Renal Cholesterol Accumulation

A Durable Response after Acute and Subacute Renal Insults

Richard A. Zager,* Takishi Andoh,[†] and William M. Bennett[‡]

From the Department of Medicine,* Fred Hutchinson Cancer Center, University of Washington, Seattle, Washington; and the Department of Solid Organ and Cellular Transplantation,[†] Legacy/Good Samaritan Hospital, Portland, Oregon

Proximal tubular cholesterol levels rise within 18 hours of diverse forms of acute renal tubular injury (eg, myoglobinuria, ischemia/reperfusion, urinary tract obstruction). These increments serve to protect against further bouts of tubular attack (so-called “acquired cytoresistance”). Whether these cholesterol increments are merely transitory, or persist into the maintenance phase of acute renal failure (ARF), has not been previously defined. Furthermore, whether subacute/insidious tubular injury [eg, cyclosporine A (CSA), tacrolimus toxicity], nontubular injury (eg, acute glomerulonephritis), or physiological stress (eg, mild dehydration) impact renal cholesterol homeostasis have not been addressed. This study sought to resolve these issues. Male CD-1 mice were subjected to glycerol-induced ARF. Renal cortical-free cholesterol (FC) and cholesterol ester (CE) levels were determined 3, 5, 7, or 14 days later, and the values contrasted to prevailing blood-urea nitrogen concentrations. The impact of 40 minutes of unilateral renal ischemia plus reflow (3 to 6 days) on mouse cortical FC/CE content was also assessed. Additionally, FC/CE levels were measured in rat renal cortex either 10 days after CSA or tacrolimus therapy, or 48 hours after induction of nephrotoxic serum nephritis. Finally, the impact of overnight dehydration on mouse renal cortical/medullary FC/CE profiles was determined. Compared to sham-treated animals, glycerol, CSA, tacrolimus, ischemia-reperfusion, and nephrotoxic serum each induced dramatic CE $\pm$ FC elevations, rising as much as 10 $\times$ control values. In the glycerol model, striking correlations ($r \leq 0.99$) between FC/CE and blood-urea nitrogen levels were observed. The FC/CE increases were specific to damaged kidney (glycerol did not raise hepatic FC/CE; unilateral renal ischemia did not alter contralateral renal FC/CE levels). Overnight dehydration raised renal CE levels, most notably in the medulla. **Conclusions:** FC/CE accumulation is a hallmark of the maintenance phase of ischemic and nephrotoxic ARF, and can

reflect its severity. That cholesterol accumulation can result from glomerular injury and dehydration suggests that it is a generic renal stress response, with potential relevance extending beyond just the phenomenon of acquired cytoresistance. (Am J Pathol 2001, 159:743–752)

In the aftermath of acute sublethal cell injury, a stress response occurs that confers partial resistance against further attack. The renal correlate of this phenomenon is known as “acquired resistance to acute renal failure,”¹ and it serves to protect the kidney from subsequent ischemic or toxic damage.^{2–11} Previously defined characteristics of this state include the following: 1) a lag time of $\sim$ 18 to 24 hours after the first renal insult must transpire before acquired resistance is expressed;^{7,9–11} 2) the protection is broad-based, such that diverse toxic, physical, or ischemic injuries can induce nonspecific protection against a broad variety of secondary insults;^{9–11} 3) it is expressed directly at the proximal tubule cell level, as evidenced by the fact that proximal tubules extracted from cytoresistant kidneys are resistant to *in vitro* damage;^{9–11} 4) the protection may not be dependent on *de novo* protein synthesis, given that protein synthesis inhibition does not prevent the emergence of cytoresistance;¹² and 5) the protection is expressed at the plasma membrane and mitochondrial levels. This is indicated by the fact that plasma membranes obtained from cytoresistant isolated tubules are relatively resistant to oxidant- and PLA₂-mediated injury,¹¹ and that corresponding mitochondria generate less free radicals during oxidant stimulated attack.¹³

In search of potential mechanism(s) for renal cytoresistance, this laboratory has found that within 18 to 24 hours after heterogeneous forms of *in vivo* or *in vitro* renal tubular cell damage, increased cellular cholesterol content develops.^{14,15} Linking these increases to acquired cytoresistance are the following observations: 1) if cholesterol levels are reduced to normal in cytoresistant tubules by chemical extraction, cytoresistance is lost;¹⁴ 2)

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Address reprint requests to Richard A. Zager, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N, Room D2-190, Seattle, WA 98109. E-mail: dzager@fhcrc.org.

lowering cholesterol by ~20% in normal cells via differing mechanisms (eg, statins, enzymatic attack) exaggerates cellular vulnerability to toxic or hypoxic damage;¹⁴ 3) chemical modification of cellular cholesterol, either via cholesterol oxidase or cholesterol esterase, causes profound mitochondrial dysfunction and cell death;¹⁶ and 4) the emergence of elevated cholesterol levels and cytoresistance temporally correlate, each being first apparent at 18 hours after the initial renal insult.¹⁴ In sum, then, considerable data indicate that cholesterol accumulation is a generic response to acute tubular injury, and can contribute to the cytoresistant state.

