PROXIMAL TUBULAR CHOLESTEROL LOADING

FOLLOWING MITOCHONDRIAL, BUT NOT

GLYCOLYTIC, BLOCKADE

Richard A. Zager, MD
Ali C.M. Johnson, BS
Sherry Y. Hanson, BS

From the Fred Hutchinson Cancer Research Center, and the University of Washington,
Seattle, WA

Address correspondence to:
Richard A. Zager, MD
Fred Hutchinson Cancer Research Center
1100 Fairview Ave. N; Room D2-190
Seattle, WA 98109
Tele: (206) 667-6549
Fax: (206) 667-6519
Email: dzager@fhcrc.org
ABSTRACT

**Background:** Diverse forms of injury cause proximal tubular cholesterol accumulation. However, underlying mechanisms, in general, and those involved with ATP depletion injury, in particular, remain poorly defined. To help elucidate this issue, cholesterol homeostasis and its determinants were assessed following partial ATP depletion states.

**Methods:** Serum-exposed HK-2 cells were subjected to mild ATP depletion, induced by mitochondrial inhibition (antimycin A; AA) or glycolytic blockade (2-deoxyglucose; DG). Four or 18 hrs later, cell cholesterol levels, HMG CoA reductase (HMGCR), the LDL receptor (LDL-R), and the ABCA1/SR-B1 cholesterol transporters were assessed.

**Results.** AA and DG each induced mild, largely sublethal ATP depletion injury. Each also caused significant HMGCR increments and SR-B1 decrements, and left ABCA1 intact. In contrast, only AA increased the LDL-R, and only AA evoked a cholesterol loading state (~25%↑). Half of this increase was statin inhibitable, and half could be blocked by serum deletion, implying that both synthetic and non synthetic (e.g., LDL-R transport) pathways were involved. The AA-induced HMGCR and LDL-R protein changes were paralleled by their mRNAs, suggesting the presence of altered transcriptional events. **Conclusions:** 1) Sublethal ATP depletion, whether induced by mitochondrial or glycolytic blockade, can upregulate HMGCR and decrease SR-B1. These changes represent a previously unrecognized ATP depletion 'phenotype'. 2) Mitochondrial blockade can also upregulate the LDL-R and evoke a cholesterol loading state. 3) The latter likely occurs via synthetic and transport pathways; and 4) the mitochondrion may be a critical, and previously unrecognized, determinant of post injury- cell cholesterol homeostasis, potentially by impacting the LDL-R.
INTRODUCTION

Cellular stress evokes a plethora of responses which appear aimed at protecting against subsequent bouts of tissue damage. Most attention in this area has focused on stress- induced cytoprotective proteins, such as heat shock proteins, heme oxygenase-1, and ferritin (1,4,9,12,18). However, alterations in lipid expression may also impart cellular resistance to further attack (e.g. unsaturated fatty acids, sphingosine, lysophosphatidic acid; ref. 5,10,21). Perhaps the most consistent cytoprotective lipid yet identified is cholesterol. This conclusion is based on a series of experiments which demonstrate that diverse forms of renal injury (ischemia, toxins, oxidative stress, sepsis, heat shock, hyperosmolality; immunologic injury, urinary tract obstruction), each evoke increases in proximal tubular cholesterol content (19,22,24-30). That these cholesterol increases are critical to cellular resistance to injury is indicated by a series of observations which demonstrate that: 1) either reversing post- injury cholesterol increments, or prevention of them (with statin or zaragozic acid therapy), cancels the cytoresistant state (11,16,22,24,29,30); and 2) alterations of cholesterol homeostasis within normal cells (e.g., via oxidation; de-esterification; interference with normal p glycoprotein- mediated cholesterol cycling) each cause cellular ATP depletion, and lethal cell damage (19,20). Of interest, chemotherapeutic injury imposed on malignant myeloid cells also induces cholesterol accumulation and a cytoresistant state (11,16). That essentially identical findings have been observed in proximal tubules and myeloid cells indicates the potential generalized nature of this post- injury cholesterol overload state.

Despite the apparent uniformity of post-injury cholesterol accumulation, the mechanisms by which it occurs may vary according to the specific cell type involved and
the type of injury sustained. The most extensive evaluation of this issue has been conducted with iron-mediated oxidative stress. Based on a series of in vivo (glycerol induced acute renal failure) and in vitro (ferrous ammonium sulfate-mediated oxidative stress) investigations from this laboratory (24,28,30), it has been hypothesized that multiple defects in cholesterol homeostasis may co-exist. These include the following: 1) increased cholesterol synthesis, based on findings of increased HMG CoA reductase (HMGCR) protein levels and activity (24); 2) increased LDL receptor (LDL-R) expression (28); and 3) Fe-induced reductions in ABCA1 and SR-B1 (28). The former can efflux free (i.e., unesterified) cholesterol from cells, whereas the latter may evoke bi-directional cholesterol transport (increasing cholesteryl ester uptake, FC efflux; ref. 8, 28). Thus, changes in their expression would be expected to alter cell cholesterol content.

