Experimental Glomerulopathy Alters Renal Cortical Cholesterol, SR-B1, ABCA1, and HMG CoA Reductase Expression

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Previous studies indicate that acute tubular injury causes free cholesterol (FC) and cholesteryl ester (CE) accumulation within renal cortex/proximal tubules. This study assessed whether similar changes occur with glomerulopathy/nephrotic syndrome, in which high-circulating/filtered lipoprotein levels increase renal cholesterol supply. Potential adaptive changes in cholesterol synthetic/transport proteins were also assessed. Nephrotoxic serum (NTS) or passive Heymann nephritis (PHN) was induced in Sprague-Dawley rats. Renal injury (blood urea nitrogen, proteinuria) was assessed 2 and 7 days (NTS), or 10 and 30 days (PHN) later. FC and CE levels in renal cortex, isolated glomeruli, and proximal tubule segments were determined. SR-B1 (a CE influx protein), ABCA1 (a FC exporter), and HMG CoA reductase protein/mRNA levels were also assessed. FC was minimally elevated in renal cortex (0 to 15%), the majority apparently localizing to proximal tubules. More dramatic CE elevations were found (~5 to 15×), correlating with the severity of proteinuria at any single time point ($r \geq 0.85$). Cholesterol increments were associated with decreased SR-B1, increased ABCA1, and increased HMG CoA reductase (HMGCR) protein and its mRNA. Tubule (HK-2) cell culture data indicated that SR-B1 and ABCA1 levels are responsive to cholesterol supply. Experimental nephropathy can increase renal FC, and particularly CE, levels, most notably in proximal tubules. These changes are associated with adaptations in SR-B1 and ABCA1 expression, which are physiologically appropriate changes for a cholesterol overload state. However, HMGCR protein/mRNA increments can also result. These seem to reflect a maladaptive response, potentially contributing to a cell cholesterol overload state. (*Am J Pathol* 2003, 162:283–291)

Previous work from this laboratory has indicated that diverse forms of renal tubular injury (eg, ischemia/reperfusion, myohemoglobinuria, sepsis syndrome, heat shock, urinary tract obstruction), can each trigger accumulation of free

cholesterol (FC) and cholesteryl esters (CE) within renal cortex.^{1–7} This seems to be mediated, at least in part, by an increase in HMG CoA reductase (HMGCR)-mediated cholesterol synthesis within proximal tubule cells.² Given the heterogeneous nature of the above injury forms, we have postulated that tissue cholesterol accumulation can be an integral component of the renal stress response.⁵ However, whether acute glomerular injury, like tubular damage, also evokes comparable changes in renal cortical/proximal tubular cholesterol levels remains ill defined. To date, this laboratory has only addressed this issue at 48 hours after induction of nephrotoxic serum (NTS) (anti-glomerular basement membrane) nephritis. Modest CE, but no FC, increases resulted,⁴ differing from the aforementioned tubular injury response.^{1–7} However, our previous NTS results were obtained at only a single early time point (48 hours), and only one disease model was studied. Hence, it remains possible that with more prolonged, or progressive, glomerular injury more dramatic renal cholesterol accumulation might result.

There are at least two major reasons why glomerulonephritis might cause renal cortical cholesterol accumulation. First, it is possible that acute glomerular injury might evoke a classic renal stress response. Because renal stress (eg, as denoted by increased heat shock protein expression) seems to correlate with an up-regulation of HMGCR,⁵ an increase in cholesterol synthesis could then result. The second possibility is that hyperlipidemia and hyperlipiduria, concomitants of glomerular injury-induced nephrotic syndrome, could facilitate glomerular and tubular cell lipid uptake. For example, mesangial cells are capable of lipid endocytosis.^{8–10} Secondly, increased filtration of FC- and CE-bearing lipoproteins might increase tubular cell cholesterol uptake, eg, via the high-density lipoprotein scavenger receptor-B1 (SR-B1) receptor.^{11–17} The latter is particularly relevant for rats, given that high-density lipoprotein, rather than low-density lipoprotein, is the primary cholesterol carrier.¹¹ An alternative scenario might be that once cholesterol accumulation within

