Proximal tubular cholesterol loading after mitochondrial, but not glycolytic, blockade

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

Fred Hutchinson Cancer Research Center and the University of Washington, Seattle, Washington 98109

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Zager, Richard A., Ali C. M. Johnson, and Sherry Y. Hanson. Proximal tubular cholesterol loading after mitochondrial, but not glycolytic, blockade. Am J Physiol Renal Physiol 285: F1092–F1099, 2003; 10.1152/ajprenal.00187.2003.—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 after partial ATP depletion states. 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 h later, cell cholesterol levels, hydroxymethylglutaryl (HMG)-CoA reductase (HMGCR), the LDL receptor (LDL-R), and ABCA1/SR-B1 cholesterol transporters were assessed. 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%↑). One-half of this increase was statin inhabitable, and one-half could be blocked by serum deletion, implying that both synthetic and nonsynthetic (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. We conclude that 1) sublethal ATP depletion, whether induced by mitochondrial or glycolytic blockade, can upregulate HMGCR and decrease SR-B1, and these changes represent a previously unrecognized or glycolytic blockade, can upregulate HMGCR and decrease SR-B1, and these changes represent a previously unrecognized determinant of postinjury cell cholesterol homeostasis, potentially by impacting the LDL-R.

Antimycin A; 2-deoxyglucose; LDL receptor; SR-B1; ABCA1

Address for reprint requests and other correspondence: R. A. Zager, Fred Hutchinson Cancer Research Ctr., 1100 Fairview Ave. N; Rm. D2–190, Seattle, WA 98109 (E-mail: dzager@fhcrc.org).

CELLULAR STRESS EVOKES A PLETHORA of responses that 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; Refs. 5, 10, 21). Perhaps the most consistent cytoprotective lipid yet identified is cholesterol. This conclusion is based on a series of experiments that demonstrate that diverse forms of renal injury (ischemia, toxins, oxidative stress, sepsis, heat shock, hyperosmolality; immunological injury, urinary tract obstruction) 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 that demonstrate that 1) either reversing postinjury cholesterol increments, or preventing 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; deesterification; interference with normal P-glycoprotein-mediated cholesterol cycling) 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 postinjury cholesterol overload state.

Despite the apparent uniformity of postinjury 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 Fe-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 coexist. These include the following: 1) increased cholesterol synthesis, based on findings of increased hydroxymethylglutaryl (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 bidirectional cholesterol transport (increasing cholesteryl ester uptake, FC efflux; Refs. 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

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terol 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 ischemia-reperfusion injury induces renal cortical cholesterol overload (22). Given the heterogeneity of cell types within the renal cortex, the present study focused on events occurring within cultured renal proximal tubular (HK-2) cells to gain cell-type-specific data. With the use of this approach, the following questions were addressed. 1) Can mild, sub-lethal ATP depletion trigger an increase in 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 predominantly via increased synthesis (e.g., as previously noted with Fe-mediated injury; Ref. 29), or might other mechanisms 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? 5) Does partial ATP depletion cause reductions in key cholesterol transport proteins (ABCA1, SR-B1) in a fashion analogous to that which follows Fe-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 T75 flasks (Costar, Cambridge, MA) with keratinocyte serum-free medium (GIBCO Life Technologies, Grand Island, NY) to which were 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 resedased on 1) additional T75 flasks (for further passage or for experiments that 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 after passage to achieve near confluence before 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 before AA or carrier addition, heat-inactivated (56°C × 20 min) normal mouse serum (100–113, Gemini Bio Products) was added to a final concentration of 2.5%. The cells were incubated under these conditions for 18 h. The flasks were then rinsed twice with HBSS (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 three times 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, and 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 per micromole of phospholipid P, in each sample (the latter determined by sample ashing and P analysis; Ref. 22).

To assess whether a second mitochondrial inhibitor might alter cell cholesterol levels, the above experiment was repeated in three control and three rotenone-treated flasks (10 or 50 μM; n = 2 and 1, respectively; in 0.05% ethanol). After 18 h, 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 the completion of an 18-h incubation, 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 RESULTS, statin therapy eliminated ~50% of AA-induced cholesterol loading, suggesting that both synthesis and possible increased cell cholesterol uptake were involved. In that case, 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 four underwent the AA challenge. The remaining four served as controls. After an 18-h incubation, cell cholesterol levels were determined.

