Time-dependent miRNA profile and renal acute phase response during septic acute kidney injury in mice

PhD thesis

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1. Introduction

Sepsis is one of the leading causes of critical illness and mortality with constantly increasing incidence. Sepsis is a severe and potentially life-threatening condition induced by a dysregulated immune response to an infection, leading to circulatory shock with consequent multi-organ dysfunction and failure. Acute kidney injury (AKI) can develop with a high incidence in septic patients, making sepsis on of the most common aetiologies of AKI.

AKI is defined as an abrupt decrease in renal function. Septic shock is accompanied by the reduction in renal blood flow and/or microvascular dysfunction leading to kidney hypoxia, which is a major mechanism of renal damage leading to AKI. Bacterial toxins and immune stimulation also contribute to AKI development.

There is an unmet need to treat septic AKI and prevent the long-term deterioration of renal function and progression to renal failure. Detailed analysis of the mechanisms can be a fruitful approach to identify possible targets of intervention.

Gram-negative bacterial cell wall endotoxin or lipopolysaccharide (LPS) administration is an established model to study the inflammatory and circulatory effects of sepsis in rodents. LPS injected mice develop AKI.

A well-known consequence of sepsis is the induction of the acute-phase response/reaction (APR) due to tissue damage and infection. Proteins are termed acute-phase proteins (APPs) if their plasma concentration changes at least 25% during inflammation. Their main role is restoring homeostasis after inflammation. APPs are known to be principally produced and secreted into the blood stream by the liver. However, they are also synthesized in other organs and tissues, thereby contributing to local defence and facilitating tissue repair. Some APPs have already been demonstrated to be produced in the kidney too.

Expression of proteins in response to inflammation is modulated by microRNAs (miRNA/miR), which are small non-coding RNAs that regulate posttranscriptional gene expression mainly via translational repression. MicroRNAs extensively regulate many cellular processes like apoptosis, cell-cycle progression and cellular differentiation. MicroRNAs also have great

influence on complex pathologic processes such as AKI. MicroRNAs have a potential both as biomarkers and in the treatment of AKI.

2. Objectives

Our aim was to study the temporal changes in the renal expression of miRNAs and proteins during LPS-induced AKI in mice at four time points in order to identify miRNAs and proteins that have not yet been related to the mechanisms of septic AKI. Furthermore, we aimed to find relationships between the changes in proteome and miRNA expression.

3. Methods

AKI was induced in male outbred NMRI mice by intraperitoneal LPS injections at the doses of 10 or 40 mg/kg bodyweight (BW). Mice were sacrificed at 1.5 and 6 h (early phase, EP) or at 24 and 48 h (late phase, LP) after LPS injection, making up the following groups:

- EP1.5h: LPS at 40 mg/kg BW, and sacrificed at 1.5 h post-injection
- EP6h: LPS at 40 mg/kg BW, and sacrificed at 6 h post-injection
- LP24h: LPS at 10 mg/kg BW, and sacrificed at 24 h post-injection
- LP48h: LPS at 10 mg/kg BW, and sacrificed at 48 h post-injection.

Control mice received equal volumes of saline.

Kidneys were removed, from which one part was processed for RNA isolation, while others were preserved for proteomic analysis. Blood was also collected and plasma samples were obtained. Plasma urea concentration was measured using a urease and glutamate-dehydrogenase enzymatic assay with colorimetric detection.

Total RNA was isolated using TRI Reagent.

The temporal miRNA expression profile of septic AKI was established using miRCURY LNA™ miRNA microarray. 1195 miRNAs were covered by the microarray. To verify the top results on the microarray, expression of miRNAs was measured with qPCR using TaqMan™ Advanced miRNA Assays. Let-7g-5p miRNA was selected as normalizing miRNA using NormFinder software.

Kidney tissue was homogenized and processed to obtain total protein extracts. Total proteome was analysed by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS). Data were analysed using database search and quantification by spectral counting. Raw data and database search files are available via ProteomeXchange with identifier PXD014664. Relative quantification of identified proteins was performed by label-free quantification (LFQ) algorithm in MaxQuant.

MirTarbase and literature were searched for validated targets of the significantly dysregulated miRNAs. MirDB and microRNA.org were used to identify predicted targets of miRNAs. Mass spectrometry results were checked for proteins that were predicted or validated targets of dysregulated miRNAs.

