Critical role of Jun N-terminal Kinase and p38 in the pathogenesis of sepsis-induced down regulation of the renal outer medullary K⁺ channel-1 expression

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Abstract

Objective: In sepsis, renal expression of the renal outer medullary K⁺ channel-1 (ROMK) is impaired via NFκB-induced cytokines. Mitogen activated protein kinases (MAPKs) are induced by lipopolysaccharide (LPS) or proinflammatory cytokines and ROMK-1 expression is downregulated via p38 or Jun N-terminal kinase (JNK).

We investigated how MAPK-inhibition of p38 or JNK affects ROMK-1 during sepsis.

Material and methods: Male C57/BL-6 mice (20–25 g) were used for in vivo and murine CCD cells (M1 cell line) for in vitro investigations. Mice were treated with LPS and MAPKs were inhibited by siRNA. M1 cells were stimulated with LPS or cytokines in addition to pathway inhibitors. In vivo, IL1β, TNFα and INFγ in blood were detected by cytometric bead immunoassay. In vitro, M1-cell line and a ROMK reporter gen assay were used.

Results: In vivo, LPS increased cytokines and decreased renal ROMK-1. Inhibition of JNK diminished LPS induction of cytokines and decrease of ROMK-1. JNK/p38 in kidney tissue were not changed after LPS nor affected by either MAPK inhibition, indicating that the regulation observed is due to cytokine-release in circulating macrophages. Incubation of M1-cells with cytokines decreased ROMK-1 expression. Inhibition of JNK/p38 had no effect on cytokine-induced down regulation of ROMK-1.

Conclusion: Inhibition of JNK decreases proinflammatory cytokines and increases renal ROMK1 in sepsis. The latter is not due to an effect on the renal level but likely on the level of macrophages.

Key words: cytokines, Jun N-terminal kinase, mitogen activated protein kinases, p38, renal outer medullary K⁺ channel-1, sepsis
List of abbreviations

ARF acute renal failure
AKI acute kidney injury
DLRA dual luciferase assay
ERK1/2 extracellular signal-regulated kinase 1/2
GFR glomerular filtration rate
IL1β interleukin 1beta
IL-6 interleukin 6
INFγ interferon gamma
JNK Jun N-terminal kinase
LPS lipopolysaccharide
MAPKs mitogen activated protein kinases
NKCC2 Na+-K+-2Cl⁻ co-transporter
NFKB nuclear factor kappaB
PBS phosphate buffered saline
ROMK(-1) renal outer medullary K⁺ channel(-1)
siRNA small interfering RNA
TNFα tumor necrosis factor alpha

Introduction

Acute renal failure (ARF) is defined as the abrupt decline in glomerular filtration rate (GFR) and tubular function affecting 5–7% of hospital patients [1, 2]. Sepsis and septic shock are important risk factors for ARF and remain the most important trigger for ARF in intensive care units [3-5]. Incidence of ARF is 20% in patients with severe sepsis and even 50% in patients with septic shock [5, 6], and the mortality rate of sepsis-related ARF is significantly high at 75% [7]. Thus, understanding the pathogenesis of sepsis-related ARF is of critical importance. Several in vivo and in vitro studies have suggested that the reduction of GFR in sepsis is secondary to altered glomerular hemodynamics [8, 9]. In contrast, the pathophysiology of sepsis-associated renal tubular dysfunction with failure in urine concentration and increased fractional sodium excretion has been poorly explained.

Urine concentration requires establishment and maintenance of a hypertonic medullary interstitium, which depends on the NaCl reabsorption [10]. Among other tubular transporting systems, the renal outer medullary K⁺ channel (ROMK), also located in the cortical collecting duct, is responsible for recycling potassium across the apical membrane, perpetuating the electrochemical gradient along the nephron, which is the moving power for continuous sodium uptake by other tubular transporters such as the Na⁺-K⁺-2Cl⁻ co-transporter (NKCC2) [3, 11-14].

