HMG–CoA Reductase Activation and Urinary Pellet Cholesterol Elevations in Acute Kidney Injury

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Summary

Background and objectives Experimental acute kidney injury (AKI) activates the HMG–CoA reductase (HMGCR) gene, producing proximal tubule cholesterol loading. AKI also causes sloughing of proximal tubular cell debris into tubular lumina. This study tested whether these two processes culminate in increased urinary pellet cholesterol content, and whether the latter has potential AKI biomarker utility.

Design, setting, participants, & measurements Urine samples were collected from 29 critically ill patients with (n = 14) or without (n= 15) AKI, 15 patients with chronic kidney disease, and 15 healthy volunteers. Centrifuged urinary pellets underwent lipid extraction, and the extracts were assayed for cholesterol content (factored by membrane phospholipid phosphate content). *In vivo* HMGCR activation was sought by measuring levels of RNA polymerase II (Pol II), and of a gene activating histone mark (H3K4m3) at exon 1 of the HMGCR gene (chromatin immunoprecipitation assay of urine chromatin samples).

Results AKI+ patients had an approximate doubling of urinary pellet cholesterol content compared with control urine samples (*versus* normal; $P < 0.001$). The values significantly correlated (r, 0.5; $P < 0.01$) with serum, but not urine, creatinine concentrations. Conversely, neither critical illness without AKI nor chronic kidney disease raised pellet cholesterol levels. Increased HMGCR activity in the AKI+ patients was supported by three- to fourfold increased levels of Pol II, and of H3K4m3, at the HMGCR gene (*versus* controls or AKI− patients).

Conclusions (*1*) Clinical AKI, like experimental AKI, induces HMGCR gene activation; (*2*) increased urinary pellet cholesterol levels result; and (*3*) urine pellet cholesterol levels may have potential AKI biomarker utility. The latter will require future testing in a large prospective trial.

Clin J Am Soc Nephrol 6: 2108–2113, 2011. doi: 10.2215/CJN.02440311

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Introduction

A seemingly constant consequence of ischemic or toxic acute kidney injury (AKI) is an upregulation of renal cortical HMG–CoA reductase (HMGCR) activity (1–10). Within 12 to 24 hours post-AKI induction, this culminates in an approximately 20% to 40% increase in renal cortical cholesterol content. Furthermore, when cultured human proximal tubular (HK-2) cells are subjected to either toxin or ATP depletion-mediated injury, increased HMGCR activity and cholesterol accumulation result (9). The significance of these *in vitro* findings is that they imply that injury-induced *in vivo* cholesterol accumulation reflects, at least in part, a direct proximal tubule cell event. The "downstream" consequences of proximal tubule cholesterol loading remain incompletely defined. However, our prior work indicates that it helps mediate the phenomenon of "ischemic preconditioning" (so-called acquired cytoresistance), whereby previously injured tubular cells become resistant to further ischemic or toxic attack (1–3). Indeed, this sequence of events is analogous to the so-called heat shock response, *i.e.*, whereby a renal stress (*i.e.*, heat shock) upregulates cytoprotective molecules (*i.e.*, heat shock proteins), which then confer a cytoresistant state (11,12). That transient hyperthermia raises both heat shock protein and renal tubular cholesterol levels directly supports this "heat shock" analogy (7).

A morphologic correlate of AKI is sloughing of proximal tubular "brush border" fragments and intact tubular cells into urine (13–16). In light of the above-noted injury-induced cholesterol loading, we hypothesized that an increase in urinary pellet cholesterol content might result. If so, such a finding might have potential value as a "biomarker" of AKI. To gain some initial experimental support for this hypothesis, we collected urine samples from mice that had been subjected to two experimental models of AKI: glycerol induced rhabdomyolysis (17,18) or the maleate model (ATP depletion induced) of ARF (19,20). Urine samples were collected at either 3 or 24 hours post-AKI induction, and, after centrifugation, the pellets were extracted in chloroform: methanol and assayed for cholesterol content (21,22). The re-

