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Activity-Based Protein Profiling of Intraoperative Serine Hydrolase Activities during Cardiac Surgery

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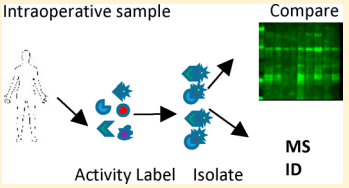
Supporting Information

ABSTRACT: The processes involved in the initiation of acute kidney injury (AKI) following cardiopulmonary bypass (CPB) are thought to occur during the intraoperative period. Such a rapid development might indicate that some of the inductive events are not dependent on de novo protein synthesis, raising the possibility that changes in activities of pre-existing enzymes could contribute to the development of AKI. Activity-based protein profiling (ABPP) was used to compare the serine hydrolase enzyme activities present in the urines of CPB patients who subsequently developed AKI versus those who did not (non-AKI) during the intra- and immediate postoperative periods. Sequential urines collected from a nested case-control cohort of AKI and non-AKI patients were reacted with a serine hydrolase activity probe, fluorophosphonate-TAMRA, and separated by SDS-PAGE. The patterns and levels of probe-labeled proteins in the two groups were initially comparable. However, within 1 h of CPB there were significant pattern changes in the AKI group. Affinity purification and mass spectrometry-based analysis of probe-labeled enzymes in AKI urines at 1 h CPB and arrival to the intensive care unit (ICU) identified 28 enzymes. Quantitative analysis of the activity of one of the identified enzymes, kallikrein-1, revealed some trends suggesting differences in the levels and temporal patterns of enzyme activity between a subset of patients who developed AKI and those who did not. A comparative analysis of affinity-purified probe reacted urinary proteins from these patient groups during the intraoperative period suggested the presence of both shared and unique enzyme patterns. These results indicate that there are intraoperative changes in the levels and types of serine hydrolase activities in patients who subsequently develop AKI. However, the role of these activity differences in the development of AKI remains to be determined.



KEYWORDS: *activity-based protein profiling, acute kidney injury, proteomics, serine hydrolase, kallikrein-1, cardiopulmonary bypass*

INTRODUCTION

Acute kidney injury (AKI) is a serious complication of cardiopulmonary bypass (CPB) resulting in loss of renal function in association with increased morbidity and mortality. Renal ischemia reperfusion injury (IRI), which occurs intraoperatively during cardiac surgery, is thought to be a major contributing factor to the subsequent development of AKI in these patients.[1] However, there is very limited detailed intraoperative observational data in humans regarding the molecular changes that occur during this period.[2] The fact that these intraoperative events occur within a window of a few hours raises the possibility that some of these processes may be mediated by activation of pre-existing molecules rather than de novo protein synthesis. Many enzymes are maintained in a latent state until catalytic activity is required allowing for rapid host responses, and such mechanisms may be relevant during the intraoperative induction phase of AKI.

Activity-based protein profiling (ABPP) was developed to selectively identify catalytically active enzymes.[3] Activity-based probes consist of a reactive group that covalently binds the enzyme catalytic site and a reporter group, which allows for the detection and isolation of labeled enzymes. The underlying premise is that enzyme activity is normally controlled by preventing substrate access to the catalytic site of an enzyme. Thus, the reaction of an activity probe with an enzyme implies that the labeled enzyme is active. This approach in conjunction with mass spectrometry offers the means to selectively isolate and identify active enzymes in a complex biological matrix.

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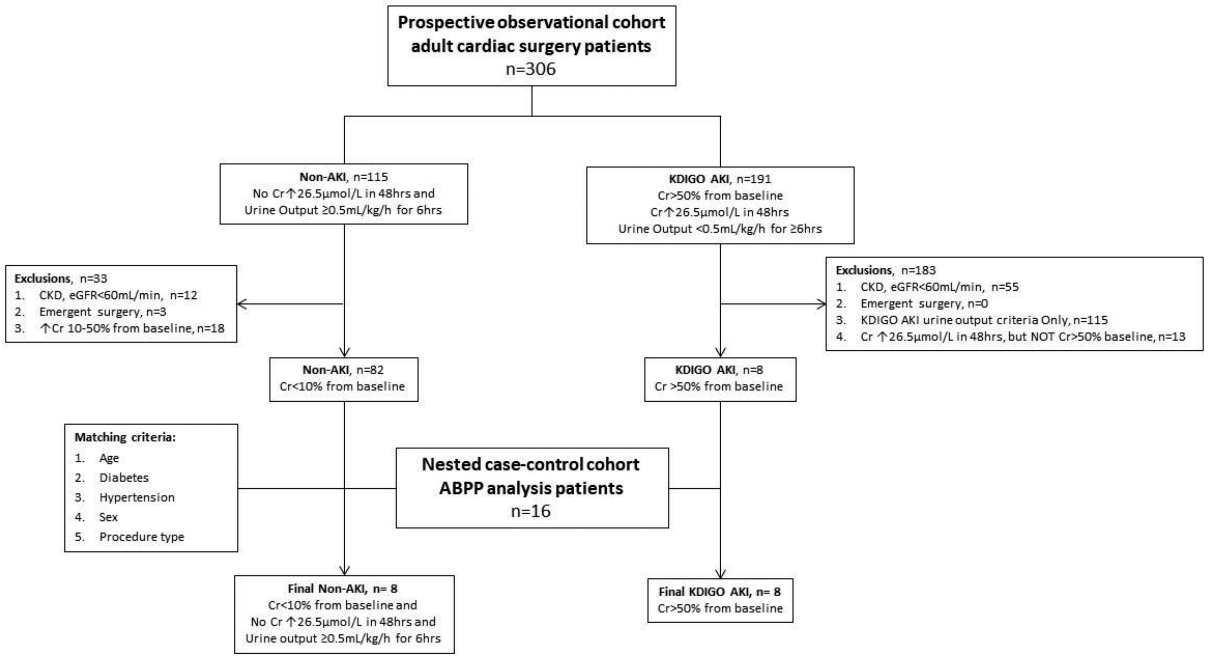

Figure 1. Nested case cohort of adult cardiac surgery patients. A nested case-controlled cohort of AKI and non-AKI patients (n = 16) was selected for in-depth ABPP analysis.