We recently described a significant down-regulation of ROMK that likely contributes to septic ARF. In previous experiments, we could further demonstrate that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, or IFN-γ are relevant in mediating regulation of ROMK expression during sepsis [14]. Because of overlapping actions of these pro-inflammatory cytokines, down regulation of sodium transporters could not be attributed to single cytokines, providing an explanation for why anti–single cytokine strategies did not improve the outcome of patients with sepsis [14-17].

In turn, during sepsis for maximal secretion, pro-inflammatory cytokines are all dependent on the transcription factor nuclear factor (NF)-κB [8, 18, 19], which will be itself activated by different mitogen-activated protein (MAP) kinases such as Jun N-terminal Kinase (JNK) or p38 [20, 21]. JNK or p38 MAP kinases may thus be an important target for new anti-inflammatory approaches for treating sepsis-induced down regulation of ROMK and tubular dysfunction during endotoxemia.

As depicted in Figure 1 in the present study, we therefore wish to investigate the role of JNK and p38 MAP kinases mediated signalling pathway on LPS-induced regulation of pro-inflammatory cytokine and ROMK expression in mice, respectively. Additionally, the impact of JNK and p38 MAP kinases on endotoxin-induced down regulation of ROMK expression as well as on promoter activity in distal tubular murine cells was explored.
Sepsis was induced by intraperitoneal injection of LPS (Sigma Aldrich, Escherichia coli 0111:B4; 10 mg/kg b.w.). In sham treated animals, same procedure was performed using the carrier (phosphate buffered saline; PBS) for injection. To ensure adequate fluid resuscitation, animals received a subcutaneous injection of lactated Ringer’s solution (50 µl/g) directly after finishing the intervention.

Twelve hours (n = 4 per group) after injection of LPS/PBS, animals were deeply anesthetized with sevoflurane, using a VIP 3000 TM (Matrix by Midmark, NY, USA), and consecutively euthanized by decollation. At that time, LPS demonstrated the strongest effect, verified in pre-studies. LPS and sham treatment were also performed in mice pre-treated with non-targeting small interfering RNA (siRNA) and siRNA silencing JNK or p38 MAP kinases (n = 4 per group). Additionally the whole in vivo setting is shown in Figure 2.

**siRNA injection**

siSTABLE modified, deprotected and in vivo processed siRNAs (siGENOME Set of 4, Dharmacon, Lafayette, CO, USA) were dissolved in RNase-free PBS. For hydrodynamic injection, synthetic siRNAs (5 mg/kg of targeting siRNA or scrambled siRNA in 1 mL PBS) or 1 mL PBS (sham) were rapidly injected into one of the tail side veins of mice (n = 4 per group) 24 hrs before LPS treatment, as this time correlated with a maximal depression of JNK or p38 MAP kinases messenger RNA (mRNA) [23].

**mRNA analysis**

In brief, RT-rPCR was performed according to iQ SYBR-Green Supermix RT-PCR system protocol (Biorad, Hercules, CA, USA). PCR amplification protocol and primers were used as recently described [14]. Quantification was performed using the \( \Delta \Delta CT \) method using GADPH as reference gene and expression in control cells was normalized to 1.
Protein analysis

For Western blot analysis, frozen kidney cortex was homogenized using a stainless steel mortar cooled by liquid nitrogen, dissolved in lysis buffer containing 25 mmol/L Tris-HCl, 7 mmol/L reduced glutathione, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2 mol/L phenylmethylsulfonyl fluoride (PMSF), 1 µmol/L leupeptin, 1 µmol/L pepstatin, 1 µmol/L trans-epoxysuccinyl-L-leucylamido butane, and 1 mg/mL trypsin inhibitor, and further minced with an ultrasonic disperser UW 70 (Bandelin Electronic, Berlin, Germany). Total protein was measured in samples using the Bradford method. 33 Samples of protein (5 to 40 µg) were analyzed by Western blot with the respective antibodies. Rabbit Anti-Kir1.1 ROMK1 polyclonal antibody (diluted 1:500) were from Acris (Acris Antibodies, Herford, Germany). Antibodies against JNK, p38 or extracellular signal-regulated kinases (ERK) 1/2 (diluted 1:1000) were all from Cell Signalling Technology (CST, Danvers, MA, USA) and against GAPDH from Abcam (Cambridge, UK). Blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Dako, Hamburg, Germany) and were developed using a chemiluminescence kit (ECL Plus) following the manufacturer’s instruction (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were analyzed densitometrically using the Quantity One software (Bio-Rad Laboratories, Philadelphia, PA, USA) and normalized for GAPDH.