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sults were expressed as a ratio to the total amount of phospholipid phosphate present, used as an internal standard. As early as 3 hours, and persisting for at least 24 hours post-AKI induction, dramatic increases in urinary pellet cholesterol content were observed compared with values observed in normal mouse urine samples (unpublished observations). To assess whether pellet cholesterol assay might be able to differentiate between structural (*i.e.*, tubular necrosis) *versus* functional (*i.e.*, prerenal) ARF, a prerenal azotemia model was created (indomethacin injection into mice that were simultaneously subjected to surgical stress, induced by abdominal laparotomy). This "prerenal" model induced substantial azotemia, but no increase in urinary pellet cholesterol levels resulted.

Based on these promising preliminary experimental data, in the present study we sought to determine (*1*) whether this same phenomenon occurs in AKI patients; (*2*) whether evidence of increased HMGCR gene activity could be documented in patients by assessing RNA polymerase II binding (the enzyme that drives transcription) to HMGCR gene fragments in pelleted urinary chromatin samples (by chromatin immunoprecipitation, ChIP, assay [10]); and (*3*) whether pellet cholesterol levels might have potential utility as a clinical AKI biomarker. If positive data were to be obtained in this "proof of concept" study, they would provide a rationale for further testing in a large prospective clinical trial.

Table 1. Patient characteristics of the ICU patient cohorts reported in this study

Characteristic	Controls (n = 15)	Acute Kidney Injury (n = 14)
Age (years)	64 ± 14	52 ± 17
Male sex	7/15 (47%)	10/14 (71%)
Caucasian	13/15 (87%)	12/14 (86%)
APACHE II score	27 ± 4	33 ± 7
Sepsis	10/15 (67%)	11/14 (79%)
Prehospital statin use	2/15 (13%)	5/14 (36%)
Hospital mortality	0/15 (0%)	4/14 (29%)
Creatinine at enrollment	0.84 ± 0.29	2.65 ± 1.16
AKIN stage at enrollment	0	2.3 ± 0.8

Clinical characteristics of the ICU patients with and without AKI at the time of enrollment. The serum creatinine concentrations at enrollment (and AKIN scores) were statistically different between the two groups. The other factors did not manifest statistical significance (overlapping 95% confidence intervals). ICU, intensive care unit; APACHE II, Acute Physiology and Chronic Health Evaluation II score; AKIN, Modified AKIN score based on serum creatinine measurements (ref 32). Continuous variables are given as means ± 1 SD.

Materials and Methods

Patient Samples

Banked, frozen urine samples were obtained from three groups of patients: (*1*) critically ill patients with AKI (AKI+; n = 14); (*2*) critically ill patients without AKI (AKI−; n = 15); and (*3*) patients with chronic kidney disease (CKD; n = 15). The AKI+ and AKI− individuals were subsets of patients who were enrolled in a large, IRB-approved prospective observational study of critically ill adults treated in multiple intensive care (ICU) units at Vanderbilt University (23–25). AKI+ was defined as a ≥50% (or >26.5 μmol/L) increase in serum creatinine concentration from baseline. All AKI+ patients had a presumptive diagnosis of "acute tubular necrosis," as judged by consulting nephrologists. The AKI− group comprised 15 critically ill ICU-hospitalized patients who had comparable overall illness severity as the AKI+ group (APACHE II scores) but who did not have AKI. The AKI+ and AKI− populations were matched for age, race, gender, and sepsis status. Demographic information for these patients and the specifics of urine sample collection has been previously described (23) (also reviewed and expanded upon in Table 1). The CKD patient population consisted of six individuals with diabetic nephropathy and nine individuals with nondiabetic CKD. These patients were enrolled in the Seattle Kidney Study, a prospective, observational IRB-approved study of subjects with CKD. Subjects are eligible if they have a Modification of Diet in Renal Disease estimated GFR <60 ml/min/1.73m^2, are not receiving dialysis, and are at least 18 years of age. The CKD subjects were matched to serum creatinine concentrations that were observed in the AKI+ group at the time of study (AKI+, 2.65 ± 1.16 mg/dl; AKI−, 0.84 ± 0.29 mg/dl; CKD, 2.2 ± 0.3 mg/dl; means ± 1 SD). In addition to these 44 patient urine samples (14 AKI+, 15 AKI−; 15 CKD), additional urine samples were obtained from 15 healthy volunteers.

