P.A.L.M. Microlaser Technology, Bernried, Germany) were fixed in acetone and stained with Mayer's hematoxylin, washed with diethyl pyrocarbonate-treated water, and air-dried. Endothelial cells from small arterioles (6×10^5 μm^2) and postcapillary venules (1.3×10^6 μm^2), as well as glomeruli (3×10^6 μm^2), were dissected using the Laser Robot Microbeam System (P.A.L.M. Microlaser Technology).

Gene expression analysis by quantitative RT-PCR. RNA was extracted from 20 × 5-μm cryosections from mouse kidney, 250 μm human kidney slices and cells, and isolated 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. RNA yield (OD 260) and purity (OD 260/OD 280) were measured by an ND-1,000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE). One microgram of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promega, Leiden, The Netherlands). The Assay-on-Demand primers (ABI Systems, Foster City, CA) used in the PCR included the housekeeping gene GAPDH (assay ID Mm99999915 _g1 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 (C_T) were averaged. According to the comparative C_T method described in the ABI manual, gene expression was normalized to the expression of the housekeeping gene, yielding the ΔC_T value. The average, relative mRNA level was calculated by $2^{-\Delta CT}$.

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 min. After drying, sections were incubated for 45 min at room temperature with primary rat anti-mouse antibodies in the presence of 5% FCS (Table 1). After washing, endogenous peroxidase was blocked by incubation with 0.1% H_2O_2 in PBS for 20 min. This was followed by incubation for 30 min at room temperature with horseradish peroxidase-conjugated secondary antibodies (Table 1). Between incubation with antibodies, sections were washed extensively with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma), and sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands). No immunostaining was observed with isotype-matched controls (Table 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 × 10-μm kidney slices were homogenized in 50 mM Tris·HCl buffer (pH 7.5) containing 150 mM NaCl and protein inhibitor cocktail (Sigma) and centrifuged at 13,000 *g* for 15 min. Total protein was determined by DC Protein Assay (Bio-Rad Laboratories, Hercules, CA), before quantification of Tie2 by ELISA (mouse Tie2 MTE200, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Tie2 levels were normalized to total protein concentrations in the tissue homogenate and expressed as picograms Tie2 per microgram total protein.

The level of soluble Tie2 in the plasma was analyzed using a commercially available Tie2 ELISAs (human DTE200 and mouse MTE200; R&D Systems, Oxon, UK) according to the manufacturer's instructions. The DTE200 ELISA kit was previously used to measure changes in soluble Tie2 in different patients groups (24, 32). During this investigation, we validated the MTE200 ELISA for suitability to measure soluble Tie2 using commercially available soluble mouse Tie2 (762-T2, R&D Systems).

Kidney function measured by albumin/creatinine ratio. To assess glomerular barrier function, the microalbumin and creatinine levels were measured in mouse urine using a commercial available kit (Exocell, Philadelphia, PA) according to the manufacturer's instructions.

Statistical analysis. Statistical significance of differences was studied by means of Student's *t*-test or ANOVA with post hoc comparison using Bonferroni correction. All statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL) and GraphPad Prism (GraphPad Software, San Diego, CA). Differences were considered to be significant when $P < 0.05$.

RESULTS

In healthy mouse kidneys, Tie2 is expressed in all vascular beds to different extents. To examine the expression pattern of Tie2 in the healthy mouse kidney, we immunohistochemically stained tissue for Tie2 protein (Fig. 1*A*). 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