Messenger RNA (mRNA) expression of Tnf-α and IL-6 levels were determined by qPCR to assess inflammation induced by LPS administration in the kidneys of mice. Renal tubular damage was assessed based on Lcn-2 gene expression. The endogenous reference gene was Gapdh. Renal gene expression of MS identified APPs was measured by qPCR too.

ROUT method (significance at the level $p = 0.01$) was performed to identify possible outliers, which were omitted from the analysis. In the miRNA microarray study, significantly changed miRNAs with a fold change above 1.5 or below 0.75 were considered differentially expressed. Only miRNAs with an average Hy3 signal intensity above 6.0 were included in the analysis. mRNA and miRNA fold changes (based on qPCR assays) were calculated by dividing each normalized expression value with the mean of the respective control group. Logarithmic transformation of data was performed in case of significant inhomogeneity of variances indicated by Bartlett's test. LFQ intensity values of proteins were log2 transformed for statistical analysis. Statistical analyses were performed using one-way ANOVA. For multiple comparisons Dunnett's post hoc test was used. GraphPad Prism (versions 6.01 and 8.0.2, GraphPad Software Inc, San Diego, CA, USA) was used for all statistical analyses and creation of graphs.

4. Results

LPS-induced renal inflammation and tubular damage

Endotoxin significantly upregulated the mRNA expression of tumour necrosis factor-α (Tnf-α) and interleukin-6 (IL-6) in the kidney at all four time points, showing that LPS induced severe renal inflammatory response (Fig. 1). Increased plasma urea concentrations and Lcn-2 expression indicated impaired renal function and ongoing, acute tubular injury, respectively (Fig. 2).
The decrease in plasma urea concentrations and mRNA expression of Lcn-2 and IL-6 at 48 h indicated the start of recovery after septic AKI in our experimental setting.

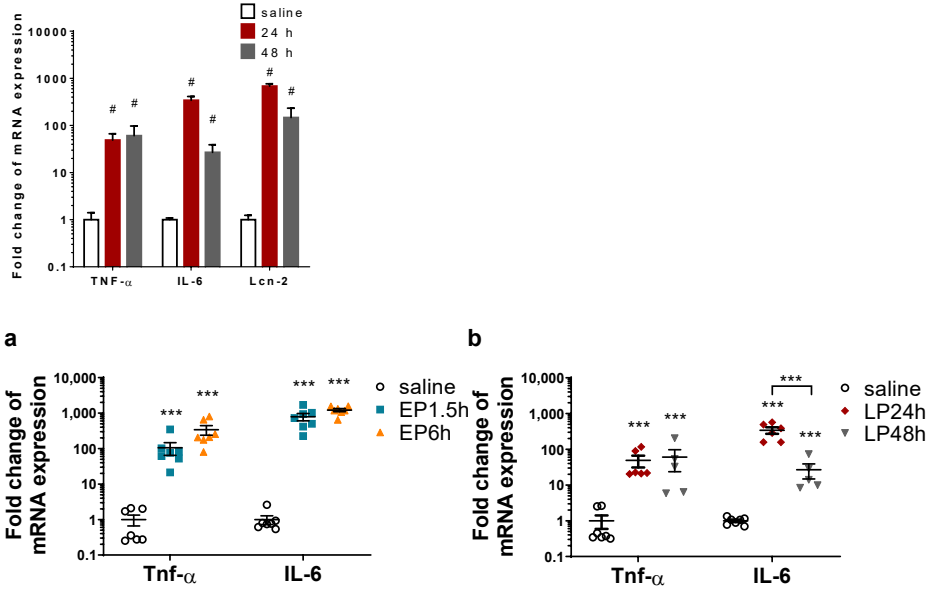

Figure 1. Relative mRNA expression of pro-inflammatory proteins after LPS injection normalized to Gapdh (fold changes vs. saline). (a, b) Tnf-α mRNA; (c, d) IL-6 mRNA; (a, c) EP, 1.5 h and 6 h, (b, d) LP, 24 h and 48 h. Data are expressed as mean ± SEM; One-way ANOVA with Dunnett's post hoc test; ***: $p<0.001$