Urine Cholesterol Assay

Urine samples were thawed at room temperature and centrifuged (12,000 rpm × 5 minutes). Immediately after thawing, the pellets underwent lipid extraction in chloroform: methanol, as described previously (1–3). The extracts were then assayed for cholesterol by the Amplex Red method (21,22). In addition, total phospholipid phosphate content was assessed, serving as an internal standard of total membrane lipid extracted (1). The cholesterol results were expressed as ratios to phospholipid phosphate content (1–3).

Assessment of HMGCR Gene Activation

We have recently demonstrated that *in vivo* gene activation can be noninvasively monitored in patients by measuring RNA polymerase II (Pol II) binding to target gene fragments in urinary pellets (23,25). Three principles underlie this approach: first, Pol II is the enzyme that drives transcription (30); second, degrees of Pol II binding to target genes is an indirect gauge of gene transcription (30,31); and third, chromatin immunoprecipitation assay (ChIP) can be successfully deployed for measuring Pol II gene binding using sheared, formalin-fixed urinary chromatin samples (23,25). Using this approach, we assessed degrees of Pol II binding to exon 1 of the HMGCR gene in urinary pellets obtained from the AKI− and AKI+ patients and from normal volunteers. In brief, the urine samples were centrifuged and the pellets were fixed in formalin. The chromatin was then sheared, and RNA polymerase II binding to the start exon (exon 1) of the HMGCR

gene was assessed by ChIP assay (23,25). The results were factored by the amount of Pol II binding to exon 1 of the β actin "housekeeping" gene.

Assessment of Chromatin Remodeling at the HMGCR Gene

We have previously demonstrated that one potential reason for increased HMGCR gene activity in response to AKI is the induction of "gene activating" histone modifications at the HMGCR gene (10). This allows for enhanced Pol II-gene binding and, hence, increased transcription. One notable example of this altered histone profile is an increase in the amount of trimethylated histone H3 at the lysine 4 position, yielding H3K4m3 (10). Thus, we sought clinical confirmation of our previous experimental findings by applying ChIP assay to urinary pellet chromatin fragments. To this end, the chromatin fragments generated for the above Pol II binding experiments were probed for H3K4m3 levels at HMGCR exon 1 by ChIP assay. The results were factored by H3K4m3 levels at the β actin gene.

Calculations and Statistics

All values are presented as means ± 1 SEM, unless stated otherwise. Comparisons were made by unpaired *t* test with Bonferroni corrections for multiple comparisons. Statistical significance was judged at a *P* value of <0.05.

Results

Clinical Characteristics of ICU Patient Cohorts

Clinical characteristics of the patients who formed the ICU/AKI− and ICU/AKI+ cohorts are presented in Table 1. Other than significantly higher serum creatinine levels and AKIN scores at enrollment for the AKI+ *versus* AKI− group, no other statistically significant differences existed between these two groups (overlapping 95% confidence intervals). Of particular note, the overall illness severity (as assessed by APACHE II scores) and the frequency of sepsis syndrome for the two groups were highly comparable.

Pol II Binding and Histone 3 Lysine 4 Trimethylation at Exon 1 of the HMGCR Gene

The results of the urine pellet chromatin assessments are presented in Figure 1. As shown in the left-hand panel, Pol II binding to exon 1 of the HMGCR gene was approximately fourfold higher in the AKI+ *versus* the AKI− group ($P < 0.025$). However, even the AKI− group had a modest increase in Pol II-HMGCR binding ($P < 0.025$) compared with control urine chromatin samples (consistent with sepsis-induced activation of the HMGCR gene, as previously shown in experimental sepsis (7).