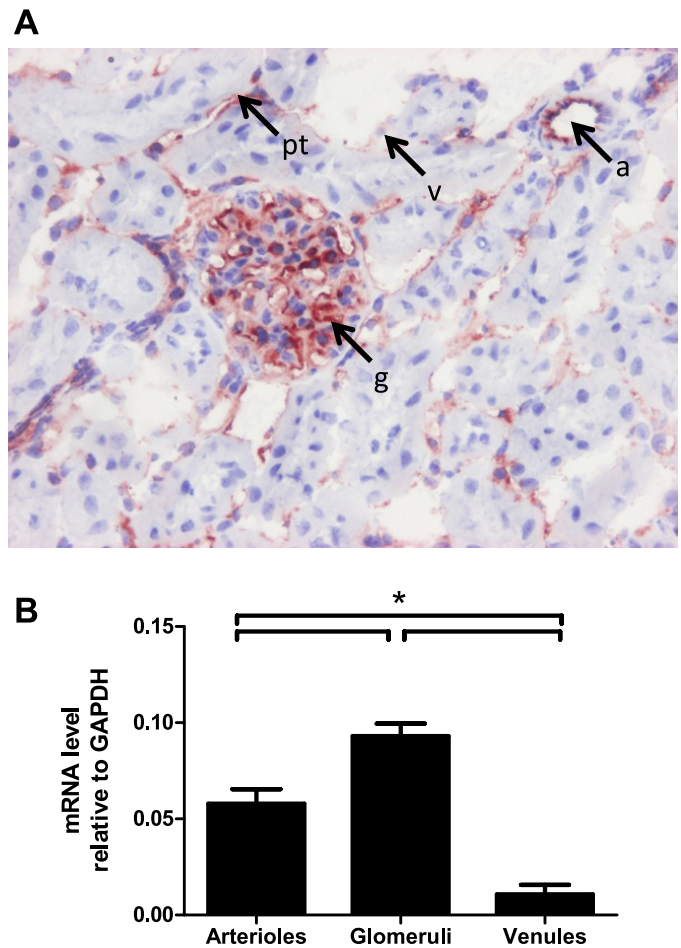


Fig. 1. Tie2 is expressed in different microvascular beds in healthy mouse kidney. *A*: protein expression detected by immunohistochemical staining. Arrows point at different microvascular beds; a, arteriole; g, glomerulus; pt, peritubular vasculature; v, venule. *B*: expression of Tie2 mRNA levels by quantitative RT-PCR (relative gene expression adjusted to GAPDH) assessed in 3 microvascular beds laser microdissected from kidney. Values are means ± SD of 3 mice/group. *$P < 0.05$.

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 before gene expression analysis. Most Tie2 mRNA was localized in glomeruli while the least was seen in venules (Fig. 1*B*).

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. Twenty-four hours after the shock insult, the mRNA in hemorrhagic shock had normalized, while an increase in mRNA was seen in the LPS-treated groups (Fig. 2*A*). The decrease in mRNA content was accompanied by a reduction in Tie2 protein levels in kidney homogenates in both models, with the most prominent reduction visible in the LPS model (Fig. 2*B*). Of note is the fact that 24 h after the shock insult, levels of Tie2 protein in both groups of shock subjected mice had normalized. Immunohistochemical detection of Tie2 revealed that the protein was lost from all vascular beds, i.e., the