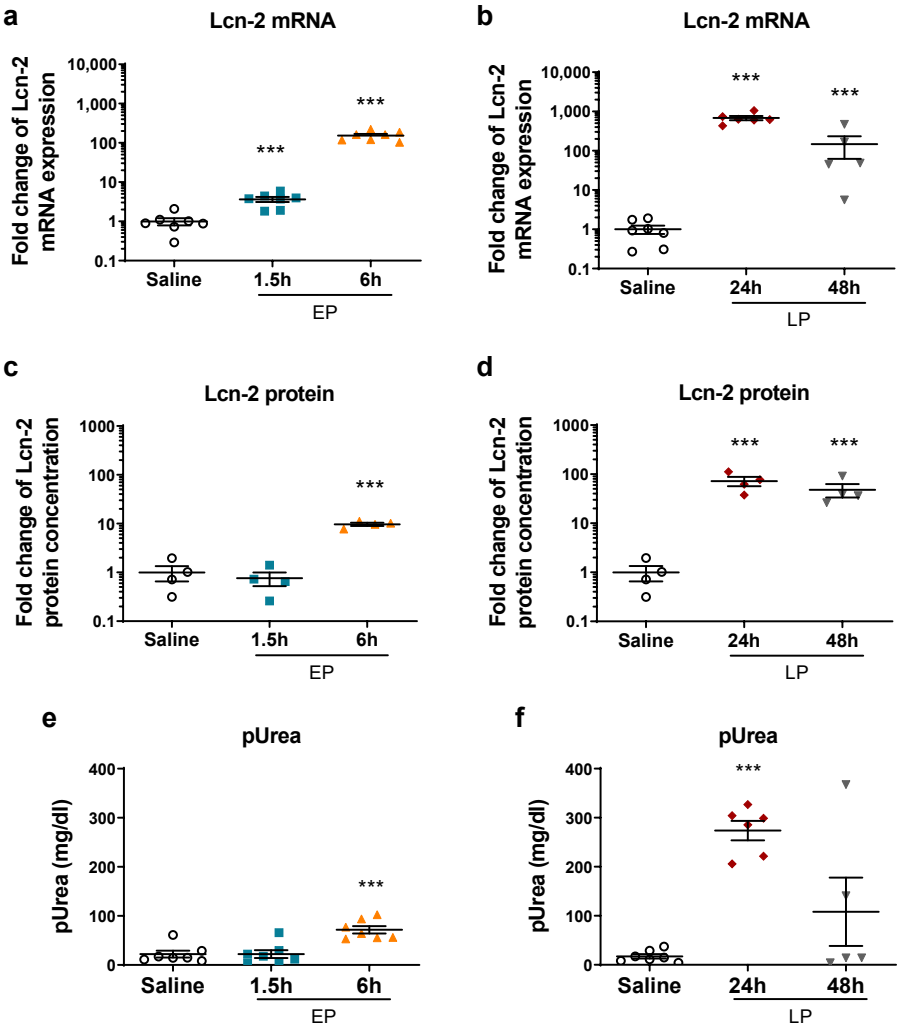

Figure 2. Acute kidney injury markers after LPS administration. (a, b) Lcn-2 mRNA expression normalized to Gapdh (fold changes vs. saline); (c, d) Lcn-2 protein expression measured by HPLC-MS/MS (fold changes vs. saline); (e, f) plasma urea concentration; (a, c, e) EP, 1.5 h and 6 h, (b, d, f) LP, 24 h and 48 h. Data are expressed as mean ± SEM; One-way ANOVA with Dunnett's post hoc test; ***: $p<0.001$

Differentially expressed miRNAs

Based on the microarray results most miRNAs were differentially regulated at the peak of the inflammatory reaction and renal injury in the EP6h (15 miRNAs were upregulated) and LP24h groups (6 miRNAs were elevated and 5 were decreased). MiRNA expression begun to normalize by 48 h.

MiRNAs showing the greatest changes on the microarray (Table 1) were verified by qPCR in all groups. Expression changes of the top upregulated microRNAs were successfully confirmed in most cases. A novel finding of our study is that miR-762 was the most upregulated microRNA at 6 h. Furthermore, the expression of the closely clustered microRNAs, miR-144-3p and miR-451a was elevated at 24 h. Our results newly indicated a potential role for these 3 microRNAs in LPS-induced AKI. In addition, our measurements confirmed previous data that the miR-21a-duplex and miR-146a-5p are induced by LPS in the kidney. Renal expression of miR-21a-3p was enhanced at 24 and 48 h. Expression of miR-21a-5p was induced at all time points. Expression of miR-146a-5p was significantly upregulated at 6 and 48 h. (Fig. 3)