It remains unknown whether these Fe mediated changes are relevant to other injury-induced cholesterol overload states. Therefore, the present study was undertaken to ascertain potential mechanisms for cholesterol accumulation in a second form of cellular injury: that evoked by partial ATP depletion. The relevance of this issue is indicated by prior observations that in vivo renal ischemic/reperfusion injury induces renal cortical cholesterol overload (22). Given the heterogeneity of cell types within renal cortex, the present study focused on events occurring within cultured renal proximal tubular (HK-2) cells in order to gain cell-type specific data. Using this approach, the following questions were addressed: 1) Can mild, sublethal ATP depletion trigger an increase cell cholesterol content? 2) If so, does the mechanism by which ATP depletion occurs (e.g., decreased mitochondrial ATP production vs. decreased glycolysis) impact the development of a cholesterol overload state? 3) Does ATP depletion injury increase
cell cholesterol solely via increased synthesis (e.g., as previously noted with iron mediated injury; ref. 29), or might other mechanism(s) also be involved? 4) If increased cholesterol synthesis results from partial ATP depletion, does increased protein prenylation, a potential by-product of increased HMGCR activity / mevalonate pathway flux (30), also result? and 5) Does partial ATP depletion cause reductions in key cholesterol transport proteins (ABCA1, SR-B1) in a fashion analogous to that which follows iron mediated oxidative stress?

**METHODS**

**Cell culture methods:** HK-2 cells, an immortalized human proximal tubular cell line established from normal human kidney (14), were used for all experiments. They were cultured in T-75 flasks (Costar; Cambridge, MA) with keratinocyte serum free medium (K-SFM; GIBCO Life Technologies; Grand Island, NY) to which was added 1 mM glutamine, 5 ng/mL epidermal growth factor, 40 µg/mL bovine pituitary extract, 25 U/mL penicillin, and 25 µg/mL streptomycin (14). The cells were passaged by trypsinization and then re-seeded into: 1) additional T75 flasks (for further passage, or for experiments which called for cell protein or adenine nucleotide analysis); 2) T25 flasks (for total cholesterol determinations); or 3) 24 well Costar plates for assessments of cellular injury / viability. The cells were cultured for 1-3 days following passage to achieve near confluence prior to the conduct of specific experiments, as described below.

**Antimycin A – induced mitochondrial inhibition: effect on cell cholesterol levels.**

Twelve T25 flasks were divided into two equal groups, as follows: 1) incubation with antimycin A (AA; 7.5 µM; dissolved in ethanol, final concentration, 0.1%; ref. 6); or 2) incubation with the AA carrier (ethanol). Immediately prior to AA or carrier addition,
heat inactivated (56º C x 20 min) normal mouse serum (Gemini Bio Products; cat. # 100-113) was added to a final concentration of 2.5%. The cells were incubated under these conditions x 18 hrs. The flasks were then rinsed x 2 with Hanks balanced salt solution (HBSS + Ca²⁺ / Mg²⁺) to remove the serum and its contaminating cholesterol. The cells were recovered by scraping the flasks with a cell scraper and then rinsing the flask x 3 with HBSS. The cells were recovered from the HBSS by centrifugation. The pellet was washed with HBSS, and then extracted in 3 mL of 1:2 chloroform: methanol (22,30). The lipid fractions were recovered and dried to a volume of 100 µL. Methanol, 10 µl, was added, the samples were sonicated and then centrifuged. They were subsequently assayed for total cholesterol using a commercially available kit (Thermo DMA, Arlington, TX), as previously performed in this laboratory (22). Results were expressed as nmoles cholesterol/µmole phospholipid phosphate (Pi) in each sample (the latter determined by sample ashing and inorganic Pi analysis; ref. 22).

To assess whether a second mitochondrial inhibitor might alter cell cholesterol levels, the above experiment was repeated in 3 control and 3 rotenone treated flasks (either 10 or 50 µM; n of 2 and 1 respectively; in 0.05% ethanol). After 18 hrs, cell cholesterol levels for the control and rotenone treated cells were compared.

**Effect of HMGCR inhibition on AA- induced cholesterol increments.**

The above experiments demonstrated that mitochondrial blockade causes cholesterol loading of serum- exposed HK-2 cells (see Results). To ascertain whether HMGCR mediated cholesterol synthesis was completely responsible for this result, the following experiment was undertaken. Eight T25 flasks of HK-2 cells were divided into two equal groups: 1) incubation with 2.5% serum + 10 µM mevastatin (in 0.1% DMSO;
ref. 29); and 2) incubation with AA + serum + mevastatin. After completing 18 hr incubations, the cells were harvested and assayed for total cholesterol content, as noted above.

**HK-2 cell cholesterol levels with the AA challenge in the absence of serum.**

As presented in the Results, statin therapy eliminated ~ 50% of AA- induced cholesterol loading, suggesting that both synthesis, and possible increased cell cholesterol uptake, were involved. If so, then if the AA challenge were conducted in the absence of serum, increased cholesterol uptake would not be possible. Hence, this would be expected to decrease the AA- induced cholesterol loading by ~50%. The following experiment tested this hypothesis. Eight flasks of HK-2 cells were cultured in the absence of serum, and 4 underwent the AA challenge. The remaining 4 served as controls. After 18 hr incubations, cell cholesterol levels were determined.