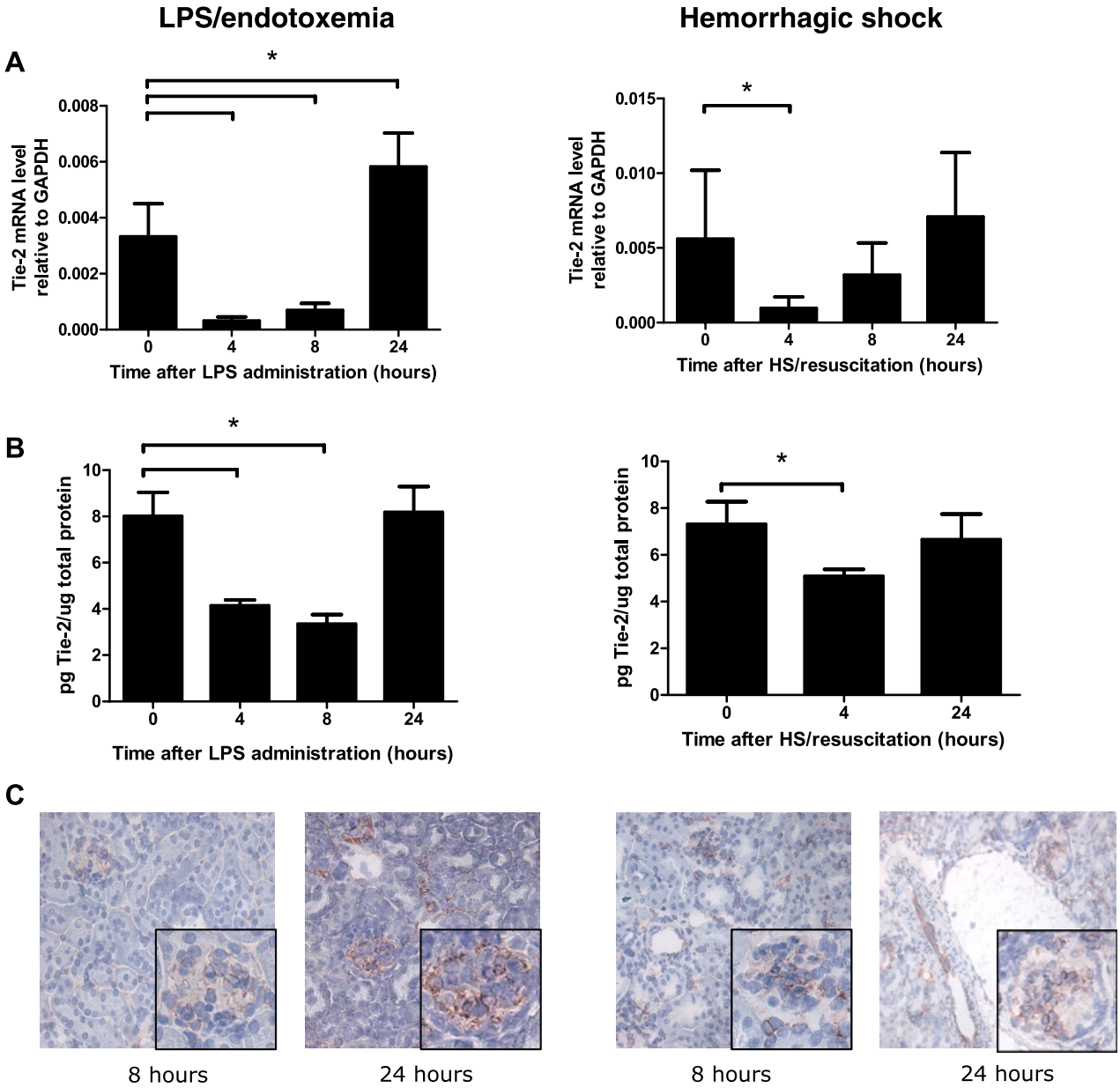


Fig. 2. Spatiotemporal changes in renal Tie2 mRNA and protein expression in mice subjected to LPS-induced shock and hemorrhagic shock followed by resuscitation. In the endotoxemia model, LPS was administered to mice at a dose of 0.5 μg/g body wt, while in the hemorrhagic shock model mice were subjected to blood withdrawal to a mean arterial pressure of 30 mmHg for 90 min, after which they were resuscitated with voluven as described in MATERIALS AND METHODS. *A*: mRNA levels shown are relative to GAPDH as a housekeeping gene and determined by quantitative RT-PCR as described in MATERIALS AND METHODS. Values are means ± SD of at least 5 mice/group. $*P < 0.05$. *B*: protein levels were measured in kidney homogenates by ELISA as described in MATERIALS AND METHODS. Values are means ± SD of at least 8 mice/group. $*P < 0.05$. *C*: representative light microscopic pictures of the microvascular localization of Tie2 protein after LPS or hemorrhagic shock, respectively, and resuscitation 8 and 24 h after induction of shock assessed by immunohistochemistry. Original magnification ×200. *Insets*: ×400. $*P < 0.05$.

arteriolar, glomerular, peritubular, and venular vasculature (Fig. 2*C*).

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 (4, 15). To determine the potential occurrence of Tie2 shedding during and after the shock period, we measured soluble Tie2 in the systemic circulation after LPS-induced shock. In mice, no increased shedding occurred during the first 24 h after LPS administration (Fig. 3*A*). Similar to the mouse model, no shedding of Tie2 into the plasma could be observed in human endotoxemia (Fig. 3*B*). 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. 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 (3) (Fig. 4, *A* and *B*). 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 proinflammatory conditions ruled out an overall nonresponsiveness 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 with glomerular endothelium, however, their Tie2 expression level was more than 100- to 1,000-fold lower, and no effect of both short-term and long-term LPS exposure on Tie2 mRNA levels could be detected (Supplementary Fig. A; all supplementary material for this article is available on the journal web site).

