

# Acute endotoxemia in mice induces downregulation of megalin and cubilin in the kidney

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Severe sepsis is often accompanied by acute renal failure with renal tubular dysfunction. Albuminuria is a common finding in septic patients and we studied whether it was due to an impairment of proximal tubular endocytosis of filtered albumin. We studied the regulation of megalin and cubilin, the two critical multiligand receptors responsible for albumin absorption, during severe experimental endotoxemia. Lipopolysaccharide (LPS) caused a time- and dose-dependent suppression of megalin and cubilin expression that was paralleled by a decrease in plasma albumin levels and an increase in the urine concentration of albumin in mice. Incubation of rat renal cortical slices with LPS also reduced the mRNA expression of megalin and cubilin. Further, LPS suppressed megalin and cubilin mRNA expression in murine primary proximal tubule cells and decreased the uptake of FITC albumin in these cells. In addition, the increase in urine levels of albumin in response to ischemia/reperfusion-induced acute renal failure was paralleled by a decrease in the expression of megalin and cubilin. Thus, our data indicate that the expression of megalin and cubilin is decreased during experimental endotoxemia and in response to renal ischemia/reperfusion injury. This downregulation may contribute, in part, to an increase in urine levels of albumin during acute renal failure.

KEYWORDS: acute renal failure; albumin; inflammation; ischemia; lipopolysaccharide

Sepsis and septic shock are a serious health problem worldwide because of high morbidity and mortality rates.<sup>1</sup> They are also one of the most important risk factors for acute renal failure (ARF), which is defined as the abrupt decline in glomerular filtration rate and tubular dysfunction. The incidence of ARF is ~43% in patients with severe sepsis and septic shock. In addition, ARF is a critical complication of sepsis because it further increases the mortality rate.<sup>2</sup> Therefore, the understanding of the pathogenesis of sepsis-related ARF is of critical importance.

Hypoalbuminemia is a common finding in septic patients,<sup>3</sup> and it is associated with higher mortality rates, not only in septic patients but also in patients with ARF.<sup>4-6</sup> Albumin is synthesized in the hepatocytes and is the most abundant plasma protein.<sup>7</sup> It contributes to the maintenance of oncotic pressure and blood volume, and it serves as a carrier for a number of substances, such as drugs, vitamins, hormones, bilirubin, and fatty acids. A decreased hepatic formation of albumin, increases in catabolism, vascular permeability, or in the urinary excretion of albumin may be reasons for hypoalbuminemia.<sup>8</sup> Under physiological conditions, the urinary loss of albumin is mainly prevented by an intact glomerular filtration barrier; thus, albuminuria is an important functional parameter of glomerular damage.<sup>9</sup> However, it has been found over the past years that albumin is also partially glomerular-filtered and then reabsorbed in the proximal tubule via receptor-mediated endocytosis.<sup>10,11</sup> Therefore, albuminuria may also result in part from an altered reabsorption by proximal tubule cells (PTCs). Two receptors, megalin and cubilin, are involved in the renal process for tubular reabsorption of albumin via a receptor-mediated endocytosis.<sup>12</sup> Megalin is a large transmembrane protein (~600 kDa) that belongs to the LDL (low-density lipoprotein) receptor family,<sup>13</sup> and cubilin, also known as the intrinsic factor/vitamin B12 complex receptor, is a peripheral membrane protein (~460 kDa).<sup>14</sup> Within the kidney, cubilin and megalin are highly expressed at the apical site of PTCs, and both proteins are critically involved in the reabsorption of several glomerular-filtered substances including albumin.<sup>12</sup> Recent findings suggest that cubilin is essential for the proximal tubular uptake of albumin, and that megalin is required for the endocytosis of the cubilin-albumin complex.<sup>15</sup> Because severe sepsis is often accompanied by ARF

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with renal tubular dysfunction, an impairment of proximal tubular endocytosis of glomerular-filtered albumin may participate in the renal loss of albumin during sepsis.

Bacterial endotoxins or exotoxins are known to stimulate the synthesis of inflammatory mediators on glomerular podocytes and tubular epithelial cells.<sup>16</sup> Administration of lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria, is an experimental approach commonly used in examining the pathogenesis of sepsis. Injection of LPS has been found to cause a decrease in plasma albumin levels and an increase in the urinary excretion of albumin in animals.<sup>17-20</sup> Recently, it has been demonstrated that LPS downregulates the expression of megalin *in vitro*.<sup>21</sup> As the multiligand receptors megalin and cubilin are of major importance for proximal tubular reabsorption of albumin, we investigated in the present study the regulation of these receptors during severe experimental endotoxemia and, in addition, on ischemia/reperfusion (I/R)-induced ARE.