Serine hydrolases constitute a large enzyme family[4] characterized by a nucleophilic serine residue in the catalytic site.[5] This shared catalytic mechanism offers a basis for simultaneously interrogating the activity status of multiple serine hydrolases with a single activity probe, and was recently used to identify serine hydrolase activities associated with electrolyte homeostasis in the urine of healthy individuals.[6] The serine hydrolase family was specifically examined as these enzymes contribute to a diversity of processes that may be altered during IRI such as innate immunity, tissue remodelling and metabolic responses.[7,8]

The objective of the present study was to determine if there were compositional and functional changes in serine hydrolase activity associated with the subsequent development of AKI and to identify the enzymes that differed.

MATERIALS AND METHODS

Patient Population

The study protocol was approved by the University of Manitoba Ethics Board and all patients provided informed consent (Ethics approval HS15221; H2012:097). A prospective, observational cohort of 306 adult cardiac surgery patients with serial urines was collected at baseline, start of CPB, 1 h CPB and arrival to intensive care unit (ICU) and has been described in detail.[9,10] Urines were centrifuged at 870g for 6 min at room temperature and supernatants were aliquoted and stored at −80 °C.

The ABPP nested case-controlled cohort consisted of 8 AKI and 8 non-AKI patients; excluding individuals with chronic kidney disease (baseline eGFR < 60 mL/min) and emergent surgery. AKI was defined using the 2012 KDIGO criteria with a serum creatinine rise >50% from baseline; and non-AKI was defined as a serum creatinine <10% from baseline and urine output ≥0.5 mL/kg/h for 6 h.[11] Non-AKI and AKI patients were matched based on age, diabetes, hypertension, sex and procedure type (CABG, valve replacement or both) (Figure 1, Table 1).

Table 1. Patient Characteristics from the ABPP Adult Cardiac Surgery Nested-Case Control Cohort (n = 16)[a,b]

variable	non-AKI (n = 8)	AKI (n = 8)	p-value
Age (years)	70 (65−76)	69 (60−77)	0.83
Gender (male)	5 (63%)	4 (50%)	1.00
Baseline eGFR (mL/min/1.72 m^2)	79.4 (70.9−94.8)	77.0 (69.2−84.2)	0.60
Baseline creatinine (µmol/L)	78 (68−95)	74 (68−93)	0.87
Diabetes mellitus	3 (38%)	3 (38%)	1.00
Chronic obstructive pulmonary disease	1 (13%)	2 (25%)	1.00
Hospitalized congestive heart failure	2 (25%)	1 (13%)	1.00
Previous myocardial infarction	3 (38%)	1 (13%)	0.57
Previous CABG	0 (0%)	0 (0%)	1.00
Peripheral arterial disease	2 (25%)	1 (13%)	1.00
Amputation or peripheral arterial bypass	0 (0%)	0 (0%)	1.00
Previous cerebrovascular accident	0 (0%)	1 (13%)	1.00
Previous transient ischemic attack	1 (13%)	1 (13%)	1.00
Type of surgery (isolated CABG)	5 (63%)	3 (38%)	0.62
THAKAR score	2 (1−3)	3 (2−3)	0.36
EURO score	1.3% (0.9−2.8 %)	1.6% (1.1−2.9 %)	0.53
Pump time (minutes)	78 (66−94)	118 (84−192)	0.04
Cross-clamp time (minutes)	49 (40−66)	82 (46−112)	0.21
Operating room duration (minutes)	252 (230−303)	264 (217−397)	0.92
Intraoperative urine output (mL)	665 (478−1265)	748 (458−1135)	0.83

[a]Values expressed as median (interquartile range) or number (percent). Continuous variables compared using Mann−Whitney Test, categorical variables compared using Chi-Square or Fisher's Exact Test. [b]Abbreviations: Coronary artery bypass grafting (CABG); European system for cardiac operative risk evaluation (EURO); THAKAR score (ref 50).

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Activity-Based Protein Profiling

Urine samples were adjusted to 50 mM Tris (pH 9.0) and reacted with 2 μM Fluorophosphonate (FP)-TAMRA (Product number 88318, Thermo Scientific, Rockford IL USA) or 0.5 mM 6-*N*-biotinylaminohexyl isopropyl phosphorofluoridate, PF biotin (Product number B394900, Toronto Research Chemicals, North York ON Canada) and incubated at 37 °C, 90 min. The reaction was stopped by adding SDS sample buffer 4× (Product number NP0007, Life technologies, Carlsbad CA USA) and heating for 5 min at 95 °C in the presence of 50 mM DTT. Proteins were then separated on Bolt 4−12% Bis-Tris plus SDS-PAGE gels (Product number NW04120BOX, Life technologies, Carlsbad CA USA) at 120volts, 80 min. Gels were washed in distilled water for 20 min before scanning fluorescence at 534 nm (Alpha Innotech, Fluorchem Q, USA).

Affinity Purification of Activity Probe-Labeled Serine Hydrolases

Urine samples from AKI patients (n = 8/time point) at 1 h CPB and arrival to ICU were each pooled separately for analysis (1 mL/patient). Pools were 0.22 μm syringe-filtered (Product number SLGV033RS, Millipore, Tullagreen, Carrigtwohill, CORK IRL) and concentrated to 2.6 mL with a centrifugal filter unit Amicon ultra-15, 3 kDa NMWL (Product number UFC900324, Millipore, Tullagreen, Carrigtwohill, CORK IRL)(15 min, 4000*g*, 20 °C). The concentrates (135 μg total protein as determined by Total protein Liquicolor CSF/urine, product number 0345−212, Stanbio laboratory, Boerne TX USA), were buffered (Tris, pH 9.0) and simultaneously labeled with 2 μM FP-TAMRA and 500 μM PF-biotin for 90 min. The sample was adjusted to 10 mM DTT heated to 55 °C, 30 min,) and alkylated (50 mM IAA, 30 min, room temperature, in the dark). Reduced samples were desalted with Zeba spin columns (Product number #89893, Thermo scientific, Rockford, IL USA). The desalting procedure was repeated with second column.