**ATP depletion from glycolysis inhibition: impact on cell cholesterol content.**

To contrast results obtained by mitochondrial inhibition, 8 flasks of HK-2 cells were prepared and divided into two equal groups: 1) addition of 2.5% serum; and 2) serum addition + 20 mM 2-deoxyglucose (to inhibit glycolysis; ref. 6). Eighteen hrs later, cell cholesterol levels were determined, as noted above.

**Impact of AA and 2-deoxyglucose on HK-2 cell viability.**

The following experiments were conducted to ascertain the extent to which AA and 2-deoxyglucose caused lethal cell injury under conditions used in the above experiments. To this end, two 24 well Costar cluster plates were seeded with HK-2 cells. At near confluence, the 24 wells/plate were each divided into the following groups (n of 6 per treatment): 1) control incubation with 2.5% serum; 2) incubation with serum + 7.5
µM AA; 3) a second set of control incubation with serum; and 4) incubation with serum + 20 mM 2-deoxyglucose. After completing 18 hr incubations, lethal cell injury was assessed by % LDH release.

**Impact of AA and 2-deoxyglucose on cellular ATP levels.**

The following experiment was undertaken to ascertain relative degrees of impairment of cellular energetics induced by AA vs. 2-deoxyglucose. To this end, 18 T75 flasks were seeded with HK-2 cells. At near confluence, they were divided into 3 groups: 1) addition of 2.5% serum; 2) addition of serum + AA; and 3) addition of serum + 2 deoxyglucose. After 4 hr incubations, adenine nucleotides were extracted in 6.66% trichloroacetic acid and then analyzed for ATP and ADP concentrations by HPLC as previously described (23). The degree of suppression of cellular energetics was assessed by calculating ATP/ADP ratios and the amount of ATP extracted per flask.

**Effect of AA on HMGCR, ABCA1, SR-B1, and LDL-R mRNAs**

Cellular cholesterol levels reflect a balance between: i) synthesis; ii) LDL-R mediated cholesterol uptake; and iii) cell free cholesterol (FC) / cholesteryl ester (CE) efflux/influx (ABCA1: FC efflux; SR-B1: CE influx/FC efflux; ref 8). To gain insights into these pathways during AA- induced injury, the mRNAs for each were determined. To this end, HK-2 cells were seeded into 36 separate T 25 flasks. Upon reaching near confluence, they were divided into 4 groups: 1) control incubation with 2.5% serum x 4 hrs (n=10); 2) incubation with 7.5 µM AA + 2.5% serum x 4 hrs (n=10); 3) control incubation with 2.5% serum x 18 hrs (n=8); and 4) incubation with AA + 2.5% serum for 18 hrs (n=8). At the completion of the incubations, the cells were recovered as noted above and immediately placed into TRIzol reagent (Invitrogen Life Technologies,
Carlsbad, CA). Total RNA was extracted as per the manufacturer's instructions. The final RNA pellet was brought up in RNase-free water to an approximate concentration of 1 mg/mL. The samples were electrophoresed x 30 min through 1.2% agarose containing ethidium bromide (Sigma Chemicals) in order to insure a lack of degradation (preservation of 18S and 28S ribosomal RNA) prior to PCR analysis (30).

ABCA1, LDL-R, and SR-B1 (as well as GAPDH) mRNAs were analyzed simultaneously by multiplexing RT-PCR using human specific primers, as previously described (28) and as shown in Table 1. [Note: The validity of this method has been previously confirmed by documenting that mevastatin therapy of HK-2 cells leads to appropriate responses of the LDL-R, ABCA1, and SR-B1 messages (a marked increase, a marked decrease, and minimal decrease, respectively (28)]. LDL-R, ABCA1, and SR-B1 values were expressed by factoring them by the GAPDH reference product.

HMGCR mRNA was analyzed separately using human specific primers and conditions listed in Table 1. Otherwise, the conditions were identical to those previously described (28; Table 1). To confirm the biologic validity of this assay, samples were obtained from 4 flasks of HK-2 cells treated with (n=2) or without (n=2) 10 µM mevastatin (to confirm a statin-induced increase in HMGCR mRNA).