## RESULTS

### LPS worsens renal function and increases urine concentration of albumin

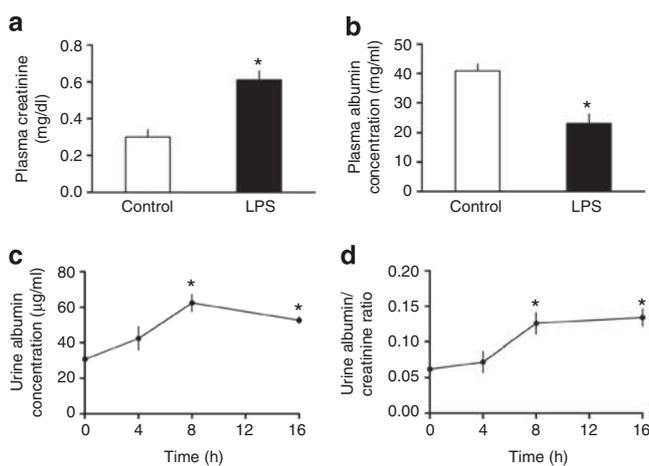
Plasma creatinine and urea levels were increased from  $0.30 \pm 0.04$  and  $24.5 \pm 2.6$  mg/dl to  $0.61 \pm 0.05$  and  $79 \pm 3.6$  mg/dl, respectively, 16 h after injection of LPS (10 mg/kg). Plasma levels of albumin were decreased from  $40.9 \pm 2.3$  to  $23.2 \pm 3.0$  mg/ml 16 h after injection of LPS (10 mg/kg). Urine albumin concentration increased 2.0- and 1.7-fold at 8 and 16 h after the injection of LPS. The ratio of urinary albumin to creatinine increased 2.0- and 2.2-fold at 8 or 16 h after the injection of LPS (Figure 1).

### LPS decreases renal megalin and cubilin expression

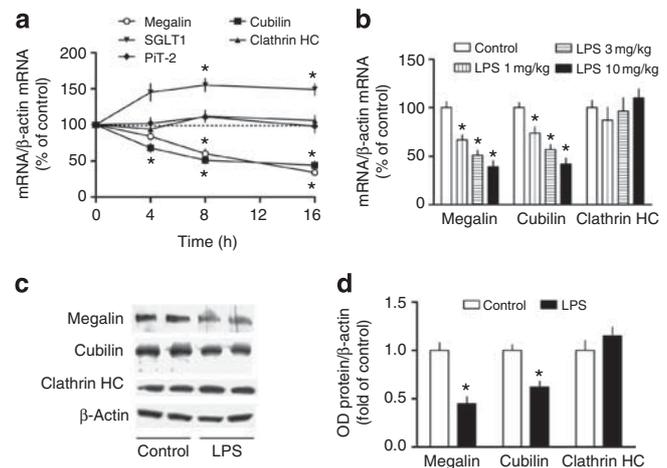
Endotoxemia, induced by the administration of a single dose of LPS (10 mg/kg), time-dependently decreased renal megalin

and cubilin mRNA abundance. Megalin mRNA was downregulated to 74%, 60%, and 34% of control levels at 4, 8, and 16 h after injection of LPS, respectively. Cubilin mRNA levels were decreased to 68%, 51%, and 44% of control levels at 4, 8, and 16 h after treatment with LPS, respectively. Clathrin heavy chain (HC) and PiT-2 mRNA levels were unaltered 4, 8, and 16 h after treatment with LPS. SGLT1 mRNA levels were increased 1.6- and 1.5-fold at 8 and 16 h after injection of LPS, respectively (Figure 2a).

Injection of increasing doses of LPS (1, 3, and 10 mg/kg) caused a dose-dependent downregulation of megalin mRNA abundance to 67%, 51%, and 37% of control levels 16 h after LPS injection, respectively. Cubilin mRNA abundance also decreased dose-dependently to 74%, 57%, and 42% of control values, respectively. Clathrin HC mRNA levels were unaltered (Figure 2b). We further investigated the expression of megalin, cubilin, and clathrin HC protein in the kidneys of vehicle- and LPS-treated animals and found that the expression of megalin and cubilin protein were decreased to 45% or 65% of control values, respectively, in animals treated for 16 h with LPS. Clathrin HC protein levels were unaltered (Figure 2c and d). As megalin and cubilin are also expressed in other tissues, we further determined the effect of LPS on the expression of megalin and cubilin in the lung and the small intestine. LPS decreased megalin mRNA expression in the lung to 49% of control values. In contrast, megalin mRNA expression was increased in the small intestine to 280% of control values. Cubilin mRNA was not detected in the lung, and was decreased in the small intestine to 11% of control values. Megalin immunoreactivity was detected in