Urine samples (n = 8/patient group/time point, 300 μL/patient)) from non-AKI and AKI patients at 1 h CPB and arrival to ICU were pooled for comparative analysis of active serine hydrolase composition. The same procedures as described above were used except that these urines were solely labeled with 250 μM PF-biotin and affinity-purified with streptavidin agarose.

The desalted samples were incubated for 1 h with rotation at room temperature with 20 μL protein G Sepharose beads 4 fast flow (Product number 17−0618−01, GE Healthcare SE-75184 Uppsala, Sweden) containing 40 μg of cross-linked anti-TAMRA antibody (Product number MA1−041, Thermo scientific, Rockford IL USA). The beads were collected by centrifugation (11 000*g*, 45 s). The supernatant was retained and the beads washed with PBS 0.02% Tween20.

FP-TAMRA probe-labeled enzymes were eluted from beads with 200 μL 1%TFA and centrifugation (11 000*g*, 45 s). After two cycles of affinity purification, the eluted FP-TAMRA probe-labeled enzymes from each extraction were pooled, dried in a speed-vac, dissolved (100 mM ammonium bicarbonate, 100 μL) and incubated overnight with trypsin (500 ng, 37 °C). The reaction was stopped with 4% TFA (50 μL), then frozen and dried in speed-vac; and was ready for mass spectrometry.

Unbound material from the FP-TAMRA purifications was pooled with material retained from the washing steps and concentrated to 250 μL with a centrifugal filter unit Amicon ultra-15, 3 kDa NMWL (Product number UFC900324, Millipore, Tullagreen, Carrigtwohill, CORK IRL) (55 min, 4000*g*, 20 °C). The concentrated sample was adjusted to 5 M urea mixed with 100 μL of PBS-washed streptavidin agarose resin (Product number #20353, Thermo scientific, Rockford IL USA). The sample was incubated at room temperature overnight with rotation. Beads were washed twice with 1 mL 1% SDS, 1 mL 6 M urea, 1 mL PBS and 500 μL 100 mM ammonium bicarbonate. Bound proteins were digested on bead overnight at 37 °C with 500 ng trypsin (Sequencing grade Modified Trypsin, V5111). The reaction was stopped with 50 μL 4%TFA and the beads were vortexed for 10 min and centrifuged (11 000*g*, 3 min). Supernatant was retained and the beads extracted a second time with 200 μL 0.1% TFA in acetonitrile. Pooled TFA eluents were dried on speed-vac, resuspended in 500 μL of 0.5% TFA and desalted with a C18-SD extraction disc cartridge (3M, USA, 4215SD) for analysis on 2D LC−MS/MS.

Nano-RPLC−MS/MS

Samples were analyzed by nano-RPLC−MS/MS using a splitless Ultra 2D Plus (Eksigent, Dublin, CA) system coupled to a high-speed Triple TOF 5600 mass spectrometer (AB SCIEX, Concord, Canada). Peptides were injected via a PepMap100 trap column (0.3 × 5 mm, 5 μm, 100 Å, Dionex, Sunnyvale, CA), and a 100 μm × 200 mm analytical column packed with 3 μm Luna C18 (2) was used prior to MS/MS analysis. Both eluents A (water) and B (98% acetonitrile) contained 0.1% formic acid as an ion-pairing modifier. The tryptic digest was analyzed in duplicate with 90 min gradient. Eluent B had a gradient from 0% to 35% over 77 min, 35% to 85% in 1 min and was kept at 85% for 5 min at a flow rate of 500 nL/min. Key parameter settings for the TripleTOF 5600+ mass spectrometer were as follows: ionspray voltage floating (ISVF) 3000 V, curtain gas (CUR) 25, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 25, declustering potential (DP) 80 V. All data was acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.6 software (ABSCIEX, USA). For IDA parameters, 0.25s MS survey scan in the mass range of 400−1250 were followed by 20 MS/MS scans of 100 ms in the mass range of 100−1600 (total cycle time: 2.3 s). Switching criteria were set to ions greater than mass to charge ratio (m/z) 400 and smaller than m/z 1250 with a charge state of 2−5 and an abundance threshold of more than 150 counts. Former target ions were excluded for 5 s. A sweeping collision energy setting of 37 ± 15 eV was applied to all precursor ions for collision-induced dissociation.

Database Analysis and Protein Identification

The MS/MS spectra analysis was performed as previously described.[6] All proteins had ≥1 peptides identified and a $\log_{10}$ expectation of less than −4. Serine hydrolases are reported based on their activities as either a metabolic or serine protease. Assignments as serine hydrolase activity are based on Uniprot;[12] the MEROPs database;[13] and the serine hydrolase list by Cravatt et al.[14]

Urine Kallikrein Enzyme Activity Assay

Urine samples were thawed on ice, vortexed and centrifuged (10 min at 10 000*g* at room temperature). Substrate H-D-Val-Leu-Arg-AFC, 8.4 mM in DMSO, (Product number 24137, ANASPEC Inc., Fremont CA 94555) was added to the reaction mixture at a final concentration of 33 μM in 50 mM Tris pH 8.0 containing 50 μL urine. Activity was monitored by measuring fluorescence at 380/500 nm in a BioTek Synergy 4 Hybrid microplate reader (BioTek Instruments, Highland park, VT