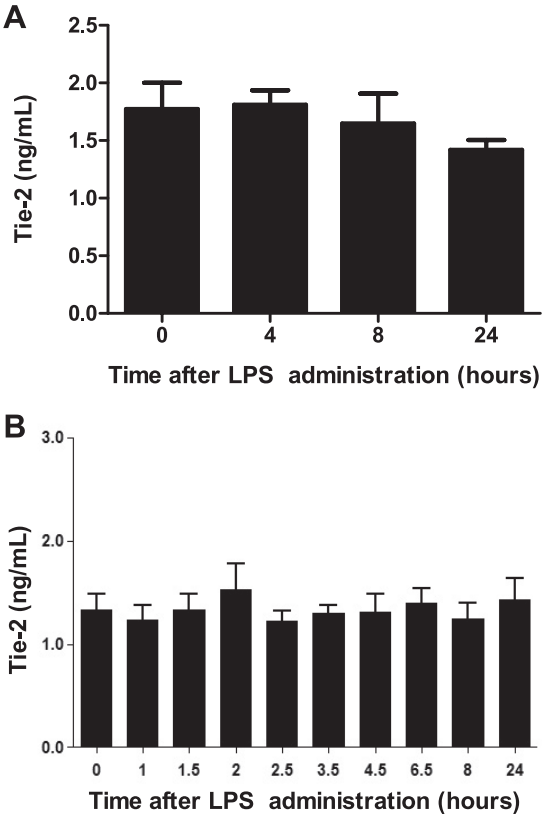


Fig. 3. Soluble Tie2 levels in plasma of mice and humans administration did not change after LPS administration. Quantitation of soluble Tie2 protein levels in plasma from LPS-challenged mice (*A*; $n = 4$) and LPS-challenged humans (*B*; $n = 6$) was performed using ELISA. Values are means ± SD. $^*P < 0.05$.

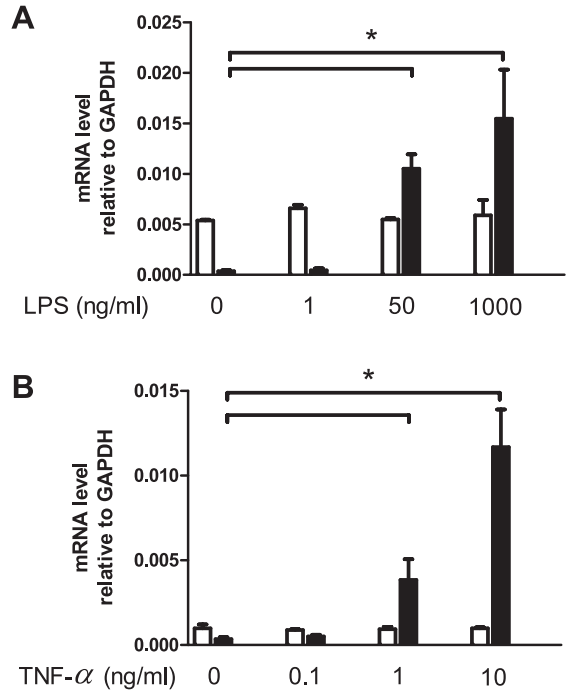
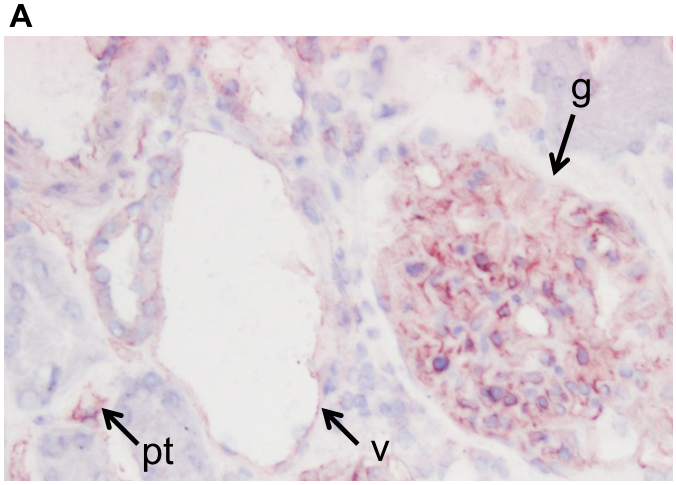


Fig. 4. In vitro, neither LPS nor TNF-α stimulation of glomerular endothelial cells affected Tie2 gene expression, while E-selectin was strongly induced. Conditionally immortalized glomerular endothelial cell (ciGENC) were exposed for 4 h to LPS (*A*) or TNF-α (*B*), at increasing concentrations. Open bars, Tie2 mRNA levels; filled bars, E-selectin mRNA levels. Values are means ± SD. $^*P < 0.05$.