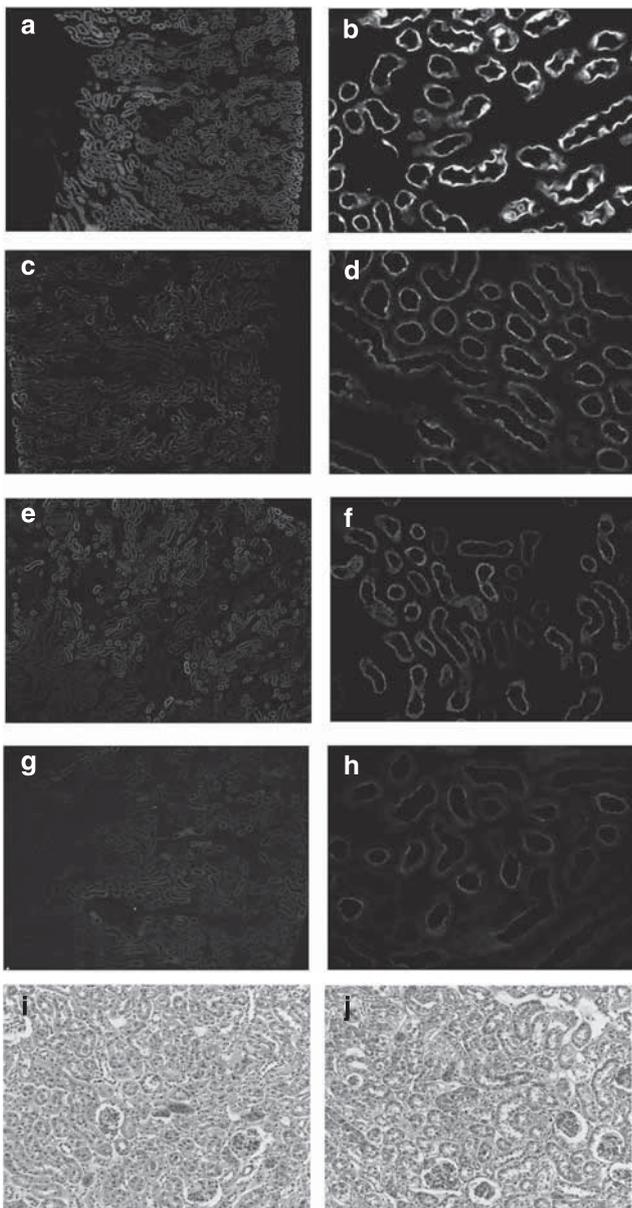


**Figure 1 | Endotoxemia induces hypoalbuminemia and increases urine concentration of albumin.** Effect of lipopolysaccharide (LPS; 10 mg/kg for 16 h) on (a) plasma creatinine concentration and (b) plasma albumin concentration. Time-dependent effect of LPS on (c) urine albumin concentration, and (d) urine albumin/creatinine ratio. Values are mean  $\pm$  s.e.m. for six animals. \* $P < 0.05$  vs. control.



**Figure 2 | Effect of lipopolysaccharide (LPS) on megalin and cubilin expression.** (a) Time-dependent effect of LPS (10 mg/kg) on renal megalin, cubilin, clathrin heavy chain (clathrin HC), PiT-2, and SGLT1 mRNA expression related to  $\beta$ -actin mRNA expression. (b) Dose-dependent effect of LPS on renal megalin, cubilin, and clathrin HC mRNA expression related to  $\beta$ -actin mRNA expression. (c) Insets show representative immunoblots for megalin, cubilin, clathrin, and  $\beta$ -actin protein expression. (d) Effect of LPS (10 mg/kg for 16 h) on megalin, cubilin, and clathrin HC protein expression related to  $\beta$ -actin protein expression. Values are mean  $\pm$  s.e.m. for six animals. \* $P < 0.05$  vs. control.

proximal tubules of vehicle-treated mice and was clearly decreased in mice treated with LPS (Figure 3a–d). Cubilin immunoreactivity was also observed in proximal tubules of vehicle-treated mice. In kidneys from LPS-treated mice, there was also a marked reduction in the staining for cubilin after 16 h (Figure 3e–h). The acute tubular necrosis score was increased in LPS-treated mice compared with vehicle-treated mice ( $2.4 \pm 0.8$  vs.  $0.5 \pm 0.2$ ,  $P < 0.05$ ; Figure 3i and j).