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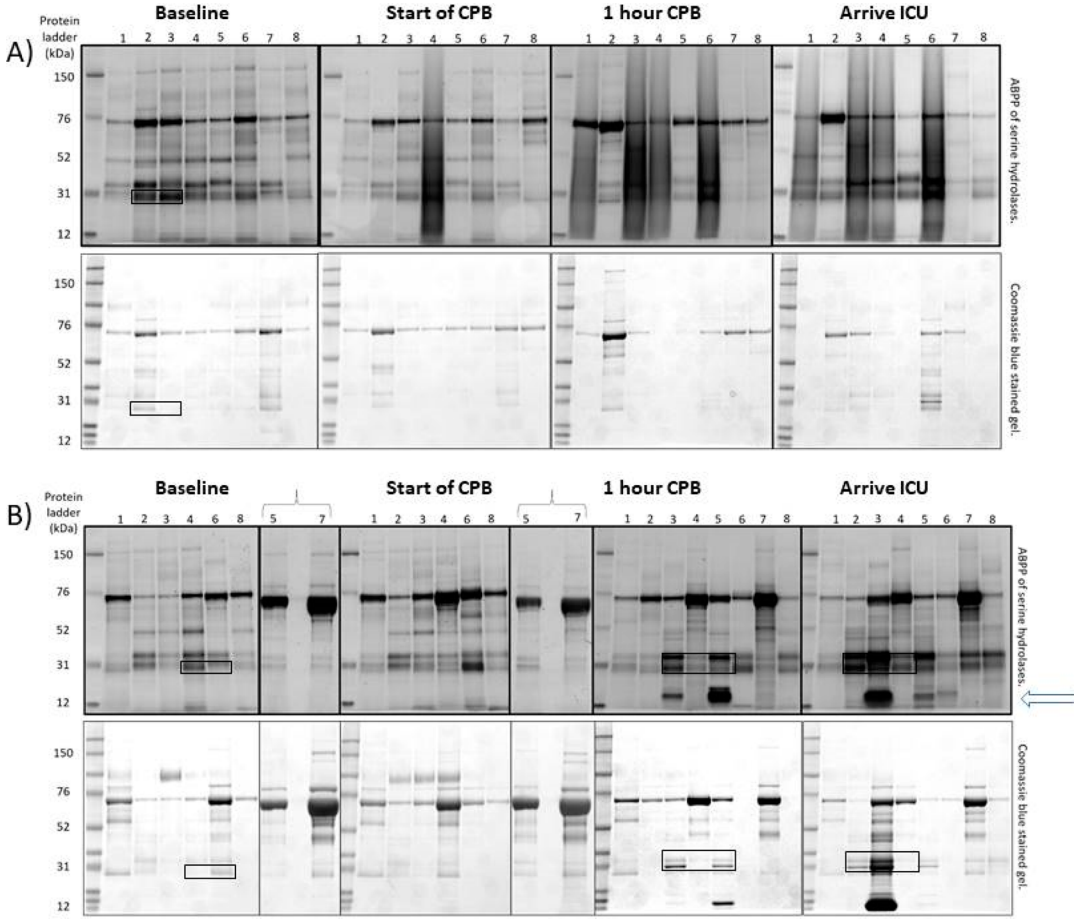

Figure 2. Urine serine hydrolase activities in individual non-AKI versus AKI patients. Serine hydrolase activity and protein profiles of urines from individual (A) non-AKI and (B) AKI patients demonstrated increased variability and temporal changes in patients who developed AKI postoperatively, compared to those who did not. Equal urine volumes (15 μL) from 8 Non-AKI and 6 AKI patients are shown at baseline, start of cardiopulmonary bypass (CPB), 1 h on CPB, arrival to the intensive care unit (ICU) and postoperative days (POD) 1 and 3−5. Two patients who developed AKI (5 and 7) had baseline proteinuria, which required significant sample dilution (4 μL) in order for the magnitude of their activity to be compared on SDS-PAGE analysis.

05404−0998) in 1 min intervals over 15 min. For each patient and time-point, blank-subtracted values for each activity interval were $\log_2$-transformed and the slope ($\log_2$ intensity per minute) was computed by simple linear regression.

Statistical Analysis

SAS (SAS Institute Inc., Cary, NC) was used for statistical analyses. For categorical data, Fisher's exact test or Pearson's chi-square test was used and data presented as counts and percentages. Parametric continuous data were analyzed by Student's *t* tests. Nonparametric continuous data were summarized as median (interquartile range) unless stated otherwise and analyzed by the Wilcoxon rank-sum or Kruskal−Wallis rank sum tests. Significant results in the Kruskal−Wallis rank sum test were further analyzed with pairwise nonparametric tests.

RESULTS

Intra- and Perioperative Changes in Urine Serine Hydrolase Activity in Patients Who Develop AKI

Serine hydrolase probe reactivity was detected in all of the urines examined. Frequently there was no direct correlation between the relative protein amounts and the levels of activity probe labeling, suggesting that some labeled enzymes were at very low concentrations (Figure 2, boxed areas). Qualitatively the patterns of activity labeling in non-AKI and AKI patients appeared to be similar at baseline. However, there were clear differences in the relative fluorescence levels of the bands, which presumably reflected quantitative differences in enzyme activities between samples. The baseline urine labeling patterns for both groups were similar to those previously reported for healthy individuals[6] with dominant bands in the 30−35, 50 and 70 kDa regions. However, the labeling patterns in two diabetic AKI patients with baseline proteinuria were significantly different. In these 2 patients, the labeling was dominated by a 70 kDa band, which correlated with a markedly elevated amount of protein in this region (Figure 2). Collectively these ABPP results suggested there were no significant differences in baseline urine serine hydrolase activities between patients who developed AKI versus those who did not.