Detection of pro inflammatory cytokines

Pro inflammatory cytokines (TNFα, IFNγ, IL1β or IL6) in serum were detected by Cytometric Bead Assay (Becton Dickinson, Heidelberg, Germany). The Assay was performed as indicated in the manufacturers instruction manual. Measurement was done with an LSRFortessa™ cell analyser (Becton Dickinson, Heidelberg, Germany).

Figure 2: Setup of the in vivo approach of the study. Male C57/BL-6 mice (20–25 g) were divided into seven groups. Sepsis was induced by intraperitoneal injection of lipopolysaccharide (LPS). Sham/control animals received phosphate buffered saline (PBS) for injection. Twenty-four hours prior to LPS or PBS injection corresponding groups were pre-treated with non-targeting small interfering RNA (siRNA) and siRNA silencing Jun N-terminal kinase (JNK) or p38. Twelve hours after injection of LPS/PBS, animals were deeply anesthetized with sevoflurane and consecutively euthanized.
Cell culture

Murine CCD cells (M1 cell line; ATCC® CRL-2038™, LGC Standards GmbH, Wesel, Germany) were incubated for 24 h to 48 h with medium (control) or LPS, single cytokines or a combination of cytokines TNF-α, IFN-γ (100 ng/mL each), and IL-1β (100 ng/mL). Additionally, cytokine-incubated M1 cells were administered with specific pathway inhibitors as indicated in the respective part of the results section.

For ROMK promoter studies, a 2.1 kB-promoter-firefly luciferase construct was created as described [24]. The construct (pGL3basic(luc+)-ROMK1) identity was confirmed by sequencing. Transfection of cells using FUGENE 6 was performed. Firefly luciferase vector was co-transfected with a plasmid containing Renilla luciferase. Cells were incubated with medium (control), TNF-α, IL-1β, or IL-6 (each 100 ng/mL) 24 h after transfection for 24 h to 48 h, and then luciferase activity was measured with a dual luciferase assay kit as described by the manufacturer. Relative luciferase activity was calculated as the ratio of firefly luciferase to Renilla luciferase. All reagents or plasmids used for reporter gen studies were purchased from Promega (Promega GmbH, Mannheim, Germany).

Statistics

Data were collected using Microsoft Excel 2010 (Microsoft, Unterschleißheim, Germany) and analysed by ANOVA or student’s t-test as appropriate. All analyses were performed using PASW Statistics software version 18 (IBM Corp., Armonk, NJ, USA) and a p-value ≤ 0.05 was considered significant.

Results

Effect of anti-JNK or anti-p38 siRNA application on ROMK1 after LPS

Injection of scrambled siRNA had no effect on the amount of ROMK1 mRNA and protein in the kidney after 24 h (Figures 3A and 4). Injection of LPS reduced ROMK1 protein as expected, which was abolished by application of anti-JNK siRNA. Compared to scrambled siRNA, anti-JNK siRNA had no effect on the amount of ROMK1 protein in the kidney. In opposite to the latter anti-p38 siRNA itself led to impaired amount of ROMK1 protein and did not increase the ROMK1 after LPS. At the moment we do not have an explanation for the effect of p38 knockdown by siRNA on ROMK1 expression. Looking at the amount of the respective mRNA the effect of LPS is much more pronounced as compared to the protein level (Figures 3A/B and 4). However, with respect to scrambled siRNA, the effect of anti-JNK siRNA is similar as to what was shown for the protein level. LPS induced reduction of ROMK1 mRNA is impaired by anti-JNK siRNA. However, mRNA is still lower as compared to anti-JNK siRNA alone. In similar to the protein level, anti-p38 siRNA alone led to a reduction of ROMK1 mRNA in the kidney, whereas LPS induced reduction of ROMK1 mRNA is impaired by anti-p38 siRNA.