**Figure 3 | Immunolocalization of megalin and cubilin.** Megalin immunolocalization in the renal cortex of (a, b) control and (c, d) lipopolysaccharide (LPS; 10 mg/kg for 16 h)-treated animals. Cubilin immunolocalization in the renal cortex of (e, f) control and (g, h) LPS (10 mg/kg for 16 h)-treated animals. Periodic acid-Schiff-stained kidney sections treated with (i) saline and (j) LPS at 16 h. Original magnification (a, c, e, g)  $\times 100$ , (i, j)  $\times 200$ , and (b, d, f, h)  $\times 400$ .

### LPS suppresses megalin and cubilin mRNA expression *ex vivo*

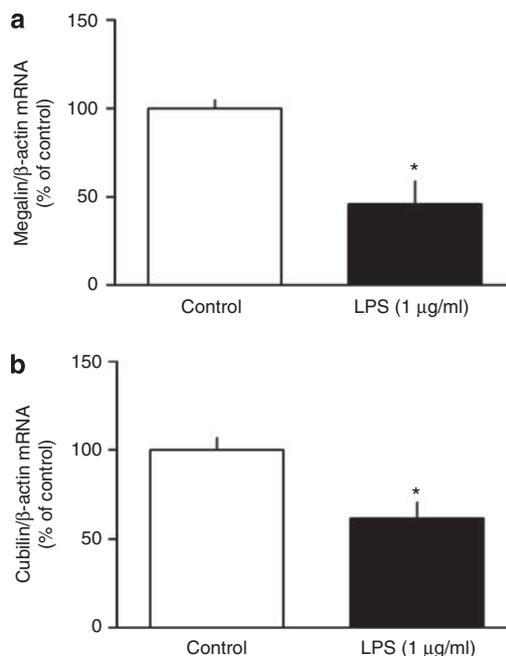
To confirm the pathophysiological relevance of our *in vivo* findings independent of systemic influences, we assessed the effect of LPS on megalin and cubilin expression *ex vivo* in rat kidney slices. LPS alone (1  $\mu\text{g}/\text{ml}$ ) significantly decreased megalin and cubilin mRNA levels after 6 h of incubation to 53% and 62% of control levels, respectively (Figure 4). There was no difference in the concentration of lactate dehydrogenase (LDH) in the supernatants of control and LPS-treated kidney slices (data not shown).

### LPS reduces megalin and cubilin mRNA expression and the uptake of FITC albumin in primary PTCs

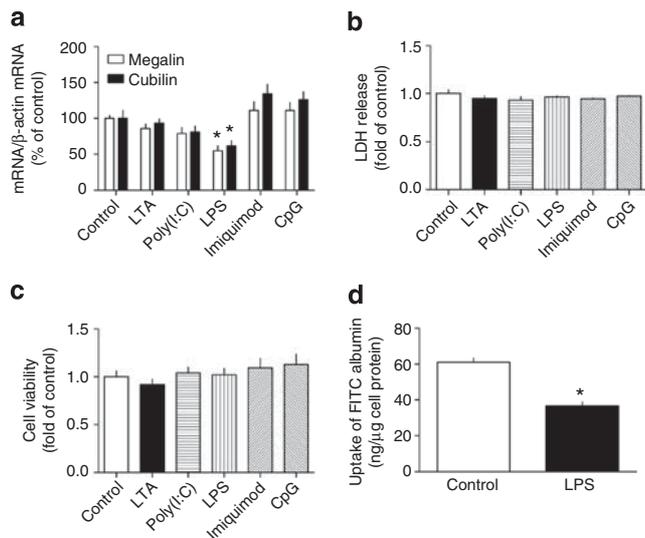
To examine a possible direct effect of Toll-like receptor (TLR) activation on megalin and cubilin expression, we incubated primary PTCs with ligands for TLR2, TLR3, TLR4, TLR7, and TLR9. We found that only the activation of TLR4 by LPS significantly downregulated megalin and cubilin mRNA to 55% and 62% of control levels, respectively, 16 h after the addition of LPS (Figure 5a). No significant accumulation of LDH in the supernatants was measured (Figure 5b). Furthermore, cellular viability was unaltered 16 h after the addition of the different TLR ligands (Figure 5c). In line with the downregulation of megalin and cubilin, the uptake of fluorescein isothiocyanate (FITC)-labeled albumin was reduced in LPS-treated cells to  $\sim 60\%$  of control values (Figure 5d).