The labeling patterns of non-AKI patients were similar at all of the time-points examined. Although there was some fluctuation in the relative intensity in the 70 kDa region of samples from this group, the levels of reactivity were generally stable. A diffuse background fluorescence was observed in some of the gels of probe labeled non-AKI urines, which impacted on the visualization of the bands in these samples (e.g., Figure 2, lane 4 start of CPB, and lanes 1, 3, 4, and 6 at 1 h CPB and arrival to

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ICU). Once this occurred for a given patient it was observed in all subsequent samples from that individual. This phenomenon was not observed in any of the AKI urines.

The labeling patterns observed in the AKI urines were substantially more dynamic than those of the non-AKI urines with marked changes in the patterns of urines collected from the same patient at different time-points. However, the detailed patterns of these shifts were not a consistent property of all of the AKI urines examined. Changes were initially observed in the 70 kDa region at the start of CPB with a subsequent shift to increased reactivity in the 30−35 kDa region. Particularly notable was the intraoperative appearance of labeling in the 17−20 kDa regions for several AKI urines (Figure 2, arrow), which was not observed in any of the non-AKI urines.

Identification of Active Serine Hydrolases in AKI Patients at 1 h CPB and Arrival to ICU

The above results indicated qualitative and quantitative differences in probe reactivity patterns of AKI urines at 1 h CPB and arrival to ICU, which evolved from a background of shared baseline activity patterns in AKI and non-AKI patients (Figure 2). To characterize these differences, AKI urines were combined on an equal protein basis to generate separate pools from 1 h CPB and arrival to ICU. Each pool was sequentially labeled with two activity based probes, fluorophosphonate-tetramethyl rhodamine (FP-TAMRA), and phosphorofluoridate biotin (PF-biotin). These probes have overlapping but nonidentical reactivity patterns and employ different affinity purification tags, potentially providing more complete coverage of the active serine hydrolases in the samples.

Probe-labeled proteins were affinity-purified sequentially with anti-TAMRA coated beads and streptavidin coated beads. Aliquots of the bead-bound material were analyzed by SDS-PAGE (Figure 3). The anti-TAMRA affinity purification enriched the labeled proteins as evidenced by the reduced amount and complexity of residual protein in the isolated fraction (Figure 3). However, our experience has been that the capture of TAMRA-labeled proteins is incomplete even with repeat cycles of anti-TAMRA based isolation suggesting that residual FP-TAMRA labeled enzymes were left in the samples. The subsequent streptavidin affinity purification step provided a near complete capture of PF-biotin labeled proteins (Figure 3). The FP-TAMRA and PF-Biotin affinity-purified proteins from each time-point were individually processed for LC−MS/MS analysis.

A total of 28 serine hydrolases were identified of which 17 were detected in both samples (Table 2). The majority of enzymes identified (n = 20) were proteases/peptidases with the remainder of the enzymes involved in metabolic processes. There was a significant enrichment in KEGG pathway "complement and coagulation cascade" components (false discovery rate, FDR 1.79×10^{-9}). These included components of the complement cascade (i.e., Factors B, D and I). The majority of proteolytic enzymes (16/22) were endopeptidases. The remaining metabolic enzymes were involved in a diversity of functions including glycerophospholipid metabolism, phospholipase activity and nonproteolytic modifications of proteins.

Urinary Kallikrein-1 Activity Levels in AKI and Non-AKI Patients

One of the identified enzymes, kallikrein-1, has a predicted molecular weight of 26.3 kDa for the active enzyme. This would place it in the gel region where urinary proteins displayed increased activity in the AKI urines at 1 h CBP and arrival to

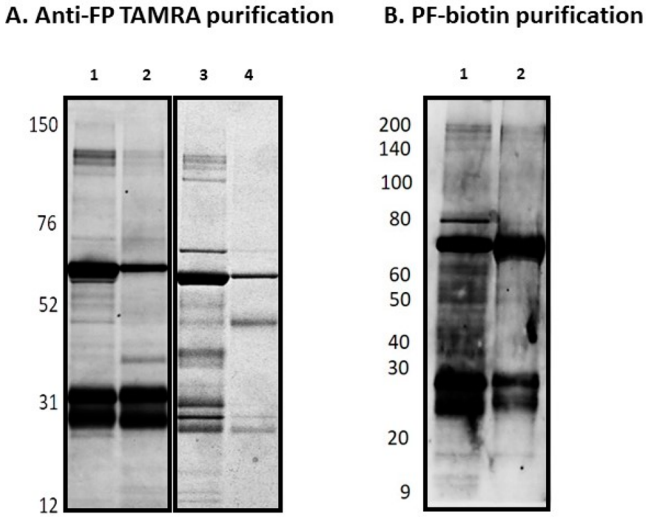

Figure 3. Sequential purification of activity probe-labeled enzymes from urine. A pooled urine sample taken at arrival to ICU from patients who subsequently developed AKI was labeled with serine hydrolase activity based probes (FP TAMRA and PF-biotin) and the labeled proteins were affinity purified. (A) Anti-FP TAMRA purification. Lane 1 shows the unbound material from an activity probe-labeled urine after incubation with anti-TAMRA beads. Lane 2 shows the proteins captured by the anti-TAMRA beads. Lanes 1 and 2 are visualized with the fluorescent TAMRA containing probe. Lanes 3 and 4 are the same gels stained with Coomassie blue to visualize the total protein content. (B) PF-biotin purification. Unbound material from the anti-FP TAMRA affinity purification was subsequently incubated with streptavidin-coated beads. Lane 1 corresponds to the material captured on the PF-biotin beads and lane 2 represents the residual unbound material following both immunodepletions.

ICU, raising the possibility that increased kallikrein-1 activity may contribute to the activity changes observed in this region of the gel. This hypothesis was directly tested by comparing the activity of kallikrein-1 in a larger nested case-control population of AKI and non-AKI patients, with KDIGO AKI defined as serum creatinine >50% from baseline or increase 26.5 μmol/L in 48 h (n = 42) (Supplemental Figure S1).

