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Potential New Markers of Inflammation-Induced Renal Injury Subside when Endotoxin Tolerance Develops in Humans

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Abstract

Purpose: Cytokines play an important role in the development of renal injury during sepsis. Because of its high mortality rate, early detection of inflammation-induced renal injury is of critical importance.

Methods: We used Surface enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI--TOF MS), to search for new biomarkers for early renal injury during acute systemic inflammation, and after development of endotoxin tolerance in humans *in vivo*.

Results: Repeated LPS administrations induced a 33 ± 7% decrease in glomerular filtration rate (p=0.02) on day 2, and an increase in serum creatinine of 11 ± 3% (p=0.002) on day 3, which was associated with the appearance of 15 peak intensities in the urinary protein profile, including an increase in ß2-microglobuline levels (p=0.04), 6 hours after the first LPS administration. Four of the 15 peak intensities on day 1 correlated with serum creatinine levels on day 3; 3950, 4445, 6723 and 7735 m/z (r=0.91, 0.97, 0.94, 0.87; p=0.03; 0.01; 0.02 and 0.05, respectively). With the development of LPS tolerance, renal function restored, reflected by a decrease in serum creatinine and ß2-microglobuline to baseline levels (p=0.2 and 0.4, respectively, between day 1 and 5), and by attenuated peak intensities in the urinary protein profile (p<0.0001 for all 15 peak intensities).

Conclusion: Renal injury occurs during repeated endotoxemia and can be predicted by new urinary markers using proteome research. The four markers that correlated with the extent of renal injury may represent potential new biomarkers for renal injury and need further identification. The inflammation-induced renal injury subsided, when LPS tolerance developed after 5 consecutive days of LPS.

Keywords: Human endotoxemia; Endotoxin tolerance; Biomarkers; ß2 microglobulin; Renal injury

Abbreviations: ICU: Intensive Care Medicine; IL: Interleukin; LPS: Lipopolysaccharide; SELDI-TOF MS: Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; TNFα: Tumor Necrosis Factor-alpha

Introduction

Renal injury is a common clinical problem in the critically ill patient, and is associated with poor outcome [1,2]. Two large multicentre cohort studies reported previously the occurrence of renal injury in an estimated 30-40% of all patients admitted to the ICU [3,4]. Furthermore, critically ill patients who develop renal injury and require renal replacement therapy, have mortality rates of 50-80% [2].

Sepsis has been identified as the most common cause of renal injury in intensive care units, although the pathophysiology is not well understood. However, in recent years, the emphasis has shifted from altered hemodynamics to a more aggravating role of pro-inflammatory cytokines, such as TNFα, IL-6 and IL1β [5-12]. Nevertheless, clinical studies using anti-cytokine therapies failed to show a survival benefit in patients with sepsis [5,13-15]. Therefore, despite many advances in therapeutic and research techniques in the past 20 years, fundamental changes in the outcome of patients with renal injury have not occurred. This limited progression may be related to many factors, including the lack of early diagnostic tests, that indicate the onset of renal injury. Currently, the diagnosis of renal injury is based on either the elevation of serum creatinine, or the occurrence of oliguria, whilst this is fraught with imprecision [16].

Since the introduction of functional genomics and proteomics, several new biomarkers have emerged [17], with high sensitivity and specificity for detecting renal injury at an early stage. Of the various methods and platforms available, the Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS) technology has proven to be a suitable platform for rapid urinary protein profiling [18,19]. This approach allows for rapid high throughput profiling of multiple urine samples, and detects low molecular weight biomarkers that are typically missed by other platforms, and even uncovers proteins bound to albumin. We previously established a SELDI-TOF MS method, useful for the discovery of new urinary biomarkers, after early stage kidney injury. This resulted in representative protein patterns, whose joint action provides improved discrimination of mild kidney injury, after a short ischemic period [20].

