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# Renal tubular triglyercide accumulation following endotoxic, toxic, and ischemic injury

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*Background.* Cholesterol accumulates in renal cortical proximal tubules in response to diverse forms of injury or physiologic stress. However, the fate of triglycerides after acute renal insults is poorly defined. This study sought new insights into this issue.

*Methods.* CD-1 mice were subjected to three diverse models of renal stress: (*1*) endotoxemia [*Escherichia coli* lipopolysaccharide (LPS), injection]; (*2*) ischemia/reperfusion (I/R); or (*3*) glycerol-induced rhabdomyolysis. Renal cortical, or isolated proximal tubule, triglyceride levels were measured ~18 hours later. To gain mechanistic insights, triglyceride levels were determined in (*1*) proximal tubules following exogenous phospholipase $A_2$ ($PLA_2$) treatment; (*2*) cultured HK-2 cells after mitochondrial blockade (antimycin A) ± serum; or (*3*) HK-2 cells following "septic" (post-LPS) serum, or exogenous fatty acid (oleate) addition.

*Results.* Each form of in vivo injury evoked three-to fourfold triglyceride increases in renal cortex and/or proximal tubules. $PLA_2$ treatment of proximal tubules evoked acute, dose-dependent, triglyceride formation. HK-2 cell triglyceride levels rose with antimycin A. With serum present, antimycin A induced an exaggerated triglyceride loading state (vs. serum alone or antimycin A alone). "Septic" serum stimulated HK-2 triglyceride formation (compared to control serum). Oleate addition caused striking HK-2 cell triglyceride accumulation. Following oleate washout, HK-2 cells were sensitized to adenosine triphosphate (ATP) depletion or oxidant attack.

*Conclusion.* Diverse forms of renal injury induce dramatic triglyceride loading in proximal tubules/renal cortex, suggesting that this is a component of a cell stress response. $PLA_2$ activity, increased triglyceride/triglyceride substrate (e.g., fatty acid) uptake, and possible systemic cytokine (e.g., from LPS) stimulation, may each contribute to this result. Finally, in addition to being a marker of prior cell injury, accumulation of triglyceride (or of its constituent fatty acids) may predispose tubules to superimposed ATP depletion or oxidant attack.

**Key words:** ischemia, endotoxemia, rhabdomyolysis, proximal tubules, HK-2 cells.

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In recent years, it has become increasingly apparent that renal cortical cholesterol accumulation is a delayed consequence of diverse forms of kidney damage. For example, within 18 to 24 hours of ischemic, toxic, obstructive, or immunologic insults, ~30% and ~300% increases in renal cortical/proximal tubular free cholesterol and cholesteryl ester levels result, respectively [1–8]. Physiologic stress (e.g., heat shock, endotoxemia, hyperosmolality) can also trigger renal cholesterol accumulation in the *absence* of tubular cell death [5]. This strongly suggests that renal cholesterol accumulation is not simply a "downstream" consequence of severe tissue damage. Rather, it more likely represents an integral component of a generic tissue "stress response." Much remains to be discerned concerning mechanisms by which cell stress evokes a cholesterol loading state. However, available data indicate that multiple pathways, including increased cholesterol synthesis/cell uptake, and decreased cell efflux, may each contribute, and in a disease-specific fashion [5–8]. Given the protean effects of membrane cholesterol on cell homeostasis, the "downstream" pathophysiologic consequences of postinjury cholesterol loading remains incompletely defined. However, one result is decreased tubular cell susceptibility to further attack [1–3, 7]. This phenomenon has been denoted by the terms "cytoresistance" and "acquired resistance to acute renal failure" [9, 10].

Unlike cholesterol, the fate of renal cortical triglyceride content following acute renal damage remains largely unknown. Given that triglyceride is the major cellular neutral lipid, the question emerges as to whether altered triglyceride expression develops following renal injury, and analogous to cholesterol, is it a component of the renal "stress response?" Indeed, a few pieces of support for this concept exist. In 1983, Tannenbaum et al reported that by 24 hours postinduction of unilateral ureteral obstruction (UUO), increased renal cortical/medullary triglyceride content resulted [11]. That triglycerides were also modestly elevated in the contralateral kidney suggests that physiologic stress (e.g., in this case, compensation for nephron loss), in addition to structural injury, might also trigger a triglyceride loading state.

In another study, Venkatachalam et al [12] found that 5 hours of mitochondrial blockade in LLC-$PK_1$ cells induced by antimycin A caused modest triglyceride elevations. However, the converse did not appear to be the case in vivo. Triglyceride levels fell, rather than rose, 1 hour postinduction of renal ischemia/reperfusion (IR) damage [13]. Thus, the fate of triglycerides following renal injury remains obscure.

Given these considerations, the present study was undertaken to ascertain whether triglycerides, like cholesterol, accumulate in response to renal injury or physiologic stress. If so, such findings would help place the previous findings of Tannenbaum, Purkerson, and Klahr [11] and Venkatachalam et al [12] into a broader pathophysiologic context. Therefore, the impacts of three highly diverse forms of in vivo renal injury/physiologic stress (renal ischemia; heme protein nephrotoxicity; endotoxemia) on renal cortical and/or proximal tubular triglyceride content were assessed. To gain initial mechanistic insights, additional experiments were performed using either isolated mouse proximal tubule segments or cultured proximal tubular (HK-2) cells subjected to different forms of stress. The results of these studies form the basis of this report.

## METHODS

### General methods

Male CD-1 mice (25 to 35 g) (Charles River Laboratories, Wilmington, MA, USA) were used for all in vivo experiments. They were maintained under routine vivarium conditions until experimentation and allowed free food and water access. The mice were sacrificed for blood and/or tissue sampling under deep pentobarbital anesthesia (~2 to 4 mg intraperitoneally). The abdominal cavity was opened via a midline abdominal incision. Blood samples were withdrawn from the inferior vena cava (IVC) using heparinized or nonheparinized syringes for obtaining either plasma or serum, respectively. Immediately post phlebotomy, the kidneys were resected, iced, and used for subsequent lipid analyses, as denoted below.

### Purified endotoxin injection: Effects on renal cortical cholesterol and triglyceride levels

It has previously been demonstrated that administration of heat killed *Escherichia coli* induces renal cholesterol loading [5, 7]. This experiment assessed whether purified endotoxin [lipopolysaccharide (LPS)] injection can reproduce these *E. coli*-induced cholesterol increments (as opposed to other bacterial components), and to determine whether there are corresponding changes in triglyceride levels. Twelve unanesthetized mice were individually placed into loose cylindric restraints and injected via the tail vein with either 0, 5, or 10 mg/kg of purified *E. coli* LPS (0111:B4; L-2630) (Sigma Chemical Co., St. Louis, MO, USA) (stock solution, 4 mg/mL saline) ($N = 4$ per treatment). The controls (no LPS) received LPS vehicle injection (0.1 mL saline intravenously). Following the injections, the mice were allowed free food and water access. Approximately 18 hours later, they were anesthetized with pentobarbital, a blood sample was withdrawn from the IVC [for blood urea nitrogen (BUN) analysis by autoanalyzer technology] (Beckman Coulter, Fullerton, CA, USA). The kidneys were then promptly resected, as noted above. Cortical tissue samples underwent lipid extraction in chloroform: methanol, and the chloroform-recovered lipids were assayed for free cholesterol and cholesteryl ester content by gas chromatography (GC) [3] and for triglyceride (see below). Tissue triglyceride, free cholesterol, and cholesteryl ester results were expressed as nmol/μmol phospholipid phosphate (Pi) [3].

