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Cholesterol ester accumulation: An immediate consequence of acute in vivo ischemic renal injury

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Background. Cholesterol is a major constituent of plasma membranes, and recent evidence indicates that it is up-regulated during the maintenance phase of acute renal failure (ARF). However, cholesterol's fate and that of the cholesterol ester (CE) cycle [shuttling between free cholesterol (FC) and CEs] during the induction phase of ARF have not been well defined. The present studies sought to provide initial insights into these issues.

Methods. FC and CE were measured in mouse renal cortex after in vivo ischemia (15 and 45 minutes)/reperfusion (0 to 120 minutes) and glycerol-induced myoglobinuria (1 to 2 hours). FC/CE were also measured in (*1*) cultured human proximal tubule (HK-2) cells three hours after ATP depletion and in (*2*) isolated mouse proximal tubule segments (PTSs) subjected to plasma membrane damage (with cholesterol oxidase, sphingomyelinase, phospholipase A_2, or cytoskeletal disruption with cytochalasin B). The impact of cholesterol synthesis inhibition (with mevastatin) and FC traffic blockade (with progesterone) on injury-evoked FC/CE changes was also assessed.

Results. In vivo ischemia caused approximately threefold to fourfold CE elevations, but not FC elevations, that persisted for at least two hours of reperfusion. Conversely, myoglobinuria had no effect. Isolated CE increments were observed in ATP-depleted HK-2 cells. Neither mevastatin nor progesterone blocked this CE accumulation. Plasma membrane injury induced with sphingomyelinase or cholesterol oxidase, but not with phospholipase A_2 or cytochalasin B, increased tubule CE content. High CE levels, induced with cholesterol oxidase, partially blocked hypoxic PTS attack.

Conclusions. In vivo ischemia/reperfusion acutely increases renal cortical CE, but not FC, content, indicating perturbed CE/FC cycling. The available data suggest that this could stem from specific types of plasma membrane damage, which then increase FC flux via aberrant pathways to the endoplasmic reticulum, where CE formation occurs. That CE levels are known to inversely correlate with both renal and nonrenal cell injury suggests the potential relevance of these observations to the induction phase of ischemic ARF.

Key words: acute renal failure, cholesterol ester cycle, myoglobinuria, progesterone, statins.

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Cholesterol is a critical component of plasma membranes, and within renal tubular cells, it exists in an approximate 1:5 ratio with plasma membrane phospholipids [1]. It interpositions itself among membrane phospholipids, affecting membrane fluidity, surface charge density, and polar head group interactions [2–6]. Cholesterol also forms tight hydrophobic interactions with plasma membrane sphingomyelin, generating so called "microdomains" ("rafts," "caveolae"), which concentrate a variety of signaling molecules [7–11]. Given these functions, it is not surprising that cholesterol exerts protean homeostatic effects [2–11]. Presumably because of this, cells tightly regulate total cholesterol content, its subcellular distribution, and the amount that is converted into cholesterol esters (CEs) [12]. Total cellular cholesterol is maintained via a balance between cholesterol synthesis (HMG CoA reductase regulated) and low-density lipoprotein (LDL) receptor-mediated LDL/cholesterol uptake [12, 13]. Under normal conditions, these two processes are inversely related, maintaining stable cholesterol levels. Regarding intracellular distribution, approximately 90% of the total cholesterol resides in the plasma membrane [14]. It reaches this location either from the endoplasmic reticulum (ER), where de novo synthesis occurs, or possibly from lysosomes [12], where LDL particles are degraded, releasing their cholesterol content. Once reaching the plasma membrane, cholesterol is not a static membrane constituent. Rather, it can cycle back to the ER, a process that may be regulated by "multidrug resistant" (MDR) P-glycoproteins [15–17]. Upon reaching the ER, acyl-CoA:cholesterol acyltransferase (ACAT) converts cholesterol into CEs. The latter can then be hydrolyzed back to free cholesterol (FC) within either the cytosol or the lysosomal system. In summary, these processes (FC transport to and from the plasma membrane, ACAT-mediated esterification, and CE hydrolysis) generate the "cholesterol ester" cycle [12, 18, 19]. CEs are widely considered to be a cytosolic cholesterol storage form [12]. However, that approximately 30% of total renal tubular cell CE content can be hydrolyzed with membrane impermeant cholesterol esterase [20] indicates that CEs can reach and insert into the tubule plasma membrane.

Dysregulated cholesterol metabolism is a hallmark of the maintenance phase of experimental acute renal failure (ARF) [1]. This conclusion stems from our previous observations that by 18 to 24 hours following diverse forms of acute renal injury (ischemia-reperfusion, glycerol-induced myohemoglobinuria, urinary tract obstruction), approximately 20% increments in renal cortical/proximal tubule total cholesterol content result [1, 21]. This is likely due, at least in part, to de novo cholesterol synthesis, given that mevastatin-induced HMG CoA reductase inhibition totally prevents cholesterol accumulation in postinjured cultured human proximal tubule (HK-2) cells [21]. All of the consequences of these postinjury cholesterol increases remain unknown. However, one that has been identified is that they contribute to the "acquired cytoresistance state" (that is, decreased tubular susceptibility to secondary attack) [1]. This conclusion is based on observations that cholesterol reductions in normal cells sensitizes them to hypoxic or toxic injury [21], and by restoring cholesterol levels back to normal in cytoresistant tubules reverses the cytoresistant state [1].

Cholesterol metabolism during the "induction phase" of ARF has remained much less well defined. In previous work from this laboratory, we were unable to document any changes in cholesterol levels during the induction phase of ischemic ARF [1]. However, only total cholesterol content was measured, precluding any conclusion as to cholesterol's relative distribution between free versus CE forms. Previous work by Molitoris et al has suggested that renal ischemia may indeed cause an acute reduction in apical plasma membrane cholesterol, relative to its total phospholipid content [22, 23]. However, whether this change reflected absolute cholesterol reductions, a conversion to CEs, or altered intracellular cholesterol distribution (for example, because of cytoskeletal disruption with altered cell polarity) was not resolved.

In light of cholesterol's critical role in cellular homeostasis, any acute changes in cholesterol/CE expression could have significant implications for the induction phase of ARF. Indeed, recent studies from this laboratory have provided direct support for this hypothesis [21]. When isolated proximal tubule (PT) segments were subjected to either acute hypoxic or oxidant (Fe-mediated) injury, approximately 35% decrements in tubule CE (but not FC) content resulted [21]. That comparable CE reductions, induced in normal tubules with cholesterol esterase, induced profound ATP depletion and ultimately cell death suggested that the previously mentioned observations had pathogenic relevance [21].

It is widely recognized that for a variety of reasons, results obtained with freshly isolated proximal tubules may not always accurately reflect changes inherent to the in vivo state [reviewed in 24]. Therefore, the present study was undertaken to explore whether altered cholesterol expression is in fact a correlate of the induction phase of in vivo ischemia/reperfusion ARF and myohemoglobinuric ARF. To elucidate potential pathogenic changes and mechanisms further, additional experiments were conducted in cultured human proximal tubule (HK-2) cells. Finally, reasons for potential discrepancies in cholesterol ester expression between in vivo versus in vitro renal tubule injury were assessed.