To date, cholesterol elevations after injury have only been documented at one time point: 18 to 24 hours after ischemic or toxic injury. However, it is well known that the state of acquired cytoresistance can persist for days to several weeks after the induction of acute renal damage.^{1,5} If cholesterol levels are, in fact, mechanistically linked to acquired cytoresistance, then one would assume that renal cortical/tubular cholesterol elevations are a durable response. Conversely, if previously documented cholesterol increases rapidly dissipate, eg, with 24 to 48 hours, this would suggest that cholesterol accumulation is only an acute stress reaction with little or no long-term implications for a postinjury state.

To shed light on this issue, in this study we have now performed a time course experiment in which renal cortical cholesterol profiles have been monitored from 3 days to 2 weeks after the induction of acute myohemoglobinuric acute renal failure (ARF), and 3 to 6 days after ischemic ARF. Additionally, we have addressed whether more insidious models of tubular injury [cyclosporine A (CSA) or tacrolimus nephrotoxicity], and a nontubular model of renal injury [acute nephrotoxic serum (NTS), anti-glomerular basement membrane, glomerulonephritis] also evoke cholesterol accumulation. Finally, we have sought to address whether a physiological, and not simply pathological, stress might also impact renal cholesterol expression. To this end, the impact of overnight dehydration on renal cholesterol levels has been determined. The result of these investigations forms the basis for this report.

Materials and Methods

Glycerol-Induced ARF

Male CD-1 mice, weighing 25 to 35 g (Charles River Laboratories, Wilmington, MA), and maintained under normal vivarium conditions with free access to food and water, were used for all experiments. On the day of experimentation, they were lightly and momentarily anesthetized with isoflurane, and then injected with 50% glycerol (total, 8.5 ml/kg) into each upper hind limb in equally divided doses. The mice were then returned to their cages and allowed continued free access to food and water. Either 3, 5, 7, or 14 days later ($n = 6, 6, 5$, and 4, respectively), the mice were deeply anesthetized with pentobarbital (~2 mg/kg; intraperitoneally). They were subjected to a midline abdominal incision, blood was

withdrawn from the inferior vena cava for blood urea nitrogen (BUN) analysis, and then the kidneys were removed. A piece of renal cortex was obtained from each kidney for subsequent free cholesterol (FC) and cholesterol ester (CE; a cholesterol storage form) analysis (see below). In addition, phospholipid profiles were assessed (see below). An equal number of normal kidneys, obtained from sham-treated normal mice, were processed simultaneously to provide control FC/CE and BUN concentrations.

Hepatic Cholesterol Levels after Glycerol-Induced ARF

The following experiment was undertaken to ascertain whether cholesterol changes after renal injury were relatively renal-specific, or whether they were part of a generalized, systemic response to ARF. To this end, hepatic tissues were collected 72 hours after glycerol injection (described above). Three normal mice provided normal hepatic tissues. These tissues were processed for FC and CE, as described below.

Postischemic Renal Damage

Although cholesterol increments have been noted during the early maintenance phase (<24 hours after injury) of postischemic ARF,^{14,17} whether these changes persist into the late period after injury has not been determined. To this end, eight mice were anesthetized with pentobarbital (~2 mg intraperitoneally), a midline abdominal incision was made, and then half of the mice were subjected to 40 minutes of left renal pedicle vascular occlusion (the right kidney was left alone to prevent subsequent death from uremia). The remaining four mice were subjected only to sham left renal pedicle occlusion. The animals were then sutured and allowed to recover from anesthesia. At either 3, 4, 5, or 6 days after surgery, one experimental (unilateral ischemia) and one control mouse were re-anesthetized, and both kidneys were removed. The renal cortices of the postischemic left kidneys, the contralateral right kidneys, and the left kidneys from the sham-operated mice were removed, the cortices isolated, and analyzed for FC and CE levels.

CSA- and Tacrolimus-Mediated Nephrotoxicity

Previously well-described models of CSA^{18,19} and tacrolimus²⁰ nephrotoxicity, performed with adult male Sprague-Dawley rats (Charles River, Wilmington, MA, 200 to 250 g), were used. They were housed in individual cages under standard vivarium conditions and fed a low-salt diet (0.05% Na; Teklad Premier, WI) with free access to water. After 7 days on this diet, weight-matched pairs of rats were randomized into four groups: 1) CSA treatment: 15 mg/kg [times 10 days, $n = 6$ rats (Sandimmune oral solution; Novartis Pharmaceuticals, East Hanover, NJ; dissolved in olive oil, 15 mg/ml); 2) CSA vehicle-treated controls ($n = 3$ rats); 3) tacrolimus

treatment: 1 mg/kg $\times$ 10 days, $n = 6$ rats (Prograf injection; Fujisawa Health Care; Deerfield IL; dissolved in sterile water 1 mg/ml); and 4) tacrolimus controls ($n = 3$). After 10 days of treatment, the rats were anesthetized with intraperitoneal ketamine. A blood sample was obtained for BUN and then the kidneys were removed. One kidney was saved for FC/CE and phospholipid analysis, as noted below. For statistical analysis, the two control groups were combined (as no significant differences between them).