### Renal I/R decreases megalin and cubilin expression

Plasma creatinine and urea levels were increased from  $0.24 \pm 0.03$  and  $25.4 \pm 2.1$  mg/dl to  $1.21 \pm 0.12$  and  $131 \pm 18.0$  mg/dl,



**Figure 4 | Effect of lipopolysaccharide (LPS) on megalin and cubilin mRNA expression *ex vivo*.** LPS (1  $\mu\text{g}/\text{ml}$  for 6 h) on (a) megalin and (b) cubilin mRNA expression related to  $\beta$ -actin mRNA expression in rat renal cortical slices. Values are mean  $\pm$  s.e.m. of three independent experiments. \* $P < 0.05$  vs. control.



**Figure 5 | Effect of Toll-like receptor (TLR) agonists *in vitro*.**

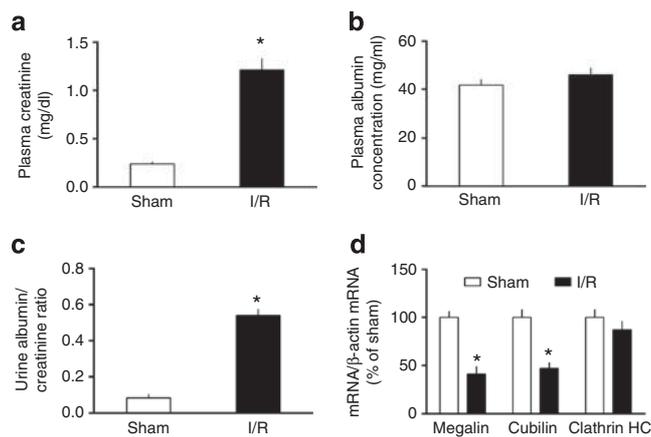
Effect of lipoteichoic acid (LTA; 1 μg/ml; TLR2 agonist), polyinosinic:polycytidylic acid (Poly (I:C); 30 μg/ml; TLR3 agonist), ultrapure lipopolysaccharide (LPS; 100 ng/ml; TLR4 agonist), imiquimod (1 μg/ml; TLR7 agonist), and type B CpG oligonucleotide ODN 1668 (CpG; 1 μg/ml; TLR9 agonist) for 16 h on (a) megalin and cubilin expression, (b) lactate dehydrogenase (LDH) release, and (c) cell viability in primary proximal tubule cells. (d) Effect of LPS on fluorescein isothiocyanate (FITC) albumin uptake in these cells. Values are mean ± s.e.m. of four to six independent experiments. \**P* < 0.05 vs. control.

respectively, after renal ischemia for 27 min and reperfusion for 16 h (I/R). Plasma levels of albumin were unchanged after I/R. Urine albumin concentration and urine albumin/creatinine ratio were increased 4.0- and 6.4-fold, respectively, in mice subjected to I/R. Megalin and cubilin mRNA were downregulated to 41% and 47% of control levels 16 h after renal ischemia for 27 min, respectively. Clathrin HC mRNA was not altered 16 h after renal ischemia for 27 min (Figure 6).

**DISCUSSION**

Several clinical studies have shown that septic patients develop albuminuria, but the mechanisms are not fully understood.<sup>22,23</sup> Within the kidney, albumin undergoes glomerular filtration and proximal tubular reabsorption.<sup>10</sup> Most findings suggest a central role for the glomerular filtration barrier, especially for podocytes, in this context.<sup>9,24</sup> However, a decrease in proximal tubular reabsorption of albumin via a decrease in the cubilin- and megalin-mediated reuptake of albumin might also contribute to the development of albuminuria as a consequence of glomerular disease. A single bolus injection of LPS in our *in vivo* model caused a marked increase in plasma levels of urea and creatinine, indicating that renal failure occurred in these mice.

We now found that endotoxemia caused a time- and dose-dependent downregulation of megalin and cubilin gene expression *in vivo*. To confirm the relevance of our *in vivo* findings, we assessed the effect of LPS on megalin and cubilin



**Figure 6 | Effect of renal ischemia reperfusion on renal function and megalin and cubilin expression.** Effect of ischemia for 27 min and reperfusion for 16 h (ischemia/reperfusion (I/R)) on (a) plasma creatinine concentration, (b) plasma albumin concentration, (c) urine albumin/creatinine ratio, and (d) megalin, cubilin, and clathrin heavy chain (HC) mRNA expression. Values are mean ± s.e.m. for six animals. \**P* < 0.05 vs. sham.