METHODS

In vivo ischemic renal injury

Forty-five–minute ischemia-reperfusion experiments. Male CD-1 mice weighing 25 to 35 g (Charles River Laboratories, Wilmington, MA, USA) and maintained under standard vivarium conditions were used for all experiments. They were anesthetized with pentobarbital (~2 mg, administered intraperitoneally), and then they were subjected to a midline laparatomy, exposing both kidneys. Body temperature was monitored with a rectal probe and maintained at 36°C to 37°C with an adjustable heating lamp. The left kidney was subjected to ischemia by total vascular pedicle occlusion. The right kidney was not subjected to ischemia, serving as a contralateral control. After completing 45 minutes of renal ischemia, three experimental groups were created: (*1*) no reperfusion, in which both the left (ischemic) and right (nonischemic, control) kidneys were removed without allowing a period of vascular reperfusion ($N = 7$ mice); (*2*) 30 minutes of reperfusion, in which the vascular clamp was removed and a 30-minute reperfusion period was permitted, followed by bilateral renal resection ($N = 6$); and (*3*) 120 minutes of reperfusion, in which a 120-minute reperfusion period was permitted prior to bilateral renal resection ($N = 6$ mice). The kidneys were immediately iced, and cortical tissue samples were dissected using a razor blade. The samples were weighed, added to four parts of cold methanol, homogenized, and extracted in chloroform:methanol (1:2) as previously described in detail [21, 25]. The samples were dried under N_2 and saved for free and esterified cholesterol analysis by gas chromatography (GC), as described later in this article.

Impact of contralateral ischemia on renal cholesterol levels. To assess whether the presence of unilateral ischemia impacts contralateral kidney cholesterol/CE levels (that is, the controls used for the previously mentioned experiments), the following additional control experiment was performed. Five mice were subjected to the previously mentioned 45-minute unilateral ischemia experiment. At its completion, both kidneys were removed. In addition, five kidneys were removed from comparably anesthetized five normal mice without their undergoing any renal ischemia. The cholesterol/CE levels between the normal kidneys, left ischemic kidneys, and the contralateral (right) nonischemic kidneys were compared.

Fifteen-minute in vivo ischemia experiments. Whereas 45 minutes of in vivo ischemia causes severe tubular necrosis and ARF [26], 15 minutes of ischemia generally causes sublethal in vivo renal tubular damage [27]. To ascertain whether the latter induces acute changes in cholesterol expression, seven mice were treated as described previously in this article, with the exception that a 15-minute, rather than a 45-minute, period of left renal pedicle occlusion was induced. In four mice, the ischemic kidneys and their contralateral controls were removed after completing the 15-minute ischemic period. In the remaining three mice, two hours of reperfusion were permitted, followed by bilateral renal resection. The samples were processed as noted previously in this article and saved for cholesterol analysis.

In vivo glycerol-induced myohemoglobinuria experiments

The following experiments were undertaken to ascertain whether acute changes in cholesterol expression occur during the induction phase of myohemoglobinuric ARF [28]. To this end, 14 mice were briefly anesthetized with isoflurane and injected with 12 mL/kg of 50% glycerol (equally divided doses into both upper hind limbs). An equal number of anesthetized mice not subjected to glycerol injection served as controls. At either one- or two-hours postglycerol injection (6 pairs and 8 pairs of mice at each respective time point), the mice were anesthetized with pentobarbital. Both kidneys were removed, and cortical tissue samples were collected. The tissues were then subjected to lipid extraction, as noted previously in this article, and saved for cholesterol analysis.

Cultured proximal tubular cell experiments

Cholesterol/cholesterol esters following simulated ischemic injury. The following experiments were undertaken to ascertain whether ATP depletion, imposed in cultured tubule cells, induces the same profile of cholesterol changes observed in the previously described ischemia experiments. To this end, HK-2 cells, an immortalized human proximal tubular cell line [29], were cultured in T75 Costar flasks (Cambridge, MA, USA) with keratinocyte serum-free medium (K-SFM; GIBCO/Life Technologies, Grand Island, NY, USA) containing 1 mmol/L glutamine, 5 ng/mL epidermal growth factor, 40 μg/mL bovine pituitary extract, 25 U/mL penicillin, and 25 μg/mL streptomycin (37°C, 5% CO_2). At near confluence, the cells were trypsinized [29], transferred to additional T75 flasks, and allowed to grow for an additional 48 to 72 hours. The flasks of cells were divided into two groups: (*1*) ATP depletion/Ca overload injury, in which eight flasks were exposed to a previously well-defined ATP depletion injury model (the addition of 10 μmol/L Ca ionophore A23187 + 7.5 μmol/L antimycin A + 20 mmol/L 2-deoxyglucose; denoted by the term "CAD") [30], and (*2*) control incubation, in which eight flasks were maintained under control incubation conditions, with the exception of 0.1% ethanol/0.2% dimethyl sulfoxide (DMSO) addition (the vehicles for the CAD addition). The cells were maintained under these conditions for three hours (corresponding to a period of severe ATP depletion, but which is prior to the development of lethal cell injury, as denoted by lactate dehydrogenase (LDH) release [30] and reconfirmed in pilot data using the current test reactants). After completing the three-hour incubations, the flasks were decanted and iced, and the cells were recovered by scraping with a rubber "policeman" in 3 mL of iced Hank's balanced salt solution (HBSS). After repeating this process two times, the cells were recovered from HBSS by centrifugation, followed by lipid extraction in 1:2 chloroform methanol [21]. The lipid phase was dried under N_2 and saved for cholesterol analysis, as described later in this article. The previously mentioned experiment was repeated in its entirety on four separate occasions, with two flasks per group per each experiment.

Impact of progesterone on HK-2 cell cholesterol profiles. Progesterone inhibits FC transport from the plasma membrane (and possibly from lysosomes) to the ER, where ACAT resides [12, 31, 32]. Thus, it serves to disrupt the normal cholesterol esterase cycle. The following experiments were undertaken to ascertain whether injury-induced cholesterol/CE changes in HK-2 cells occur via increased cholesterol flux through these normal (progesterone-inhibited) pathways. To this end, T75 flasks (N = 12), prepared as described previously in this article, were equally divided into three groups: (*1*) continued control incubation conditions × four hours (with the addition of 0.15% DMSO; the progesterone vehicle); (*2*) incubation × four hours with 15 μg/mL progesterone (P-0130; dissolved in DMSO; Sigma, St. Louis, MO, USA); or (*3*) incubation with progesterone × one hour, followed by the three-hour CAD challenge (in the continued presence of progesterone). After a total of four hours, the cells were harvested, and the lipids were extracted and saved for cholesterol/CE analysis.