To determine whether a possible interplay between cells determined the main 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 (Fig. 5*A*). Upon ex vivo incubation of the slices in normal medium for 8 h, Tie2 mRNA levels significantly dropped compared with levels in control kidney snap- frozen directly before slice production. These lower mRNA levels were still well above the detection limit of the analytic 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 h, however, had no extra effect on Tie2 mRNA levels (Fig. 5*B*).

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 h (Fig. 6*A*). This inflammatory response was accompanied by a loss of



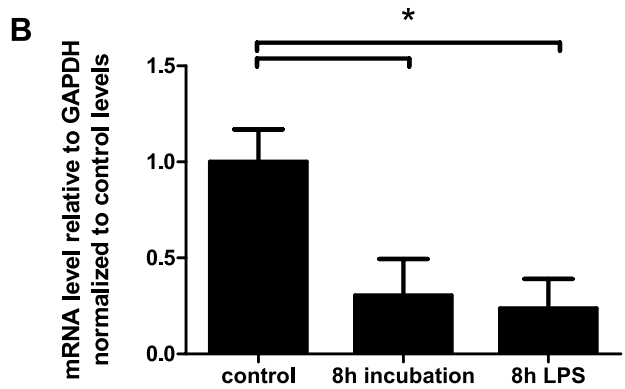


Fig. 5. Tie2 expression in human kidney slices does not change under the influence of LPS. *A*: immunohistochemical detection of Tie2 in the human kidney. Tie2 protein expression is visible in glomeruli (g), peritubular (pt), and postcapillary venule (p) endothelial cells. *B*: Tie2 expression in human kidney tissue in a controlled, ex vivo precision-cut tissue slice incubation system. After incubation for 8 h in medium with and without 50 μg/ml LPS, slices were harvested and processed for mRNA expression analysis. Tie2 mRNA expression decreased significantly upon incubation in medium for 8 h, but no additional effect of exposure to LPS on Tie2 mRNA expression levels was observed. $*P < 0.05$.

glomerular barrier integrity, as evidenced by the occurrence of a gradual increase in urinary albumin/creatinine ratio from 0 to 24 h after the initiation of the insult (Fig. 6*B*).

By semiquantitative analysis, we showed that neutrophils represent the main responding white cell population in this model. Glomerular neutrophil influx was at a maximum at 4 h after LPS injection (Fig. 7*B*). Leukocyte-endothelial cell interactions can contribute to changes in the molecular status of the endothelium and represent 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 is often as small as, or even smaller than, the diameter of the white blood cells passing by (23). 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 before LPS administration and studied the consequences for Tie2 expression and proteinuria. FACS analysis of whole blood of mice 24 h after injection of an NIMP antibody demonstrated that the mice had become severely neutropenic, with only 2.1 ± 1.5% of total white blood cell count being neutrophils vs. 23.7 ± 9.1% in mice treated with control IgG antibody ($P < 0.001$). Interestingly, neutrophil depletion did not block the LPS-induced Tie2 downregulation, either at the mRNA or at the protein level (Fig. 8*A*). 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 (Supplementary Fig. B-A), corroborating previous reports (27). Eight hours after LPS administration, no differences in VEGF-A or VEGFR-2 between the neutrophil-depleted and neutrophil-competent mice could be observed (Supplementary Fig. B-B). At the same time, neutrophil depletion did diminish the occurrence of proteinuria in response to LPS administration (Fig. 8*B*).

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 endotoxemia 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 the maintenance of the glomerular filtration barrier in acute shock.

Tie2 protein loss can be explained by shock-induced Tie2 degradation. Bogdanovic et al. (4) elegantly showed in human umbilical vein endothelial cell cultures that in response to Ang-1, Tie2 is rapidly internalized and degraded, while Ang-2 mildly induced Tie2 degradation. In healthy human volunteers subjected to LPS, a systemic increase in Ang-2 levels was observed, with a maximum peak of five times control values at 4.5 h after LPS challenge, while Ang-1 remained relatively unchanged (22). Were a similar change in serum levels to 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 analytic 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 in the expression of the orphan receptor Tie1 (5). If this were 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 iv 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