Urinary kallikrein-1 activity was detectable in both patient groups with the levels in the AKI group showing greater variability. Kallikrein-1 activity was generally lower and more variable within the AKI population compared to the non-AKI group (Figure 4) but these trends were not statistically significant. There was a transient increase in the kallikrein-1 activity in a subset of non-AKI patients at 1 h CPB but the activity in this group returned to near baseline levels by arrival to ICU. A transient rise in kallikrein-1activity was also observed but this was delayed relative to the non-AKI peak.

It was noted that kallikrein-1 activity of the AKI patients displayed a broader range of activities at each time point relative to those patients that did not develop AKI. The activities in AKI patients ranged from no detectable activity to levels that were in the highest quartile of activities observed for the non-AKI patients. Although these results did not support our original hypothesis they did demonstrate the presence of active serine hydrolases in urine, which confirms the results of the probe analysis

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Table 2. Serine Hydrolases Identified in the Activity-Based Probe-Labeled AKI Urines[a]

Enzyme	Uniprot	Molecular weight	1h CPB	Arr ICU
Metabolic hydrolases, n=8				
Acylamino-acid-releasing enzyme	P13798	81.2	■	■
Acyl-protein thioesterase 1	O75608	24.7		■
Alpha/beta hydrolase domain-containing protein 14B	Q96IU4	22.3		■
Bile salt-activated lipase	P19835	78.3		■
Group XV phospholipase A2	Q8NCC3	46.6	■	■
Isoamyl acetate-hydrolyzing esterase 1 homolog	Q2TAA2	27.6		■
Phosphatidylcholine-sterol acyltransferase	P04180	49.5		■
Sialate O-acetylesterase	Q9HAT2	58.3	■	■
Serine proteases, n=20				
Apolipoprotein(a)	P08519	226.4	■	■
Complement factor D	P00746	27	■	
Complement factor I	P05156	66.6	■	■
Complement C1 r subcomponent-like protein	Q9NZP8	53.5	■	■
Dipeptidyl peptidase 2	Q9UHL4	54.3	■	■
Dipeptidyl peptidase 4	P27487	88.2	■	■
Haptoglobin	P00738	45.2	■	■
Kallikrein-1	P06870	28.9	■	■
Lactotransferrin	P02788	78.1	■	■
Lysosomal Pro-X carboxypeptidase	P42785	55.8	■	■
Lysosomal protective protein (cathepsin A)	P10619	56.2	■	■
Mannan-binding lectin serine protease 2	O00187	75.7	■	■
Plasminogen	P00747	90.5	■	■
Probable serine carboxypeptidase CPVL	Q9H3G5	54.1	■	■
Prostasin	Q16651	36.4	■	
Prostate-specific antigen	P07288	28.7		■
Retinoid-inducible serine carboxypeptidase	Q9HB40	50.8	■	
Transferrin	P02787	77	■	■
Tripeptidyl-peptidase 1	O14773	61.2	■	■
Urokinase-type plasminogen activator	P00749	48.5		■

[a]Serine hydrolase activity probe-labeled proteins were affinity-purified from AKI patients at 1 h CPB and arrival to ICU. The shaded boxes indicate enzyme was detected in the samples and unshaded indicates that the protein was not detected. All protein identifications are based on at least 2 unique peptides and a $\log_{10}$ expectation value ≤ -3 for the protein. Molecular weight estimates refer to the unprocessed protein.

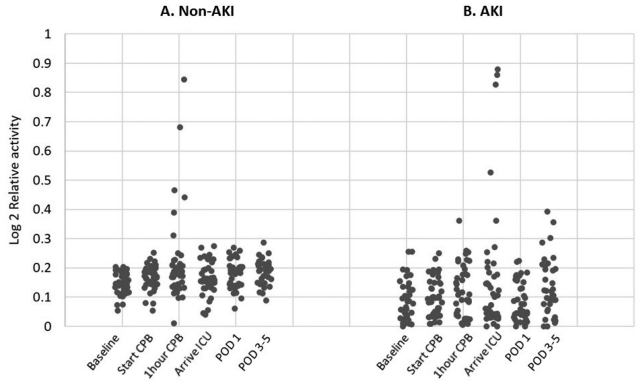

Figure 4. The detection of kallikrein-1 activity in urine. Kallikrein-1 activity was assayed in Non-AKI (N) and AKI (A) patients at time points baseline, start of cardiopulmonary bypass, 1 h on cardiopulmonary bypass, arrival to the intensive care unit (ICU), postoperative day 1 and postoperative day 3−5.

Comparison of Intraoperative Active Serine Hydrolases in AKI and Non-AKI Patients

The initiating events that lead to AKI postoperatively are thought to occur during the intraoperative period. The comparative gel analysis of FP-TAMRA labeled urinary proteins (Figure 2) also indicated there were intraoperative changes in the urines of patients who developed AKI. Based on this information, a comparative study was made of the affinity-purified probe-labeled enzymes from the urines of non-AKI and AKI patients at 1 h CPB and arrival to ICU. Pooled samples consisted of the same patient samples examined by ABPP comparative gel analysis. The urines were labeled with 250 μM PF-biotin probe as the preliminary studies demonstrated the labeling spectrum of this probe was similar to the FP-TAMRA probe. The FP-TAMRA probe was less efficient in terms of affinity recovery, and the concern was that this probe would potentially compromise the breadth of the affinity isolation.

A total of 27 enzymes were identified, 19 of which were identified in the previous analysis of the AKI urines from these time-points (Table 3). Several enzymes were detected in each of the time points in both patient groups (e.g., CEL, LCAT, PLG, SIAE, TF). There were also a limited number of enzymes that appeared to be restricted to one of the patient groups (i.e., AKI or non AKI) at one or both of the time points examined. In the case of the AKI patients these included ABHD12; activated complement and coagulation components; phospholipase A2 group 15 and plasma kallikrein B1 (KLKB1). The non-AKI urines contained lactotransferrin, acylaminoacyl-peptide hydrolase, kallikrein 3 and urokinase, which were not detected in the AKI samples. Collectively these results suggested that there may be differences in the activated urine serine hydrolases in patients who do or do not develop AKI postoperatively.