Impact of HMG CoA reductase inhibition on HK-2 cell cholesterol profiles. The previously mentioned HK-2 cell experiment was repeated exactly as detailed with the exception that mevastatin (10 μmol/L, in 0.1% ethanol, M2537; Sigma Chemicals) was substituted for progesterone treatment. By so doing, the impact of cholesterol synthesis on the cell injury-mediated cholesterol changes could be assessed.

Isolated proximal tubule segment experiments

Impact of glycine and pH on cholesterol/cholesterol ester levels in hypoxic PT segments. (1) Hypoxic cell injury in the absence of cell death. As previously noted, hypoxic injury imposed on isolated mouse PT segments causes simultaneous decrements in CEs and marked LDH re-

lease [21]. The goal of the following experiment was to ascertain whether the observed CE losses were merely secondary, nonspecific consequences of hypoxic cell death. To this end, cortical PT segments were prepared from six mice as previously described [21] and suspended in experimentation buffer (in mmol/L: NaCl, 100; KCl, 2.1; $NaHCO_3$, 25; KH_2PO_4, 2.4; $MgSO_4$, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.2; glucose, 5; alanine, 1; Na lactate, 4; Na butyrate, 10; 36 kD dextran, 0.6%; gassed with 95% O_2/5% CO_2; final pH, 7.4). After allowing a 15-minute rewarming period to 37°C (isolation is performed at 4°C), the tubule preparations were divided into four equal aliquots within 10 mL Erlenmeyer flasks (1 mL of tubule preparation, 2 to 4 mg protein/mL) creating four experimental groups: (1) control incubation × 45 minutes (95% O_2/5% CO_2); (2) incubation with O_2/CO_2 with the addition of 2 mmol/L glycine; (3) 30 minutes of hypoxia (95% N_2/5% CO_2; followed by 15 minutes of reoxygenation; O_2/CO_2); and (4) 30 minutes of hypoxia + 15 minutes of reoxygenation in the presence of 2 mmol/L glycine. The rationale for using glycine is that it blocks lethal cell injury/plasma membrane disruption without altering the ATP depletion state [33]. At the completion of the 45-minute incubations, lethal cell injury was assessed by the percentage of LDH release, and then the tubules were subjected to lipid extraction [20] and cholesterol/CE analysis.

(2) Hypoxic injury at 7.4 versus 6.8 pH. A fundamental difference between in vivo ischemia and in vitro hypoxic injury is the prevailing pH (falling with ischemia, but not hypoxia) [24]. The following experiment assessed whether this difference impacts cholesterol expression during ATP depletion injury. Four sets of PTS were each divided into four aliquots: (*1*) control incubation × 30 minutes, pH 7.4; (*2*) control incubation × 30 minutes, pH 6.8; (*3*) 15 minutes of hypoxia, followed by 15 minutes of reoxygenation, pH 7.4; and (*4*) hypoxia/reoxygenation, pH 6.8. The standard incubation buffer was used in both limbs of the experiment (the pH being reduced to 6.8 with HCl prior to tubule addition). After the 30-minute incubations, the percentage of LDH release was determined, followed by tubule extraction for cholesterol analysis.

Effect of plasma membrane injury on cholesterol/cholesterol ester expression

The following experiments assessed whether selected forms of plasma membrane injury, induced in isolated tubules, give rise to CE increments.

Cholesterol oxidase treatment. Five sets of isolated tubules were each divided into two aliquots: (*1*) control incubation × 30 minutes and (*2*) incubation with 0.5 U/mL of cholesterol oxidase × 30 minutes (Sigma; C-7149). Of note, cholesterol oxidase is believed to specifically attack plasma membrane FC residing in sphingomyelin/cholesterol microdomains [34]. At the completion of the 30-minute incubations, the percentage of LDH release was determined, and then the tubules were processed for cholesterol/CEs.

Sphingomyelinase treatment. The previously mentioned cholesterol oxidase experiment was repeated as detailed previously in this article, substituting 0.5 U/mL of sphingomyelinase (S-8633; Sigma) for cholesterol oxidase treatment. The incubations were conducted for 45 rather than 30 minutes.

Phospholipase A_2 treatment. Since ischemia/hypoxia-mediated plasma membrane injury arises in part via phospholipase A_2 (PLA_2) activation [reviewed in 25], the previously mentioned experiment was repeated substituting Naja Naja PLA_2 (0.05 U/mL; P-7653; Sigma Chemicals) [35] for the previous test reactants. The incubations were conducted for 30 minutes.

Cytochalasin B treatment. Because ischemic/hypoxic plasma membrane reorientation is mediated in part by cytoskeletal disruption [22, 23], the previously mentioned experiments were repeated substituting cytochalasin B (100 μmol/L, C-6762; Sigma; in order to disrupt the cytoskeleton) [36] for the former test agents. The incubations were conducted for 45 minutes.

Impact of elevated cholesterol esters on the severity of in vitro hypoxic tubular injury

The following experiment was done to assess whether an acute increase in CEs might impact the severity of hypoxic injury to isolated tubules. To this end, five sets of proximal tubules were each divided into the following four treatments: (*1*) control incubation × 30 minutes; (*2*) incubation with 0.5 U/mL cholesterol oxidase × 30 minutes; (*3*) incubation under normal oxygenated conditions for 15 minutes, followed by 15 minutes of hypoxia; and (*4*) 15 minutes of cholesterol oxidase exposure, followed by 15 minutes of hypoxia. After completing the incubations, the percentage of LDH release was determined.

Measurement of free cholesterol and cholesterol esters

Free and esterified cholesterol were measured by GC using a recently described method from this laboratory [21]. In brief, the dried lipid extracts obtained from the previously mentioned experiments were reconstituted in 1 to 2 mL hexane, followed by sonication and vortexing to dissolution. To analyze FC, a 100 μL sample of each extract was transferred to a glass culture tube containing 50 μL of an internal standard solution (stigmasterol, 100 μg/mL in ethyl acetate, EtOAc). The sample was dried under N_2 and reconstituted in 100 μL of bis-(trimethylsilyl)trifluoroacetamide (BSTFA, 25% vol/vol EtOAc; Sigma). They were then transferred to an injection vial, sealed, and heated for one hour at 60°C. After completion of BSTFA derivatization, a 1 μL sample was applied to a Hewlett Packard 5890 Series II gas chromatograph

fitted with a flame ionization detector and a 30 m × 0.32 mm DB-5 (0.25 μm) column (J & W Scientific, Folsom, CA, USA). The initial temperature (100°C) was maintained for three minutes, after which it was increased by 40°C per minute to 290°C and thereafter by 5°C per minute to 300°C for five minutes. The trimethylsilyl ether of cholesterol eluted at 12.5 minutes and that of the internal standard at 13.6 minutes.

To quantitate CEs, they were first separated from the FC pool. This was achieved by adding samples of the hexane aliquots (700 μL, for HK-2 cells; 400 μL, for renal cortex or isolated tubules) to an amino solid-phase extraction column (3 mL, 500 mg; Varian Bond Elut, Harbor City, CA, USA) previously washed with hexane. The column eluant, containing esterified, but not FC, was saved [21]. 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 × 45 minutes [21]. 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 described previously in this article. Previously performed validation studies confirmed the complete absence of any contaminating FC within the CE eluant and 100% efficiency in CE recovery [21]. Hence, the amount of recovered FC resulting from CE hydrolysis was taken as the amount of CE present in the original sample. Free and esterified cholesterol results were expressed as nmol/μmol phospholipid phosphate (P_i) in the original lipid extract [21].