NTS Nephritis

Previous studies from this⁸ and another laboratory³ have demonstrated that acquired cytoresistance, as expressed at the tubular cell level, can be induced by acute anti-glomerular basement membrane-mediated glomerulonephritis. However, it has not previously been ascertained whether this form of cytoresistance is associated with altered cholesterol expression. To address this issue, three Sprague-Dawley rats (~250 g) were lightly anesthetized with ether and injected via tail vein with 0.2 ml/kg of anti-glomerular basement membrane NTS.²¹ Three additional rats injected with an equal amount of nonimmune serum served as controls. From 36 to 48 hours after serum injection, a timed urine collection was completed to assess proteinuria, using the sulfosalicylic acid method.²² At the completion of the experiment, the rats were deeply anesthetized with pentobarbital, both kidneys were resected, and the cortices from each rat were dissected, combined, and frozen (1 sample per animal). The tissues were then processed for cholesterol analysis, as noted below.

Cortical/Medullary Cholesterol and CE Analysis

The following experiment was undertaken to assess whether a physiological, rather than a pathophysiological, stress might also alter renal FC/CE profiles. To this end, 14 mice were divided into two groups: normal mice ($n = 7$) and mice subjected to overnight dehydration (withdrawal of water, but not food; $n = 7$). The next morning, the mice were anesthetized with pentobarbital, and both kidneys removed. Cortical and medullary tissues were dissected from each kidney. They were then processed for FC and CE to address two questions: 1) does a cortical-medullary cholesterol gradient exist within the kidney, consistent with the normally occurring osmotic gradient? and 2) if so, does increasing medullary osmolality increase medullary cholesterol profiles?

Tissue Cholesterol Analysis

The kidneys were placed on an iced plate and cortical tissue samples were dissected using a razor blade. The samples were weighed, added to four parts cold methanol, homogenized, and extracted in chloroform:methanol (1:2) as previously described in detail.^{15,16} Hepatic tissues were treated in the same manner. The extracts were dried under N_2 and saved for FC and CE analysis by gas

chromatography.¹⁵ In brief, the dried lipid extracts obtained from the above experiments were reconstituted in 1 to 2 ml of hexane, followed by sonication and vortexing to dissolution. To analyze FC, a 100- μ l sample of each extract was transferred to a glass culture tube containing 50 μ l of an internal standard solution (stigmastanol, 100 μ g/ml in ethyl acetate, EtOAc). The sample was dried under N_2 and reconstituted in 100 μ l of bis-(trimethylsilyl)trifluoroacetamide [BSTFA; Sigma (St. Louis, MO); 25% v/v EtOAc]. They were then transferred to an injection vial, sealed, and heated for 1.0 hours at 60°C. After completion of BSTFA derivatization, a 1- μ l sample was applied to a Hewlett Packard 5890 Series II gas chromatograph fitted with a flame ionization detector and a 30 m $\times$ 0.32 mm DB-5 (0.25 μ m) column (J&W Scientific, Folsom, CA). The initial temperature (100°C) was maintained for 3 minutes, after which it was increased by 40°C/minute to 290°C, and thereafter by 5°C/minute to 300°C for 5 minutes. The trimethylsilyl ether of cholesterol eluted at 12.5 minutes and that of the internal standard at 13.6 minutes. To quantify CEs, they were first separated from the FC pool. This was achieved by adding 400- μ l samples of the hexane aliquots to an amino solid-phase extraction column (3 ml, 500 mg; Varian Bond Elut, Varian, Harbor City, CA) previously washed with hexane. The column eluant, containing CE, but not FC, was saved.¹⁵ After two subsequent column washings, all of the eluants were combined, the internal standard was added, and the sample was evaporated to dryness. The esters were hydrolyzed in EtOH/30% KOH at 55°C for 45 minutes.¹⁵ The samples were added to 2.0 ml of water, and then 3.0 ml of hexane were added. The hexane phase was dried under N_2 and then assayed for FC, as above. Previously performed validation studies confirmed the complete absence of any contaminating FC within the CE eluant, and 100% efficiency in CE recovery.¹⁵ Hence, the amount of recovered FC resulting from CE hydrolysis was taken as the amount of CE present in the original sample. FC and CE results were each expressed as nmol/ μ mol phospholipid phosphate (P_i) in the initial lipid extract.¹⁵

Phospholipid Analysis

To assess whether changes in membrane phospholipids accompanied changes in cholesterol expression, renal cortical lipid extracts from all glycerol-treated mice, post-ischemic mice, tacrolimus/CSA-treated rats, NTS nephritis rats, and each group's corresponding controls were analyzed for phospholipids by two-dimensional thin layer chromatography, as previously described in detail.²³ In brief, the five dominant plasma membrane phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, or sphingomyelin) were assessed. Individual phospholipids were quantified by scraping the individual spots from the silica thin-layer chromatography plates and measuring their phosphate content.²³ Results were expressed as the percent to which each phospholipid contributed to the total phospholipid mass (the sum of phosphatidylcholine plus phosphatidylserine plus phosphatidylethanolamine plus