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Endotoxin (lipopolysaccharide, LPS) is a glycolipid that constitutes the major portion of the outermost membrane of Gram negative bacteria, and when administered to healthy volunteers, evokes a diverse spectrum of biological activities similar to that seen during early sepsis, including renal injury [21,22]. When healthy volunteers are challenged with repeated LPS administrations, a phenomenon called 'endotoxin tolerance' develops [21]. Interestingly, endotoxin tolerance provokes "cross-tolerance" against other forms of injury in animals, for example renal ischemia-reperfusion injury [23,24]. However, whether LPS tolerance provides protection against inflammation-induced renal injury is a matter of debate. It was shown that instead of attenuated renal TNFα levels upon an LPS re-challenge during repeated LPS administrations, renal TNFα concentrations were about twice as high in the LPS preconditioned group compared to the LPS challenged naïve controls, but still with less kidney injury [24,25]. Up to now, human studies are not available.

The aim of the current study was to search for potential new early markers of renal injury during acute endotoxemia, and to investigate whether renal injury can be ameliorated by the induction of LPS tolerance.

Materials and Methods

Subjects

The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki, including current revisions, and the European Good Clinical Practice guidelines. Written informed consent was obtained from all study participants. Five male volunteers participated in the experiments (NCT 00246714). All volunteers had normal physical examinations, ECG and routine laboratory studies, before start of the experiment. Volunteers were not taking any prescription medications, and they were negative for Hepatitis B surface antigen and Human Immunodeficiency Virus (HIV) infection.

Study design

Five subjects received i.v. bolus injections of 2 ng/kg/day *Escherichia coli* LPS for 5 consecutive days, with a 24 hour observation period on day 1, followed by a 6 hour period of observation on day 2-5. The subjects fasted overnight, before the start of the experiment. Before each LPS administration, the subjects were prehydrated with 1500 ml glucose/saline infusion [26], and after LPS administration, a continuous intravenous drip was started at 150 ml/hr during six hours.

Endotoxin

U.S. Reference *E. coli* endotoxin (lot Ec-5, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD) was used in this study. *Ec-5* endotoxin, supplied as a lyophilized powder, was reconstituted in 5 ml saline; 0.9% for injection, and vortex mixed for at least 5 minutes after reconstitution. The endotoxin solution was administered as a single intravenous bolus injection during 1 minute, at a dose of 2 ng/kg of body weight. This dose of Ec-5 endotoxin was pyrogenic, and elicited subjective side effects in healthy non-tolerant male volunteers in our previous studies [21,27,28].

Hemodynamic and inflammatory parameters

During the 5-day experiment, hemodynamic (heart rate and blood pressure) and inflammatory (temperature, C-reactive protein and cytokine levels) parameters were closely monitored. Heart rate (ECG) and mean arterial pressure (arterial catheter) were continuously monitored on day 1 and day 5, and non-invasively on day 3. Temperature measurements were obtained, every 30 minutes after LPS administration from day 1, until day 5, using a tympanic thermometer (°C). C-reactive protein levels were measured before each LPS administration, during the 5-day experiment, by use of routine laboratory techniques. Circulating levels of TNFα, IL-6 and IL-10 were determined before (t=0) and after (t=30, 60, 90 min, 2, 4, and 6 hrs) administration of LPS on day 1 and 5, by use of the Luminex Assay (Bioplex-kits: BioRad Laboratories, Hercules, California USA), in accordance with manufacturers' instructions. The lower limits of detection was for TNFα 36 pg/ml; IL-6 8 pg/ml, and for IL-10 8 pg/ml.

Renal function

Serum creatinine levels were measured before each LPS administration during the 5-day experiment, by use of routine laboratory techniques. The glomerular filtration rate was calculated by use of creatinine levels in urine samples (mmol/L), with the amount of urine (mL), and the serum creatinine levels (μmol/L), before and during 3-hour periods after LPS administration on day 1, until day 5.

Urine sample collection and preparation

Freshly collected urine samples (before and until 6 hrs after LPS administrations) were briefly centrifuged (10 min, 2000 g) and stored with protease inhibitors [29], in small aliquots at -80°C, to minimize freeze-thaw cycles. A master pool reference sample of healthy volunteers was prepared by mixing together 50 urine samples containing 0.2 mmol creatinine each [20].