Statistics

All values are given as means ± 1 SEM. Statistical comparisons were performed by either the paired or unpaired Student *t* test. If multiple comparisons were made, the Bonferroni correction was applied.

RESULTS

Cholesterol levels following in vivo ischemia

As shown in Figure 1A, a threefold to fourfold increase in CE levels was noted by the completion of 45 minutes of ischemia (I) compared with values observed in contralateral control kidneys. This difference persisted during vascular reperfusion, being observed at the completion of both 30- and 120-minute reflow (R). CE accumulation was also observed after 15 minutes of ischemic challenge (Fig. 1B). However, in contrast to the 45-minute ischemic challenge, these ester elevations were fully reversed during a two-hour reperfusion period. As shown in Figure 2, in no instance did ischemia or ischemia/reperfusion induce a significant change in FC concentrations.

The previously mentioned differences in CEs could not simply be attributed to a reduction in CEs in the contralateral (nonischemic) kidneys, rather than a rise in CEs in the ischemic/postischemic kidneys. This is because there was no significant difference in CEs between completely normal kidneys and the contralateral (control) kidneys taken from mice with unilateral ischemia (5.0 ± 0.2 vs. 5.1 ± 0.2, respectively). The ischemic kidneys from this experiment once again manifested striking CE elevations (10.8 ± 2, $P < 0.03$ vs. either nonischemic kidney group). FC values did not differ among these three kidney groups (analogous to the data shown in Fig. 2).

Glycerol-induced myohemoglobinuria

As shown in Figure 3, no significant changes in either CE (Fig. 3A) or FC content (Fig. 3A) were observed following the induction of myohemoglobinuria. This was true at both one and two hours after the glycerol injection.

HK-2 cell culture experiments

Impact of the ATP depletion/Ca ionophore (CAD) challenge. Acute cell injury, induced by CAD, caused an approximate 40% increase in CE content (Fig. 4A). Conversely, FC levels were not affected by CAD treatment (216 ± 4 and 221 ± 4, CAD and control values, respectively, P = NS; data not shown). As previously noted, a three-hour CAD challenge is insufficient in length to produce lethal cell injury, as defined by LDH release.

Progesterone treatment. Progesterone (Prog) caused a significant reduction in CEs in normal HK-2 cells (Fig. 4B), as predicted by its ability to inhibit plasma membrane FC cycling to the ER. However, progesterone did not block the CAD-mediated CE increments, with CEs rising by 70% (compared with progesterone treatment alone; Fig. 4B). FC was not significantly affected by progesterone treatment (controls, 254 ± 12; progesterone, 242 ± 10; progesterone + CAD; 241 ± 10, all P = NS; data not shown).

Mevastatin treatment effects. As shown in Figure 4C, mevastatin caused a significant reduction in HK-2 cell CE content ($P < 0.001$). However, mevastatin did not block CAD-mediated CE increments (rising 67% compared with their mevastatin controls, $P < 0.005$; Fig. 4C). Mevastatin caused a slight but significant lowering of FC levels in noninjured cells (293 ± 5 vs. 283 ± 3, $P < 0.05$; data not shown). However, mevastatin had no effect on FC levels in the presence of the CAD challenge (283 ± 3 for both the mevastatin and the mevastatin + CAD groups).

Isolated proximal tubular segment experiments

Effect of glycine on cholesterol/CE expression during hypoxia. Glycine exerted a profound cytoprotective effect against hypoxic injury, reducing LDH release from approximately 65 to 20% (Fig. 5A). This change was

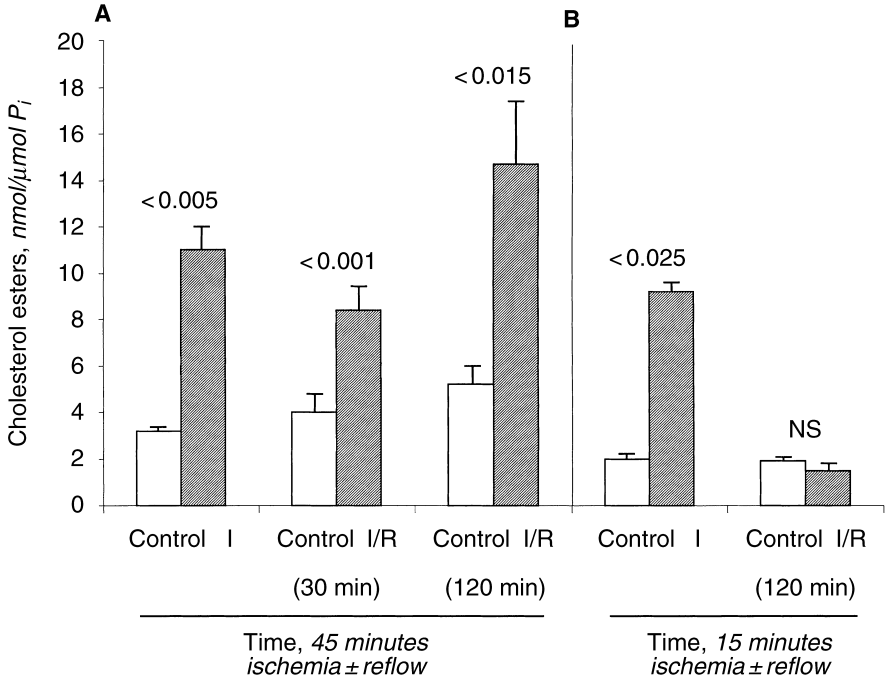

Fig. 1. Cholesterol esters (CEs) following either 45 minutes of ischemia (*A*) or 15 minutes (*B*) of ischemia + vascular reperfusion. Both 15 and 45 minutes of ischemia (I) caused significant increases in CE levels compared with values found in contralateral control (cont) kidneys. In the case of the 45-minute ischemic insult, the CE elevations persisted for 30 and 120 minutes of reperfusion (I/R). However, in the case of 15 minutes of ischemia, the CE increments were dissipated during reperfusion.