Table 3. Serine Hydrolases Identified in the Activity-Based Probe-Labeled Urines of AKI and Non-AKI Patients[a]

Identifier	Description	AKI 1h CPB	Non AKI 1h CPB	AKI Arr ICU	Non AKI Arr ICU
Q8N2K0	abhydrolase domain containing 12	■			
H0YAC1	kallikrein B1	■			
O00748	Cocaine esterase			■	
Q9UHL4	dipeptidyl-peptidase 7			■	
P09958	furin			■	
Q2TAA2	isoamyl acetate-hydrolyzing esterase 1 homolog			■	
P06870	kallikrein 1			■	
Q9NZP8	complement component 1, r subcomponent-like	■		■	
Q8NCC3	phospholipase A2 group XV	■		■	
P00734	coagulation factor II, thrombin	■		■	
P05156	complement factor I	■		■	■
P10619	cathepsin A	■		■	■
Q16651	protease, serine 8	■		■	■
P19835	carboxyl ester lipase	■	■	■	■
P04180	lecithin-cholesterol acyltransferase	■	■	■	■
P00747	plasminogen	■	■	■	■
Q9HAT2	sialic acid acetylesterase	■	■	■	■
P02787	transferrin	■	■	■	■
P00738	haptoglobin	■	■	■	
P00751	Complement factor B	■	■	■	
P27487	dipeptidyl peptidase 4	■	■		
P09871	complement component 1, s subcomponent		■	■	
P08519	lipoprotein, Lp(a)		■	■	
P02788	lactotransferrin		■		■
P13798	acylaminoacyl-peptide hydrolase				■
P07288	kallikrein related peptidase 3				■
P00749	plasminogen activator, urokinase				■

[a]Serine hydrolase activity probe-labeled proteins were affinity-purified from pooled AKI or non-AKI patients at 1 h CPB and arrival to ICU. The shaded boxes indicate enzyme was detected in the samples and unshaded indicates that the protein was not detected. All protein identifications based on at least 2 unique peptides and a $\log_{10}$ expectation values ≤ -3 for the protein.

DISCUSSION

This study provides several novel pieces of information regarding the intraoperative processes occurring in patients undergoing CPB and developing AKI. Quantitative and qualitative intraoperative changes in the serine hydrolase activity were demonstrated in the urines of all patients. Patients who developed AKI postoperatively displayed the greatest intra-

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operative changes in urinary serine hydrolase activity. A number of enzyme activities were identified that appeared to be associated with AKI. Significantly there did not appear to be any discernible differences between the baseline activities of the non-AKI and AKI patient groups, suggesting that initially both groups had similar types and levels of urinary enzyme activities prior to the initiation of ischemia reperfusion injury during CPB. Although the present results do not provide any evidence of a causal relationship between the changes in enzyme activity and the subsequent development of AKI, the data does suggest that there are changes that may differentiate the two patient groups intraoperatively and prior to the development of AKI.

An initial protein identification was performed on affinity-purified probe-labeled urine from AKI patients after 1 h of CPB and arrival to ICU as these urines appeared to contain the majority of bands present in the non-AKI urines plus some additional species. The AKI urines offered a means of capturing a potentially more complete repertoire of enzymes relevant to both patient groups. This was significant as it was not clear whether differences in the intensities of homologous regions of the gels of AKI and non-AKI urines represented higher activities of enzymes common to both patient groups or the activation of novel species. The direct assay of KLK1, one of the enzymes isolated from these samples, confirmed the activity of this enzyme in AKI and non AKI urines and validated the utility of the approach. All 28 enzymes identified in the AKI urines have been identified in normal urine by mass spectrometry[15] but only 10 of these were detected in an active state by ABPP in normal urine.[6] This is despite the fact that greater than 8-times more normal urine was used for the isolation of active enzymes.[6] The implication is that the enzymes identified in the AKI urines are also present in normal urines either at lower levels or not in an active state.

A direct comparative analysis of the same AKI and non-AKI patients used in the initial ABPP gel analysis identified a comparable number of urine enzymes with significant overlap between the enzyme identifications in the two analyses (Table 2). Enzymes shared between AKI and non-AKI patients included members of the complement and coagulation systems as well as a number of enzymes that have previously been identified in normal urine.[6] These enzymes may be involved in normal physiological processes or stress responses induced by cardiac surgery. There was a group of enzymes that were only identified in the AKI group. These included additional elements of the complement system and other proteases (e.g., kallikrein B1, cathepsin A, Furin, DPP7, kallikrein-1) and enzymes involved in various processes (e.g., PLA2G15, CES2, and IAH1). Conversely there was a set of enzymes that were only detected in the non-AKI patients (e.g., LTF, APEH, KLK3, PLAU). Conceivably this last group of enzymes might be involved in processes that are renoprotective during ischemia reperfusion injury, or normal stress responses that failed to activate in the AKI group.

It is important to note that ~1/3 of the sample quantity that was used for the original analysis (Table 2) was used for enzyme isolation in the comparative analysis (Table 3). This was necessary due to the limited quantities of remaining patient samples. Thus, the differential expression of an enzyme activity in the comparative analysis may be a reflection of relative levels of activity between samples rather than the absolute presence or absence of an active species. This was demonstrated in the case of KLK1 activity, which was directly measured in the AKI urines (Figure 4) but only identified in the one of the AKI urine samples, arrival to ICU. Notably this time point was the one at which the greatest KLK1 activity was detected (Figure 4). These observations highlight the need for the use of quantitative enzyme activity assays to validate qualitative comparative enzyme differences.