Figure 1. BUN concentrations at 3, 5, 7, or 14 days after induction of glycerol-mediated myohemoglobinuric ARF. The **closed bars** and **shaded bars** represent glycerol-treated and control mice, respectively. Decreasing azotemia is noted in the glycerol-treated mice, with near recovery being observed by 14 days after glycerol injection.

phosphatidylinositol plus sphingomyelin). By so doing, relative changes in membrane phospholipids could be assessed and contrasted with changes in cortical cholesterol content.

Calculations and Statistics

All values are given as means $\pm$ 1 SEM. Statistical comparisons were made by either paired or unpaired Student's *t*-test: paired analysis was used if left *versus* right kidney values from a given set of animals was used; unpaired analysis was used for comparison between values obtained from different animals. If more than one comparison with any set of data were made, the Bonferroni correction was applied.

Results

Time Course of ARF after Glycerol

As shown in Figure 1, glycerol-induced myohemoglobinuria induced severe azotemia, as first assessed 3 days after glycerol injection. With each subsequent time point, the severity of azotemia decreased, but remained significantly elevated compared to simultaneous controls. There was considerable variability in the severity of azotemia in the postglycerol-treated mice, with coefficients of variation being 60, 58, 90, and 28% at 3, 5, 7, and 14 days, respectively, after the induction of ARF. These differences allowed for a comparison between the degree of azotemia *versus* the extent of FC/CE changes, as discussed immediately below.

Cholesterol Levels after Glycerol-Induced Renal Injury

At each assessed time point, both CE (Figure 2), and in most instances FC (Figure 3), were dramatically higher in the postglycerol *versus* the control renal tissues. The greatest relative increase was in CE, which for the first 7

Figure 2. CE levels corresponding with the time-course experiment presented in Figure 1. The **closed bars** and **shaded bars** represent the glycerol and control mice, respectively. The *P* values represent unpaired Student's *t*-test analysis at each time point. Within each glycerol-treated group, there was significant variation in CE and BUN values: the *r* values illustrate striking positive correlations between these two parameters at each time point. Individual values for the day 7 and day 14 results are presented in Table 1.

days reached values that were $\sim$ 10 times higher than was observed in control tissues. The CE/FC elevations persisted almost completely unabated for the first 7 days. At day 14, CE levels still remained elevated, whereas FC had returned to near normal values.

A striking relationship existed between the degree of azotemia in each postglycerol-treated mouse and the degree of FC/CE elevations (see *r* values presented above each set of bars in Figures 2 and 3). This relationship was particularly noteworthy at 7 and 14 days, where *r* values of ≥ 0.90 were observed. This relationship is further presented in Table 1 (individual postglycerol BUN, FC, and CE values at day 7 and day 14 are presented).

Hepatic Cholesterol Levels after Glycerol Injection

At 3 days after glycerol injection, the BUNs for the three test mice were 76, 98, and 220 mg/dL (compared to BUNs of 25, 28, and 34 for their normal controls). Despite the severe ARF in the postglycerol mice, no significant elevation in hepatic FC or CE was observed (FC: 120 ± 8 *versus* 132 ± 21 ; CE: 12.5 ± 2.7 *versus* 13.3 ± 1.3 ; control *versus* postglycerol group, respectively). This was de-

Figure 3. FC levels corresponding with the time-course experiment presented in Figure 1. The results with FC analysis primarily mimicked those observed with CE analysis (see Figure 2 legend for explanations).

Table 1. Correlation between the Severity of Azotemia and Cholesterol/Ester Accumulation

BUN, mg/dL	Free cholesterol (nmol/ μ mol Pi)	Cholesterol ester (nmol/ μ mol Pi)
Day 7		
36	300	15.4
46	299	19.4
58	338	21.8
66	364	36.8
224	492	97.6
	$r = 0.98$	$r = 0.99$
Day 14		
33	250	4.9
44	273	10.8
56	267	13.0
66	353	34.0
	$r = 0.93$	$r = 0.90$

This table presents individual BUN, free cholesterol, and cholesterol ester values observed in each individual mouse obtained at either 7 or 14 days after induction of glycerol-mediated ARF. The values are arranged in the order of rising BUNs. As can be noted, there is a striking correlation between the extent of azotemia and cholesterol/cholesterol ester elevations.

spite the fact that there was a dramatic elevation in renal cholesterol pools in these same postglycerol-treated animals (FC, 213 ± 7 versus 319 ± 25 ; $P < 0.025$; CE: 4.8 ± 1 versus 39.3 ± 11 ; $P < 0.025$; controls versus postglycerol, respectively). It was noteworthy that normal kidney and liver had differing FC/CE profiles: liver had $\sim 50\%$ lower ($P < 0.01$) FC, and an approximate fourfold higher ($P = 0.05$) CE content (values given above). This indicates that in liver, a relatively greater amount of cholesterol is stored in the CE pool.