Triglyceride analysis was performed on plasma and on renal cortical lipid extracts [3] using a commercially available enzymatic assay (#2780-400H) (Thermo Electron, Arlington, TX, USA). This method utilizes a lipase to deacylate tissue triglycerides following their recovery in the chloroform extraction phase. The generated free glycerol was then assayed. Of note, any endogenous free glycerol which might exist in tissue *prior to* tissue extraction is not measured by this assay because free glycerol enters the methanol, not the chloroform (lipid) extraction phase (confirmed in this laboratory by testing spiked glycerol additions). It is possible that mono- and diglycerides could also be detected by this assay, following deacylation to glycerol. However, preliminary studies using a lipid affinity column (BondElut $NH_2$) (Varian, Harbor City, CA, USA), which separates triglycerides from other glycerol containing moieties confirmed that 85% to 95% of the tissue triglyceride reactants reflected true triglyceride content (higher percentages in injured vs. control tissues; as would be expected with a triglyceride loading state). Hence, the values were taken as triglyceride levels.

### Effect of LPS injection on isolated tubule triglyceride, cholesterol, and injury responses

By 18 to 24 hours postinjection of heat-killed *E. coli*, proximal tubular cells manifest "cytoresistance" to either hypoxic or oxidative injury, as assessed in isolated proximal tubule segments [9]. The following experiment was conducted to (*1*) ascertain whether purified LPS injection elicits this same cytoprotective response; and (*2*) to determine corresponding triglyceride and cholesterol levels directly at the proximal tubular cell level. Eight mice were subjected to either 10 mg/kg LPS or LPS vehicle injection ($N = 4$ each). Eighteen hours later, the kidneys were removed, iced, and cortical proximal tubular segments were prepared by collagenase digestion, sieving,

and Percoll centrifugation [14]. The recovered tubules from each mouse were divided into three equal aliquots (~2 mg proximal tubular protein/mL of experimentation buffer) [14], and rewarmed over 15 minutes from isolation temperature (4°C) to 37°C. The aliquots from each mouse were then subjected to one of the following incubations: (*1*) control incubation × 20 minutes (95% $O_2$/5% $CO_2$); (*2*) 10 minutes of hypoxic incubation (95% $N_2$/5% $CO_2$); or (*3*) incubation × 20 minutes with an oxidant insult [ferrous ammonium sulfate complexed to the siderophore hydroxyquinoline (FeHQ), 25 μmol/L of each] [15]. At the completion of the incubations, cell injury was assessed by% tubule lactate dehydrogenase (LDH) release [14]. Those tubules which had been subjected to control incubation conditions were extracted in chloroform: methanol and used for free cholesterol, cholesteryl ester, and triglyceride analysis ($N = 4$ pairs of samples from control and post-LPS–treated mice). Lipid results were expressed as nmol/μmol Pi.

### Renal cortical triglyceride levels following I/R injury

By 18 to 24 hours post in vivo I/R, renal cortical cholesterol increments result [1]. The following experiment assessed whether this form of injury also alters renal triglyceride levels. Three mice were anesthetized with pentobarbital, subjected to right nephrectomy through a midline laparotomy, and then the left renal vascular pedicle was occluded × 15 minutes with an atraumatic vascular clamp. Body temperature was maintained at ~37°C with a heating lamp. Following vascular clamp removal and restitution of blood flow, the abdominal incision was sutured with two layers of 4-0 chromic sutures. The mice were allowed to recover from anesthesia with free food and water access. Approximately 18 hours later, they were reanesthetized, the abdominal incisions were opened, and blood was obtained for BUN and triglyceride analysis. The postischemic kidneys were resected, lipid extracts prepared, and assayed for triglyceride content.

Three groups of control mice were established for the above experiment. These included (*1*) four normal kidneys resected from mice without prior interventions; (*2*) three mice subjected to sham surgery without ischemia or uninephrectomy; and (*3*) three mice subjected to unilateral (right) nephrectomy followed by left renal resection 18 hours later. Renal cortex was then assayed for triglyceride content.

### Effect of rhabdomyolysis on renal cortical triglyceride levels

To further assess the impact of renal injury on triglyceride levels, a third model of acute renal failure was chosen: glycerol-induced rhabdomyolysis [4, 10]. Six mice were briefly anesthetized with isoflurane and then injected with either 8, 8.5, or 9.0 mL/kg of 50% glycerol ($N = 2$ at each dose; glycerol administered intramuscularly in divided doses into both upper hind limbs). Six mice, briefly anesthetized with isoflurane, served as controls. Eighteen hours later, the mice were anesthetized with pentobarbital, plasma was obtained (for BUN and triglyceride determination), and then the kidneys were removed to assess renal cortical triglyceride levels. The latter were correlated with both the glycerol dosage and the severity of BUN elevations. [Note: Since exogenous glycerol could potentially provide the "backbone" for triglyceride synthesis (following its phosphorylation to glycerol-3-phosphate), correlations between glycerol dosage, triglyceride levels, and severity of injury, were assessed. However, exogenously administered glycerol would not directly alter the triglyceride assay, since any free glycerol would not enter the chloroform phase during lipid extraction, as noted above.]

### Impact of acute cell injury on isolated tubule triglyceride levels

The following two sets of experiments were undertaken to gain possible insights into mechanisms by which tubular injury might evoke triglyceride increments.

*Impact of phospholipase $A_2$ ($PLA_2$) on tubular cell triglyceride levels.* Four sets of isolated tubules were prepared from four normal mice and divided into four equal aliquots: aliquot 1, control incubation for 30 minutes; aliquots 2 to 4, incubation with bovine pancreatic $PLA_2$ (P8913) (Sigma Chemical Co.), in dosages of either 5, 10, or 25 U/mL. At the end of the incubations,% LDH release was determined, the tubule suspensions were extracted in chloroform:methanol, and assayed for triglyceride content.