Table 1. Fold changes of the mostly upregulated miRNAs after LPS administration relative to the control kidneys as measured by the microarray technique. Data in EP (1.5 and 6 h) and LP (24 and 48 h) were subject to separate statistical analyses using one-way ANOVA with Dunnett's post hoc test (separated by double line in the middle of the table), *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ns: not significant

miRNA	1.5 h	6 h	24 h	48 h
miR-762	1.19 ± 0.27 (ns)	2.69 ± 0.34 ***	1.16 ± 0.32 (ns)	1.24 ± 0.22 (ns)
miR-144-3p	1.37 ± 0.87 (ns)	1.10 ± 0.24 (ns)	2.56 ± 1.04 **	1.65 ± 1.43 (ns)
miR-451a	1.63 ± 0.98 (ns)	1.08 ± 0.33 (ns)	3.76 ± 1.56 **	2.22 ± 2.41 (ns)
miR-21a-5p	1.07 ± 0.2 (ns)	1.39 ± 0.18 *	4.44 ± 0.66 ***	4.59 ± 1.34 **
miR-21a-3p	1.36 ± 0.12 (ns)	2.00 ± 0.20 ***	1.88 ± 0.18 ***	1.34 ± 0.17 (ns)
miR-146a-5p	1.10 ± 0.08 (ns)	0.98 ± 0.08 (ns)	1.51 ± 0.14 **	1.58 ± 0.21 **

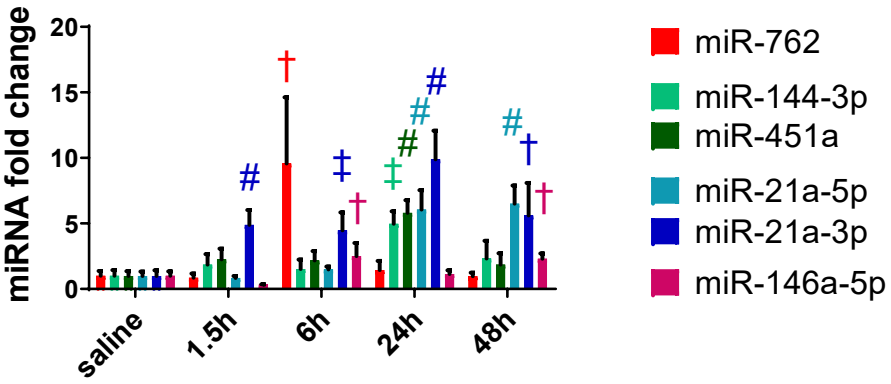

Figure 3. qPCR validation of top upregulated miRNAs on the microarray. The relative expression of miRNAs was normalized to let-7g-5p (fold changes vs. saline). Data are expressed as mean ± SEM; One-way ANOVA with Dunnett's post hoc test; †: $p<0.05$, ‡: $p\leq0.01$, #: $p\leq0.001$

Validated/predicted microRNA targets identified by mass spectrometry

Among the predicted targets of miR-762, the miRNA newly associated with septic AKI there was one, the secretion associated Ras related GTPase 1B (Sar1b) or GTP-binding protein Sar1b that seemed to be a relevant association with miR-762, as its concentration was significantly suppressed 6 h after LPS administration, at the time of the peak upregulation of miR-762 (Fig. 4).

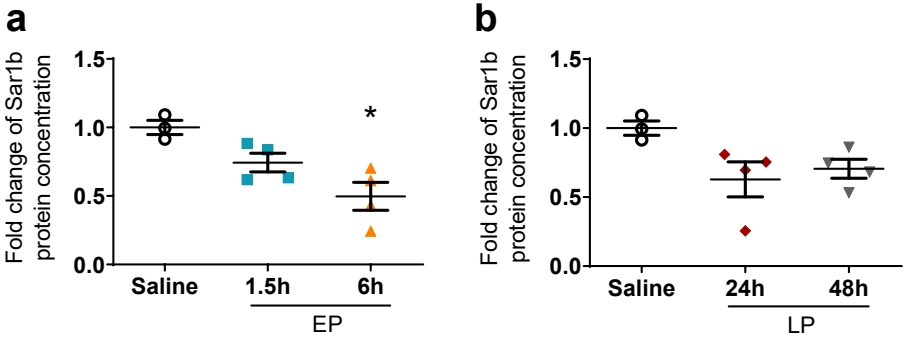

Figure 4. Fold change of Sar1b protein expression in the kidneys of mice after LPS administration. Protein expression measured by HPLC-MS/MS (fold changes vs. saline), (a) EP, 1.5 h and 6 h, (b) LP, 24 h and 48 h. Data are expressed as mean ± SEM; One-way ANOVA with Dunnett's post hoc test; *: $p<0.05$