mRNA expression *ex vivo* in rat kidney slices, where we used a high concentration of LPS (1 μg/ml) to imitate the condition of septic shock.<sup>25</sup> In agreement with the results obtained in our *in vivo* experiments, we found that LPS decreased megalin and cubilin mRNA expression *ex vivo*. This finding may indicate that the downregulation of megalin and cubilin in response to LPS is independent of systemic and renal hemodynamic or tubular alterations, such as hypotension, reduced renal blood flow, and reduced glomerular filtration, which we and others observed in this *in vivo* model.<sup>26</sup> In subsequent *in vitro* studies in primary PTCs, endotoxin was clearly shown to decrease the cellular uptake of FITC-labeled albumin, which is in line with previous observations.<sup>21,27</sup> The decrease in the cellular uptake of FITC-labeled albumin was paralleled by a decrease in cubilin and megalin mRNA expression. This finding is in accordance with previous *in vitro* reports, showing a decreased expression of megalin in response to LPS or plasma from septic patients.<sup>20,23,24</sup> We now found that cubilin mRNA expression is also decreased in response to LPS. As proximal tubules uptake LPS *in vivo*,<sup>28</sup> these findings may indicate a possible direct effect of LPS on the expression of megalin and cubilin in PTCs, which could be likely mediated via activation of extracellular-signal-regulated kinases 1 and 2 (ERK1/2).<sup>21</sup> As the expression of several TLRs has been reported for renal tubule cells,<sup>29,30</sup> we further investigated the expression of megalin and cubilin in response to ligands for other TLRs. In contrast to the TLR4 ligand LPS, we found that typical ligands for TLR2, TLR3, TLR7, and TLR9 did not alter the expression of megalin and cubilin in primary PTCs. Therefore, our data suggest that megalin and cubilin are affected specifically by TLR4 ligands. Taken together, our data show that LPS directly reduces megalin and cubilin expression. It cannot be completely excluded that the observed changes are a result of cellular damage. However, several lines of evidence indicate that LPS directly reduces

megalyn and cubilin expression. (1) in accordance with previous observations, we found only a modest renal tubular injury in our *in vivo* model of LPS-induced ARF;<sup>31,32</sup> (2) our *ex vivo* and *in vitro* findings, which are in accordance with previous findings, indicate that there is no cell damage, at least *ex vivo* and *in vitro*;<sup>33,34</sup> and (3) the observation that the expression of clathrin HC, which is involved in endocytosis, and of the apical sodium cotransporters SGLT1 and PiT-2 is unaltered or even increased.<sup>35</sup> In addition, the latter finding also argues against a global decrease in proximal tubular apical membrane carriers and ion channels in response to LPS. However, the precise mechanism for the repression of megalin and cubilin gene expression in response to LPS *in vivo* has to be addressed in further studies.

To prove the functional consequence for the downregulation of megalin and cubilin gene expression, we further investigated plasma and urine levels of albumin. In line with previous observations, we found that the injection of LPS caused a decrease in plasma albumin concentration,<sup>19,20</sup> and an increase in the urine concentration of albumin. This observation is in contrast to a recent report, where rather a decrease in urine albumin concentration has been found,<sup>36</sup> but it strongly supports other findings reporting an increase in the urine concentration of albumin.<sup>17,18,36,37</sup> The increase in urine albumin concentration and urine albumin/creatinine ratio occurred in parallel with the decrease in megalin and cubilin expression. Thus, our data support a rather sustained increase in urine albumin/creatinine ratio, and do not support a transient proteinuria and/or albuminuria as it has been assumed by others.<sup>18,37,38</sup>

To exclude extrarenal factors and to prove the importance of the kidneys for the resulting increased urine albumin levels in response to endotoxemia, we further investigated the effect of I/R injury on renal megalin and cubilin expression and on albumin levels. We found that megalin expression was decreased in response to I/R injury. This finding fits very well with another study reporting decreased megalin mRNA expression in response to I/R.<sup>39</sup> As renal I/R induces the renal formation of cytokines,<sup>40</sup> and may also activate the ERK1/2 pathway,<sup>41,42</sup> it seems likely that the decrease of megalin in response to I/R could also be due to an activation of cytokines and/or the ERK1/2 pathway. In addition, we now found that cubilin expression is also decreased in response to I/R injury. The downregulation of megalin and cubilin was paralleled by an increase in the urine concentration of albumin, confirming previous observations.<sup>36</sup> Although we could not discriminate between an increase in glomerular-filtered albumin and a decrease in tubular-reabsorbed albumin, a decreased tubular absorption of albumin could likely be involved in the increase of urine albumin concentration in this model, which has been mainly linked to an altered glomerular function,<sup>43,44</sup> and also in the endotoxemic mouse model.

We further observed that plasma levels of albumin were not altered after I/R-induced injury. Thus, the resulting hypoalbuminemia in response to LPS may be rather due to

alterations in the extrarenal handling of albumin, such as a decrease in hepatic albumin synthesis, increased catabolism of albumin, or an increase in the vascular permeability of albumin.<sup>8,45,46</sup> However, as the plasma half-life of albumin is ~2 weeks, a hepatic failure may be of less importance in this context.