Effect of anti-JNK or anti-p38 siRNA application on pro inflammatory cytokines in plasma after LPS

It is known that JNK as well as p38 are involved in NFκB mediated secretion of pro inflammatory cytokines in circulating monocytes/macrophages [25-28] induced in sepsis and/or after LPS. Therefore, we investigated whether application of the respective inhibitory siRNAs has any influence on the increase of pro inflammatory (TNFα, IL1β, IFNγ, IL6) cytokines in the plasma of mice treated with LPS (Figure 5). For all pro inflammatory cytokines measured LPS led to a
Figure 3: Effect of lipopolysaccharide (LPS) and/or anti-Jun N-terminal kinase (JNK) or anti-p38-small interfering RNA (siRNA) on the protein level of the renal outer medullary K⁺ channel-1 (ROMK1) in renal tissue after 12 h.

Protein was extracted from kidney tissue. The amount of ROMK1 protein expression in renal cortex 12 h after induced acute kidney injury was detected. The respective inhibitory si-RNAs were administered as described in the methods section (3A).

(3B) Single most typical western blot.

* indicates statistically significant difference between clamp group of each drug intervention and the corresponding sham group.

# indicates statistically significant difference to the LPS group. n is given in the figure.
partly tremendous increase in plasma levels which was seriously inhibited by application of inhibitory siRNA against JNK or p38. With respect to IL1β, IFNγ or IL6 inhibition of JNK even totally abolished a significant increase due to LPS (Figure 5B-D). Neither scrambled siRNA nor antiJNK/p38 siRNA alone had any effect on the respective cytokine levels in plasma. Therefore we conclude that application of inhibitory siRNAs against JNK or p38 is sufficient to seriously impair LPS induced increase of the cytokine. May in part explain why ROMK1 protein amount after LPS is not affected by anti-p38 siRNA. As it is known from former studies [14] that pro inflammatory cytokines impair expression of ROMK1 in kidney tissue this may in principle be a sufficient explanation of the effect observed on expression of ROMK1 in the kidney.

Effect of anti-JNK or anti-p38 siRNA application on MAPK amount in renal tissue after LPS

In order to investigate whether application of inhibitory siRNA also affects the amount of JNK or p38 in the kidney we did western blots against the respective proteins (Figure 6). No effect of LPS was detected on the amount of JNK or p38 in renal tissue. The same was true for scrambled siRNA, in-
hibitory siRNA or its combination with LPS. Actually, anti-JNK siRNA led to a minor increase of p38. Therefore we conclude that in our case the application of inhibitory siRNAs did not affect its targets (JNK, p38) in renal tissue but rather in circulating blood cells (as already mentioned above). We moreover conclude that the effect of LPS on renal expression of ROMK1 is mediated largely via pro-inflammatory cytokines. The fact that TNFα, IL1β, IFNγ (or IL6) decrease the expression of ROMK1, is in accordance with what we have shown before [14].

**Effect of TNFα, IL1β, and IFNγ on expression of ROMK1 in distal tubular M1 cells**

In order to gain detailed insight into the mechanism that led to cytokine induced impairment of ROMK1, we investigated the ROMK1 mRNA in M1 cells after application of TNFα, IL1β or IFNγ alone or in combination (100ng/ml each). As shown in Figure 7A application of the cytokines together led to a decreased amount of ROMK1 mRNA as detected by qPCR. IFNγ alone also impaired ROMK1 after 48h, whereas TNFα and IL1β showed a tendency to reduce ROMK1 but did not reach statistical significance. ROMK1 mRNA only shows minor expression level

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**Figure 5**: Effect of lipopolysaccharide (LPS) and/or anti-Jun N-terminal kinase (JNK)- or anti-p38-small interfering RNA (siRNA) on the proinflammatory cytokine concentration [TNF-alpha (5A), IL-1beta (5B), IFN-gamma (5C), IL-6 (5D)] in murine plasma after 24 h.