We identified a seemingly strong association between miR-144-3p and one of its validated targets, aquaporin-1 (Aqp1). The HPLC-MS/MS analysis detected that Aqp1 protein concentration significantly increased in EP and decreased at 24 h (Fig. 5). Aqp1 changed inversely to miR-144-3p at 24 h after treatment with LPS as miR-144-3p was upregulated (Fig. 3) while Aqp1 was downregulated.

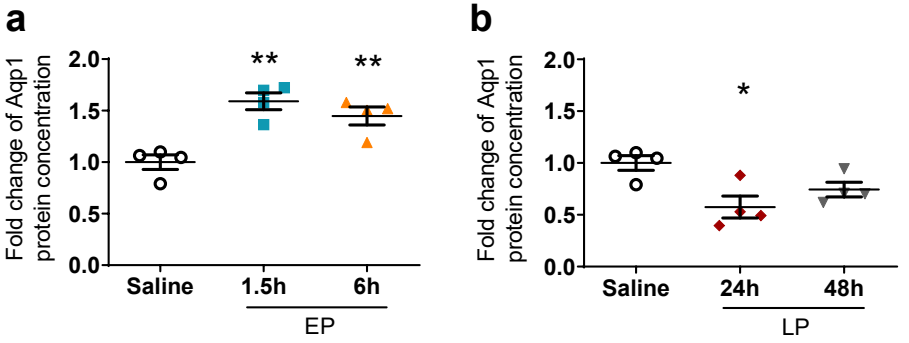

Figure 5. Fold change of Aqp1 protein expression in the kidneys of mice after LPS administration. Protein expression measured by HPLC-MS/MS (fold changes vs. saline), (a) EP, 1.5 h and 6 h, (b) LP, 24 h and 48 h. Data are

expressed as mean ± SEM; One-way ANOVA with Dunnett's post hoc test; *: $p<0.05$, **: $p<0.01$.

Acute-phase proteins were the most upregulated proteins in the kidney in the late phase after LPS administration

Proteome changes were the most significant and abundant at 24 h (Fig. 6). At 24 h forty-seven, while at 48 h forty-four proteins were upregulated at least 4-fold (log2FC=2).

APPs were abundantly present among the most upregulated proteins in LP (Fig. 6). In EP the established AKI marker Lcn-2 was the only APP upregulated more than 4-fold. However, at 24 h 47%, while at 48 h 39% of the proteins upregulated at least 4x were APPs.