In summary, we provide evidence that experimental endotoxemia causes a decrease in renal megalin and cubilin expression, which is paralleled by an increase in the urine concentration of albumin. We further found that the expression of megalin and cubilin is decreased in response to renal I/R injury. Our findings indicate that this downregulation could be partly involved in the increase in urine albumin concentration during ARF. Therefore, this study contributes to our pathophysiological understanding about the development of albuminuria under ARF.

## MATERIALS AND METHODS

### Experimental animals

All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal protection committee. Male C57BL/6J mice (20–25 g) were obtained from Charles River (Sulzfeld, Germany). Mice were acclimated for at least 1 week in the animal facility with free access to food and water. Controls received intraperitoneal injections of 250  $\mu$ l of 0.9% saline. LPS-treated mice received a single intraperitoneal injection of 250  $\mu$ l of LPS (1, 3, or 10 mg/kg from *Escherichia coli* serotype 0111:B4; Sigma Aldrich Chemical, Taufkirchen, Germany) dissolved in 0.9% of saline. The animals ( $n = 6$  per group) were killed 4, 8, or 16 h after injection of LPS or saline. Blood was collected and the kidneys, lung, and small intestine were quickly removed, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until extraction of total RNA. For induction of renal I/R injury, renal arteries of mice ( $n = 6$  per group) were totally occluded for 27 min with microaneurysm clamps, followed by reperfusion for 16 h. In sham controls, renal arteries were only touched with forceps.

### Measurement of renal and blood parameters

Spot urine was obtained 4, 8, or 16 h after the injection of LPS. Plasma and urine levels of creatinine and plasma concentration of urea were determined using commercially available kits (BioAssay Systems, Hayward, CA). Plasma and urine levels of albumin were determined using a commercially available mouse albumin ELISA (Immunology Consultants Laboratory, Newberg, OR).

### Primary PTCs

Primary PTCs were isolated from mouse renal cortex, based on the method of Vinay *et al.*<sup>47</sup> In brief, the cortex was minced and digested in Hank's balanced salt solution (Invitrogen GmbH, Darmstadt, Germany) containing 10% collagenase II and 25% of bovine serum albumin for 20 min. After filtration through a 150- $\mu$ m cell strainer, the suspension was washed in cold phosphate-buffered saline. The tubule fragments were separated on a 55% Percoll gradient in  $2 \times$  phosphate-buffered saline containing 5 mmol/l glucose in which the proximal tubules form the lowest band. This band was removed and washed three times in cold phosphate-buffered saline. The proximal tubule fragments were cultured in a hormone-defined (1% of Insulin-Transferrin-Selenium-A Supplement (Invitrogen GmbH),

50 nm hydrocortisone, 5 nM triiodothyronine, and 5 nM epidermal growth factor) Dulbecco's modified Eagle's medium/F-12 medium supplemented with 1% fetal calf serum. After adhesion in ~4 h, the medium was changed to remove fibrocytes. The cells were grown in culture medium until confluent and then in serum-free medium for 24 h before use. Thereafter, cells were treated with or without the TLR2 ligand-purified lipoteichoic acid from *Staphylococcus aureus* (1 µg/ml), the TLR3 ligand polyinosinic:polycytidylic acid (poly(I:C))-LMW (30 µg/ml), the TLR4 ligand ultrapure LPS from *E. Coli 0111:B4* (100 ng/ml), the TLR7 ligand imiquimod (1 µg/ml), and the TLR9 ligand type B CpG oligonucleotide ODN 1668 (CpG; 1 µg/ml) for 16 h. All chemicals were purchased from InvivoGen (San Diego, CA). Uptake of FITC-conjugated bovine serum albumin was determined as described.<sup>48</sup> In brief, cells were washed three times with phosphate-buffered Ringer's solution (pH 7.4) and were incubated with 0.5 mg/ml FITC-conjugated albumin in Ringer's solution for 15 min at 37 or 4 °C. Thereafter, cells were rinsed four times with ice-cold Ringer's solution, and were lysed with Triton X-100 (0.1% vol/vol in Ringer's solution). Fluorescence was determined in cell lysates using a NanoDrop 3300 Fluorospectrometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm, and normalized for total protein in the samples measured by BCA protein assay (Pierce, Rockford, IL). Fluorescence was corrected for extracellular binding and unspecific adhesion to the cells by subtraction of fluorescein counts on cells at 4 °C.