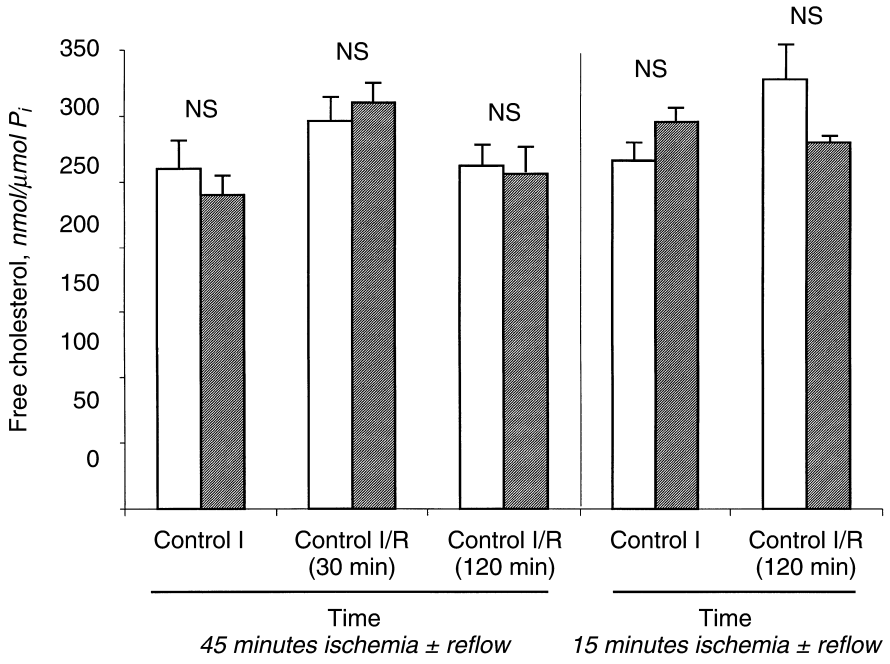

Fig. 2. Free cholesterol (FC) following either 45 minutes of ischemia (*A*) or 15 minutes (*B*) of ischemia + vascular reperfusion. The values presented were derived from the same experiments presented in Figure 1. No significant changes in FC levels were observed following ischemia (I) or ischemia/reperfusion (I/R) compared with values observed in contralateral controls.

observed in the absence of any independent glycine effect on LDH release (as reflected by the oxygenated "control" tubule results; Fig. 5B). As previously demonstrated [20], hypoxia caused an approximate 30% decrease in CE in isolated tubules ($P < 0.015$ vs. controls; Fig. 5B). Despite the fact that glycine almost completely blocked lethal tubule injury, as assessed by LDH release, it had no impact whatsoever on hypoxia-induced CE decrements (Fig. 5B). Neither hypoxia nor glycine had any significant effect on FC levels (controls, 232 ± 19; glycine, 245 ± 92; hypoxia, 246 ± 22; hypoxia + glycine, 244 ± 27; data not depicted).

Impact of pH on tubule cholesterol expression during hypoxia. Incubating isolated tubules under oxygenated conditions at pH 6.8 versus 7.4 had no impact on LDH release (Fig. 6A). However, the extent of hypoxia-induced

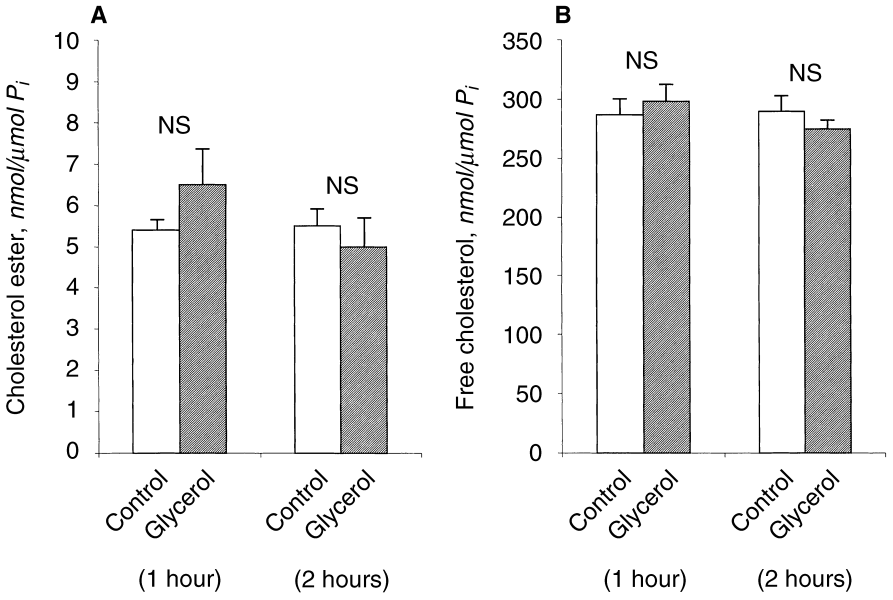

Fig. 3. Cholesterol ester (*A*) and free cholesterol (*B*) levels in renal cortex either one or two hours after glycerol-induced myohemoglobinuria. In no case were altered levels observed, in contrast to values observed in control mouse kidneys.

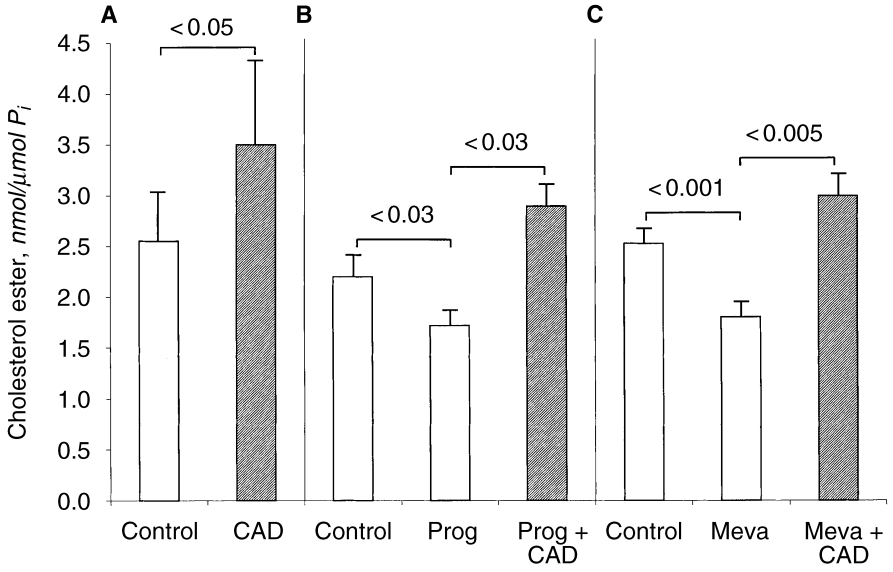

Fig. 4. Cholesterol ester levels in control human proximal tubule (HK-2) cells, in HK-2 cells challenged with ATP depletion/Ca overload injury (CAD), and CAD injury conducted in the presence of either progesterone or mevastatin. (*A*) The CAD injury protocol (×3 hours) caused a significant (~40%) increase in CE levels. (*B*) Despite the fact that progesterone (Prog) significantly decreased CEs under otherwise normal (control) conditions, it did not attenuate CAD-mediated CE elevations (rising 70% over their progesterone controls). (*C*) Mevastatin (Meva) significantly lowered CEs under normal (Control) culture conditions. However, it did not block CAD-mediated CE accumulation (rising 67% over their Meva controls).

LDH release was reduced by approximately 35% when conducted at pH 6.8 versus 7.4 ($P < 0.001$). This is consistent with previously reported findings [37–39].