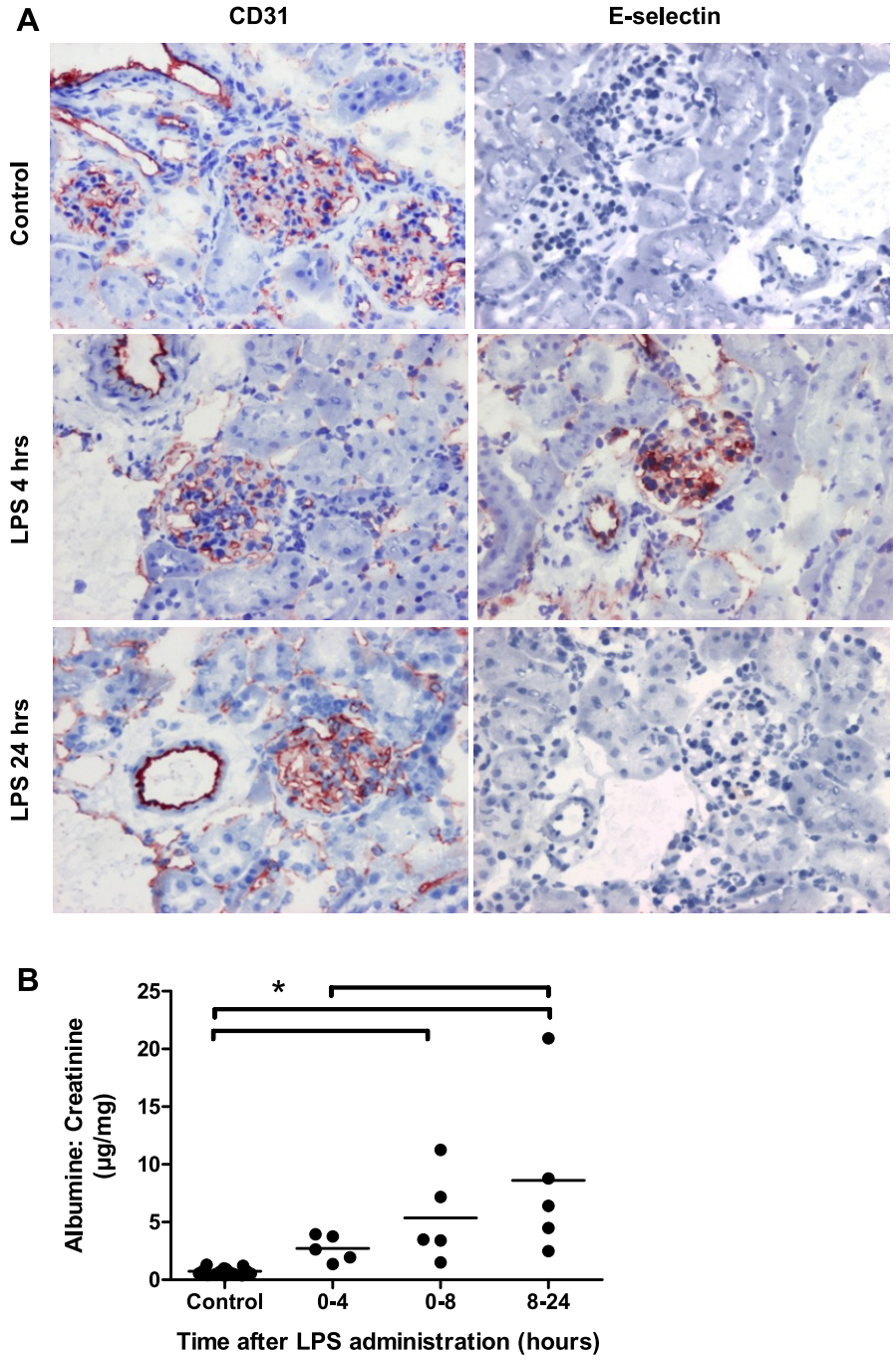


Fig. 6. LPS administration to mice induced proinflammatory microvascular endothelial cell activation in parallel with proteinuria due to loss of glomerular barrier function. *A*: immunohistochemical staining of CD31 and E-selectin at 2 different time points after ip LPS administration shows a minor loss 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 ×200. Representative sections of biopsies from 5 mice/group are shown. $*P < 0.05$. *B*: after LPS administration to mice (0.5 μg/g), loss of glomerular barrier function became visible by an increase in urine albumin/creatinine ratio.

inhibitor resulted in Tie2 mRNA rescue upon TNF-α challenge (Kuldo JM, Molema G, unpublished observations).

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 knockouts to affect specific kinases. Whether a causal relationship exists between Tie2 loss and changes in Po_2 or shear stress, immune cell-expressed Tie2- microvascular endothelial cell angiopoietin/Tie2 interactions (39), or e.g., interleukin-18 (41), and what the functional consequences of Tie2 loss would 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 (18). 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 vs. the neutrophil-incompetent mice.