SELDI-TOF MS analysis

We previously established a solid practical procedure for the preparation of urinary protein samples for downstream proteomic analysis [20]. We have compared urine samples from a defined group of intensive care unit patients with mild tubular injury after ischemia, during coronary bypass graft surgery, with those of healthy volunteers, and observed significant differences associated with early renal injury [20]. In the present study, a similar approach was used, in which urine samples were thawed, vortexed and diluted with ultraPURE™ DNAse/RNAse free distilled water (Invitrogen, Breda, the Netherlands), to obtain constant creatinine concentrations (2.0 mmol/L). Subsequently, 250 μl urine was 10 times concentrated and desalted with centrifugal ultra-filtration (45-60 min, 13.000 g 3 kDa Microcon filters, Millipore, Billerica, USA).

The preparation procedure was based on protocols from Ciphergen [19,30], and described elsewhere in detail [20]. Briefly, we used an 8-spot weak cation-exchange chip (ProteinChip CM10; Ciphergen Biosystems, Fremont, CA). All spots were pretreated two times with 200 μl binding buffer (0.1 M ammonium acetate, pH 4.0), for 5 minutes on a shaking platform (500 rpm), in a bioprocessor. After pretreatment, 5 μl concentrated urine samples (100 nmol creatinine) were applied on the chip, and incubated in the humid chamber for 30 min. Samples were removed with a pipette, and spots were washed three times for 5 min with binding buffer (6 μl). Spots were washed with distilled water and air dried for 10 min. Subsequently, 0.8 μl of a saturated solution of sinapinic acid in 0.5% (vol/vol) trifluoroacetic acid and 50% (vol/vol) acetonitrile, used as an energy-absorbing matrix was applied to each spot surface, allowed to air-dry, and reapplied. The chip was analyzed in a PBS IIc SELDI mass spectrometer (Ciphergen Biosystems, Fremont, CA). Data were collected with standard operational settings,

including starting laser intensity at 200, warming positions with 2 shots set at laser intensity 205, no warming shots included, high mass at 200 kDa, optimized from 1 kDa to 21 kDa in the low range, and optimized from 20 kDa to 71 kDa in the middle range, detector sensitivity at 9. External mass calibration was performed with all in 1 protein standard II proteins (Ciphergen Biosystems). In the range of 3-20 kDa: hirudin BHVK (6964 kDa), bovine cytochrome C (12230 Da), equine myoglobin (16951 Da). In the range of 20-70 kDa: bovine carbonic anhydrase (29023 Da), enolase for *Saccharomyces cerevisiae* (46671 Da), and bovine albumin (66433 Da). The timeline of every patient sample, together with the masterpool, is measured on the same chip in the same run. Inter-chip samples series were normalized by the masterpool. Masterpool samples were similar to those used previously [20]. Due to the standardized sample treatment and storage with a cocktail of protease inhibitors (in detail explained [20]), samples were stable for longer time. Obtained data were analyzed automatically using a minimal threshold peak of 30% of all spectra, a signal/noise first pass of 20, and a signal/noise second pass of 10.

β2-microglobulin assay

β2-microglobulin levels were measured with a non-competitive two-sided ELISA. The polyclonal rabbit anti-human β2-microglobulin was from DakoCytomation, Clostrup, Denmark. No protease inhibitors were added to this urine samples. Normal β2-microglobulin value in urine is <0.3 mg/L.

Statistical analysis

Only clear peaks were selected, and intensities of these manual detected peaks (Ciphergen ProteinChip Software 3.2.0) were exported to excel and corrected for masterpool intensities. A heatmap of these intensities was generated using the CIMminer algorithm (http://discover.nci.nih.gov/cimminer). Color-coded clustered image maps, "heat maps", to visualize differences in protein expression profiles, were generated using the CIMminer algorithm (http://discover.nci.nih.gov/cimminer). Statistical analysis with regard to the correlation between peak intensities and serum creatinine levels was performed by use of Graphpath Prism® (version 4.03 for Windows; Graphpath Software, San Diego, CA, USA).