Hypoxia at pH 7.4 caused the usual 30% decrease in CEs (Fig. 6B). However, when hypoxia was induced at pH 6.8, there was a complete block in CE reductions (Fig. 6B).

Buffer pH had no apparent effect on FC levels under oxygenated conditions (203 ± 3 and 200 ± 6, pH 7.4 and 6.8, respectively). However, under hypoxic conditions, pH 6.8 resulted in slightly increased FC levels (224 ± 7 vs. 203 ± 5, respectively, at pH 6.8 and 7.4, respectively, $P < 0.01$; data not depicted).

Effect of plasma membrane/cytoskeletal injury on tubule cholesterol/ester expression. Cholesterol oxidase treatment caused a striking (15- to 20-fold) increase in CE levels, with essentially reciprocal reductions in FC content being apparent (Table 1). This was despite any increase in lethal cell injury, as assessed by LDH release (Table 1). Attacking cholesterol/sphingomyelin microdomains with sphingomyelinase also caused significant but far more modest CE increments (~50%). Although a reciprocal reduction in FC was also observed, it did not achieve statistical significance. Plasma membrane alterations induced with either PLA_2 or with the cytoskeletal disrupting agent cytochalasin B tended to slightly decrease, rather than increase, CE levels (sug-

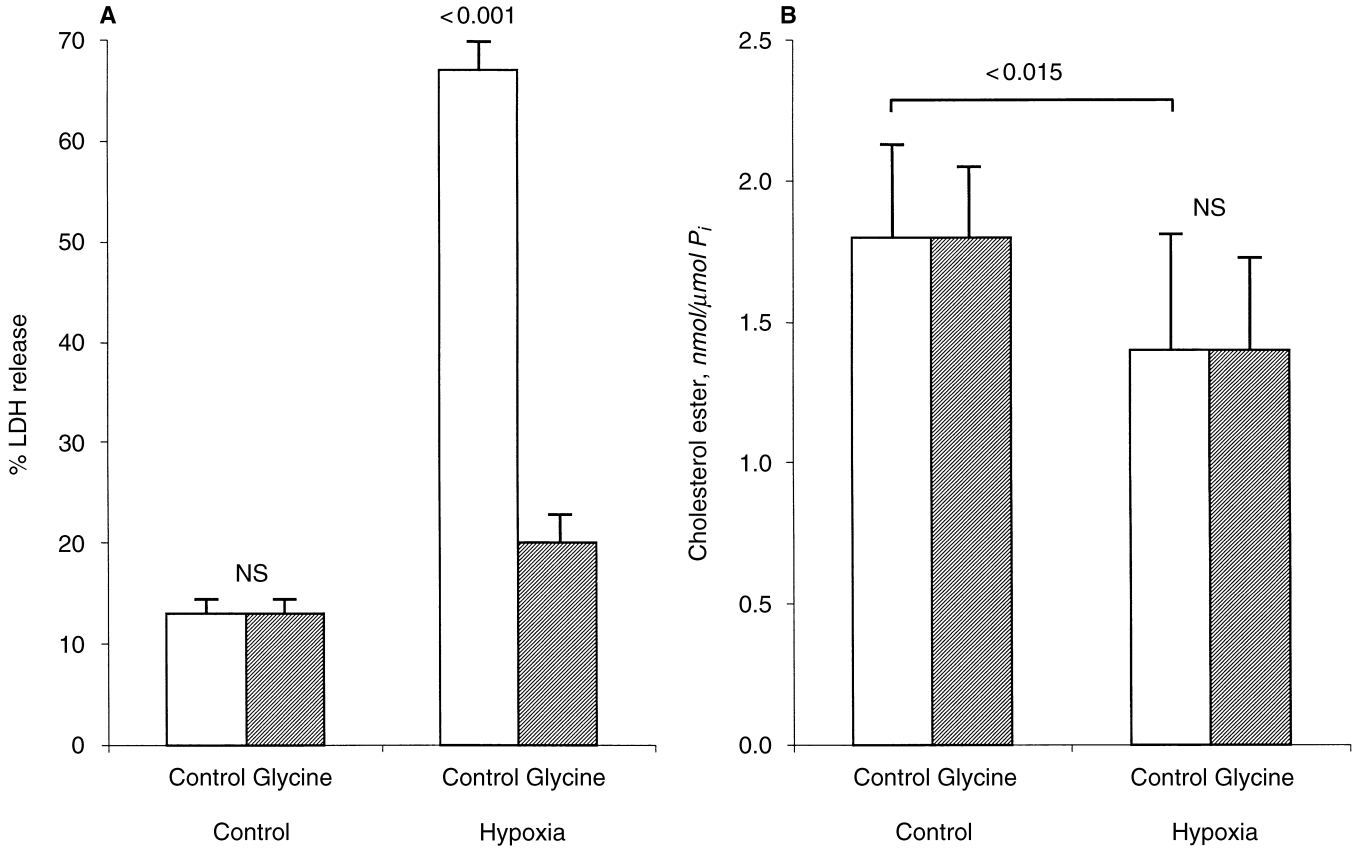

Fig. 5. Effect of glycine on lactate dehydrogenase (LDH) release and cholesterol ester (CE) changes during hypoxic injury to isolated tubules. (*A*) Administration of 2 mmol/L glycine (▩) almost completely blocked hypoxia-mediated LDH release without exerting an independent effect under oxygenated (control; □) conditions. Despite this protective effect, glycine did not attenuate hypoxia-mediated CE reductions (*B*).

gesting suppression rather than stimulation of FC movement from the plasma membrane to the ER; Table 1).

Impact of cholesterol oxidase on tubule hypoxic injury. Cholesterol oxidase had no significant effect on LDH release under oxygenated conditions, reproducing the results given in Table 1. However, pretreatment of tubules with this enzyme for 15 minutes decreased tubule vulnerability to superimposed hypoxic attack (decreasing percentage LDH release from 61 ± 4% to 37 ± 4%, $P < 0.04$). Of note, the dose of cholesterol oxidase used in this experiment, 0.5 U/mL, contrasts with the cholesterol oxidase dose that our laboratory had previously found to induce lethal cell injury to isolated tubule cells (2 to 4 U/mL) [20].

DISCUSSION

A previous report from our laboratory documented that by 18 to 24 hours following diverse forms of renal injury, total renal cortical/tubular cell cholesterol levels rise by approximately 20% and that this change mechanistically contributes to the "acquired cytoresistance" state [1]. However, when cholesterol levels were quantitated in the immediate aftermath of ischemic injury [1], no change in cholesterol content was observed. Thus, it was concluded that renal tubular cholesterol accumulation is a hallmark of the maintenance phase, but not the induction phase, of ARF. It is noteworthy that in that previous study [1], cholesterol was measured with an assay that quantifies only total cholesterol content. Given that ≤1% of tubular cholesterol is in its ester form [21], the employed assay did provide a useful gauge of total cellular cholesterol content. However, by its nature, it cannot assess CEs and, hence, the integrity of the "CE cycle." In a more recent study using GC [21], we observed that CEs may, in fact, be highly relevant to the induction phase of ischemic ARF. This conclusion was based on the finding that acute hypoxic injury imposed on isolated proximal tubules caused an abrupt 30% decline in CE content. When this same degree of CE reduction was reproduced in isolated tubules with exogenous cholesterol esterase, lethal cell injury resulted [21]. Thus, these findings led to the conclusion that CE reductions could be a concomitant and potential mediator of hypoxic injury, at least in freshly isolated proximal tubules [21].