Cholesterol/CE Values after Unilateral Renal Ischemia

As shown in Figure 4, both FC and CE were dramatically elevated in the left, postischemic kidneys that were harvested 3 to 6 days after surgery (all values at each time point are presented as a single postischemia group). This was true whether the postischemic values were con-

Figure 4. FC and CEs in sham-operated control (cont) mice, and in mice subjected to left (Lt) renal ischemia with the contralateral right (Rt) kidney left in place. Induction of unilateral left renal ischemia induced profound elevations in both FC and CE values, as assessed 3 to 6 days after ischemia (all postischemic values treated as a single group; see text). Note that there was no difference in FC/CE values between kidney samples obtained from the sham-operated (cont) group and the right kidney harvested from mice with left renal ischemia. Thus, the presence of left renal ischemic/reperfusion injury did not impact contralateral CE/FC values.

Figure 5. FC and CE values in rat kidneys after 10 days of either CSA or tacrolimus (Tacr.) therapy. Both CSA and tacrolimus (Tacr.) induced dramatic CE increments in renal cortex, compared to vehicle-treated controls (cont). In contrast, only small, but statistically significant, increases in FC values were observed (*, $P < 0.03$; Δ , $P < 0.05$ versus controls).

trasted to their own right (Rt) nonischemic kidney, or to kidneys harvested from sham-operated control (cont) mice. Each individual FC and CE value was elevated irrespective of whether the kidneys were harvested at 3, 4, 5, or 6 days postischemia (ie, each $>99\%$ confidence band for control values).

It is notable that the right, nonischemic, kidney cholesterol/CE values were not higher than values obtained from normal kidneys (obtained either from sham-operated mice or from completely normal mice, eg, the controls used for the glycerol experiments). This indicates that surgical stress, *per se*, or postoperative weight loss that results from it or from ARF, cannot explain the increase in renal cholesterol accumulation observed in these experiments.

Cholesterol/CE Values after CSA and Tacrolimus Treatment

Ten days of CSA or tacrolimus each caused only slight, but significant, increases in FC content (Figure 5, left). Far more dramatic CE elevations were observed with each agent, reaching values that were ~ 8 to $10\times$ those observed in control tissues (Figure 5, right). (Note: this is consistent with the fact that a large increase in cholesterol storage, ie, ester formation, can be observed under conditions of only slight increases in FC levels.) Both CSA

Figure 6. FC and CE at 48 hours after induction of NTS nephritis. NTS (anti-glomerular basement membrane antibody) mediated glomerulonephritis caused a striking increase in CE levels. Although FC levels were slightly higher as well, this did not achieve statistical significance.

Table 2. Phospholipid Analysis during the Maintenance Phase (Day 3) of Glycerol-Induced ARF

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 6)	12.2 (1.1)	46.6 (0.5)	8.0 (0.4)	6.5 (0.3)	26.8 (1.3)
Glycerol (<i>n</i> = 6)	15.9 (1.6)	48.5 (0.3)	8.4 (0.2)	5.1 (0.2)	22.2 (1.3)
<i>P</i>	<0.005	<0.02	NS	<0.01	<0.001

This table presents the percentage to which each individual phospholipid contributed to the total documented phospholipid mass (the sum of each of the five phospholipids presented). Values = means (1 SEM in the parentheses).

SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

The assessments were made at 3 days after glycerol injection. *n*, number of mice in the control and experimental groups.

and tacrolimus induced mild azotemia during the course of the experiments (controls: 17 ± 1 mg/dL; CSA, 31 ± 1 mg/dL; tacrolimus, 29 ± 1 mg/dL; each group, $P < 0.01$, versus the control group).

NTS Nephritis

As shown in Figure 6, injection of NTS caused a profound CE increase, reaching values that were approximately four times those seen in control rats. Conversely, only a very small, and nonsignificant ($P = 0.08$), increase in FC levels was observed. NTS injection caused marked proteinuria, with protein excretion rates of 1.25 ± 0.25 and 23.5 ± 0.6 mg/hour being observed in control and NTS-treated rats, respectively ($P < 0.0001$).

Phospholipid Assessments after Renal Injury Protocols

Glycerol-Induced ARF in the Mouse

At 3 days after glycerol injection, a significant increase in percent sphingomyelin content was observed (Table 2). This was associated with reciprocal decreases in phosphatidylserine and phosphatidylethanolamine content. The relative increase in sphingomyelin was also noted at 5 days after glycerol injection (Table 3), and dissipated thereafter (Tables 4 and 5).

CSA/Tacrolimus-Treated Rats

Neither CSA nor tacrolimus induced any detectable change in phospholipid expression (Table 6). Thus, the cholesterol changes were observed in the absence of any other discernible change in membrane lipids (although it is recognized that small, but significant differences might have been observed had a larger number of samples been assessed).