LPS-induced changes on different hemodynamic and inflammatory parameters between time points were tested for significance, using ANOVA with repeated measures. Maximum levels of cytokines and β2-microglobulin levels were tested for significance by use of the Wilcoxon's signed rank test for non-parametric data, and expressed as median levels with interquartile ranges. All other data is expressed as mean ± SEM. A $p<0.05$ was considered statistically significant. In view of the explorative nature of this study, statistical analyses were not adjusted for multiple testing.

Results

Baseline characteristics of the volunteers

The age, weight, height, heart rate and blood pressure of the volunteers were 21 ± 1 years, 72 ± 2 kg, 186 ± 0.2 cm, 71 ± 4 bpm, and 123 ± 6/73 ± 4 mmHg, respectively.

Hemodynamic and inflammatory parameters

Endotoxemia resulted in the expected and transient flu-like symptoms, with a general increase in heart rate, temperature, C-reactive protein, pro-inflammatory cytokines (TNFα and IL-6), and anti-inflammatory cytokine IL-10, with concomitant decrease in mean arterial pressure. After 5 consecutive days of LPS administrations to induce LPS tolerance, all parameters measured were significantly attenuated, indicating the development of LPS tolerance (difference between day 1 and 5; $p<0.001$ for all parameters, except mean arterial pressure $p=0.015$, Table 1).

Renal function

Serum creatinine levels increased from 73 ± 5 μmol/L, before the first LPS administration to a peak concentration of 82 ± 6 μmol/L, after 3 consecutive days of LPS administrations ($p=0.01$), which returned to baseline levels of 70 ± 6 μmol/L on day 5 ,when LPS tolerance developed ($p=0.2$ between baseline levels on day 1 compared to day 5, Figure 1). Glomerular Filtration Rate (GFR) measured every day decreased with 33 ± 7%, after 2 consecutive days of LPS administrations ($p=0.02$ between day 1 and 2), and returned to baseline levels on day 5, when LPS tolerance developed ($p=0.2$ between day 1 and 5).

Urine profiles by SELDI-TOF MS in individual urine samples

Protein profiles of individual subjects showed peaks abundantly appearing in urine collected 6 hours after the first LPS administration, as compared to baseline, and after LPS administration on day 5 (Figure 2). The abundance of urinary protein peaks is shown in figure 3. In

Table 1: Hemodynamic and inflammatory parameters during endotoxemia and endotoxin tolerance.

Hemodynamic and inflammatory parameters of 5 healthy volunteers during 5 consecutive days of 2 ng/kg LPS administrations to induce a LPS tolerant state. During the first and last day an arterial cannula registered mean arterial pressure (MAP) and heart rate continuously. On day 3 this was monitored non-invasively every half hour. Temperature levels were obtained every half hour by use of a tympanic thermometer. C-reactive protein levels were measured once a day before LPS administration and circulating cytokines were measured every hour on day 1 and 5, and before LPS administration on day 3. The response to LPS administration with regard to above mentioned parameters were tested for significance by use of ANOVA for repeated measures. Data are expressed as mean ± SEM whereas the data concerning cytokine levels are expressed in median with interquartile range. "np" indicates 'not performed' and * indicates $p<0.05$.

		Day 1	Day 3	Day 5
Heart rate (/min)				
	baseline	61 ± 2	66 ± 3	57 ± 2
	peak level	97 ± 3*	86 ± 9	78 ± 2
MAP (mmHg)				
	baseline	98 ± 3	87 ± 2	97 ± 3
	peak level	83 ± 4*	86 ± 4	86 ± 2
Temperature (°C)				
	baseline	36.2 ± 0.2	35.9 ± 0.2	36.1 ± 0.1
	peak level	37.6 ± 0.1*	36.7 ± 0.2	36.6 ± 0.1
C-reactive protein (mg/mL)				
	baseline	<5 ± 0	30 ± 3	24 ± 2
	after 24 hours	26 ± 2*	27 ± 2	
TNFα (pg/mL)				
	baseline	8	8	8
	peak level	484[366-1124]*	np	13[8-14]
IL-6 (pg/mL)				
	baseline	8	8	8
	peak level	2153[1674-2236]*	np	118[57-222]
IL-10 (pg/mL)				
	baseline	8	8	8
	peak level	186[155-265]*	np	26[18-34]