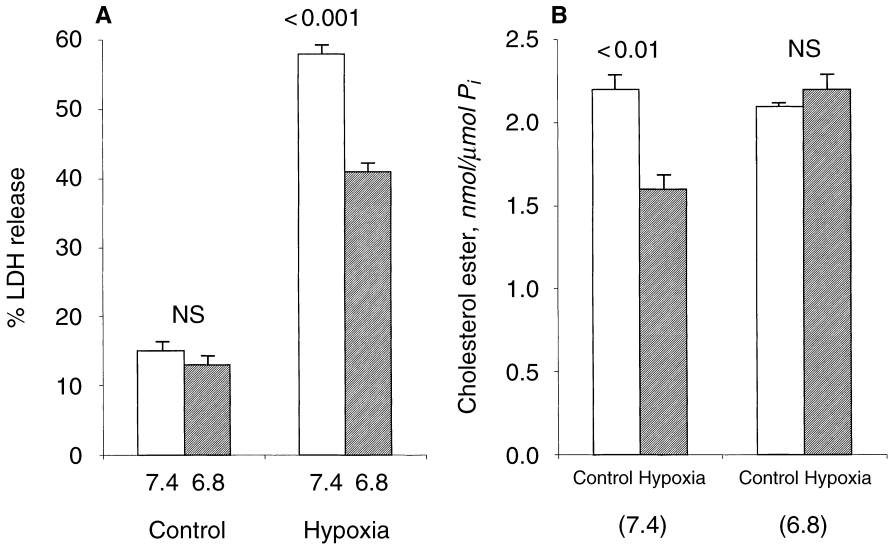

Fig. 6. Effect of pH on LDH release and CE changes during hypoxic injury to isolated tubules. Incubating tubules at pH 6.8 (vs. 7.4) attenuated hypoxic injury (LDH release; ▨) without exerting an independent effect under oxygenated (control, □) conditions (*A*). Whereas hypoxia caused CE reductions at pH 7.4, it failed to do so at pH 6.8 (indicating that pH is a determinant of CE expression in isolated tubules).

Table 1. Cholesterol ester and free cholesterol levels in isolated tubules subjected to diverse forms of plasma membrane or cytoskeletal damage

Group	Cholesterol esters	Free cholesterol	LDH release
Cholesterol oxidase	44 ± 3[b]	291 ± 11[a]	14 ± 1%
Control	2.5 ± 0.1	327 ± 12	12 ± 1%
Sphingomyelinase	3.3 ± 0.4[a]	298 ± 17	17 ± 2%[a]
Control	2.1 ± 0.1	320 ± 13	13 ± 1%
PLA_2	1.5 ± 0.2	251 ± 20	24 ± 1%[a]
Control	2.0 ± 0.3	253 ± 20	18 ± 2%
Cytochalasin B	2.2 ± 0.1[a]	355 ± 25	19 ± 2%
Control	2.9 ± 0.1	350 ± 18	18 ± 2%

All values are given as nmol/μmol inorganic phosphate in isolated tubule extracts (mean ± 1 SEM). For experimental details, see text.
[a] $P<0.05$, [b] $P<0.01$

Given the potential caveats inherent to studies employing isolated PTSs (for example, isolation artifacts [24]), the goal of the present investigation was to define better the fate of CEs during in vivo ischemic/reperfusion injury. To this end, two lengths of ischemia (15 and 45 min) were employed to induce sublethal and lethal tubular damage, respectively [26, 27]. As shown in Figure 1, each of these ischemic protocols induced dramatic CE increments by the end of the ischemic period. Interestingly, a comparable degree of CE elevation was noted at the end of the 15- and 45-minute ischemic challenges, suggesting that it is the presence of severe ATP depletion and not necessarily its length that determines initial CE accumulation. With the 15-minute ischemic insult, the CE elevations were dissipated during early reperfusion, consistent with the fact that this is a readily reversible form of tubular damage [27]. Conversely, with the 45-minute ischemic challenge, the CE elevations persisted unabated for up to two hours of reperfusion. Whether the latter finding stems from more severe initial ischemic damage (as dictated by a longer duration of ischemia) or from more severe reperfusion injury remains unknown. It is noteworthy that 12 mL/kg glycerol injection, which induces profound myohemoglobinuric renal injury, but only modest ATP depletion [40], had no impact whatsoever on CE content during the induction injury phase (0- to 2-h post-glycerol injection). This suggests that CE accumulation during ischemia is relatively specific to this form of injury, rather than simply reflecting acute cell injury per se.

That CEs rose without a concomitant increase in FC indicates a disturbance of the CE cycle. To our knowledge, this is the first documentation that this phenomenon is an acute consequence of in vivo ischemic renal damage. The CE increments seem somewhat paradoxical in two regards: First, cholesterol's fate is opposite to that of phospholipids during ischemia. Whereas PLA_2-mediated phospholipid de-esterification occurs, in the case of cholesterol, esterification results. Second, during hypoxic injury imposed on isolated proximal tubules, CEs acutely fall, not rise, as noted previously in this article. Whereas multiple potential explanations can be offered for the differing fate of CEs versus phospholipids during in vivo ischemia (for example, nothing would necessarily dictate parallel changes in these highly different membrane constituents), the reason(s) for CE decrements versus increments during in vitro hypoxic versus in vivo ischemic tubular damage remains far more obscure. Hence, additional experiments were performed in an attempt to partially elucidate this issue.

The first possibility considered is that hypoxic injury is so rapidly expressed in isolated tubules (for example, ~60% cell death within 15 minutes) that insufficient time exists for CE accumulation to result. Stated differently, the observed CE reductions during hypoxic tubular in-

jury could simply be an epiphenomenon, reflective of necrotic cell death. In contrast, in vivo ischemic tubule necrosis generally requires hours, not minutes, to become fully expressed [26, 27]. To explore this potential explanation for differing in vitro versus in vivo results, isolated tubules were subjected to hypoxic injury with and without 2 mmol/L glycine, a potent cytoprotectant [33]. Glycine almost completely prevented lethal hypoxic injury, as assessed by LDH release. In spite of this, CE reductions remained fully expressed. This makes it clear that CE loss during in vitro hypoxic injury is not simply a secondary consequence of necrotic cell death.