#### LDH and MTT assay

Cell injury was determined by the release of LDH into the incubation medium using a commercially available assay kit (Cayman Chemical, Ann Arbor, MI). The values were presented as fold of the LDH release that was observed in control incubated cells. Cell viability was assayed by the MTT assay in accordance with the manufacturer's instructions (Cayman Chemical).

#### mRNA extraction and real-time PCR analysis

Total tissue and cell RNAs were extracted from homogenized tissue with TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. Total RNA was reverse transcribed into cDNA according to standard protocols as described previously.<sup>49</sup> Real-time PCR for megalin, cubilin, and β-actin was performed in a LightCycler 480 (Roche, Mannheim, Germany). All PCR experiments were conducted using the LightCycler DNA Master SYBR Green I kit provided by Roche Molecular Biochemicals (Mannheim, Germany) as described previously. The following primers were used: mouse Megalin (NM\_001081088) sense: 5'-GGAGGAACCAATCTGTTGTAATGT-3', antisense: 5'-GATGGTTGCCTGGAGGG-3'; rat Megalin (NM\_030827) sense: 5'-ACCGCCGAATGCCGCTGACT-3', antisense: 5'-TGCCCCAATGCCATAGGTAACGA-3'; mouse Cubilin (NM\_001081084) sense: 5'-AGCTCAACCTCCATTCAATCATA-3', antisense: 5'-GTGCAATCTGTGCTGCTT-3'; rat Cubilin (NM\_053332) sense: 5'-TGCATGTCACCTTACGTTTT-3', antisense: 5'-TGTAAGCCTCTCCACTCC-3'; mouse/rat β-actin (NM\_007393) sense: 5'-CCGCCCTAGGCACCAGGGTG-3', antisense: 5'-GGCTGGGGTGTGAAGGTCTCAAA-3'; mouse clathrin heavy polypeptide (NM\_001003908) sense: 5'-ATCGCCCAGCTGTGTGAGAA-3', antisense: 5'-TCTTGCAAGCCGCTGAATA-3'; mouse SGLT1 (NM\_019810) sense: 5'-CGGAAGAAGGCATCTGAGAA-3', antisense: 5'-AATCAGCAGGAGGATGAACA-3'; mouse PiT-2 (NM\_011394) sense:

5'-AGGAGTGCAGTGGATGGAGC-3', antisense: 5'-ATTAGTATGACACGACGCGCCG-3'.

#### Protein preparation and immunoblotting

Protein preparation and immunoblotting were performed as described previously.<sup>50</sup> In brief, protein samples were electrophoretically separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes, which were blocked overnight in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20, and then incubated for 1 h at room temperature with antibodies against megalin (1:500), β-actin (Sigma Aldrich; 1:5000), clathrin HC (Cell Signaling, Danvers, MA; 1:1000), or cubilin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500). After being washed, the membrane was incubated for 2 h with the secondary antibody (Santa Cruz Biotechnology; 1:2000) and subjected to a chemiluminescence detection system.

#### Tubular injury

Renal tissue injury was assessed in 10% phosphate-buffered, formalin-fixed, paraffin-embedded, and periodic acid-Schiff base-stained tissue sections. A semiquantitative score for tubular injury was calculated for each animal by a blinded observer. The percentages of tubules that displayed cellular necrosis, loss of brush border, interstitial edema, vacuolization, and tubule dilatation were scored as follows: 0 = none, 1 = <30%, 2 = 30-60%, and 3 = >60%. For each animal, at least 10 fields were examined.

#### Immunohistochemistry for megalin and cubilin

Kidneys from sham-operated and LPS-treated mice were fixed in 4% paraformaldehyde solution by retrograde perfusion through the abdominal aorta. Immunolabeling was performed on 5-µm paraffin sections as described previously. In brief, after being boiled in a microwave oven followed by cooling, kidney sections were incubated with an anti-megalin antibody (1:500) or an anti-cubilin antibody (Santa Cruz Biotechnology; 1:200) overnight at 4 °C, followed by incubation with a secondary antibody. As a negative control, we used the secondary antibody without incubation with the primary antibody.

#### Preparation of rat kidney slices

Male Sprague Dawley rats (200-250 g body weight; Charles River) were anesthetized and the kidneys were removed. Slices (0.5 mm thick) were cut with a McIlwain Tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). The slices (three slices per experimental condition) were then incubated in Hanks' balanced solution at 37 °C for 6 h alone or in the presence of LPS (1 µg/ml).

#### Statistical analyses

Data were analyzed by analysis of variance with multiple comparisons followed by the *t*-test with Bonferroni adjustment. *P* < 0.05 was considered significant.

#### DISCLOSURE

All the authors declared no competing interests.

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