*Impact of hypoxia/reoxygenation and iron-mediated oxidative stress.* Four sets of tubules were prepared and divided into the following six aliquots: 1 and 2, control incubation × 20 minutes ± 2 mmol/L glycine; aliquots 3 and 4, incubation with ferrous ammonium sulfate, complexed with equimolar amounts of the siderophore hydroxyquinoline (FeHQ ± 2 mmol/L glycine) to induce oxidative stress [15]; and aliquots 5 and 6, 10 minutes of hypoxic incubation (95% $N_2$/5% $CO_2$) + 10 minutes of reoxygenation (95% $O_2$/5% $CO_2$) ± 2 mmol/L glycine. At the completion of the incubations,% LDH release was assessed, followed by triglyceride analysis. [Note: Glycine was added to half the incubations to mitigate lethal cell injury, such that triglyceride levels could be assessed in the presence/absence of cell death [16].]

### Cultured proximal tubular (HK-2) cell experiments

*Impact of "septic serum."* The following experiment was undertaken to ascertain whether serum from septic mice (containing inflammatory cytokines) would alter

tubular cell triglyceride content. If so, this would help to dissociate LPS-mediated in vivo tubular cell triglyceride changes from possible LPS-derived renal hemodynamic effects. Six T-25 Costar flasks were seeded with HK-2 proximal tubular cells in keratinocyte serum-free medium (K-SFM), as previously described [17]. After reaching near confluence in 2 days, they were divided into two treatment groups: (*1*) addition of 12.5% control serum (obtained from normal pentobarbital anesthetized mice; followed by complement heat inactivation at 56°C × 30 minutes); and (*2*) addition of 12.5% "septic" serum (obtained from three mice 2 hours after 10 mg/kg LPS injection; heat inactivated, as above). The cells were incubated under routine culture conditions for 18 hours and then the media was removed and saved for triglyceride analysis (to assess the amount of triglyceride exposure resulting from serum addition). The flasks were iced and washed × 3 with Hanks balanced salt solution (+Ca/Mg). The cells were detached with a cell scraper, recovered by centrifugation, the lipids extracted in chloroform:methanol, and the extracts assayed for triglyceride content, as noted above.

*Mitochondrial inhibition with antimycin A: Impact on HK-2 cell triglyceride levels.* When HK-2 cells are subjected to mitochondrial electron chain inhibition, largely sublethal cell injury results, due to partial support of adenosine triphosphate (ATP) levels by glycolysis [18]. The following experiment assessed the impact of this form of cell injury on triglyceride levels. Twenty T-75 costar flasks of HK-2 cells, cultured over 2 to 3 days to near confluence, were divided into four experimental groups ($N = 5$ per group) as follows: (*1*) control incubation; (*2*) incubation with 7.5 μmol/L antimycin A (in 0.1% ethanol final concentration); (*3*) incubation with 5% heat inactivated mouse serum (Gemini Bioproducts, Woodland, CA, USA); and (*4*) incubation with 5% mouse serum + antimycin A. After completing 18-hour incubations under these conditions, the cells were washed, recovered, the lipids extracted, and assayed for triglyceride content. The antimycin A challenges were conducted in the absence vs. the presence of serum in an attempt to dissociate de novo triglyceride synthesis (serum absent) vs. possible triglyceride/fatty acid uptake (serum present).

*Effect of "septic" serum and antimycin A treatments on cell viability (LDH release).* To assess the impact of the above treatments on HK-2 cell viability, two 24-well Costar plates were seeded with HK-2 cells, as follows: (*1*) control incubation; (*2*) incubation with 12.5% heat inactivated septic serum; (*3*) incubation with 12.5% control heat inactivated serum; (*4*) incubation with 7.5 μmol/L antimycin A; (*5*) incubation with 5% normal heat inactivated mouse serum (Gemini Bioproducts); and (*6*) incubation with 5% heat inactivated serum + antimycin A. After 18 hours,% LDH release was determined. In order to make this determination, the amount of LDH in the serum which was added to the culture medium (i.e., exogenously added LDH) was subtracted from the media LDH content after completing each of the challenges.

*Effect of fatty acid supplementation on HK-2 cell triglyceride content.* The following experiment was undertaken to test whether exogenous fatty acid supplementation leads to an increase in HK-2 cell triglyceride content. Sixteen T-75 flasks were seeded with HK-2 cells. After achieving near confluence (2 days), they were divided into four groups (four flasks each): group 1, 5% fatty acid free bovine serum albumin (BSA) addition; or groups 2 to 4, BSA with either 0.25 mmol/L, 0.5 mmol/L, or 1 mmol/L oleic acid (O-1008) (Sigma Chemical Co.) addition. After 18-hour incubations, the cells were washed with Hanks solution and harvested for triglyceride analysis.% LDH release was also determined.

*Fatty acid–mediated TG loading: Effect on cellular resistance to superimposed attack.* The following experiment was undertaken to ascertain whether triglyceride loading, like cholesterol loading, induces a cytoresistant state. To this end, six 24-well Costar plates were divided into the following treatment groups: group 1, incubation with 5% BSA; group 2, incubation with BSA + 0.5 mmol/L oleate; and group 3, incubation with BSA + 1 mmol/L oleate. After 18-hour incubations, the plates were washed using normal culture medium (K-SFM) to remove the BSA and fatty acid additions. Next, the cells were subjected to one of the following treatments: (*1*) control incubations × 18 hours; (*2*) ATP depletion/$Ca^{2+}$ ionophore addition ("CAD" challenge = 7.5 μmol/L calcium ionophore A23187 + 7.5 μmol/L antimycin A + 20 mmol/L 2-deoxyglucose); or (*3*) oxidant injury (10 μM FeHQ). After completing the challenges, cell injury was assessed by% LDH release. [Thus, these experiments allowed assessment of the effects of two oleate doses (with resulting two degrees of triglyceride loading; see Results section) on HK-2 cell susceptibility to two forms of superimposed attack (CAD or FeHQ).]

### Calculations and statistics

All values are presented as means ± 1 SEM. Statistical comparisons were made by paired or unpaired Student *t* testing. If multiple comparisons were made, the Bonferroni correction was applied. Significance was judged by a $P$ value of $< 0.05$.