NTS Nephritis in the Rat

As with the tacrolimus- and CSA-treated rats, the NTS nephritis-induced cholesterol increments were unaccompanied by any discernible changes in membrane phospholipids (Table 7). (As noted above, it remains possible that small, but significant, differences might have been observed had a larger number of samples been assessed).

Postischemic Renal Injury in the Mouse

Despite dramatic cholesterol increments, no major changes in phospholipid profiles were observed. The only significant difference was a slight, but significant, decrease in percent phosphatidylserine expression, relative to the other phospholipids (see Table 8).

Cortical and Medullary Cholesterol/CE Levels Plus Dehydration

As shown in Figure 7, a cortical-medullary CE gradient was apparent in normal mouse kidney, with medullary values being $\sim 40\%$ higher than those observed in renal cortex ($P < 0.001$). With overnight dehydration, both cortical and medullary CE levels increased, compared to control values ($P < 0.001$). However, there was a preferential increase within the medulla: in the normal kidneys, the absolute difference between medullary versus cortical CE values was 1.67 ± 0.3 nmol/ μ mole Pi; in contrast, the difference in values for the dehydrated mice was 2.7 ± 0.2 nmol/ μ mole Pi; ($P < 0.01$).

In contrast to CE, there was no gradient in FC values within the kidney (cortex, 246 ± 6 nmol/ μ mole Pi; medulla, 252 ± 8 nmol/ μ mole Pi; NS). These values did not significantly change with dehydration (233 ± 3 and 236 ± 4 nmol/ μ mole Pi; respectively; NS).

Table 3. Phospholipid Analysis during the Maintenance Phase (Day 5) of Glycerol-Induced ARF

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 4)	13.0 (0.6)	41.9 (2.2)	9.4 (0.7)	8.7 (1.1)	27.0 (1.4)
Glycerol (<i>n</i> = 4)	16.8 (0.4)	39.5 (1.7)	10.7 (0.6)	9.4 (1.0)	25.5 (1.2)
<i>P</i>	<0.005	NS	NS	NS	NS

As per Table 2 legend, with the assessments being made at 5 days after glycerol injection.

Table 4. Phospholipid Analysis during the Maintenance Phase (Day 7) of Glycerol-Induced ARF

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 6)	12.6 (0.8)	43.2 (0.5)	7.6 (0.3)	7.9 (0.5)	28.7 (0.4)
Glycerol (<i>n</i> = 6)	12.9 (1.2)	44.8 (1.0)	7.7 (0.2)	8.9 (1.4)	25.7 (0.9)
<i>P</i>	NS	NS	NS	NS	<0.025

As per Table 2 legend, with the assessments being made at 7 days after glycerol injection.

Discussion

Acute ischemic and toxic renal injury have long been recognized to induce acute alterations in plasma membrane phospholipid expression, the most notable change being phospholipid deacylation with concomitant lysophospholipid and free fatty acid accumulation.^{23–25} Because the phenomenon of acquired cytoresistance is expressed at the plasma membrane level,¹¹ we have previously questioned whether some fundamental change in membrane phospholipid composition might underlie this state. By analyzing cortical tissues extracted from cytoresistant renal cortex at 18 to 24 hours after injury, only quantitatively trivial and inconsistent changes in membrane phospholipid content have been observed.²³ This caused us to reject the hypothesis that altered plasma membrane phospholipid composition is responsible for the cytoresistant state. Unlike phospholipids, we consistently observed an ~35% increase in membrane ceramide content within cytoresistant renal tissues.^{26–28} However, ceramide, an effector molecule within the sphingomyelin signaling pathway, is generally considered to be a pro-apoptotic, not a survival, factor.^{29,30} Furthermore, when exogenous ceramide has been added to either isolated tubules or to cultured human proximal tubule (HK-2) cells, increased, or in some instances decreased, susceptibility to superimposed hypoxic and toxic injuries have been observed.^{26–29} These considerations caused us to also reject ceramide accumulation as a prime determinant of the cytoresistant state.

Although the above considerations make it unlikely that ceramide is a key determinant of cytoresistance, we have considered its consistent elevation in cytoresistant tissues as a possible clue to more pathogenetically relevant factor(s). Because ceramide is derived from sphingomyelin, and because sphingomyelin tightly associates with cholesterol in membrane microdomains (DRMs, caveolae, rafts),^{31–35} we questioned if cholesterol accumulation might accompany the ceramide increases, and potentially mediate the cytoresistant state. Indeed, in each model of cytoresistance tested to date, a 20 to 40% increase in total cholesterol content has been observed.¹⁴ However, unlike ceramide, cholesterol does

seem to be linked to acquired cytoresistance, based on the following observations: 1) decreasing cholesterol content in normal cells (either by statin-induced synthesis blockade, enzymatic degradation, or chemical extraction) sensitizes them to superimposed hypoxic and toxic injuries¹⁴; and 2) lowering cholesterol levels to normal values in cytoresistant tubules restores normal cellular resistance to attack.¹⁴ It is notable that cholesterol exists within cells either as FC or as CEs. The latter, which normally comprises only ~1 to 2% of total renal cortical cholesterol,¹⁵ has shown the greatest percent increase in cytoresistant tissues, rising as much as 10-fold more than basal values.¹⁵ That specific reductions in cellular CE levels results in profound ATP depletion and lethal cell injury¹⁶ suggest that the CE pool may have prime importance in mediating the cytoresistant state. The mechanism(s) by which increased cholesterol pools enhance cellular resistance to damage remains incompletely defined. However, previous studies from this laboratory suggest that cholesterol's ability to increase plasma membrane rigidity is at least partly responsible.¹⁴