Moreover, in the acute shock conditions in both mice and humans 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

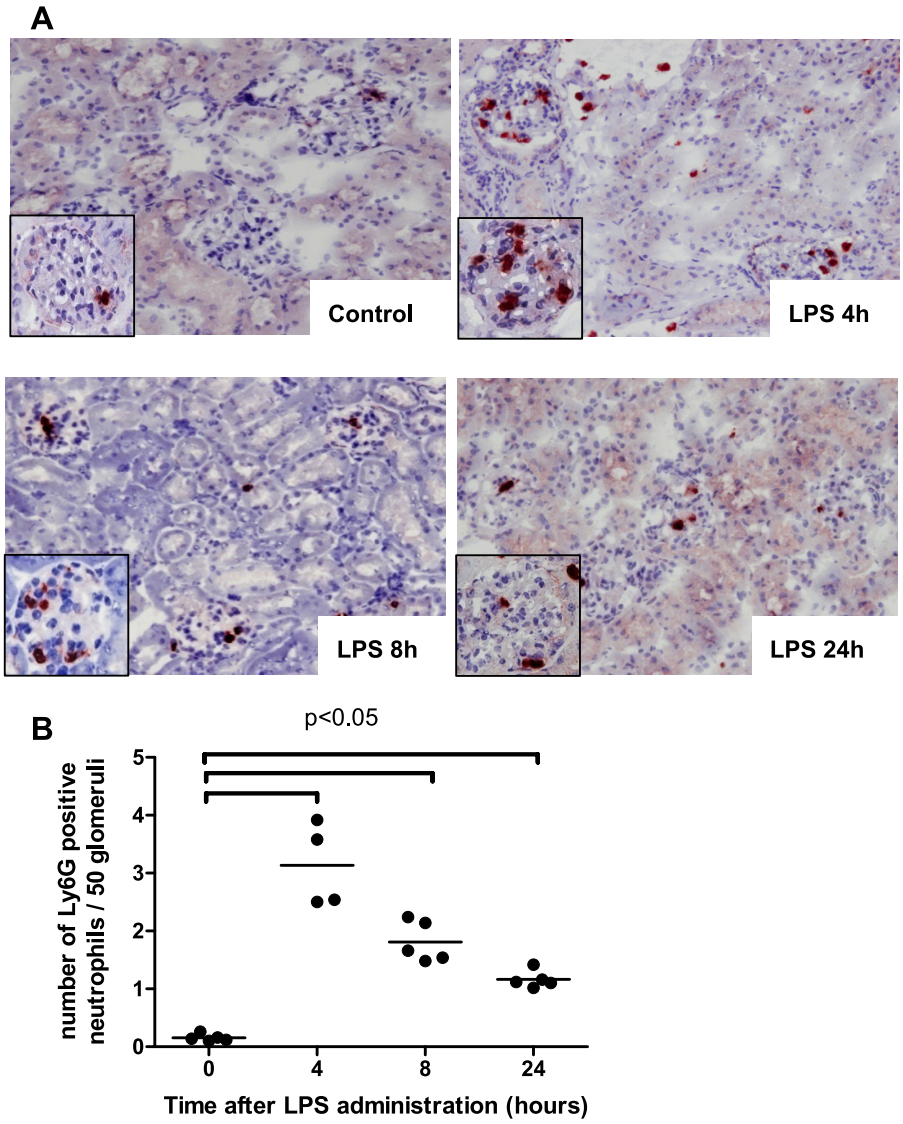


Fig. 7. Kinetics of neutrophil influx in kidneys of mice subjected to LPS challenge. *A*: renal infiltrating neutrophils were detected by Ly6G immunohistochemical staining in mouse kidneys at different time points after LPS administration. Original magnification ×200. *Insets*: glomeruli at original magnification of ×400. *B*: quantification of the extent of neutrophil influx was assessed by counting 50 randomly chosen glomeruli per biopsy at ×400 magnification. *$P < 0.05$.