**Antimycin A effects on ABCA1, SR-B1, LDL-R, and HMGCR protein expression.**

Eight T25 flasks were seeded with HK-2 cells. Upon reaching near confluence, they were equally divided into two groups: 1) addition of 2.5% serum + AA; or 2) addition of 2.5% serum + 0.1% ethanol (the AA vehicle). Eighteen hrs later, cell protein extracts from each flask were prepared as previously described and used for Western blotting for SR-B1, ABCA1, LDL-R, and HMGCR (8,24,28). In brief, for SR-B1
detection, 5 µg of protein extract were electrophoresed through a 12% Bis-Tris acrylamide Nupage gel (Invitrogen, Carlsbad, CA) and probed with rabbit anti-SR-B1 antibody (catalog number NB-400-104; Novus Biologicals, Littleton, CO). For ABCA1 detection, a 25 µg protein extract sample was electrophoresed through a 4-12% gradient Bis-Tris acrylamide Nupage gel. Rabbit anti-ABCA1 (catalog # NB-400-15; Novus Biologicals) was used as the primary antibody. Secondary detection for both anti-SR-B1 and anti-ABCA1 antibodies was performed using horseradish peroxidase-labeled donkey anti-rabbit IgG (catalog number NA 934; Amersham-Pharmacia, Piscataway, NJ) and enhanced chemiluminescence (ECL Kit; Amersham-Pharmacia; ref. 10). For detection of LDL-R, 25 µg protein extract samples were electrophoresed through a 4-12% gradient Bis-Tris acrylamide Nupage gel. Mouse anti-LDL-R (catalog number LP02: Oncogene Research Products, Boston, MA) was used as the primary antibody. Secondary detection was performed with horseradish peroxidase-labeled sheep anti-mouse IgG (catalog # NA 931; Amersham-Pharmacia, Piscataway, NJ) and ECL (8). HMGCR was probed in 25 µg protein samples electrophoresed into the above 4-12% Nupage gel, using rabbit anti-HMGCR (a gift from PA Edwards, Los Angeles, CA). Detection was performed with donkey anti-rabbit IgG (24). Nonspecific secondary antibody staining in the above Western blots has been excluded by the fact that the secondary antibody, in the absence of the primary antibody, does not identify the relevant protein band(s). Equal protein loading/transfer with these protocols has been confirmed by India ink staining. Relevant protein band quantitation was performed by optical density scanning (ABCA1, 220 kDa; SR-B1, 82 kDa; non glycosylated LDL-R, 120 kDa; glycosylated LDL-R, 160 kDa; HMGCR, ~45 kDa; ref. 24).
Deoxyglucose effects on ABCA1, SR-B1, LDL-R, and HMGCR protein expression.

To ascertain whether ATP depletion induced by 2-deoxyglucose causes a different pattern of cholesterol homeostatic protein expression than does AA, the above experiment was repeated, with the exception that 2-deoxyglucose challenge was substituted for AA treatment.

Effect of AA-induced cholesterol loading on HK-2 cell Rho and Ras prenylation.

To ascertain whether AA-induced changes in the mevalonate pathway impacts protein prenylation, 4 control samples and 4 samples obtained 18 hrs post AA treatment were probed for Rho and Ras by Western blotting as previously described (30). In the case of Rho, prenylation was assessed by comparing the ratio between the prenylated (lower) band and unprenylated (top) band on the gel following their quantitation by ECL. The relative position of these two bands has previously been determined by treating HK-2 cells with mevastatin, which causes a marked decrease of the lower (prenylated), but not of the upper (unprenylated) band (30). Thus, the ratio of lower/upper (prenylated/unprenylated) band serves as an indication of Rho prenylation. In the case of Ras, a dominant unprenylated band appears and a second (upper) band develops with prenylation (30). Hence, the latter was sought following AA treatment.

Calculations and Statistics. All values are presented as means ± 1 SEM. Statistical comparisons were performed by unpaired Student’s t test. Significance was judged by a p value of <0.05.
RESULTS

HK-2 cell cholesterol levels in response to AA with and without mevastatin.

As shown in Fig. 1, the AA challenge caused a marked increase in cell cholesterol content, rising from 255±6 to 307±13 nmoles/µmole Pi. When this same experiment was performed in the presence of mevastatin (in both the control and AA incubations), AA still induced a statistically significant cholesterol increase. To contrast the relative degree of AA-induced cholesterol increases in the presence vs. absence of mevastatin, the % increase in cholesterol between the two sets of flasks were compared. The % increase in cholesterol induced by AA in the absence of mevastatin was 25±3%, compared to 11±1% in the presence of AA + statin (p<0.005).

AA-induced cholesterol accumulation in the absence of serum.

The above results are consistent with the view that ~50% of the AA-induced cholesterol accumulation was due to synthesis (i.e., statin inhibitable) and 50% was statin resistant (i.e, likely due to increased uptake ± decreased efflux). If so, then eliminating uptake by removing serum should reduce the AA-induced cholesterol loading by ~50%. Indeed, this is precisely what was found with AA treatment of cells. In the absence of serum, AA induced a 12% cholesterol increase (control cells, 264 ± 2; AA, 295 ± 4; p<0.001). Given that AA cause a 25% cholesterol increase in the presence of serum, the withholding of serum decreased cholesterol loading by ~50% (i.e., from 25% to 12%).

Rotenone effects on HK-2 cell cholesterol levels.

To test whether a second model of mitochondrial inhibition would raise cholesterol levels in the presence of serum, rotenone effects were assessed. Rotenone caused a smaller (~10%), but still significant (p=0.01), increase in total cellular
cholesterol, compared to the increase observed with AA (~25%). [Of note however, these rotenone and AA results cannot be directly compared because both rotenone doses, unlike antimycin, caused ~40% cell detachment, consistent with greater injury (e.g., more dead cells which would not synthesize or transport cholesterol)]. Because of this cell detachment, this model was not chosen for further experiments.

**Effect of 2-deoxyglucose on HK-2 cell cholesterol levels.**

Unlike AA, treatment of cells with 2-deoxyglucose did not significantly alter cellular cholesterol levels. Total values for the control and 2-deoxyglucose treated cells were 264±11 and 262±15 nmoles/µmole Pi, respectively.