the range of 3-20 kDa, 15 out of 59 automatically detected *m/z* values showed a significant increase in peak intensities from baseline after the first LPS administration, which returned to baseline levels after 5 consecutive days of LPS administrations ($p<0.001$ for all 15 measured peak intensities, Table 2). Four peak intensities measured on day 1 (Figure 4A), correlated significantly with the maximal raise in serum creatinine levels on day 3; 3950 *m/z* (r=0.91, p=0.03), 4445 *m/z* (r=0.97, p=0.01), 6723 *m/z* (r=0.94, p=0.02), and 7735 *m/z* (r=0.88, p=0.05), depicted in figure 4. To eliminate interindividual variabilities, we normalized the values and intensities for each individual measured at baseline.

Urinary β2-microglobulin levels

LPS administration induced an increase in urinary β2-microglobuline levels of 103% (64-155) 6 hours after LPS administration on day 1 (p=0.04), which returned to baseline levels 6 hrs after LPS

Urine production (mL)	2128± 385	930± 84	1368± 213	1120± 187	783± 112

Figure 1: Serum creatinine levels (µmol/mL) and amount of urine produced by 5 healthy volunteers during 5 consecutive days of LPS administrations to induce a LPS tolerant state. Creatinine levels were measured before each LPS administration and urine samples were collected 24hrs on the first day and 6 hrs after the following LPS administrations. The response of LPS administration with regard to maximum serum creatinine levels were tested for significance by use of a one-way ANOVA. Data is expressed as mean±SEM. * indicates $p<0.05$ compared to day 1.

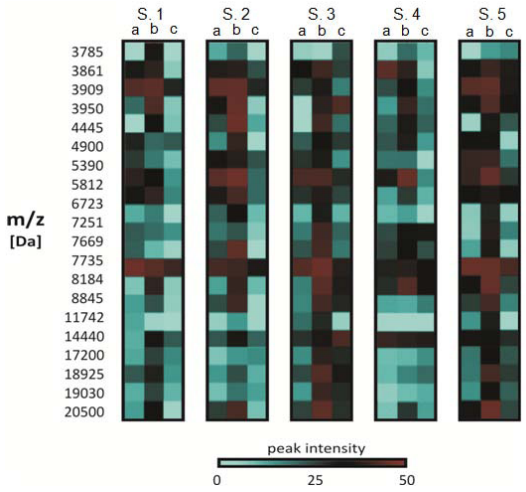

Figure 2: Color-coded clustered image maps or "heat maps" visualize differences in protein expression. Twenty peak intensities of the manual detected peaks within the 3-22 kDa range are shown generated in a heatmap. Red color means high intensities of the peaks and blue corresponds with low intensities. 'S' indicates subject and the number indicates the 5 different healthy volunteers. 'a'=baseline day 1, 'b'= 6 hours after the first LPS administration (day 1) and 'c'= 6 hours after the last LPS administration (day 5).