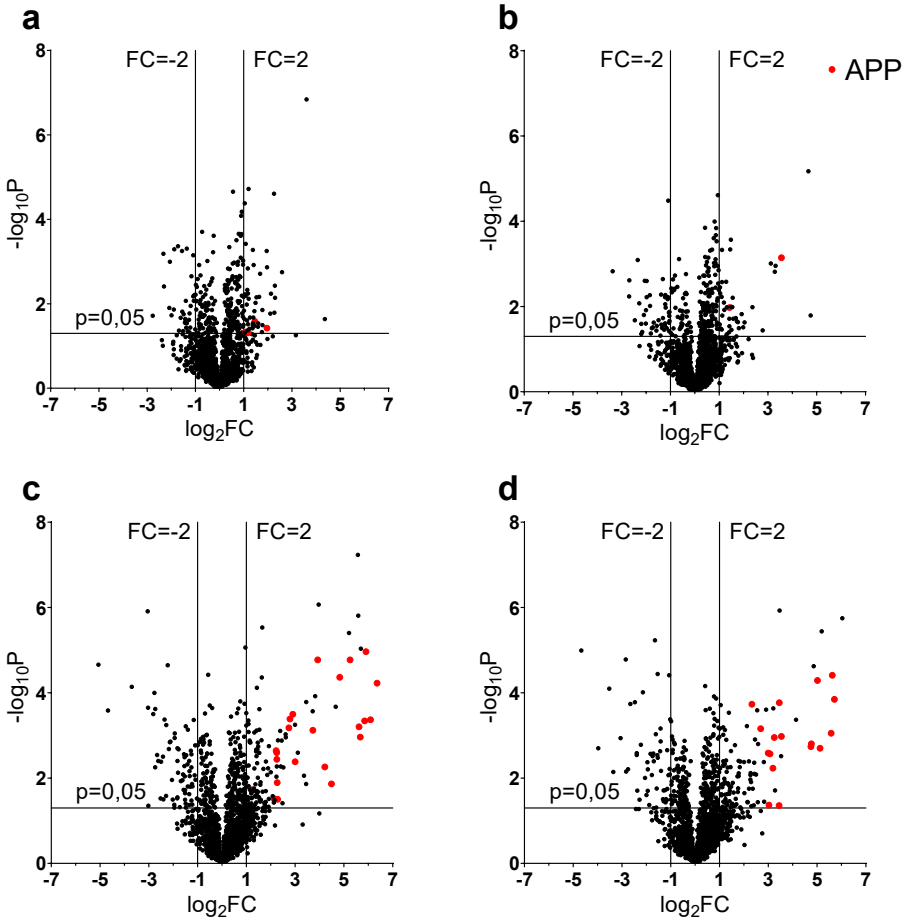

Figure 6. Renal proteome changes after LPS administration. The level of significance (given as –log10p values) is plotted against the fold changes (given as log2FC). Vertical lines mark 2-fold changes, while horizontal lines mark the significance level of P=0.05. Red dots indicate APPs. (a) 1.5 h, (b) 6 h, (c) 24 h, (d) 48 h.

The following APPs were enriched in the kidneys after LPS administration in LP (at either of the two or at both time points): complement C3 (C3), fibrinogen-α, -β, -γ (Fga, Fgb, Fgc), haptoglobin (Hp), hemopexin (Hpx), inter alpha-

trypsin inhibitor heavy chain 4 (Itih4), inter-alpha-trypsin inhibitor heavy chain H1 (Itih1), two isoforms of serum amyloid A (Saa1 and Saa2), ceruloplasmin (Cp), transferrin (Tf), ferritin heavy chain (FHC), alpha-1-antitrypsin (Serpina1), serine protease inhibitor A3K (Serpina3k), serine protease inhibitor A3N (Serpina3n), alpha-2-macroglobulin (A2m), beta-2-microglobulin (B2m), apolipoproteins A1 (ApoA1) and E (ApoE), vitamin D-binding protein or Gc-globulin (DBP), alpha-1-acid glycoprotein (A1AGP), von Willebrand factor A domain-containing protein 5A (Vwa5a) and serum albumin (Alb).

Acute-phase protein synthesis took place in the kidney after LPS administration

We performed qPCR analysis to verify that the source of upregulated APPs was the kidney. We found that LPS significantly upregulated the renal mRNA expression of several APPs (Fig. 7). Gene expression of ceruloplasmin and haptoglobin was induced already at 1.5 h. Expression of complement C3, fibrinogen-α, -β, -γ, serum amyloid A, ceruloplasmin, haptoglobin, hemopexin, inter alpha-trypsin inhibitor heavy chain 4, and ferritin heavy chain was upregulated by LPS at 6, 24 and 48 h. Transferrin expression was upregulated in the kidneys only in LP.

mRNA expression of fibrinogen-α, -β, -γ, serum amyloid A, ceruloplasmin, hemopexin and ferritin heavy chain decreased form 24 h to 48 h. Expression of complement C3 and transferrin also showed a decreasing tendency at 48 h, though the difference was not statistically significant.