**Extent of cell injury induced by AA and 2-deoxyglucose.**

AA treatment did not cause a significant increase in % LDH release (controls 7±1%; AA, 8±1%; NS). However, total adherent cell LDH content (a reflection of total viable cell mass; ref. 6,7) of the AA exposed cells was decreased by 12% (16.4±2 units vs. 14.6±0.1 units; p<0.001), clearly indicating that cell injury (most likely an anti-proliferative response ± a small amount of apoptosis) did occur. In contrast to AA, 2-deoxglucose did cause a small, but significant increase in % LDH release 6±1% vs. 10±1%; p<0.001). Thus, in both injury models, very slight, but significant, and comparable, injury could be detected. [Note, since 2.5% serum contains a small amount of LDH, this value was subtracted from the total supernatant values prior to determining % LDH release].

**Cellular energetics following AA or 2-deoxyglucose treatment.**

Cellular energetics, as assessed by ATP/ADP ratios, were significantly depressed with both AA and 2-deoxyglucose treatment (control ATP/ADP ratios, 18.3±0.6; with
AA, 11.8±1.6; with 2-deoxyglucose, 8.5±0.4) (p<0.001 vs. controls; NS vs. each other). A comparison of absolute ATP concentrations per flask showed that 2-deoxyglucose caused a slightly greater depression in ATP than did AA treatment (64±2 μmoles vs. 79±5 μmoles, respectively, p<0.03; control values, 90±4 μmoles). Thus, despite the fact that AA, but not 2-deoxyglucose, treatment raised cell cholesterol levels, the degree of ATP reduction was at least comparable, or slightly worse, with the latter agent.

**HMGCR, LDL-R, ABCA1, and SR-B1 mRNA levels following the AA challenge.**

The results of RT-PCR analyses are presented in Table 2. In brief, HMGCR mRNA values were slightly, but statistically, higher at both 4 hrs and 18 hrs after AA addition. As a physiologic control for the HMGCR mRNA assay, levels were measured in control and statin treated cells, as noted in the Methods section. Statin therapy induced an approximate 7 fold increase in HMGCR values (6.5±0.2 vs. 1.0±0.2, statin vs. control values; p<0.005).

AA also induced modest, and statistically significant, elevations in LDL-R mRNA values at each of the two time points. Conversely, ABCA1 message was suppressed by ~25% at 4 hr post AA addition. Although ABCA1 mRNA values returned to ‘normal’ by 18 hrs of AA treatment, these values should be considered physiologically abnormal, given that cholesterol loading at 18 hrs would be expected to increase the ABCA1 message (and protein) levels to help restore normal cholesterol content. SR-B1 mRNA levels were trivially, but significantly, elevated at both the 4 and 18 hr time points.

**AA effects on HMGCR, LDL-R, SR-B1, and ABCA1 protein expression.**

Representative Western blots and statistical comparisons between control cells (open bars) and 18 hr AA treated cells (shaded bars) are presented in Fig. 2. As shown at
the far left, HMGCR protein expression was significantly increased in the AA treatment group, compared to controls (p<0.025). AA also caused an approximate 33% increase in unglycosylated LDL-R protein levels (lower band; p<0.02). In contrast, there was a slight, but non significant, increase in the amount of the LDL-R glycosylated moiety (upper band; not depicted in bar graph). Unlike the results with HMGCR and LDL-R, AA had no significant effect on ABCA1 expression. However, AA induced a ~35% decrease in SR-B1 (p<0.001). A corollary of the SR-B1 reductions was the appearance of a lower molecular weight protein band, consistent with an SR-B1 degradative product (Fig. 2). This band was not seen in control HK-2 cell protein samples.

2-deoxyglucose effects on HMGCR, LDL-R, SR-B1, and ABCA1 protein expression.

After 18 hrs of 2-deoxyglucose exposure, a significant increase in HMGCR protein was noted (230±24 vs. controls, 151±29; p<0.01). On a relative basis, this was highly comparable to the % increase observed with AA treatment. Also like AA, 2-deoxyglucose caused a significant decrease in SR-B1 levels (126±10 vs. controls, 205±20; p<0.01), again comparable to that observed with AA (although with 2-deoxyglucose, a second apparent degraded band did not appear). ABCA1 was not significantly affected by 2-deoxyglucose, again recapitulating the AA results. However, in striking contrast to AA treatment, 2-deoxyglucose failed to increase LDL-R expression. Rather, a non significant trend towards decreased, rather than increased, LDL-R protein expression resulted [(upper band: 96±28, control; 69±15, deoxyglucose; NS); lower band: 183±12, control; 168±6, deoxyglucose; NS). Thus, of the 4 proteins tested, only LDL-R expression differed between the AA and 2-deoxyglucose treatment. [All values are presented as densitometry units].
Rho and Ras prenylation.

Western blot probes of Rho and Ras are presented in Fig. 3. Two Rho bands were observed, with the top and bottom representing the unprenylated and prenylated moieties, respectively. No obvious differences in their expression were observed between the control (open bars) and AA treated samples (shaded bars). Furthermore, the ratios of the prenylated / unprenylated band did not significantly differ for the control and AA treatment groups (as depicted by the bars). The Ras probe revealed only a single band which did not differ between the control (open bar) and AA treated (shaded bar) groups (i.e., no evidence of a prenylated band was apparent in either group).