## RESULTS

### LPS injection effects on renal cortical cholesterol and triglyceride levels

As shown in Figure 1, left panel, the 5 and 10 mg/kg LPS injections induced ~15% and 22% increases in renal cortical free cholesterol content, respectively. LPS induced even greater% increases in cholesteryl esters,

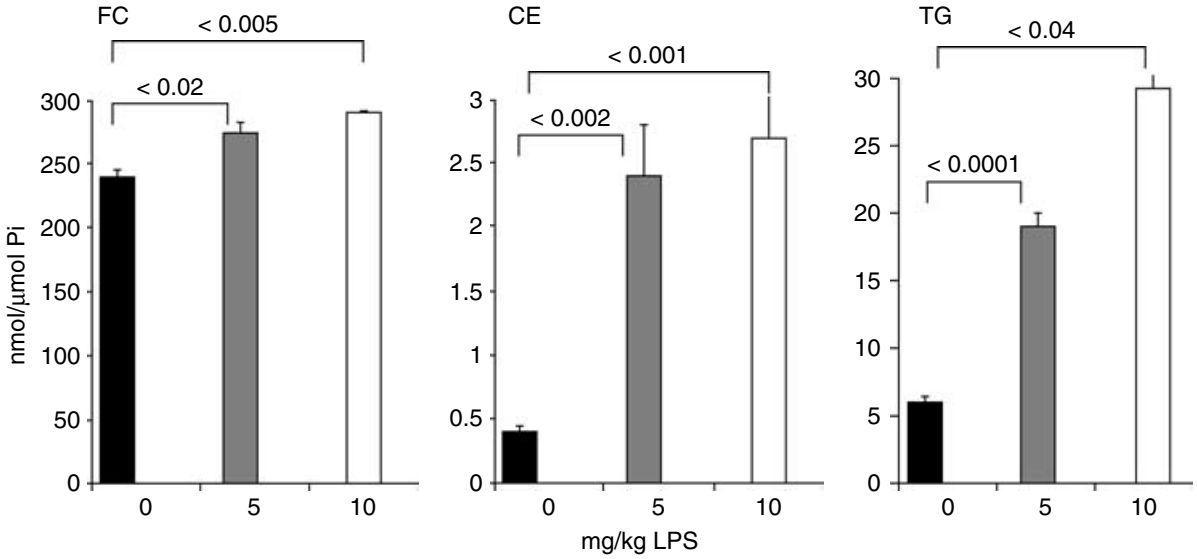


**Fig. 1. Renal cortical free cholesterol (FC), cholesteryl esters (CE), and triglyceride (TG) levels 18 hours following endotoxin [lipopolysaccharide (LPS)] injection.** LPS exposure caused significant, dose-dependent, increases in both free cholesterol and cholesteryl esters (left and middle panels). As shown in the right panel, quantitatively more dramatic, and dose-dependent, increases in renal cortical triglyceride accumulation occurred following LPS injection.

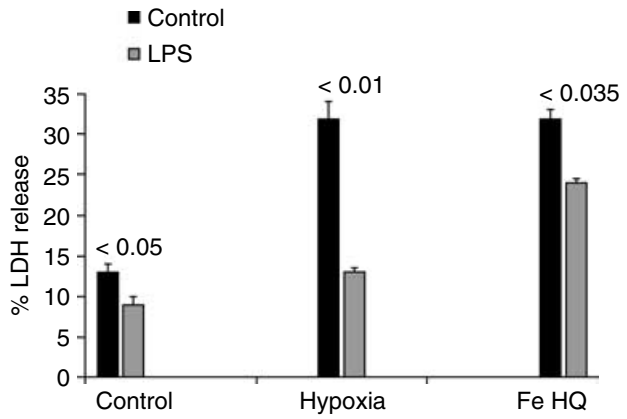


**Fig. 2. Lipopolysaccharide (LPS) injection induces proximal tubular cytoresistance.** Isolated proximal tubules were extracted from either LPS-exposed, or control mice and then tested for cytoresistance (response to either hypoxia or FeHQ-mediated oxidative stress). As shown, the post-LPS-exposed tubules manifested significant reductions in lactate dehydrogenase (LDH) release with either the hypoxic or iron (Fe) challenge, indicating that cytoresistance was present.

rising ~500% and ~600% with the 5 and 10 mg/kg LPS doses (Fig. 1, middle panel). [Thus, these results indicate that previously documented renal cortical cholesterol increments following heat killed *E. coli* injection [5, 7] arose as a consequence of LPS exposure, rather than as a response to alternative bacterial components (e.g., proteases).]

LPS also induced dramatic, dose-dependent, increases in renal cortical triglyceride concentrations (Fig. 1, right panel). At the higher LPS dose, an approximate 400% triglyceride increase was apparent, rising from ~7 to ~30 nmol/μmol Pi.

LPS induced dose-dependent azotemia (BUN concentrations 29 ± 2, 60 ± 15, and 94 ± 20 mg/dL with 0, 5, and 10 mg/kg LPS injections, respectively) ($P < 0.015$, 10 mg/kg vs. controls). Plasma cholesterol levels were not significantly impacted by LPS injection (120 ± 15; 138 ± 10; and 132 ± 20 mg/dL for 0, 5, and 10 mg/kg LPS dosages, respectively). Conversely, plasma triglyceride levels were elevated at 18 hours post-LPS injection (289 ± 7 mg/dL vs. controls, 92 ± 20 mg/dL) ($P < 0.01$).

## LPS effects on proximal tubular resistance to superimposed injury

As shown in Figure 2, proximal tubules isolated from mice treated 18 hours earlier with 10 mg/kg LPS manifested the cytoresistance phenomenon [1, 7]. This was denoted by almost complete protection against hypoxic injury (NS vs. control incubations), and by a 35% reduction in iron-mediated % LDH release. (Thus, these results indicate that prior observations of tubule cytoresistance following heat-killed *E. coli* injection [7] can be recapitulated with purified *E. coli* LPS treatment.)

## LPS effects on isolated tubule cholesterol and triglyceride levels

The tubules from the above experiments which underwent control incubations were subjected to free cholesterol, cholesteryl esters, and triglyceride analyses. Significantly higher values for each were observed in the tubules harvested from the LPS-treated vs. control mice (see Fig. 3).