ATP depletion from glycolysis inhibition: impact on cell cholesterol content. To contrast results obtained by mitochondrial inhibition, eight 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 hours 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 = 6/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 an 18-h incubation was completed, lethal cell injury was assessed by %lactate dehydrogenase (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 three groups: 1) addition of 2.5% serum, 2) addition of serum +AA, and 3) addition of serum +2-deoxyglucose. After a 4-h incubation, 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 among 1) synthesis; 2) LDL-R-mediated cholesterol uptake; and 3) 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 in 36 separate T25 flasks. On reaching near confluence, they were divided into four groups: 1) control incubation with 2.5% serum × 4 h (n = 10); 2) incubation with 7.5 μM AA+2.5% serum × 4 h (n = 10); 3) control incubation with 2.5% serum × 18 h (n = 8); and 4) incubation with AA+2.5% serum for 18 h (n = 8). At the completion of incubation, the cells were recovered as noted above and immediately placed into TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted as according to 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 for 30 min through 1.2% agarose containing ethidium bromide (Sigma) to ensure a lack of degradation (preservation of 18S and 28S ribosomal RNA) before 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. 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 humanspecific primers and conditions listed in Table 1. Otherwise, the conditions were identical to those previously described (28; Table 1). To confirm the biological validity of this assay, samples were obtained from four 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).

**Effects of AA on ABCA1, SR-B1, LDL-R, and HMGCR protein expression.** Eight T25 flasks were seeded with HK-2 cells. On 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 (AA vehicle). Eighteen hours 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 (NB-400–15, Novus Biologicals). HMGCR detection, 5 μg of protein extract samples were electrophoresed through a 4–12% gradient Bis-Tris acrylamide Nupage gel. Rabbit anti-ABCA1 (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 (NA 934, Amersham-Pharamacia, Piscataway, Nj) and enhanced chemiluminescence (ECL Kit, Amersham-Pharamacia; Ref. 10). For detection of LDL-R, 5-μg protein extract samples were electrophoresed through a 4–12% gradient Bis-Tris acrylamide Nupage gel. Mouse anti-LDL-R (LP02, Oncogene Research Products, Boston, MA) was used as the primary antibody. Secondary detection was performed with horseradish peroxidase-labeled sheep anti-mouse IgG (NA 931, Amersham-Pharmacia) and ECL (8). HMGCR was probed in 25-μg protein samples electrophoresed into the above 4–12% Nupage gel. Rabbit anti-HMGCR (a gift from P. A. Edwards, Los Angeles, CA) was detected 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; nonglycosylated LDL-R, 120 kDa; glycosylated LDL-R, 160 kDa; HMGCR, ~45 kDa; Ref. 24).

**Effects of deoxyglucose 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 the 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, four control samples and four samples obtained 18 h 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 (bottom) band and unprenylated (top) band on the gel after their quantitation by ECL. The relative position of these two bands has previously been determined by treating

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**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, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGCR</td>
<td>5’T-CTC TCC CTT GCT GAT GGG AGG TTG-3’</td>
<td>94°C–60 s, 55°C–60 s, 72°C–60 s; 30 cycles</td>
<td>747</td>
</tr>
<tr>
<td></td>
<td>5’T-CCA CGA GCA TAG ATG AGC-3’</td>
<td>94°C–60 s, 55°C–60 s, 72°C–60 s; 23 cycles</td>
<td>260</td>
</tr>
<tr>
<td>LDL-R</td>
<td>5’T-GGT GGT AGA TGT CAT-3’</td>
<td>94°C–60 s, 55°C–60 s, 72°C–60 s; 23 cycles</td>
<td>215</td>
</tr>
<tr>
<td>SR-B1</td>
<td>5’T-GGT GGT AGA TGT CAT-3’</td>
<td>94°C–60 s, 55°C–60 s, 72°C–60 s; 30 cycles</td>
<td>310</td>
</tr>
<tr>
<td>ABCA1</td>
<td>5’T-GGT GGT AGA TGT CAT-3’</td>
<td>94°C–60 s, 55°C–60 s, 72°C–60 s; 23 cycles</td>
<td>490</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’T-GTT TCC ACC ACC ATG GAG AGG-3’</td>
<td>94°C–60 s, 55°C–60 s, 72°C–60 s; 23 cycles</td>
<td>490</td>
</tr>
</tbody>
</table>

Primer and RT-PCR conditions used to quantitate mRNAs are shown. Hydroxymethylglutaryl CoA reductase (HMGCR) analyses were run separately (with GAPDH), whereas SR-B1, LDL receptor (LDL-R), ABCA1, and GAPDH were multiplexed.
HK-2 cells with mevastatin, which causes a marked decrease in the bottom (prenylated), but not in the top (unprenylated) band (30). Thus the ratio of the bottom to the top (prenylated/unprenylated) band serves as an indication of Rho prenylation. In the case of Ras, a dominant unprenylated band appears and a second (top) band develops with prenylation (30). Hence, the latter was sought after AA treatment.