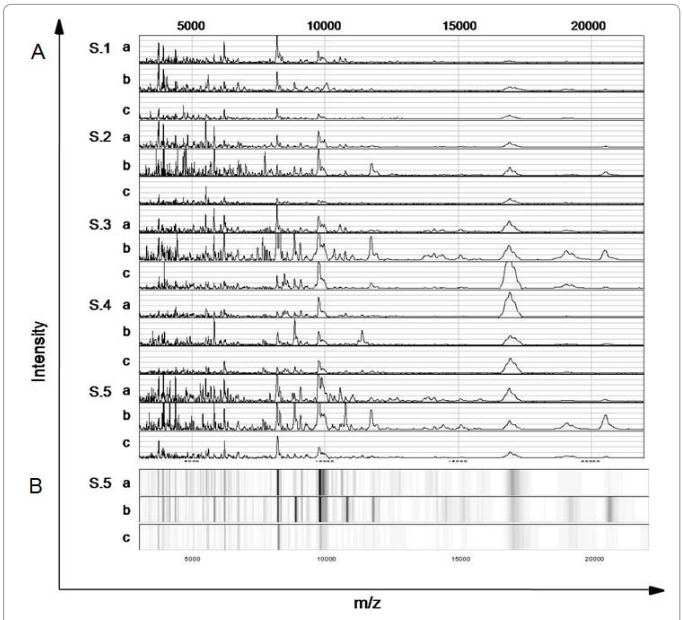

Figure 3: A) Spectra of surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) analysis of 5 volunteers of which peak intensities within the 3-22 kDa range are shown. 'S' indicates subject and the number indicates the 5 different healthy volunteers. 'a'=baseline day 1 (negative control), 'b'= 6 hours after the first LPS administration (day 1) and 'c'= 6 hours after the last LPS administration (day 5).The Seldi-TOF MS spectra show more peak intensities 6 hours after the first LPS administration (b) compared to baseline (a) and an attenuation of most peak intensities 6 hours after the 5th LPS administration when LPS tolerance developed (c). **3B)** Representative gel view of one volunteer (S.5) as in Figure 3A, also showing downregulation of most of the peak intensities after 5 consecutive days of LPS administrations when LPS tolerance developed.

Table 2: Detected protein masses after SELDI-TOF MS profiling.
Area under the curve generated data on all 15 intensities (m/z) which were detected in the urine proteome profile of 5 healthy volunteers before (t=0, day 1), 6 hours after the first and fifth LPS administration (t=0-6 day 1 and 5. respectively). # indicates a significant ($p<0.05$) increase in peak intensity after the first LPS administration. Data was tested for significance by use of the one-way ANOVA and is expressed as mean ± SEM.

Intensities (m/z;Da)	a t=0, day 1 (negative control)	b t=0-6 hrs (after LPS injection), day 1	c t=0-6 hrs (after LPS injection), day 5
3785#	0.3 ± 0.1	1.8 ± 0.8	0.7 ± 0.4
3861#	7.8 ± 3.2	8.2 ± 0.7	2.1 ± 0.9
3909#	11.9 ± 4.0	15.0 ± 4.3	2.8 ± 0.8
3950#	2.4 ± 1	13.8 ± 3.2	4.1 ± 2.7
4445#	0.8 ± 0.4	10.4 ± 5.1	1.2 ± 0.2
4900	2.4 ± 0.6	4.7 ± 1.1	1.1 ± 0.5
5390	3.9 ± 1.3	4.4 ± 1.5	1.0 ± 0.3
5812#	10.3 ± 2.8	21.4 ± 5.9	1.8 ± 0.4
6723#	3.4 ± 0.8	6.7 ± 1.5	1.9 ± 0.6
7251#	0.8 ± 0.2	2.6 ± 0.7	0.4 ± 0.1
7669#	1.4 ± 0.3	5.0 ± 1.7	1.6 ± 0.6
7735#	1.7 ± 0.3	8.8 ± 3.9	0.8 ± 0.4
8184	22.1 ± 8.0	19.2 ± 5.1	7.5 ± 1.5
8845#	2.0 ± 0.7	15.4 ± 3.3	2.6 ± 1.0
11742#	1.4 ± 0.3	6.9 ± 2.3	1.2 ± 0.5
14440	0.7 ± 0.2	1.5 ± 0.7	0.1 ± 0.1
17200	3.8 ± 1.5	4.9 ± 0.6	5.0 ± 2.1
18925#	0.9 ± 0.14	3.1 ± 1.2	1.6 ± 0.4
19030#	1.3 ± 0.2	6.7 ± 2.9	2.1 ± 0.8
20500#	2.0 ± 0.6	13.4 ± 4.7	1.3 ± 0.5