In addition to increased AKI induced Pol II binding, a doubling of the gene-activating mark, H3K4m3, was observed at exon 1 of the HMGCR gene compared with that observed in either the control or AKI− patient urine samples (Figure 1, right panel; $P < 0.03$). Indeed, the H3K4m3 levels were essentially identical for the control and ICU / AKI− groups.

Urine Pellet Cholesterol Levels

As shown in Figure 2, the ICU/AKI– patient population had essentially the same urinary pellet cholesterol/phospholipid phosphate ratios to those observed in the control

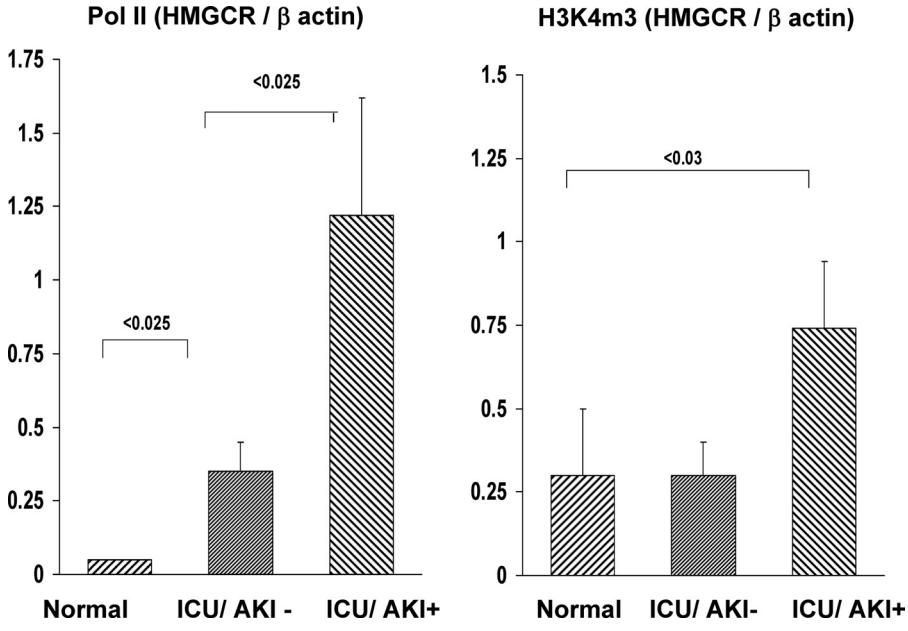

Figure 1. | Patients with AKI manifest increased levels of RNA polymerase II (Pol II) and of histone H3 lysine 4 trimethylation (H3K4m3) at exon 1 of HMG–CoA (HMGCR) gene in urine chromatin samples. Urine pellet samples from normal subjects, intensive care unit/acute kidney injury (ICU/AKI)+ patients, and ICU/AKI− patients were probed for Pol II and H3K4m3 at exon 1 of the HMGCR gene (ChIP assay; all results factored by amounts of Pol II and H3K4m3 at exon 1 of the house keeping gene β-actin). Increased Pol II levels were observed in both ICU cohorts, *versus* normal controls, but the levels were approximately fourfold greater in the AKI+ patients *versus* the AKI− controls. The AKI+ patients had a twofold increase in H3K4m3 levels at the HMGCR gene, whereas no increase was seen in the ICU/AKI− population. Thus, these data support the concept that AKI causes increased expression of the HMGCR gene. Values are presented as means ± 1 SEM.