* indicates statistically significant difference from control group.

# indicates statistically significant difference from group treated with LPS alone. n is given in the figure.
(CT value in controls around 34), which makes detection by qPCR highly delicate and elaborate. Therefore we decided to investigate the regulation of ROMK1 expression by using a reporter gene assay consisting of the putative promoter region of ROMK1 cloned in a luciferase-plasmid (see methods section). The resulting ROMK1 plasmid (ROMK1-luc) was transfected into M1 cells as described in the methods section and dual luciferase assay (DLRA) was performed. As shown in Figure 7B application of the cytokines together led to a decreased activity of the putative ROMK1 promoter. IFNγ or IL1β alone also impaired ROMK1 after 48h, whereas TNFα did not affect ROMK1 promoter activity. Thus, the impairment of mRNA after TNFα, IL1β or IFNγ alone or in combination as detected by qPCR is highly likely due to impairment of expression as is indicated by the DLRA data shown. Therefore, we decided to investigate the cytokine-induced regulation of ROMK1 in the latter system in more detail.

**Effect of MAPKs on cytokine induced regulation of ROMK1 expression**

In order to answer the question whether JNK or p38 are involved in expression regulation of ROMK1 on the cellular level, we applied the specific inhibitors (JNK inhibitor II or

![Image](image.png)

*Figure 6: Effect of lipopolysaccharide (LPS) and/or anti-Jun N-terminal kinase (JNK)- or anti-p38-small interfering RNA (siRNA) on the protein level of mitogen activated protein kinases (JNK, p38, extracellular signal-regulated kinase 1/2 - ERK1/2) in renal tissue after 12h. Protein was extracted from kidney tissue. The amount of the renal outer medullary K⁺ channel-1 (ROMK1) protein expression in renal cortex 12 h after induced acute kidney injury was detected. The respective inhibitory si-RNAs were administered as described in the methods section. n is given in the figure.*

JNK (6A), p38 (6B), ERK1/2 (6C).
Figure 7: Effect of pro inflammatory cytokines on the expression of the renal outer medullary K⁺ channel-1 (ROMK₁) in cortical collecting duct M1 cells after 48h.

(7A) Effect of pro inflammatory cytokines on the mRNA of mitogen activated protein kinases in cortical collecting duct M1 cells after 48h.
Total RNA was generated from M1 cells. The amount of ROMK₁ mRNA expression in renal cortex 24 h after induced acute kidney injury was normalized to the respective GAPDH signal.
* indicates statistically significant difference from control group. n is given in the figure.

(7B) Effect of pro inflammatory cytokines on the reporter signal of pGL3basic(luc⁺)-ROMK1 transfected into cortical collecting duct M1 cells after 48h.
M1 cells were transfected and dual luciferase assay was performed as described in the methods section.
* indicates statistically significant difference from control group.
# indicates statistically significant difference from group treated with TNFα, IL1β, IFNγ in combination. n is given in the figure.
SB203580) alone or together with the combination of TNFα, IL1β, and IFNγ. In order to investigate the major MAPKs together we additionally applied a specific inhibitor for ERK1/2 (U0126). As indicated in Figure 8, the inhibition of JNK, p38 or ERK1/2 alone did not influence promoter activity of ROMK1 nor did any of these inhibitors affect the cytokine-induced impairment of ROMK1 promoter activity. As LPS itself did not decrease ROMK1 promoter activity (Figure 9A), we conclude that LPS-induced down regulation of renal ROMK1 in mice is due the induction of pro inflammatory cytokines (namely TNFα, IL1β, and IFNγ) by LPS rather than via a direct effect of LPS in renal tissue or on distal tubular cells. Importantly, neither TNFα, IL1β nor IFNγ in combination nor LPS impaired the reporter signal using the DLRA vector without the putative ROMK1 promoter insert (pGL3basic(luc+)) (Figure 9B), which clearly indicates that the effects described in the mentioned experiments are due to the promoter insert.