DISCUSSION

All forms of in vivo renal injury tested to date (22,24-30) have demonstrated a number of common features vis à vis the development of a cholesterol overload state. These include the following: i) an 18 hr 'lag' time is required for cholesterol loading to develop; ii) both unesterified (free)- and esterified cholesterol participate in this response; iii) these changes are fully expressed within proximal tubules; and iv) cholesterol loading confers cellular resistance to further attack. Despite this common post- injury cholesterol 'phenotype', less consistent information exists regarding the in vivo mechanisms which initiate, and maintain, this cholesterol overload state. For example, following glycerol- or heat shock- mediated injury, increased renal cortical HMGCR protein expression results (24). Conversely, although sepsis syndrome and renal ischemia induce marked cholesterol loading, renal cortical HMGCR protein levels remain at control levels (24,26). Studies of corresponding HMGCR mRNA levels following renal injury further suggest different operant pathways. For example, glycerol- induced myohemoglobinuria
induces stepwise increases in renal cortical HGMCR mRNA (30). However, following renal ischemia, renal cortical HMGCR mRNA decrements result (30). These discrepancies suggest that multiple, and potentially disease specific, pathways mediate the injury-initiated cholesterol loading state.

A major caveat to assessments of cellular mechanisms performed using in vivo tissues is the heterogeneity of cell types within renal cortex. This precludes evaluation of proximal tubule cell-specific events. To circumvent this problem, this laboratory has explored pathways of cholesterol accumulation in cultured HK-2 cells subjected to Fe mediated oxidative attack (28,30). These studies further suggest that multiple pathways may contribute to in vitro cholesterol accumulation, including increased synthesis and possibly decreased cholesterol efflux due to reductions in the ABCA1 and SR-B1 free cholesterol efflux proteins. However, HMGCR driven-synthesis appears to be primary in Fe challenged cells, based on the fact that statin therapy completely blocks the cholesterol loading state (30). However, two significant caveats to these prior HK-2 cell experiments exist (28,30): First, they were conducted under serum free conditions, thereby precluding the possibility of cholesterol uptake from the extracellular space (e.g., via the LDL receptor); and second, the iron challenge caused significant cell death (~30-40% LDH release). Hence, the relative partitioning of cholesterol between viable vs. non-viable cells could not be ascertained. Therefore, to explore mechanisms of injury-induced tubular cholesterol accumulation under conditions devoid of these two caveats, the current study employed a sublethal ATP depletion model conducted in the presence of serum. Because partial ATP depletion exists during the recovery phase of ischemic
ARF (e.g., ref. 17), it was hoped that the results obtained might provide insights into mechanisms of renal cholesterol accumulation with post ischemic kidney damage.

The first notable result stemming from the current studies is that sublethal ATP depletion, when induced by mitochondrial blockade, can initiate the cholesterol loading state. Although this is the first demonstration of this phenomenon, it does not seem particularly surprising, given prior observations that multiple forms of injury can induce this result (19,22, 24-30). However, what is surprising is that the pathway by which partial ATP depletion occurs appears to be critical. As detailed in the Results section, AA caused an approximate 25% cholesterol increase. Rotenone also induced a cholesterol overload state. However, when a comparable, if not greater, degree of ATP depletion was induced by glycolytic blockade with 2-deoxyglucose, absolutely no cholesterol loading did result. These findings indicate that ATP decrements alone are insufficient to evoke cholesterol increments. Rather, some unidentified consequence(s) of mitochondrial dysfunction (e.g., potentially cytochrome C release, free radical generation, loss of Ca buffering capacity, etc.) appear to be required.

A second notable result of the current investigations is the demonstration that increased HMGCR-mediated synthesis is not necessarily the sole mechanism by which injury evokes a cholesterol loading state. In contrast to prior studies of in vitro Fe mediated injury in which mevastatin completely blocked cholesterol accumulation (29,30), in the present study, statin treatment only blocked ~50% of the AA-induced cholesterol overload state. Thus, this finding indicates that although cholesterol synthesis was clearly operative, alternative (statin resistant) pathway(s) were also involved. There are at least three potential candidates in this regard: First, AA treatment significantly
increased LDL-R expression. Given that serum was present, increased cholesterol uptake was a plausible result. That AA caused ~50% less cholesterol uptake in the absence (vs. the presence) of serum is consistent with this concept. Second, SR-B1 was decreased with AA treatment. Although this transporter can mediate cholesteryl ester influx, as well as free cholesterol efflux, the latter function appears dominant in proximal tubular cells (8). Thus, AA-induced SR-B1 reductions could have contributed to the cholesterol loading state; and third, despite AA-induced cholesterol accumulation, a failure of a normal compensatory increase in the ABCA1 cholesterol transporter did not result. Thus, the finding of 'normal' ABCA1 levels in the setting of AA-induced cholesterol increases represents an aberrant response.