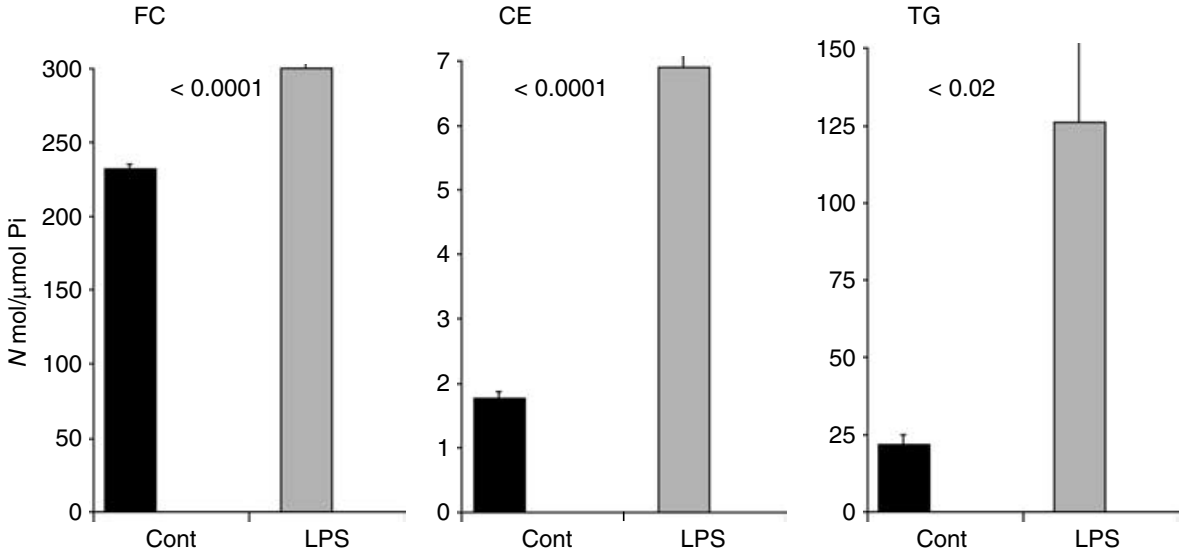


**Fig. 3. Quantitation of free cholesterol (FC), cholesteryl esters (CE), or triglycerides (TG) in isolated proximal tubules harvested from either control (cont) mice or mice which were 18 hours postlipopolysaccharide (LPS) injection.** The tubules obtained from the post-LPS-treated mice showed significant free cholesterol, cholesteryl esters, and triglyceride increases. The latter were by far the greatest, as assessed on a molar basis (~100 nmol/µmol increase for triglyceride vs. ~35 and ~5 nmol/µmol increase for free cholesterol and cholesteryl esters, respectively).

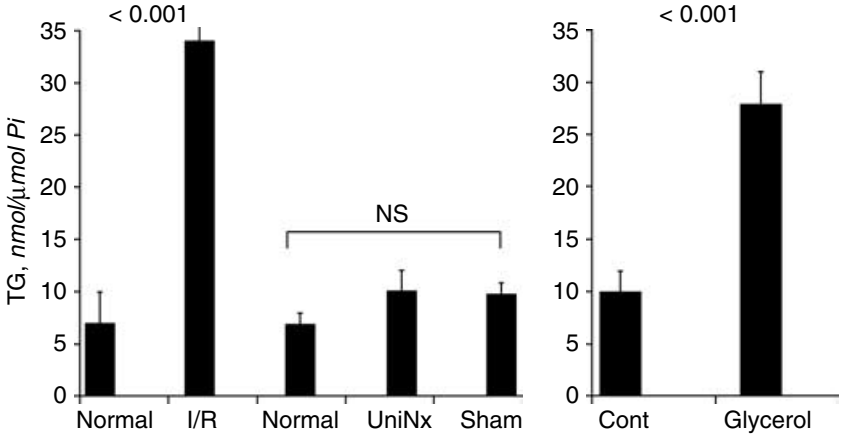


**Fig. 4. Triglyceride (TG) levels in renal cortex from normal mice, mice subjected to renal ischemia/reperfusion (I/R), unilateral nephrectomy (UniNx), or sham surgery (sham) (left panel).** By 18 hours post-I/R, triglyceride levels had increased ~fourfold in renal cortex. This could not be explained by either contralateral nephrectomy or by the stress of surgery, as neither of these controls had elevated triglyceride levels, compared to normal mouse kidney cortex. Right panel, triglyceride levels in renal cortex 18 hours postglycerol-induced rhabdomyolysis, compared to controls. Rhabdomyolysis evoked ~3× increases in renal cortical triglyceride levels, compared to matched controls.

### Effect of renal ischemia on renal cortical triglyceride levels

Renal ischemia + 18 hours of reflow, performed on uninephrectomized mice, caused an approximate fivefold increase in renal cortical triglyceride levels (Fig. 4) ($P < 0.0001$). This increase could not be attributed to either to the stress of surgery or to the effects of unilateral nephrectomy because neither sham surgery nor uninephrectomy significantly altered renal cortical triglyceride levels (Fig. 4). Postischemic terminal plasma triglyceride values were markedly *reduced* at the time of renal triglyceride analysis (controls, 92 ± 13 mg/dL; postischemia, 39 ± 1 mg/dL) ($P < 0.02$). [Thus, the higher renal cortical triglyceride levels in the postischemic kidney could not simply be ascribed to potential blood-trapping effects.] The BUNs for the three postischemic mice were 35, 56, and 110 mg/dL (normal values <30 mg/dL). The degree of azotemia in these three mice strongly correlated with the corresponding degree of renal cortical triglyceride accumulation (32, 33, and 37 mg/dL, respectively; $r$, 0.99).

### Effects of rhabdomyolysis-induced acute renal failure on renal cortical triglyceride levels

As shown in Figure 4, right panel, glycerol-induced rhabdomyolysis caused an approximate 3× increase in renal cortical triglyceride levels, compared to sham-treated controls. Glycerol injection also induced severe acute renal failure (BUNs 152 ± 8 mg/dL; vs. controls, 28 ± 2 mg/dL) ($P < 0.001$). The renal cortical triglyceride levels for each mouse correlated much better with the severity of azotemia which was observed in each animal (BUN range 128 to 184 mg/dL; $r$, 0.66 vs. cortical triglyceride level),

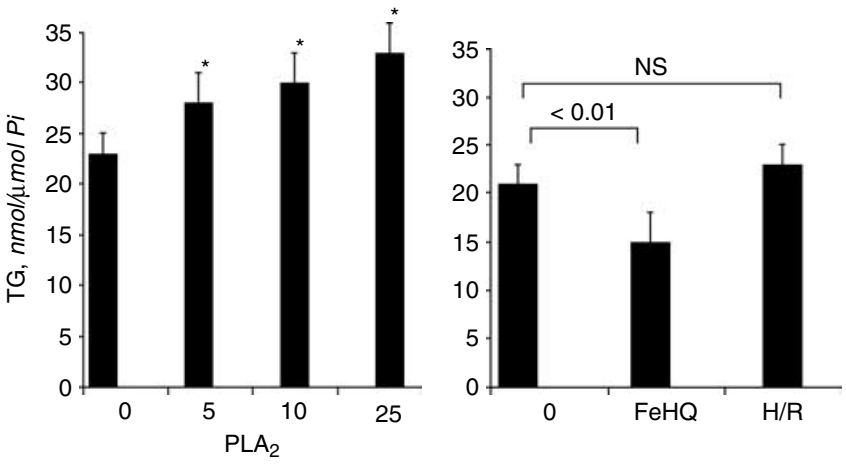


**Fig. 5. Effects of exogenous phospholipase $A_2$ ($PLA_2$), iron-mediated oxidant injury (FeHQ), and hypoxia/reoxygenation (H/R) injury on triglyceride (TG) levels in isolated mouse proximal tubules.** Left panel, $PLA_2$ addition to isolated tubules caused dose-dependent triglyceride increments. Right panel, conversely, FeHQ-mediated oxidant stress [ferrous ammonium sulfate/hydroxyquinoline (FeHQ)], significantly decreased tubule triglyceride levels. H/R had no discernible triglyceride effect.

than with the absolute dose of glycerol which each mouse had received ($r$, −0.12). [This suggests that the triglyceride accumulation was a reflection of the severity of injury, rather than the exact dose of glycerol injected.]