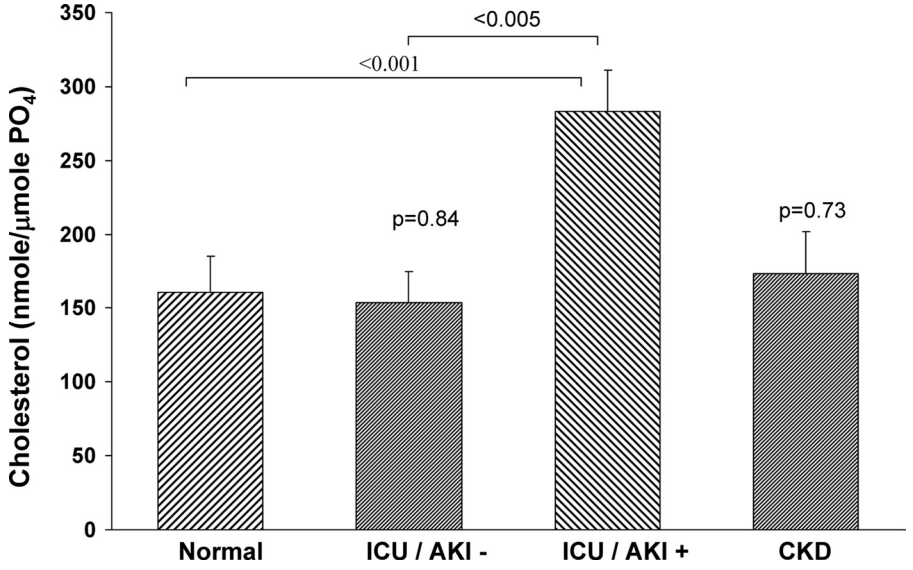

Figure 2. | Urinary pellet cholesterol levels are elevated in patients with acute kidney injury (AKI) but not in critically ill intensive care unit (ICU) patients without AKI or in patients with chronic kidney disease (CKD). Urine samples from 15 normal volunteers, 15 ICU patients without AKI, 14 ICU patients with AKI, and 15 patients with CKD were assayed for urinary pellet cholesterol levels. The results were factored for lipid extract phospholipid phosphate levels. The AKI+ patients had an approximate doubling of pellet cholesterol levels, whereas the AKI− and CKD patients manifested normal levels (*versus* normal values).

urine samples. Conversely, the ICU/AKI+ patients had an approximate doubling of pellet cholesterol levels ($P < 0.005$). The urine creatinine concentrations for the AKI− and AKI+ groups did not statistically differ (100 ± 16 mg/dl and 137 ± 26 mg/dl, respectively, $P = 0.23$). There was no significant relationship between the urine creatinine concentrations and the pellet cholesterol/phospholipid ratios in the AKI+ group ($r = 0.14$). This is consistent with the fact that creatinine excretion reflects GFR, whereas pellet cholesterol levels reflect tubular cell cholesterol loading and tubular cell membrane sloughing. Of note, however, the urine pellet cholesterol levels did significantly correlate with the serum creatinine concentrations ($r = 0.5$; $P < 0.01$), suggesting that it could potentially be a reflection of tubular injury severity.

As shown in Figure 2, the CKD patients did not manifest any increase in urinary pellet cholesterol content compared with control urine pellet values. Of interest, eight of the 15 CKD patients were receiving statin treatment, and yet their urinary pellet cholesterol levels were slightly, but not statistically, higher than values observed in non-statin-treated CKD patients (206 ± 40 *versus* 128 ± 34, respectively; $P = 0.17$).

Discussion

The results of this study provide clinical support for a sequence of events that has been noted in experimental studies of AKI, namely, that (*1*) acute tubular injury activates the HMGCR gene; (*2*) this results in increased cholesterol synthesis and proximal tubular cell cholesterol loading; and (*3*) upon release of tubular cells and tubular cell debris into the urinary space, increases in urinary pellet cholesterol content result. We have previously demonstrated that, unlike AKI, experimental *chronic* nephropathy does not activate these pathways (29). Thus, we hypothesized that an increase in urinary pellet cholesterol might have utility as an AKI biomarker.