Several of the identified serine proteases regulate renal sodium homeostasis via cleavage and activation of the epithelial sodium channel (ENaC) to promote sodium reabsorption.[16,17] Kallikrein-1 cleaves the ENaC receptor γ-subunit to promote sodium reabsorption[18] and is also implicated in renal potassium and calcium homeostasis.[19,20] Urokinase-type plasminogen activator cleaves plasminogen into plasmin, a serine protease that activates ENaC.[21] These enzymes are present in healthy urines and may be endogenously active.[6] Another ENaC regulator, prostasin,[17] was identified in AKI patients but not healthy individuals.[6] It is tempting to speculate that there may be differential intraoperative responses to ENaC activation and sodium reabsorption, potentially moderating the subsequent risk for developing AKI. However, it is possible these findings may also be due to the greater depth of affinity purification achieved using two sequential activity-based probes versus FP-TAMRA alone.[6]

Notably, kallikrein-1 has been shown to ameliorate AKI in a rat IRI model through decreased inflammatory cell infiltration, apoptosis, nitric oxide, and oxidative stress.[22] These findings are supported by rat gentamicin and cisplatin AKI models, which also demonstrate that kallikrein-1 inhibits inflammatory cell recruitment, apoptosis and development of fibrotic lesions through suppression of oxidative stress, and these effects are mediated via bradykinin 2 receptor pathways.[23−25] We detected urinary kallikrein activity in both groups, but with higher trending levels in non-AKI patients, suggesting the possibility that kallikrein may be renoprotective in human AKI. These findings are consistent with low observed urinary kallikrein activity in ICU patients with incipient AKI,[26] but need to be further validated.

Several of the active serine hydrolases identified are components of the innate immune system.[27] Activation of the alternate pathway (complement factors B, D and I) and the lectin pathway (mannan-binding lectin serine protease 2, MASP-2) indicate that the complement system is activated.[28] Urinary MASP-2 levels are elevated in patients following CPB,[29] and this protein has been shown to be a critical mediator of tissue injury in renal IRI in animals.[30] The role of complement C1r subcomponent-like protein remains unclear as it has been suggested to be either a negative regulator of complement-mediated cytotoxicity[31] or activator of the classical pathway by cleaving pro-C 1s. These activities are consistent with mouse models of renal IRI that demonstrate a pathogenic role for the alternate[32−34] and lectin[30,35] pathways, but not the classical pathway,[36] and further extend these observations to human renal IRI.

Identification of lactotransferrin, transferrin and haptoglobin suggests that iron-binding proteins may be relevant in mediating the response to IRI, potentially by reducing oxidative stress and ferroptosis.[37,38] The comparative analysis of the patient groups suggests that active lactotransferrin may be more active in the non-AKI patients (Table 3) potentially providing more effective renoprotection. Other iron-sequestering proteins such as neutrophil-gelatinase associated lipocalin and hepcidin-25 are independent predictors of AKI following cardiac surgery[9,39−41] and renoprotective when administered in mouse models of AKI.[42−45] Interestingly, this ABPP approach identified urinary

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dipeptidyl peptidase 4 (DPP4) in AKI patients, which may degrade hepcidin-25 to its less active isoforms.[46,47] This raises the intriguing possibility that luminal enzyme activity may play an in vivo regulatory role by inactivating hepcidin-25.

A recent report using a porcine IRI-AKI model described a transient increase in DPP4 activity at 4 h postreperfusion that returned to baseline by 11 h postoperatively.[48] This study observed no correlation between relative DPP4 concentration and enzymatic levels, highlighting the importance of functional analysis. Interestingly, patients with biopsy-proven acute rejection have elevated urinary DPP4, suggesting the possibility that it plays a role across different renal injury phenotypes.[49]

This study demonstrates qualitative and quantitative differences in urine serine hydrolase activities in patients who subsequently develop AKI versus those who do not. These changes begin intraoperatively and precede the clinical onset of AKI. The urine serine hydrolase patterns of AKI patients did not show a consistent pattern as a group. There are several potential explanations for this: (1) there is heterogeneity in the processes leading to AKI such that serine hydrolase activity changes may only be relevant to a subset of AKI patients; (2) the kinetics and duration of enzyme activity changes may be very transient and not fully captured by the prespecified time-points of this study; (3) alternatively, these observations may be epiphenomenon and not directly relevant to AKI development.

In conclusion, this study provides novel information regarding some of the earliest intraoperative events in human ischemia reperfusion injury during CPB and suggests differences in the levels and types of serine hydrolase activities that may be associated with AKI development. Whether such correlates play a direct role in the development of AKI remains to be determined. However, these results suggest that quantitation of serine hydrolase activities might provide a basis for early intraoperative identification of patients who will develop postoperative AKI. These possibilities remain to be tested and will be dependent upon the development and application of enzyme-specific assays for the comparative quantification of activity in larger cohorts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00500.

Figure S1: Nested case cohort of adult cardiac surgery patients. A larger nested case-controlled cohort of AKI and non-AKI patients (n = 42) was selected for urinary kallikrein activity analysis (PDF)

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Notes

The authors declare the following competing financial interest(s): A patent application is submitted for the 32 serine hydrolase enzymes identified in acute kidney injury urines.

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■ ABBREVIATIONS

ABPP, activity-based protein profiling; AKI, acute kidney injury; PF biotin, 6-*N*-biotinylaminohexyl isopropyl phosphorofluoridate; CABG, coronary artery bypass graft; CPB, cardiopulmonary bypass; Cr, creatinine; DPP4, dipeptidyl peptidase 4; eGFR, estimated glomerular filtration rate; ENaC, epithelial sodium channel; FDR, false discovery rate; FP TAMRA, fluorophosphonate 5-Carboxytetramethylrhodamine; ICU, intensive care unit; IRI, ischemia reperfusion injury; kDa, kilodalton; KDIGO, Kidney Disease Improving Global Outcomes; LC−MS/MS, liquid chromatography−tandem mass spectrometry; MASP-2, mannan-binding lectin serine protease 2; MDRD eGFR, Modified Diet in Renal Disease estimated glomerular filtration rate; POD, postoperative day; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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