In vitro hypoxia is generally induced at a physiologic pH (that is, a buffer pH of 7.4). However, during ischemia, tissue acidosis (pH ~6.8) rapidly develops. It has been noted by multiple laboratories that pH has a profound effect on cellular responses to hypoxic injury, culminating into a modest cytoprotective effect [38, 39]. Given these considerations, we tested the hypothesis that differences in pH during in vitro versus in vivo oxygen deprivation injury might explain disparate CE expression. To this end, isolated tubules were exposed to hypoxia at either 7.4 or 6.8 pH. The acidotic milieu completely prevented the hypoxia-mediated CE losses and slightly, but significantly, raised tubule FC content. Based on these results, the following conclusions can be drawn: (*1*) pH is clearly an important determinant of cholesterol/CE expression during hypoxia, and (*2*) pH appears to be only a partial explanation for the differences in cholesterol expression observed during in vivo versus in vitro injury, given that acidosis, while preventing CE loss, did not allow CE increments to develop.

The next possibility that was considered is whether tubule isolation injury might, in some way, prevent ester accumulation during an ATP depletion state. To pursue this issue, CE levels during acute cell injury were assessed in cultured proximal tubule epithelial (HK-2) cells. Despite their obvious differences with the in vivo state, they do permit experiments to be conducted in the absence of membrane isolation artifacts. When the HK-2 cells were subjected to the acute ATP depletion challenge, the same qualitative cholesterol profiles to those observed during in vivo ischemia resulted, namely, CE increments without a change in FC content. It is notable that the magnitude of the CE elevations was less impressive than those resulting from in vivo ischemia. A large number of theoretical explanations for this quantitative difference can be surmised (for example, in vivo tubules have a much more extensive brush border/plasma membrane surface, possibly allowing for more cholesterol cycling; the pH issue noted previously in this article). Nevertheless, having identified an in vitro model system that qualitatively recapitulated the cholesterol changes found during in vivo ischemia, it was employed in an attempt to gain mechanistic insights into the pathways involved.

At least three perturbations could explain increased CE accumulation in response to cell injury: (*1*) increased cholesterol synthesis, followed by rapid esterification, (*2*) decreased CE hydrolysis, or (*3*) plasma membrane injury, triggering increased FC trafficking to the ER, where rapid esterification occurs. The first possibility has been excluded, given that mevastatin completely failed to block HK-2 cell injury-mediated CE increments. That mevastatin decreased free and esterified cholesterol in coincubated control cells indicates that the drug did exert its expected biologic effect. The second possibility, decreased CE hydrolysis, cannot be excluded at this time. Nevertheless, it appears an unlikely explanation for CE accumulation, given the rapidity and degree of the CE increments (for example, threefold to fourfold within 15 min of in vivo ischemia). Furthermore, cholesterol esterase activity should rise, not fall, during ischemia, given its pH optimum of 6.5 [41]. In light of these considerations, increased CE formation derived from pre-existent plasma membrane cholesterol pools, rather than de novo cholesterol synthesis or decreased CE hydrolysis, seems most likely.

It is well recognized that substrate (FC) availability is the prime determinant of CE formation [12]. Thus, if cell injury were to increase FC delivery from the plasma membrane to the ER, increased ACAT-mediated CE formation should result. Given that cholesterol is concentrated within plasma membrane cholesterol-sphingomyelin microdomains, we tested whether specific damage to these regions with either cholesterol oxidase or sphingomyelinase would acutely increase tubule cell CE content (implying increased plasma membrane-ER trafficking). Indeed, this appeared to be the case since both agents, particularly cholesterol oxidase, increased CE levels. It is noteworthy that both cytochalasin B (used to simulate ischemia-induced cytoskeletal disruption with secondary plasma membrane changes) and PLA_2 each tended to decrease, not increase, tubule CE content. These results imply that damage to cholesterol-rich domains (caveolae, rafts), rather than plasma membrane damage per se is most effective in triggering plasma membrane cholesterol/ER trafficking. Interestingly, progesterone did not block cell injury-mediated CE increments, either in HK-2 cells or during in vivo ischemia experiments (data not presented). This implies that the hypothesized increase in plasma membrane-ER cholesterol trafficking occurs via an aberrant (nonprogesterone inhibitable) pathway. Indeed, this result does not seem surprising, given that extensive plasma membrane reorganization, including brush border internalization [42], is known to result from ischemic renal damage.

Finally, it is interesting to speculate about whether CE accumulation during in vivo ischemia could potentially modulate the acute evolution of tubular damage. Several pieces of evidence provide indirect support for such a pos-

sibility: First, it has already been established that when CE levels are experimentally depressed in tubular cells (with cholesterol esterase), extensive cell injury results [20]. Second, approximately 6× CE elevations correlate with the emergence of the acquired cytoresistant state [21]. Third, several studies performed in non-renal cells have suggested that CE formation can have a cytoprotective effect [12, 43, 44], and fourth, in the present study, when CEs were acutely raised in isolated tubules with cholesterol oxidase, a substantial decrease in hypoxic isolated tubule injury resulted. It is noteworthy that other ischemia-inducible abnormalities, most notably acidosis [38, 39] and PLA_2-mediated unsaturated fatty acid accumulation [35, 45], decrease ischemic/hypoxic cell damage. Perhaps CE accumulation is just one additional example of injury-evoked adaptive responses which serve as a "break," slowing the evolution of ischemic cell death.

In conclusion, this study demonstrates that striking CE accumulation is a previously unrecognized consequence of acute in vivo renal ischemic-reperfusion injury. It occurs in the absence of a parallel increase in FC content, indicating an acute alteration of normal cholesterol-CE cycling. Based on a series of in vitro studies, the most likely mechanism appears to be acute plasma membrane injury (possibly directed at cholesterol-sphingomyelin microdomains), resulting in increased cholesterol flux to the ER where esterification occurs. That this process is not progesterone inhibitable suggests that an injury-evoked (that is, nonphysiologic) transport pathway is involved. This study adds to a growing body of information that indicates that changes in cholesterol expression are hallmarks of both the induction and the maintenance phases of ischemic ARF. It is notable that in the latter instance, completely comparable data are observed whether in vivo, cell culture, or isolated tubule experiments are employed. Conversely, during the acute injury phase, isolated tubules may not accurately reproduce all in vivo events. Finally, different mechanisms underlie cholesterol changes at differing points of the injury process (for example, increased cholesterol synthesis during the maintenance, but not the induction, injury phase). These model-specific and timing differences need to be considered when designing studies to explore cholesterol homeostasis during the evolution of experimental ARF. Finally, the subcellular localization of these cholesterol/CE changes will need to be addressed to define their biological impacts more fully.

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APPENDIX

Abbreviations used in this article include: ACAT, acyl-CoA:cholesterol acyltransferase; ARF, acute renal failure; ATP, adenosine 5′-triphosphate; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; CAD, Ca + A23187 + antimycin A + deoxyglucose; CE, cholesterol ester; ER, endoplasmic reticulum; FC, free cholesterol; GC, gas chromatography; HBSS, Hank's balanced salt solution; HK-2, human proximal tubule; HMG-CoA, 3-hydroxy-3-methlglutaryl coenzyme A; I, ischemia; K-SFM, keratinocyte serum-free medium; LDH, lactate dehydrogenase; LDL, low density lipoprotein; MDR, multi-drug resistant; PLA_2, phospholipase 2; Prog, progesterone; PT, proximal tubule; R, reflow.

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