Plasma triglyceride levels varied greatly in the glycerol-treated mice (from 20 to 130 mg/dL; mean, 82 ± 35 mg/dL; control values, 100 ± 8 mg/dL). These variations could not be explained by the dose of glycerol which was administered (dose vs. plasma triglyceride levels, $r = 0.16$). However, when the plasma triglyceride values (square roots) were contrasted with the prevailing renal cortical triglyceride elevations, a strong *inverse* correlation was observed ($r$, 0.80, $P < 0.05$) (i.e., the lower the plasma triglyceride level, the greater the cortical triglyceride content).

### Isolated proximal tubule-acute injury experiments

*$PLA_2$ treatment.* Incubating proximal tubules with $PLA_2$ caused dose-dependent increases in triglyceride levels (see Fig. 5, left panel), each dose being statistically different from controls ($P < 0.025$). These changes occurred without any lethal cell injury, as denoted by% LDH release (controls, 11 ± 1%; $PLA_2$, 11 ± 1%, 12 ± 1%, and 10 ± 1% for the 5, 10 and 25 U/mL $PLA_2$ treatments, respectively).

*Iron-mediated oxidative stress.* Iron treatment significantly lowered, rather than raised, triglyceride levels by ~25% (Fig. 5, right panel) whether or not the incubations were conducted with 2 mmol/L glycine to mitigate cell death. [Hence, for presentation, the ± glycine triglyceride results are combined.]% LDH releases were controls, 9 ± 1%; iron, 24 ± 2%; and iron + glycine 17 ± 1%.

*Hypoxia/reoxygenation (H/R) injury.* H/R did not alter triglyceride levels, whether or not glycine was present (for presentation, the triglyceride results are combined) (Fig. 5, right panel). This was despite the fact that glycine completely blocked H/R-induced cell death (LDH release controls, 9 ± 1%; H/R 23 ± 1%; and H/R + glycine 10 ± 1%). [Thus, in both the iron and H/R experiments, triglyceride levels were not impacted by the extent of cell death.]

### Cultured HK-2 cell experiments

*Septic serum exposure.* Cells exposed to 12.5% septic serum had an approximate 25% increase in triglyceride content, compared to 12.5% serum-incubated controls (415 ± 25 vs. 315 ± 10 nmol/μmol Pi, respectively) ($P = 0.037$) (not shown in figures). This difference could not be explained by a difference in serum triglyceride content (and hence cell triglyceride exposure following serum addition), given that the 2-hour postseptic serum and control mouse serum had comparable triglyceride concentrations (controls 165 ± 20; 2-hour septic serum 153 ± 1 mg/dL). [Note: This is different than the hypertriglyceridemia which developed at 18 hours post-LPS injection, and hence it was the reason for harvesting serum from LPS-treated mice prior to the development of high serum triglyceride levels.] The septic serum exerted a mild cytotoxic effect, raising% LDH release from 7.1 ± 0.2% (control serum) to 10.5 ± 0.6% ($P < 0.001$).

*Antimycin A/HK-2 cell experiments.* As shown in Figure 6, incubating HK-2 cells with antimycin A in the absence of serum caused triglycerides to increase by ~50 nmol/μmol Pi ($P < 0.002$). Exposing the cells to 5% serum alone (in absence of antimycin A) led to marked HK-2 cell triglyceride increases ($P < 0.001$). When antimycin A was added to serum-exposed cells, it further raised triglyceride content by ~120 nmol/μmol Pi. When comparing the antimycin A-induced triglyceride increases in the presence vs. the absence of serum, it is apparent that the increase was approximately $2\frac{1}{2}$ times as great (~50 vs. 120 nmol/μmol Pi) when the antimycin A challenge was conducted with serum present. Antimycin A induced a minor degree of cell death in the absence of serum (controls 4.9 ± 0.1%; antimycin A 8.2 ± 0.3% LDH release) ($P < 0.001$), and in the presence of serum

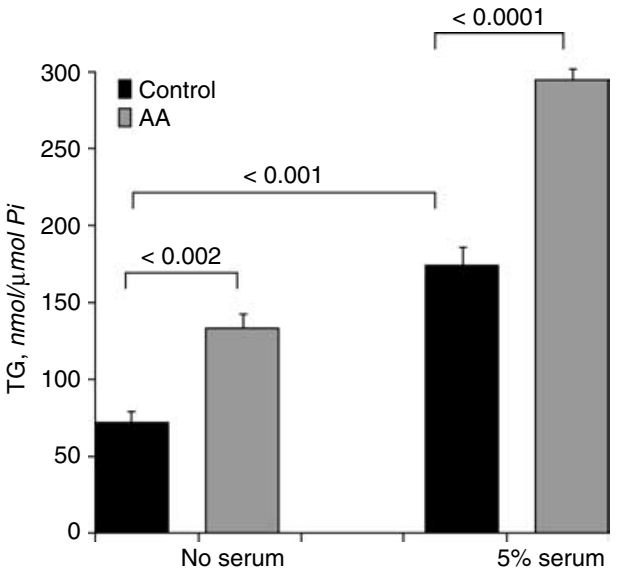


**Fig. 6. Antimycin A (AA) effects on HK-2 cell triglyceride (TG) content in the absence or presence of 5% serum.** Antimycin A caused an approximate 50 nmol/μmol increase in triglyceride content when the cells were maintained in serum-free medium (left two bars) ($P < 0.002$), However, when serum was present, antimycin A caused a 125 nmol/μmol triglyceride increase, compared to their serum-matched controls. This indicates that antimycin A was able to induce far more triglyceride loading in the presence, vs. the absence, of serum. Serum addition alone also raised triglyceride levels (vs. no serum) ($P < 0.001$), indicating presumptive triglyceride uptake, or triglyceride substrate incorporation, into cellular triglyceride pools.

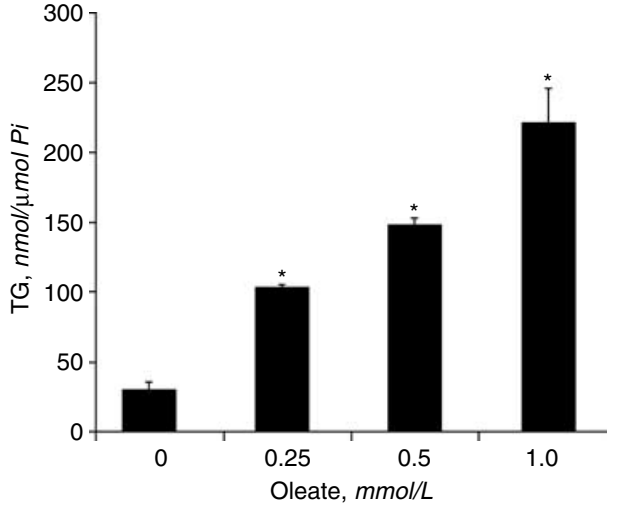


**Fig. 7. Effect of fatty acid (oleate) addition on HK-2 cell triglyceride (TG) levels.** Addition of increasing doses of oleate, added in the presence of 5% bovine serum albumin (BSA), caused dramatic, stepwise increases in triglyceride content. All values were statistically different. $*P < 0.001$, compared to the controls (no oleate addition).