To test these hypotheses, we sought evidence for increased HMGCR activity in patients with AKI by measuring degrees of RNA polymerase II binding to urinary chromatin fragments of the HMGCR gene. As shown in the left-hand panel of Figure 1, a marked increase in Pol II binding was observed in the AKI+ patients compared with that observed in either the control or ICU/AKI− urine samples. Because the degree of Pol II-gene binding correlates with rates of gene transcription, this finding provides clinical support for the experimental observation that AKI increases HMGCR gene activity. That a gene activating histone mark, H3K4m3, was also elevated at the HMGCR gene in the AKI+, but not in the AKI−, patient urine samples further supports the concept that AKI increases HMGCR gene transcription.

With this information in hand, we directly tested the hypothesis that an increase in urinary pellet cholesterol levels might be found in clinical AKI and that these values would be able to differentiate AKI *versus* critical illness without AKI, and *versus* CKD. As shown in Figure 2, this appears to be the case. Patients with AKI had an approximate doubling of urinary pellet cholesterol content compared with values observed in control urine samples. Conversely, critically ill patients without AKI manifested normal pellet cholesterol levels. Furthermore, urine pellet cholesterol values were normal in the patients with CKD. Noteworthy in regard to this latter finding is that many urinary protein AKI biomarkers, *e.g.*, NGAL, KIM-1, and MCP-1, are typically elevated with both acute as well as chronic kidney disease (26–28). That pellet cholesterol levels were elevated in the AKI+, but not in the CKD, cohort suggests potential differential diagnostic utility in this regard.

Of note, statin therapy, which is common in patients with CKD, could theoretically be a confounding variable in interpreting urinary pellet cholesterol levels. However, we have previously demonstrated that statins do not alter renal cortical cholesterol content (8). Indeed, eight of the 15 CKD patients in the present study received statin therapy, and yet their urinary pellet cholesterol levels tended to somewhat higher (albeit nonsignificantly), not lower, than those found in the non-statin-treated CKD patients (see Results). Thus, it appears most unlikely that statin treatment would negate the potential utility of pellet cholesterol levels as a useful AKI biomarker.

Conclusions

In sum, these studies provide clinical support for a novel concept that has emerged from our experimental studies of AKI: that AKI activates the HMGCR/cholesterol axis and that, with release of tubular cells and tubular cell debris into urine, increases in urinary pellet cholesterol levels result. Particularly noteworthy in this regard are the following: (*1*) prior experimental data demonstrate that pellet cholesterol levels rise within 3 hours post-AKI induction, indicating potential utility in detecting early AKI; (*2*) pellet cholesterol levels remain normal with experimental prerenal azotemia; and (*3*) pellet cholesterol levels appear to be able to differentiate patients with acute *versus* chronic renal disease. Of note, the current emphasis in AKI biomarker research is to develop a *panel* of biomarkers that reflect differing injury-induced pathways. Thus, the addition of a *lipid* biomarker to a variety of urinary *protein* biomarkers might be quite useful in this regard. It is premature to draw conclusions as to the clinical utility of measuring urinary pellet cholesterol levels. Clearly, this will require a large prospective clinical trial to assess. However, this study does provide clinical support for conclusions derived from our previous experimental AKI studies, *i.e.*, that AKI activates the HMG–CoA reductase gene and that renal tubular cholesterol loading results. Whether these "bench to bedside" observations will ultimately find clinical application, either in terms of deploying a new biomarker or in terms of better understanding AKI pathogenesis, remains to be defined.

Acknowledgments

We thank Mr. John Ruzinski and Mr. Ernie Ayers, Kidney Research Institute, Seattle; Ms. Nancy Wickersham, Vanderbilt University; and Ms. Jill Corson, Puget Sound Blood Center, Seattle, for their assistance with acquiring test urine samples. We also thank Ms. Kirsten Becker for her technical assistance. This work was supported by grants from the National Institutes of Health (DK384342; R21-DK083315; HL081332; HL-103836; DK082192, and HL 070938), and from nonrestricted research funds from the Kidney Research Institute, Seattle.

Disclosures

None.

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Received: March 15, 2011 **Accepted:** May 18, 2011

Published online ahead of print. Publication date available at www.cjasn.org.