The effects on ROMK1 induced by inhibition (expression) of JNK (and the minor effects induced by p38) by inhibitory siRNA in vivo additionally are most likely due to impairment of cytokine release and are not due to inhibition of cell signalling pathways in the renal tissue (distal tubule).

Discussion

Potassium channels in renal tubular epithelia greatly contribute to the homeostasis of salts and water [29, 30]. One of their crucial roles...
is the formation of the cell-negative potential, which serves as a driving force for the electrogenic passive transport of solutes, such as apical sodium entries through Na-glucose co-transporter in the proximal tubule cells and the epithelial Na⁺-channel in principal cells of the cortical collecting duct [29, 30]. We recently described a significant down regulation of ROMK that likely contributes to septic ARF. In previous experiments, we could further demonstrate that pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, or IFN-γ are relevant in mediating regulation of ROMK expression during sepsis [14]. This is in line with other reports addressing the effects of cytokines on renal tubular K⁺ channels [31]. Because of overlapping actions of these pro-inflammatory cytokines, down regulation of sodium transporters could not be attributed to single cytokines, providing an explanation for why anti–single cytokine strategies did not improve the outcome of patients with sepsis [14-17]. In this issue we aimed to further investigate the mechanisms whereby LPS as a bacterial cell wall molecule, which provokes severe sepsis in experimental animals and causes a time-dependent pronounced hypotension, tachycardia, and AKI, impairs formation of the cell-negative potential along the renal tubule by down regulation of ROMK expression. The NF-κB pathway, a major pro-inflammatory signaling pathway, is locally activated during sepsis, being in authority for maximal secretion of pro-inflammatory mediators. The transcription factor NF-κB itself will be activated by different mitogen-activated protein (MAP) kinases such
as Jun N-terminal Kinase (JNK) or p38 [20, 21]. Mitogen activated protein kinases (MAPKs) are a family of protein kinases involved in many cellular programs such as cell proliferation, cell differentiation and cell death and an abnormal activation of MAPKs has been observed in pathological circumstances such as sepsis. The most important groups of MAPKs are the p38, c-Jun-N-terminal kinase (JNK1/2) and extracellular signal-regulated kinase (ERK1/2) MAPK [32]. MAPK have been extensively implicated in mediating the pathophysiological consequences of sepsis. A prominent feature of LPS signaling is the induced expression of immune and inflammatory mediators through the activation of mitogen-activated protein kinases [33-36]. Activation of MAPK by LPS in a variety of cell types leads to the activation of transcription factors that regulate the induction of genes encoding cytokines and other proteins involved in the inflammatory response, including tumor necrosis factor-α, IL-1, IL-6, and Cox-2 [25, 33, 35-39]. However, the role of the MAPK signal transduction pathway in sepsis-induced renal tubular dysfunction with down regulation of ROMK expression has not been investigated. Previous studies already discovered the blockade of MAPKs as a promising target to avoid non-renal organ dysfunction such as intestinal motor disturbances during sepsis [40].

In our experiments, LPS induced reduction of ROMK mRNA and protein levels were impaired by anti-JNK and anti-p38 siRNA. As it is known that JNK as well as p38 are involved in NF-kB-mediated secretion of proinflammatory cytokines in circulating monocytes/macrophages induced in sepsis [25-28], we investigated whether application of the respective inhibitory siRNAs has any influence on the increase of pro-inflammatory cytokines in the plasma of mice treated with LPS.

For all pro-inflammatory cytokines measured LPS led to a partly tremendous increase in plasma levels which was seriously inhibited by application of inhibitory siRNA against JNK or p38. This is in line with previous experiments [41]. Therefore, we conclude, that application of inhibitory siRNAs against JNK or p38 is sufficient to seriously impair LPS induced increase in TNFα, IL1β, IFNγ or IL6 in plasma, which is most likely to happen via inhibition of the respective pathways in circulating monocytes/macrophages. Thus, these data represent an indirect readout for the fact that siRNA application leads to inhibition of its target structures in circulating blood cells.