In sum, the above findings point to multiple AA-induced defects in cholesterol homeostasis: 1) increased HMGCR protein expression and cholesterol synthesis; 2) increased LDL-R expression / cholesterol uptake; 3) decreased SR-B1 levels; and 4) a failure to physiologically up-regulate ABCA1. To ascertain whether these alterations could have stemmed, at least in part, from transcriptional events, the mRNAs for each of these proteins were assessed at two time points: i) during the initiation phase of cholesterol accumulation; and ii) when cholesterol overload was fully developed (4 and 18 hrs post AA addition, respectively). In the cases of HMGCR and the LDL-R, modest, yet statistically significant, mRNA increments were observed. In contrast, ABCA1 message at the 18 hr time point remained at 'normal' values. Thus, in each case, protein and message levels qualitatively paralleled each other, suggesting that altered transcriptional regulation of each was likely involved. In contrast, SR-B1 reductions appeared to be dissociated from its message levels, which were very slightly, but
significantly, increased above control levels. This suggests that post transcriptional events (e.g. increased SR-B1 catabolism), rather than reduced synthesis, was more likely involved.

Given that AA and 2-deoxyglucose each induced ATP depletion, but only the former evoked a cholesterol loading state, potential differences in HMGCR, LDL-R, ABCA1 and SR-B1 protein expression with these two challenges were sought. By so doing, a distinction between *ATP dependent changes*, and *changes evoked by mitochondrial inhibition, per se*, could be assessed. Interestingly, 2-deoxyglucose induced comparable HMGCR protein increments to those which followed AA treatment. Deoxyglucose also decreased SR-B1 levels, while leaving ABCA1 intact. Again, these results are consistent with those which followed AA treatment. However, what did differ between the two forms of ATP depletion injury was that 2-deoxyglucose tended to *decrease, rather than increase*, the LDL-R. While tenable, it is premature to conclude that this difference in LDL-R expression fully explains why AA, but not 2-deoxyglucose, caused the cholesterol loading state. However, this difference does underscore our basic hypothesis: that mitochondrial dysfunction, per se, and not simply ATP reductions, is a critical determinant of post-injury cholesterol homeostasis.

The final goal of this study was to ascertain whether partial ATP depletion-mediated cholesterol enrichment is associated with changes in the prenylation of signaling molecules, notably Ras and Rho. The relevance of this issue is that prenylation allows for Ras/Rho translocation to the plasma membrane where they can contribute to cellular resistance to stress (2,3,13,15). Since isoprenoids (e.g., farnesyl and geranylgeranylpyrophosphate) are intermediary products of the mevalonate pathway, it is
conceivable that injury-induced alterations in cholesterol homeostasis could directly impact prenylation events. For example, an increase in HMGCR activity could increase isoprenoid production. Alternatively, increased cholesterol uptake via LDL-R mediated transport, coupled with decreased ABCA1 or SR-B1 mediated free cholesterol efflux, should cause 'feedback' inhibition of the HMGCR axis, potentially decreasing isoprenoid synthesis, and hence, prenylation. Therefore, Ras and Rho were probed by Western blot in AA-treated and control HK-2 cells. Despite the above considerations, no changes in their expression were observed. Of note, in previous studies conducted on renal cortex following Fe mediated oxidative stress (30), cholesterol accumulation was also dissociated from changes in Ras / Rho appearance. Thus, when these past in vivo, and current in vitro, data are viewed together, they indicate that degrees of mevalonate pathway activity and protein prenylation are not necessarily linked. Because Ras and Rho activation may enhance cell survival, the finding that an increase in cell cholesterol can be dissociated from Ras/Rho prenylation helps to underscore that cholesterol, per se, is the dominant mevalonate pathway product which helps to confer the post-injury cytoresistant state.

In conclusion, the present study documents for the first time that partial ATP depletion increases, and decreases, the HMGCR axis, and SR-B1 expression, respectively. That two disparate mechanisms for inducing ATP depletion, i.e., mitochondrial and glycolytic blockade, each evoke these two changes suggests that they represent a previously unrecognized ATP depletion-induced 'phenotype'. However, despite these changes, cellular cholesterol accumulation need not result, presumably due to offsetting homeostatic mechanisms. However, with concomitant mitochondrial
blockade, cholesterol loading does result, possibly due to an associated increase in the LDL-R. This suggests that multiple defects must exist in order for cholesterol accumulation to result. The specific mechanism(s) by which mitochondrial inhibition conditions cholesterol accumulation, beyond ATP depletion-related changes, remains unknown. However, a mitochondrial initiated increase in the LDL-R expression appears to be one likely candidate in this regard.
ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (RO-1 DK38432; RO-1 DK54200), and from an unrestricted research grant from Abbott Laboratories, North Chicago, IL.
REFERENCES


16. Stirewalt DL, Appelbaum FR, Willman CL, Zager RA, Banker DE: Mevastatin can increase toxicity in primary AMLs exposed to standard therapeutic agents, but statin efficacy is not simply associated with RAS hotspot mutations or overexpression. *Leukemia Res* 1572: 1-13, 2002


FIGURE LEGENDS

Figure 1. **Left hand panel.** Cholesterol levels in HK-2 cells following 18 hr incubations under control (cont) conditions (2.5% serum in K-SFM) or under the same conditions + antimycin A (AA) treatment. As seen in the two left hand bars, AA caused an approximate 50 nmole/µmole Pi increase in cholesterol values. When this same experiment was repeated in the presence of mevastatin (statin; in both control and AA treated cells), a still significant, but smaller, AA-induced increase in cholesterol resulted. **Right hand panel.** Percent increase in cholesterol caused by AA treatment in the presence or absence of mevastatin. Although statin therapy blunted >50% of the AA-induced cholesterol increase, a significant increase still resulted. This indicates that both synthetic and non synthetic pathways were involved in AA-induced cholesterol loading.