**Calculations and statistics.** All values are presented as means ± SE. 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 nmol/μmol P. 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 percent increase in cholesterol between the two sets of flasks was compared. The percent increase in cholesterol induced by AA in the absence of mevastatin was 25 ± 3% compared with 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 caused a 25% cholesterol increase in the presence of serum, the withholding of serum decreased cholesterol loading by ~50% (i.e., from 25 to 12%).

**Effects of rotenone on HK-2 cell cholesterol levels.** To test whether a second model of mitochondrial inhibition would raise cholesterol levels in the presence of serum, the effects of rotenone were assessed. Rotenone caused a smaller (~10%), but still significant (P = 0.01), increase in total cellular cholesterol, compared with the increase observed with AA (~25%). [Of note, however, these rotenone and AA results cannot be directly compared because both rotenone doses, unlike AA, caused ~40% cell detachment, consistent with greater injury (e.g., more dead cells that would not synthesize or transport cholesterol). Because of this cell detachment, the 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 nmol/μmol P, respectively.

**Extent of cell injury induced by AA and 2-deoxyglucose.** AA treatment did not cause a significant increase in %LDH release [control 7 ± 1%; AA, 8 ± 1%; not significant (NS)]. However, total adherent cell LDH content (a reflection of total viable cell mass; Refs. 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 antiproliferative response ± a small amount of apoptosis) did occur. In contrast to AA, 2-deoxyglucose 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, because 2.5% serum contains a small amount of LDH, this value was subtracted from the total supernatant values before determination of %LDH release].

**Cellular energetics after 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 vs. 79 ± 5 μmol, respectively, P < 0.03; control values, 90 ± 4 μmol). 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 after 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
and 18 h after AA addition. As a physiological control for the HMGCR mRNA assay, levels were measured in control and statin-treated cells, as noted in METHODS. Statin therapy induced an approximate sevenfold 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 h post-AA addition. Although ABCA1 mRNA values returned to “normal” by 18 h of AA treatment, these values should be considered physiologically abnormal, given that cholesterol loading at 18 h 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-h time points. Conversely, ABCA1 message was suppressed by 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.

### DISCUSSION

All forms of in vivo renal injury tested to date (22, 24–30) have demonstrated a number of common fea-

### Table 2. mRNA values after 4 or 18 h of control incubations or incubation with antinymycin A

<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-h 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-h 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-h 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-h 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>

Values are means ± SE and are presented as a ratio of the individual product, factored by the concomitant GAPDH value. n, No. of samples/group; AA, antinymycin A; Cont, control; NS, not significant. HMGCR, LDL-R, ABCA1, and SR-B1 mRNA values after either 4 (n = 4) or 18 h (n = 18) of control or AA treatment (both in the presence of serum) are shown.

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 bands 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 prenylated/unprenylated band ratios did not significantly differ for the control and AA treatment groups (as depicted by the bars). The Ras probe revealed only a single band that did not differ between the control (open bars) and AA-treated (shaded bars) groups (i.e., no evidence of a prenylated band was apparent in either group).
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Fig. 3. Western blots of Rho and Ras from control (open bars) and AA-treated cells (shaded bars) after 18-h incubation. Prenylated and unprenylated Rho appear as the bottom and top band, respectively (at ~20–24 kDa). The ratios of the 2 (prenylated/unprenylated) are depicted in the bars and did not statistically differ for the control and AA treatment groups. Ras appears as a single, unprenylated band at ~21 kDa, which was slightly, but not significantly, decreased by AA treatment.
the latter function appears dominant in proximal tubular cells (8). Thus AA-induced SR-B1 reductions could have contributed to the cholesterol-loading state. 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 upregulate 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: 1) during the initiation phase of cholesterol accumulation; and 2) when cholesterol overload was fully developed (4 and 18 h 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-h 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, were 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 that followed AA treatment. Deoxyglucose also decreased SR-B1 levels, while leaving ABCA1 intact. Again, these results are consistent with those that 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 postinjury 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). Because 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 analysis 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 in renal cortex after Fe-mediated oxidative stress (30), cholesterol accumulation was also dissociated from changes in Ras/Rho appearance. Thus when these past in vivo and present 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 that helps to confer the postinjury cytoprotective 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 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 mitochondria-initiated increase in LDL-R expression appears to be one likely candidate in this regard.

DISCLOSURES

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REFERENCES