(serum alone 7.1 ± 0.2%; serum + antimycin A 10.5 ± 0.6% LDH release) ($P < 0.005$).

*Oleic acid supplementation: Effect of HK-2 cell triglyceride content.* None of the oleic acid doses caused any lethal cell injury, as assessed by LDH release (<3% for all treatment groups). As shown in Figure 7, BSA-treated HK-2 cells had a triglyceride level of 30 ± 6 nmol/μmol Pi. When oleate was added along with BSA, a steep dose-response relationship with triglyceride levels resulted (rising to 221 ± 25 nmol/μmol Pi) ($P < 0.001$).

*Effects of oleic acid supplementation/triglyceride loading on cell susceptibility to injury.* As shown in Figure 8, oleate treatment of HK-2 cells did not affect their viability under baseline conditions. However, when the cells were pre-treated with oleate (followed by its washout), it predisposed to both iron and ATP depletion/calcium ionophore-induced cell death (Fig. 8). This injury-sensitizing effect was dose dependent, being greater with the 1 mmol/L vs. the 0.5 mmol/L oleate pretreatments (comparing results in the two Figure 8 panels).

## DISCUSSION

As previously noted, UUO has been documented to evoke significant triglyceride accumulation in renal medulla, and to a lesser extent, in renal cortex [11]. However, whether these triglyceride increments were specific for obstructive renal injury, or whether they were reflective of a more generalized renal injury response, was not defined. The current investigations indicate that the latter situation is almost certainly the case. This conclusion is based on observations that three highly divergent forms of in vivo renal injury, notably endotoxemia, I/R, and rhabdomyolysis, each evoked striking, and comparable, degrees of renal cortical triglyceride accumulation. The results obtained with LPS injection are particularly noteworthy. Unlike ureteral obstruction, I/R, or rhabdomyolysis, each of which causes marked renal histologic damage, endotoxemia induces a hemodynamic, or "prerenal," form of acute renal failure [5]. Thus, that renal triglyceride loading developed following LPS injection indicates that structural renal injury is not a prerequisite for this response. Rather, when viewing all of the available data together, it appears that triglyceride accumulation seemingly reflects a previously unrecognized component of a generic renal *"stress response"* (i.e., analogous to an up-regulation of cholesterol levels). Indeed, on a quantitative (molar) basis, triglycerides increase to a much greater extent than does total cholesterol (~300% vs. ~25% increases, respectively) in each form of injury tested to date [1–8].

Given the heterogeneity of cell types within renal cortex, the structures which account for, or participate in, postinjury triglyceride accumulation cannot be ascertained by whole tissue analysis. Therefore, to ascertain whether proximal tubules are prominently involved, they were isolated from LPS-treated and control mice and triglyceride levels were assessed. As with renal cortex, ~3 to 4× higher triglyceride levels were observed in post-LPS-exposed isolated tubules, compared to their

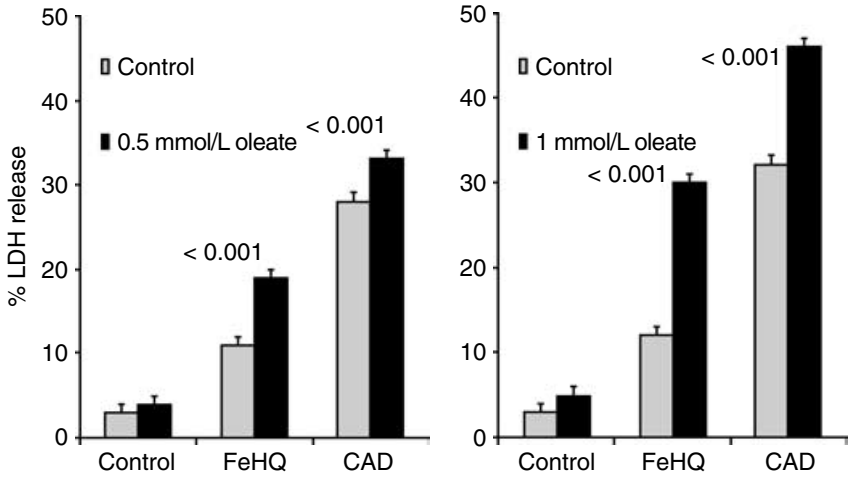


**Fig. 8. Impact of oleate-induced triglyceride loading on HK-2 cell susceptibility to superimposed oxidant or adenosine triphosphate (ATP) depletion/calcium ionophore-induced attack.** Cells were treated × 24 hours with either 0.5 or 1 mmol/L oleate/bovine serum albumin (BSA) to enhance cell triglyceride levels. This was followed by cell washing to remove the oleate/BSA from the medium prior to the addition of the challenges (FeHQ = ferrous ammonium sulfate/hydroxyquinoline complex; CAD = calcium ionophore A23187 + antimycin + 2-deoxyglucose). Both forms of injury were intensified by prior oleate exposure/triglyceride supplementation. This injury-potentiating effect was oleate dose-dependent (> with 1 mmol/L vs. 0.5 mmol/L oleate pretreatment).

matched isolated tubule controls. Of further note, *absolute* triglyceride levels in both control and experimental isolated tubules were ~ twice as high, on a molar basis, as their corresponding renal cortical tissue samples. This suggests that the proximal tubule are relatively triglyceride enriched, compared with other cortical structures. If true, then the proximal tubule compartment, which comprises ~70% of cortical mass, must account for the bulk of postinjury triglyceride accumulation in renal cortex. Of note, endotoxemia caused hypertriglyceridemia by the time of tissue sampling (18 hours postinjection). However, that isolated (blood-free) tubules from LPS-treated mice had three to four times higher triglyceride levels than did control tubules indicates that possible plasma triglyceride contamination of tissue samples cannot explain the higher cortical triglyceride levels. This point is further underscored by the rhabdomyolysis and I/R injury results. Plasma triglyceride levels were significantly *suppressed* in these animals, and yet, three- to fourfold renal cortical triglyceride elevations were observed.

Cellular triglyceride levels reflect a balance between synthesis, substrate catabolism, and possible endocytic transport across the plasma membrane [19–25]. The available data suggest that multiple mechanisms, rather than any single pathway, likely account for the presently documented injury-induced renal triglyceride loading state. The following possibilities appear particularly relevant in this regard, based on the available data.