Figure 2. **Comparison of Western blot results from control cells (open bars) and AA treated cells (shaded bars) after 18 hr incubations.** HMG CoA reductase (HMGCR) appeared as a single band at ~45 kDa and was significantly increased by AA treatment. LDL receptor (LDL-R) appears as a non glycosylated and a glycosylated band at 120 and 160 kDa, respectively. AA induced a statistically significant increase in the non glycosylated band (depicted by the bars). A trend, but not a statistically significant increase, in the glycosylated band existed (not depicted by bar graph). ABCA1 appears as a broad band at ~220 kDa, the appearance of which was not significantly altered by AA treatment. Conversely, AA induced a significant decrease in SR-B1 (seen at ~82 kDa) with the concomitant appearance of a lower molecular weight, possibly degradative, product which was not observed in the control cell protein extracts.
Figure 3. Western blots of Rho and Ras from control (open bars) and AA treated cells (shaded bars) after 18 hr incubations. Prenylated and unprenylated Rho appear as the lower and upper band, respectively (at ~20-24 kDa). The ratios of the two (prenylated/unprenylated) are depicted in the bars and did not statistically differ for the control and AA treatment groups. Ras appears as a single, non prenylated, band at ~21 kDa which was slightly, but not significantly, decreased by AA treatment.
Table 1. Primers and conditions used for PCR analyses.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences</th>
<th>PCR Conditions</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCR</td>
<td>5'-TTA CTC CTT GGT GAT GGG AGC TTG-3'</td>
<td>94°C – 60sec, 55°C – 60sec, 72°C – 60sec; 30 cycles</td>
<td>747 bp</td>
</tr>
<tr>
<td></td>
<td>5'-TCC TGT CCA CAG GCA ATG TAG ATG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-R</td>
<td>5'-CAA TGT CTC ACC AAG CTC TG-3'</td>
<td>94°C – 60sec, 55°C – 60sec, 72°C – 60sec; 23 cycles</td>
<td>260 bp</td>
</tr>
<tr>
<td></td>
<td>5'-TCT GTC TCG AGG GGT AGC TG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR-B1</td>
<td>5'-CTG TGG GTG AGA TCA TGT GG-3'</td>
<td>94°C – 60sec, 55°C – 60sec, 72°C – 60sec; 23 cycles</td>
<td>215 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GCC AGA AGT CAA CCT TGC TC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA1</td>
<td>5'-CAA CTA CAA AGC CCT CTT TG-3'</td>
<td>94°C – 60sec, 55°C – 60sec, 72°C – 60sec; 30 cycles</td>
<td>310 bp</td>
</tr>
<tr>
<td></td>
<td>5'-CTT GGC TGT TCT CCA TGA AG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GTC TTC ACC ACC ATG GAG AAG-3'</td>
<td>94°C – 60sec, 55°C – 60sec, 72°C – 60sec; 23 cycles</td>
<td>490 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GCT TCA CCA CCT TCT TGA TGT CAT C-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1 legend:** Primers and RT-PCR conditions used to quantitate mRNAs. HMGCR were run separately (with GAPDH), whereas SR-B1, LDL-R, ABCA1, and GAPDH were multiplexed.
Table 2: mRNA values after 4 hr or 18 hrs of control (cont) incubations or incubation with antimycin A (AA).

<table>
<thead>
<tr>
<th>Group</th>
<th>HMGCR</th>
<th>LDL-R</th>
<th>ABCA1</th>
<th>SR-B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hr AA *</td>
<td>1.22 ± 0.17</td>
<td>0.24 ± 0.005</td>
<td>0.35 ± 0.04</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>4 hr Cont *</td>
<td>1.08 ± 0.13</td>
<td>0.21 ± 0.005</td>
<td>0.44 ± 0.03</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.02</td>
<td>&lt;0.001</td>
<td>&lt;0.005</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>18 hr AA +</td>
<td>1.38 ± 0.17</td>
<td>0.23 ± 0.01</td>
<td>0.46 ± 0.01</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>18 hr Cont +</td>
<td>1.24 ± 0.13</td>
<td>0.18 ± 0.004</td>
<td>0.45 ± 0.02</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.025</td>
<td>&lt;0.0002</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 2 legend. HMGCR, LDL-R, ABCA1 and SR-B1 mRNA values after either 4 or 18 hrs of antimycin A (AA) treatment (both in the presence of serum). *n = 10, + n = 8 samples per group. All values are presented as a ratio of the individual product, factored by the concomitant GAPDH value. Statistics compare the results at either 4 or 18 hrs.
Figure 1.
Figure 2.
Figure 3.