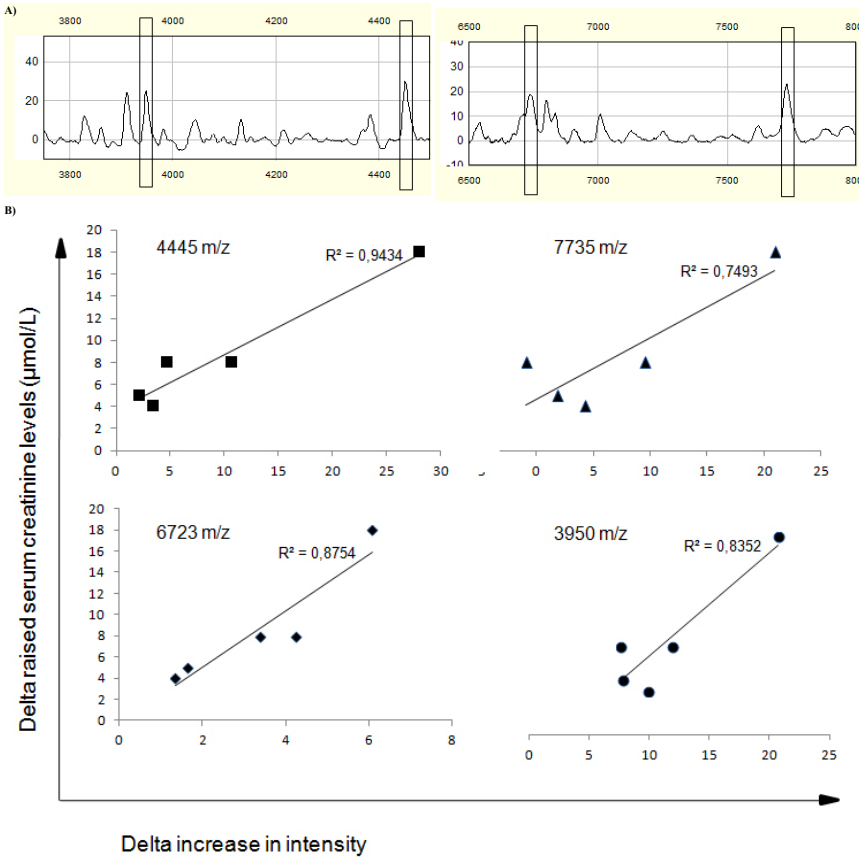

Figure 4: Representative urine protein profiles of m/z values versus peak intensity in the 3000-8000 Da range of one volunteer 6 h after the first LPS gift (b; panel A), showing four discriminative peak intensities 3950 m/z, 4445 m/z, 6723 m/z and 7735 m/z, which correlated significantly to the increase in serum creatinine levels of 5 volunteers (panel B). Maximum levels of serum creatinine were found on day, 3 and data is tested for significance by use of Graphpad Prism 4, and expressed as R^2 values.

administration of the 5th day, when LPS tolerance developed (p=0.4 between day 1 before LPS administration, and 6 hours after the fifth LPS administration on day 5).

Discussion

The present work indicates the existence and the ability to detect subclinical renal injury during repeated experimental endotoxemia in humans, at an early stage, by urinary proteomics. More importantly, we found 4 peak intensities of unknown origin, that correlated with the increase in serum creatinine levels 2 days later, and therefore, may act as new potential biomarkers for inflammation-induced renal injury. During repeated LPS administrations to induce LPS tolerance, renal protein loss subsided, which may suggest that increased cytokine levels present during acute endotoxemia, but not during LPS tolerance, are responsible for the tissue injury observed during inflammation. Because of its high mortality rate, early detection and understanding the mechanisms of inflammation-induced renal injury is of critical importance. Up to now, the diagnosis of renal injury is either based on observed increase in serum creatinine, or the presence of oliguria. As serum creatinine levels may underestimate the extent of acute renal injury, the use of urinary proteome research seems promising. Moreover, by use of various biomarkers, it has been shown that renal injury occurs in advance of any changed biochemical or clinical parameter. However, all biomarkers exert advantages and shortcomings in predicting renal injury at an early stage, and the ideal biomarker has not been discovered yet [16].