### $PLA_2$ activation with free fatty acid channeling into triglycerides

It is well recognized that diverse forms of cell injury, including those utilized in this study, activate $PLA_2$ [26–28]. This results in fatty acid mobilization from phospholipid pools, presumably increasing their availability for triglyceride formation via diacylglycerol acyltransferase (DGAT) activity. To prove that $PLA_2$-mediated free fatty acid mobilization can promote triglyceride formation, we tested whether a nonlethal dose of exogenous $PLA_2$ might increase triglyceride content in isolated tubules. As illustrated in Figure 5 (left panel), a tight dose-response relationship between $PLA_2$ dosage and resulting tubule triglyceride levels was observed. Thus, these new observations prove that $PLA_2$ activity can, indeed, increase tubular triglyceride content. However, it should also be noted that when *endogenous* $PLA_2$ was activated by either H/R or iron-induced oxidant stress, no isolated tubule triglyceride increments resulted. This indicates that other injury-induced pathways must modulate the extent to which $PLA_2$ activity is able to enhance cellular triglyceride pools (e.g., possible loss of DGAT activity). Indeed, this is consistent with previous in vivo observations of Matthys et al [13]. These investigators observed that renal cortical triglyceride levels did not increase, but rather slightly decreased, immediately post-I/R tissue damage. Thus, other factors must also be involved.

### Decreased mitochondrial consumption increases free fatty acid availability

It is well known that renal tubular metabolic work declines with reductions in glomerular filtration rate (GFR). This is due to decreased sodium filtration, and hence, decreased sodium reabsorption. For example, with severe acute renal failure, renal $O_2$ consumption falls by ~80% [29]. Since free fatty acids are the dominant tubular

mitochondrial substrate [30], it follows that a decrease in tubular sodium reabsorption/metabolic work decreases free fatty acid utilization, thereby increasing fatty acid availability for possible triglyceride formation. Given that endotoxemia, rhabdomyolysis, and I/R injury each caused marked reductions in GFR (as denoted by azotemia), a decrease in mitochondrial respiration, and hence, fatty acid consumption/beta oxidation, would be expected to provide additional free fatty acid for triglyceride formation. Clearly, a reduction in GFR does not pertain to the current in vitro (HK-2; isolated tubule) experiments. However, it is noteworthy that both antimycin A and $PLA_2$ directly, and indirectly, decrease mitochondrial respiration [31], respectively. Thus, decreased mitochondrial fatty acid utilization might well have contributed to triglyceride accumulation even in the current in vitro experiments.

### Increased triglyceride/triglyceride substrate uptake from the circulation

In the previously discussed study by Tannenbaum et al [11], increased accumulation of intravenously administered $^{14}$C14-labeled fatty acid (oleate, arachidonate) was observed in unilaterally obstructed kidneys. However, it could not be determined whether these increased levels reflected increased uptake and/or decreased fatty acid metabolism (e.g., due to a corresponding near cessation of GFR). The present study provides direct evidence supporting the notion that increased triglyceride/triglyceride substrate uptake does, in fact, contribute to an injury-evoked triglyceride accumulation state. When HK-2 cells were challenged with antimycin A in the *absence* of serum, increased triglyceride levels occurred. However, when this same protocol was conducted in the *presence* of serum (with its constituent triglycerides and fatty acids), exaggerated triglyceride accumulation resulted (i.e., above that which could be accounted for by either antimycin A alone, or serum addition alone). Furthermore, when HK-2 cells were incubated with increasing doses of oleic acid, dramatic, dose-dependent, triglyceride formation resulted. Thus, it appears that injured tubular cells can rapidly increase their uptake of exogenous triglyceride/triglyceride substrate (fatty acids), resulting in an enhancement of cellular triglyceride pools. Whether cell injury causes an increase in direct triglyceride endocytosis, or simply triglyceride substrate (i.e., fatty acid) uptake, remains to be defined. However, it is noteworthy that following both in vivo I/R and rhabdomyolysis, a striking inverse correlation was noted between circulating triglycerides and renal cortical triglyceride content. This provides strong, albeit indirect, support for the concept advanced above: that circulating triglycerides (or their derived fatty acids) may well contribute to an renal injury-evoked triglyceride loading state. Finally, it appears that such processes may be enhanced by circulating cytokines, given that serum from septic mice caused far greater HK-2 cell triglyceride accumulation than did control serum despite comparable serum triglyceride levels.

The final issue which was addressed in this study was whether postinjury triglyceride loading, or the processes leading to it, might impact subsequent injury responses, or the emergence of the previously discussed "cytoresistant state." To address this issue, cells were triglyceride loaded via oleate treatment, and then the oleate was washed out of the culture medium prior to the application of an oxidant (iron) or ATP depletion challenge. The importance of fatty acid washout *prior* to assessing susceptibility to injury resides in the fact that unsaturated fatty acids can exert selected cytoprotective effects [32–36]. Despite this consideration, oleate-induced triglyceride supplementation induced striking, dose-dependent, increases in susceptibility to oxidant and ATP depletion-induced attack. Given the complexity of fatty acid uptake and the "downstream" consequences thereof, it is impossible to conclude at this time that postinjury triglyceride loading, per se, exerts a direct injury-provoking effect. For example, free fatty acids can exert a variety of pronecrotic, as well as apoptotic influences, mediated via direct membrane lytic effects, alterations in pro- and antiapoptotic proteins, mitochondrial cytochrome-c release, cytotoxic ceramide generation, altered cell signaling (e.g., via protein kinase C) [35–38]. Thus, it is possible that direct adverse oleate effects on cell homeostasis, rather than the resulting triglyceride accumulation, was responsible for the proinjury effect. Indeed, it has been speculated in the literature that triglyceride formation may represent a *protective* response, due to a shuttling cytotoxic fatty acids into neutral lipid [35, 36]. On the other hand, it would seem prudent to leave open the possibility that triglyceride loading, per se, predisposes to cell injury, given that oleate treatment, by itself, caused no overt HK-2 cytotoxicity, and because exogenous oleate was removed from the cell culture medium following triglyceride loading. Thus, although cellular "lipotoxicity" [35, 36] is generally considered a consequence of fatty acid toxicity rather than the resulting triglyceride formation, the present data suggest that the latter possibility may still exist. Indeed, if true, then excess injury-induced triglyceride formation could have a biphasic response: first, protecting against cytotoxic fatty acid accumulation, and second, potential delayed adverse effects, potentially mitigating a post injury cytoresistant state. Whether or how cellular triglyceride overload, per se, might impact cell injury responses remains a largely unexplored issue at this time.

## CONCLUSION

The present study demonstrates that diverse forms of renal injury evoke dramatic triglyceride accumulation

in renal cortex and in proximal tubular cells. That this occurs with heterogeneous insults, and whether or not structural renal damage results, suggest that triglyceride accumulation is a previously unrecognized component of the generic renal "stress response." Multiple mechanisms likely contribute to this triglyceride accumulation, including excess $PLA_2$ activity, cytokine stimulation, increased triglyceride synthesis, and increased triglyceride or triglyceride substrate (e.g., fatty acid) uptake. The contribution(s) of each to triglyceride loading, and the downstream consequences thereof, represent interesting scientific issues which could have potentially important implications for both cell injury and/or repair.

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