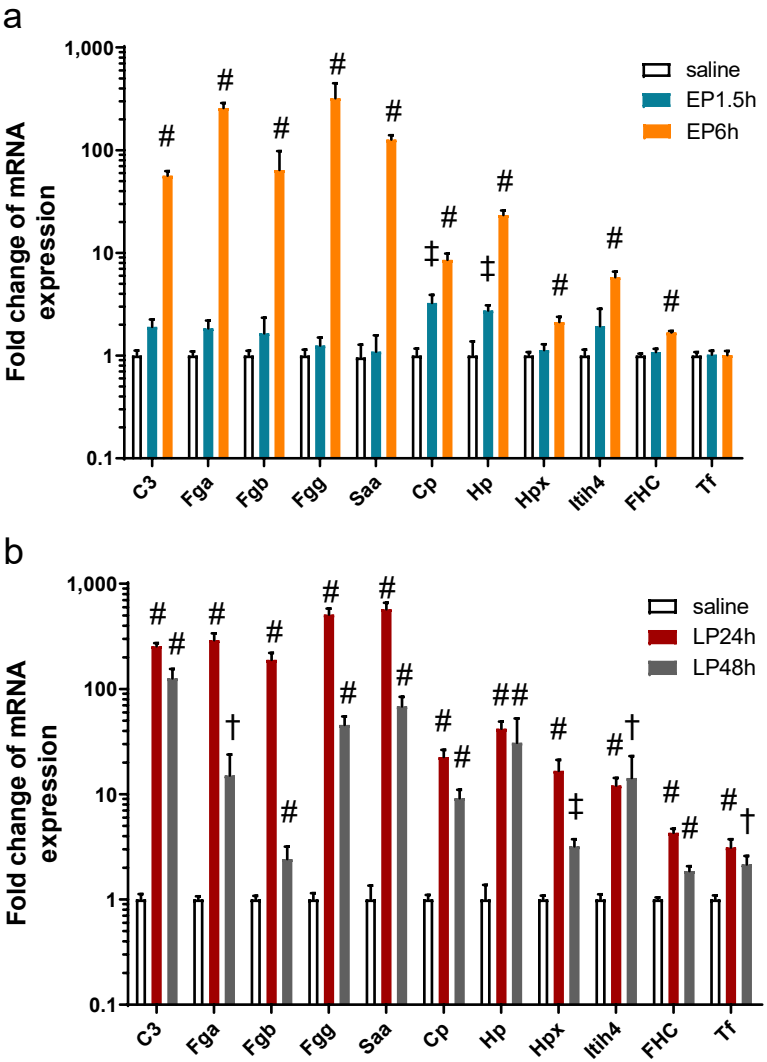

Figure 7. Fold changes of mRNA expression of APPs relative to the respective control kidneys in mice after LPS administration. †: $p<0.05$, ‡: $p\leq0.01$, #: $p\leq0.001$. C3: complement C3, Fga: fibrinogen-α, Fgb: fibrinogen-β, Fgg: fibrinogen-γ, Saa: serum amyloid A, Cp: ceruloplasmin, Hp: haptoglobin, Hpx: hemopexin, Itih4: inter alpha-trypsin inhibitor heavy chain 4, FHC: ferritin heavy chain, Tf: transferrin. (a) 1.5 h, 6 h. (b) 24 h, 48 h.

5. Conclusions

The major findings of this study are the following:

- Bacterial lipopolysaccharide (LPS) administration caused serious septic AKI characterized by severe functional impairment of the kidney, upregulation of several inflammatory markers and marked changes in the expression of regulatory microRNAs (miRNAs) and large number of proteins. Both the renal miRNA and protein expression changes were modest at 1.5 h and peaked at 6 and 24 h after LPS-administration. Recovery could be detected by 48 h.
- MiRNA microarray indicated that expression of 71 miRNAs changed significantly at 1.5 and 6 h and 39 at 24 and 48 h. Expression of miR-762 was most upregulated at 6 h after LPS administration, which is a newly identified miRNA that was upregulated in septic AKI. MiR-762 and other upregulated miRNAs may have a role in renal inflammation by attenuating the LPS-induced immune response.
- Aqp1 is assumed to be regulated by miR-144-3p. Our data support a pathogenic role for Aqp1 and miR-144-3p in LPS-induced AKI.
- Acute-phase proteins (APPs) were among the significantly upregulated proteins. APPs were synthesized locally in the kidney as demonstrated by renal mRNA expression of most detected APPs, suggesting that a local acute phase reaction is also a characteristic response of the kidney to massive systemic inflammation.

6. Bibliography of the candidate's publications

Publications related to the thesis

1. Tod P, Róka B, Kaucsár T, Szatmári K, Vizovišek M, Vidmar R, Fonovič M, Szénási G, Hamar P. Time-Dependent miRNA Profile during Septic Acute Kidney Injury in Mice. Int J Mol Sci [Internet]. 2020;21:5316. doi: 10.3390/ijms21155316. (IF: 5.924, rank: Q1)
2. Róka B, Tod P, Kaucsár T, Vizovišek M, Vidmar R, Turk B, Fonović M, Szénási G, Hamar P. The Acute Phase Response Is a Prominent Renal Proteome Change in Sepsis in Mice. Int J Mol Sci [Internet]. 2020;21:200. doi: 10.3390/ijms21010200. Cited: in: : PMID: 31892161. (IF: 5.924, rank: Q1)

Publications not related to the thesis

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