The results of the present studies extend on our understanding of cholesterol accumulation after injury in a number of important ways, as follows:

First, we have documented for the first time that cholesterol increments after injury represent a durable, and not simply, transitory event. The data demonstrate that they persist for at least 2 weeks after glycerol-induced injury, and for at least 6 days after unilateral renal ischemic damage. That the cytoresistant state can persist for days to weeks after injury^{1,5,7} suggests the potential mechanistic importance of these durable cholesterol increments.

Second, the present results are the first to demonstrate a striking correlation between the severity of tissue injury/organ failure and the extent of cholesterol accumulation. This is indicated by the extraordinarily tight correlation between BUN and cholesterol/CE elevations in the post-glycerol ARF model (eg, $r \geq 0.90$ at 7 to 14 days after injury).

Third, the cholesterol elevations seem to be a direct result of tubular injury, and not simply a result of the uremic state. Stated differently, cholesterol accumulation

Table 5. Phospholipid Analysis during the Recovery Phase (Day 14) of Glycerol-Induced ARF

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 4)	14.8 (0.1)	40.6 (0.3)	8.5 (0.3)	8.2 (0.2)	27.9 (0.4)
Glycerol (<i>n</i> = 4)	14.4 (0.6)	41.6 (0.3)	8.7 (0.4)	8.3 (0.5)	28.0 (0.4)
<i>P</i>	NS	NS	NS	NS	NS

As per Table 2 legend, with the assessments being made at 14 days after glycerol injection.

Table 6. Phospholipid Analysis after 10 Days of CSA or Tacrolimus (TLM) Treatment

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 6)	21.1 (0.6)	40.3 (1.4)	8.5 (0.3)	9.7 (0.4)	21.1 (2.0)
CSA (<i>n</i> = 6)	19.9 (1.1)	42.5 (1.3)	8.1 (0.3)	9.1 (0.2)	20.4 (2.0)
Tacr. (<i>n</i> = 6)	20.6 (1.3)	43.2 (1.2)	8.1 (0.4)	9.7 (0.4)	19.3 (2.0)
<i>P</i>	NS	NS	NS	NS	NS

See Table 2 legend. The results for the CSA- or tacrolimus (Tacr.)-treated rats were contrasted to a single control group.

Table 7. Phospholipid Analysis 48 Hours after the Induction of Nephrotoxic Serum Nephritis

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 3)	18.4 (1.0)	35.3 (3.8)	7.9 (0.1)	9.8 (0.2)	28.7 (2.7)
NTS (<i>n</i> = 3)	18.0 (0.4)	37.6 (3.0)	8.9 (0.5)	8.6 (0.3)	26.9 (1.6)
<i>P</i>	NS	NS	NS	<0.03	NS

As per Table 2 legend, comparing the phospholipid results between control *versus* nephrotoxic serum nephritis (NTS) rats.

after injury seems to be an organ-specific, injury-induced, phenomenon. This is underscored by observations that: 1) hepatic cholesterol levels remained normal in uremic (postglycerol-treated) mice that manifested massive renal cholesterol accumulation; and 2) postischemic cholesterol accumulation in mouse kidney was unaccompanied by any cholesterol increase in the contralateral control kidney. Previous observations that unilateral ureteral obstruction also raises cholesterol levels in the absence of uremia¹⁰ further illustrates the dissociation between uremia and tissue cholesterol increments.

Fourth, this is the first study to demonstrate that insidious renal tubular injury (in this case induced by either tacrolimus or CSA) can, like acute massive renal damage (eg, ischemia/reperfusion, rhabdomyolysis), induce cholesterol/ester accumulation. These observations thereby extend the potential importance of altered cholesterol metabolism to a much broader range of renal insults than previously recognized (eg, possibly involving aminoglycosides, radiocontrast agents, NSAIDs, and so forth). Whether cholesterol accumulation during the course of insidious nephrotoxic injury might serve as a defense mechanism that helps stem further tissue damage remains an intriguing, but unresolved, issue as of this time.