Of possible clinical importance, multiple peak intensities were identified 6 hours after LPS administration in our experiments, when serum creatinine levels were still unchanged. More importantly, we discovered 4 peak intensities of unknown origin, which correlated significantly with the subsequent raise in serum creatinine levels. The question remains whether a single marker can fulfill the requirements to reliably detect damage, as early as possible. An alternative strategy will be the identification of several markers, which on their own do not present high specificity and sensitivity, but as a panel, may work in concert and show a specific fingerprint that will help to distinguish between the various types and pathogenesis of renal injury [17,31].

In the present study, we used a panel ranging from 3-20 kDa, as no significant LPS-induced proteins were found in the 20-70 kDa range (data not shown). Furthermore, we did not find any of the peaks significantly altered in our previous study, with coronary artery bypass graft-induced renal injury [20]. This suggests that indeed different pathologic mechanisms were triggered, and it is likely that the protein masses found in the present study, related to renal injury, are highly sensitive as experimental human endotoxemia, and its systemic inflammatory response exerts a relatively mild and short insult on the kidneys. The biomarkers currently in use, e.g. α1-microglobulin, adenosine deaminase binding protein, cystatin C, renal tubular epithelial

antigen-1, N-acetyl-β-glucosaminidase, alanine-aminopeptidase, α/π-glutathione-S-transferase, γ-glutamyl transpeptidase, platelet activating factor, IL-18, kidney injury molecule-1 and Na/H exchanger isoform-3 [32], were not detected. Obviously, the four proteins that were found to be associated with endotoxin-associated acute kidney injury in this study need to be identified and validated for their predictive value.

The marker β2-microglobulin [33,34] consists of 99 amino acids with one disulfide bridge, and has a molecular weight of 11.7 kDa. Production of β2-microglobulin is very stable, and its urinary excretion inversely relates to glomerular filtration rate [34]. The present study shows that ß2-microglobulin levels increased during renal injury and were detected within the first 6 hours after LPS administration, while serum creatinine levels remained unchanged within this time period. β2-microglobulin levels tend to decrease over time, resulting in cleaved β2-microglobulin forms, that were not detectable by available immunoassays [33,35].

There is mounting evidence that suggest that pro-inflammatory cytokines are also involved in the development of renal injury in septic patients [5-12]. However, data on whether LPS tolerance also protects against renal injury in humans are lacking, therefore, the aim of the present study was to induce LPS tolerance in healthy volunteers, to accomplish attenuated levels of pro-inflammatory cytokines, by administration of LPS on 5 consecutive days, and to monitor renal function by use of the urinary proteome. Indeed, LPS tolerance is associated with less renal injury, as demonstrated adequately by urinary proteomics. Whether the protective properties result from either an indirect effect through inflammatory changes on neutrophils and renovascular endothelial cell, or from a direct effect on renal cytokine receptors, remains unclear. A weakness of the current study is the small cohort size. In experimental intensive care medicine, studies are very labor-intensive and demanding for the subjects, therefore, sample sizes are usually small as translational experiments are aimed at providing the basis for confirmatory study designs, endpoints and methodologies. Nevertheless, such explorative studies may facilitate larger future trials.

Animal studies have shown that LPS tolerance protects against subsequent renal injury; however, the pathological mechanism appears to be different than expected [24,25]. In contrast to attenuated systemic TNFα levels measured in LPS tolerant animals, increased renal production of pro-inflammatory cytokine TNFα was found. The finding of increased renal TNFα levels combined with less renal damage, suggest that down regulation of renal TNFα receptors may occur [36]. Renal TLR-4 expression was also measured, and it appeared that expression of this receptor was increased, and not down regulated during LPS tolerance, indicating a renal 'stress response', rather than reflecting a hyporesponsive state [25]. Although in humans the mechanism is still unclear, our study demonstrates that repeated LPS administrations are associated with less renal injury.

In conclusion, the present study shows that following the administration of LPS to human's urinary proteomics revealed potential new markers that were associated with a subsequent increase in plasma creatinine. This underlines the role of biomarkers to forecast the approach of inflammation-induced renal injury at an early stage. During the development of LPS tolerance, urinary excretion of markers of renal injury subsides, and glomerular filtration rate normalizes.

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