In order to investigate, whether application of inhibitory siRNA also affects the amount of JNK or p38 in renal tissue, we performed western blots against the respective proteins. Surprisingly, no effect of LPS was detected on the amount of JNK or p38 in renal tissue. Other studies investigating MAPK-mediated regulation of Na+/H+ exchanger 3 (NHE-3) and tubular HCO3- absorption, demonstrating direct effects of MAP on renal tubular cells are not in contrast to our results, as on the one hand, these experiments were with exception performed ex vivo or in vitro, so that no conclusions can be drawn from these studies to the complex pathophysiological circumstances in vivo [39, 42, 43]. On the other hand, siRNA targeting JNK and p38 in our experiments were able to effectively block MAPKs in plasma, however siRNA silencing did not sufficiently inhibit MAPK-formation in renal tissue, which is in line with previous siRNA studies [44]. Therefore we conclude that application of inhibitory siRNAs did not affect its targets (JNK, p38) in renal tissue but rather in circulating blood cells. Moreover, our data indicate that the effects of LPS on renal expression of ROMK, being controlled by the MAPK pathway, are subsequently mediated largely via pro-inflammatory cytokines [14].

To verify our hypotheses and to gain detailed insight into the mechanism that led to cytokine induced impairment of ROMK1, we investigated the ROMK1 mRNA in M1 cells after application of pro inflammatory cytokines. In accordance to previous studies [14], application of cytokines led to a decreased amount of ROMK mRNA and – re-
revealing for the first time in the literature – to a decreased activity of the putative cloned ROMK promoter as well.

In order to answer the question whether JNK or p38 are involved in expression regulation of ROMK on the cellular level, experiments with specific MAPK inhibitors were performed using again the reporter gen assay consisting of the putative promoter region of ROMK cloned in a luciferase-plasmid. Inhibition of MAPKs alone did not influence promoter activity of ROMK nor did any of these inhibitors affect the cytokine-induced impairment of ROMK1 promoter activity. As LPS itself also did not decrease ROMK promoter activity though being featured with LPS binding options, we conclude that LPS induced down regulation of renal ROMK1 in mice is due to the induction of pro-inflammatory cytokines by LPS and not via a direct effect of LPS in renal tissue or on distal tubular cells which is in accordance with other studies investigating the absorptive mechanisms of the thick ascending limb of the kidney [31]. The effects on ROMK being provoked by inhibition of JNK or p38 additionally are most likely due to impairment of cytokine release and are not due to inhibition of cell signalling pathways in the renal distal tubule tissue.

Conclusion

Taken together, our data demonstrate a JNK and p38 MAP kinases mediated liberation of pro-inflammatory cytokines, leading in turn to a significant down regulation of ROMK expression as well as on promoter activity in distal tubular M1 cells and in murine renal tissue. Therefore, inhibition of MAP kinases JNK and p38 might be a promising therapeutic option for treatment of sepsis-induced tubular dysfunction.

Speculations

Up to now the treatment of sepsis is based on a fast and adequately calculated antibiotic therapy, balanced fluid and catecholamine therapy and if possible a surgical intervention, but other anti-inflammatory therapies have not been promising until now (e.g. anti-cytokine therapies).

Based on our results further detailed investigations focussing MAPKs in macrophages are necessary, which could be an alternative new therapeutic option in sepsis and septic shock and the avoidance of ARF.

Pizzino and colleagues recently investigated the JNK-pathway inhibition, one major target of our study, in a rodent model of cecal legation and puncture (CLP)-derived sepsis. They were able to demonstrate reduced cytokine levels and an improvement in outcome when SP600125 was administered [45].

Taken together we need to investigate mechanistic more in detail followed by reliable and well-conducted randomized clinical trials using MAPKs-pathway inhibitors like JNK or p38.

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Disclosure of potential conflicts of interest

Conflict of interest

The authors have not disclosed any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.
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**Statement on the welfare and ethical principals of used animals**

All procedures performed in the study involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted and according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Animal Protection Committee approved the animal study as specified in the material and methods section.

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