Fifth, although both our previous¹⁴ and present data document cholesterol accumulation in response to a variety of direct proximal tubular insults, the present study is the first to demonstrate that glomerular injury can also induce this result. Noteworthy in this regard are previous observations from Nath and colleagues³ and from this laboratory⁸ that the NTS (anti-glomerular basement membrane) nephritis model induces a tubular cytoresistant state. Nath and colleagues³ have ascribed this cytoresis-

tance to heme oxygenase-1 (HO-1) induction. The present results suggest that an up-regulation of cholesterol expression may also be involved. The mechanistic link between glomerular injury and renal cortical cholesterol accumulation, and whether the cholesterol increments are glomerular and/or tubular in location, remain to be defined.

Sixth, the present study underscores that after injury cholesterol increments typically occur in the absence of any obvious, or uniform, change in membrane phospholipid composition, at least as assessed by two dimensional-thin layer chromatography. Although we have previously noted a dissociation of cholesterol accumulation from altered phospholipid expression at 18 to 24 hours after injury, that this same lack of relationship also exists during the maintenance phase of renal injury underscores the relative specificity of the cortical cholesterol changes. Thus, it can now be stated that a hallmark of the cytoresistant state, and the maintenance phase of ARF, is not simply a cholesterol elevation; rather there is an increase in the ratio of cholesterol to individual phospholipids. This would be expected to confer increased membrane rigidity at a time of superimposed tubular cell attack.^{14,36}

Seventh, the present study provides the first evidence that perturbations in cholesterol/CE expression may extend to physiological, and not just pathophysiological, stress. This is evidenced by observations that overnight water deprivation was a sufficient renal challenge to induce a significant increase in both cortical and medullary CE content. It is notable that within the kidney, a cortical-medullary CE gradient exists, and it is enhanced by overnight water deprivation. These two observations suggest that the balance between free and esterified chole-

Table 8. Phospholipid Analysis after Unilateral Renal Ischemia

Group	% SM	% PC	% PI	% PS	% PE
Controls (<i>n</i> = 4)	18.0 (0.7)	40.6 (1.3)	9.6 (0.4)	8.1 (0.2)	23.7 (1.2)
I/R (<i>n</i> = 4)	22.2 (1.9)	43.3 (1.7)	9.6 (0.3)	7.1 (0.5)	17.8 (21.2)
<i>P</i>	NS	NS	NS	NS	<0.01

As per Table 2 legend. The phospholipid results are compared for left kidneys which were subjected to 40 minutes of ischemia + 3 to 6 minutes of reflow, I/R, *versus* the right contralateral control (nonischemic kidney). Not shown, the right kidney results did not differ from kidneys obtained from sham-operated kidneys.

Figure 7. CE levels in mouse renal cortex and medulla under normally hydrated conditions and after overnight dehydration. Under either condition, the medulla demonstrated significantly higher CE values *versus* cortical values (*P* by paired Student's *t*-test). Overnight dehydration significantly increased CE elevations in both cortex and medulla (*P* values by unpaired Student's *t*-test).

terol in renal tissues may be partly under osmoregulation. Although purely speculative, it is interesting to hypothesize that an increase in CE content might confer medullary cytoprotection, helping medullary cells to withstand hyperosmotic stress. That dehydration increased medullary CE content is at least consistent with this concept.

Finally, the mechanism(s) responsible for cholesterol/CE accumulation after cellular stress remain to be completely defined. At least three possibilities exist: increased synthesis, decreased cellular efflux, and/or increased extraction from blood via the low-density lipoprotein receptor.^{37–39} To date, we have demonstrated that statins can completely block cholesterol accumulation in the aftermath of cell injury in cell culture experiments.¹⁵ This indicates an important role for increased synthesis. However, statin therapy does not completely abrogate cholesterol accumulation *in vivo* (unpublished data from this laboratory). This suggests that decreased cellular cholesterol efflux, and/or increased low-density lipoprotein cholesterol uptake, are also likely to be involved. Multiple pathways exist by which of these processes may occur, and their relevance to the present results are subjects of ongoing investigations. However, one possibility that has been excluded is a loss of ABCA-1, a key cholesterol efflux protein.⁴⁰ This is because pilot data indicate that ABCA-1 is not normally expressed in normal mouse renal cortex, as assessed by Western blot.⁴⁰ This makes it very difficult to ascribe increased cholesterol accumulation to a specific derangement in this cholesterol efflux pathway. Clearly, more work is required to both define the operative cholesterol accumulation pathway(s), and the molecular species that trigger them (eg, a specific cytokine, free radical, and so forth) after tissue damage.

In conclusion, the present studies indicate the following: 1) cholesterol/CE accumulation represents a durable adaptive response to either acute or subacute forms of renal tubular cell injury; 2) the degree of cholesterol ele-

vations can closely mirror the extent of renal damage; 3) the cholesterol accumulation is directly caused by tissue damage, rather than being a secondary consequence of uremia; and 4) heterogeneous insults, eg, acute glomerulonephritis, or modest dehydration, can trigger renal cholesterol accumulation. This implies that altered cholesterol/CE expression, and presumably concomitant changes in the underlying mevalonate synthetic/signaling pathway,⁴¹ likely have broad-reaching biological implications, potentially extending well beyond acute tubular necrosis and its attendant cytoresistant state.

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