under pathological conditions in both mice and humans (11, 32). For example, sTie2 plasma levels are elevated in Crohn's disease (10), critical limb ischemia (16), and acute myocardial infarction (25). Lowering of sTie2 is furthermore a prognostic marker in the treatment of renal carcinoma (19), a tumor type associated with elevated VEGF production, which was identified as one of the triggers for Tie2 shedding from the endothelial membrane (15). Proteinuria is present early in septic patients and a prognostic factor for the development of sepsis in postoperative patients (9), 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 (29) reported on the downregulation of Tie2 protein in the liver, lungs, and diaphragm of LPS-challenged mice. 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 h after LPS administration, in the kidneys they do. These deviations may be explained by the fact that the effects of LPS are dosage, and LPS and mouse strain dependent (30). 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:B6l at 5 mg/kg, respectively). The considerable heterogeneity in basic microvascular endothelial cell behavior in organ-specific microenvironments (1) may contribute to differences in molecular control of the observed changes. As Mofarrahi and colleagues (29) 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 a resemblance to patients with trauma hemorrhage, while LPS-induced shock is certainly a laboratory model for human sepsis. Shock-induced organ failure is a multistep and time-dependent process, and in our models the full development of 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 clinically relevant animal models for sepsis and

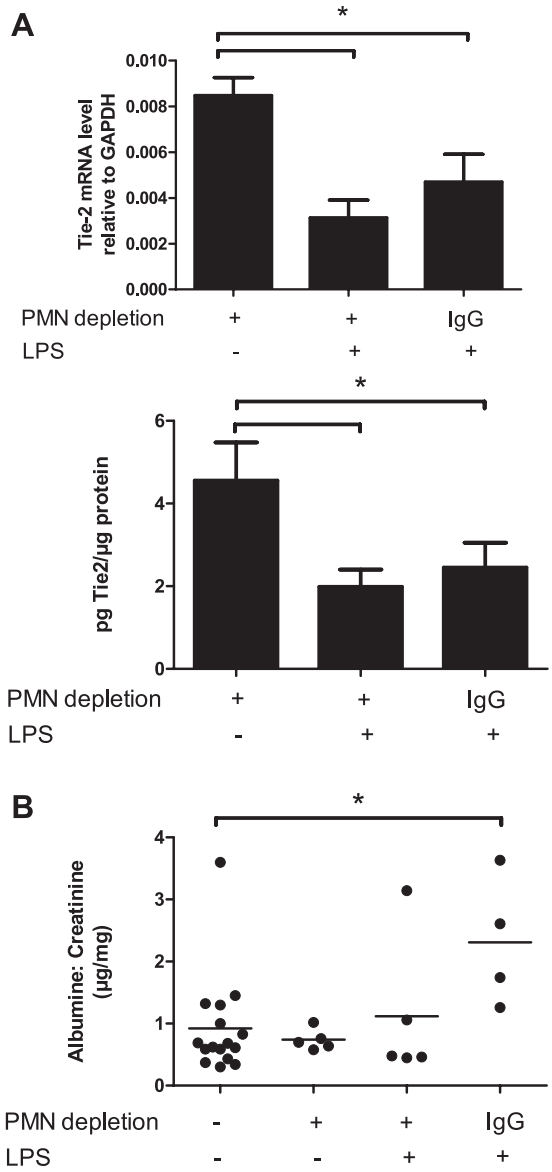


Fig. 8. Neutrophil depletion did not affect LPS-induced loss of Tie2, yet abolished loss of glomerular barrier function. *A*: effect of LPS on Tie2 mRNA (*top*) and protein (*bottom*) expression in neutrophil-depleted mice. Mice were treated with anti-NIMP antibody 24 h before LPS challenge to deplete polymorphonuclear neutrophils (PMN) or control IgG antibody that did not affect PMN count, as assessed by flow cytometry (not shown). At *time 0*, mice were ip challenged with LPS at 0.5 μg/g and killed 8 h later. Values are means ± SD; $n = 5$. *$P < 0.05$ by ANOVA with post hoc comparison of all groups using Bonferroni correction. *B*: neutrophil depletion by pretreatment of mice with anti-NIMP antibody abolished LPS-induced proteinuria, while pretreatment with control antibody did not affect this pathophysiological process. All mice were housed in a metabolic cage before the experiment (PMN−, LPS− group), while after the LPS injection mice were housed for 8 h in a metabolic cage before they were killed. Mean and individual values are shown. *$P < 0.05$ by ANOVA with post hoc comparison of all groups using Bonferroni correction.

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. However, it does not affect our findings per se that also Tie2 can be dynamically controlled.

To study Tie2 downregulation 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 (17). 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 h 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 h 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 endotoxemia without a concurrent sTie2 level increase. Loss of Tie2 could not be directly related to the occurrence of proteinuria.

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