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glomerular and/or tubular cells is initiated, secondary compensatory mechanisms come into play that act to hold further cholesterol accumulation in check. Such compensatory mechanisms might include: 1) down-regulation of SR-B1; 2) reduced HMGCR-mediated cholesterol synthesis; or 3) up-regulation of ABCA1 (ATP-binding cassette transporter), a dominant cellular FC efflux pathway.^{18–22}

Cholesterol accumulation could have potential pathogenic relevance for progression of renal disease. For example, renal cholesterol loading, such as induced by high-lipid diets, has been reported to accelerate glomerulosclerosis in rats.^{23,24} Cholesterol-lowering agents have been purported to attenuate the progression of experimental nephropathy.^{25–29} Finally, this laboratory has demonstrated that proximal tubular cell cholesterol levels can impact mitochondrial function⁷ and cellular susceptibility to toxic or ischemic attack.^{1,2} These considerations suggest that further definition of renal cholesterol homeostasis in the setting of glomerulopathy is worthy of investigation.

Hence, the present studies were undertaken to address the following questions: 1) Does glomerular disease evoke progressive renal cortical cholesterol accumulation? (In this regard, it is notable that the extent of tubular injury strongly correlates with the degree of the cholesterol overload state.⁴) 2) If renal cortical cholesterol overload does develop after glomerular injury, are these changes expressed predominantly in glomerular or proximal tubular cells? 3) Might cholesterol accumulation after induction of glomerulopathy evoke counterregulatory pathways, potentially capable of limiting the cholesterol overload state?

Materials and Methods

Induction of Experimental Glomerulonephritis

Two different models of experimental glomerulonephritis, induced in the rat, were selected for study: NTS nephritis and passive Heymann nephritis (PHN). These two models were chosen because they reflect differing mechanisms and histological patterns of injury as follows: NTS, an acute inflammatory injury model induced by anti-glomerular basement membrane antibody;³⁰ and 2) PHN, a chronic noninflammatory model of membranous nephropathy.³¹ In both instances, nephrotic range proteinuria and hypoalbuminemia result.^{32–34} In addition, the PHN model induces an ~50+% increase in serum cholesterol levels, another manifestation of the nephrotic syndrome (previous unpublished observations from this laboratory). Male Sprague-Dawley rats weighing 200 to 250 g (Simonsen Labs, Gilroy, CA), housed in metabolic cages with free access to standard rat chow and water, were used for all *in vivo* experiments.

NTS Nephritis

NTS was induced in rats by injection of anti-glomerular basement membrane serum, raised in male sheep by repeated immunization with 75 mg of lyophilized rat glo-

meruli, emulsified in complete Freund's adjuvant.³⁰ The rats were lightly anesthetized with ether and injected via the tail vein with either anti-glomerular basement membrane serum or with nonimmune sheep serum (dose of 1 ml/kg body weight; $n = 24$ rats). The NTS and control rats were studied simultaneously at either 2 or 7 days after serum injection (corresponding to severe, and then resolving, renal disease, respectively; six rats per each group at each time point). At the appropriate time points, the rats were placed in metabolic cages and urine was collected for ~14 hours. Urinary protein excretion was assessed by the sulfosalicylic acid method,^{30,31} with the results being expressed as mg/hour $\times$ 24 hours. The day after urine collection, the rats were deeply anesthetized with ether and ~2 ml of blood was obtained by cardiac puncture for subsequent blood urea nitrogen (BUN) analysis (Beckman Analyzer 2; Beckman Instruments, Palo Alto, CA). The kidneys were then resected through a midline abdominal incision and the cortices were dissected with a razor blade. Some cortical samples were snap-frozen at -70°C and saved for subsequent extraction of protein (for Western blotting) or RNA (for polymerase chain reaction) analysis as discussed below. The remaining cortical tissues were extracted in chloroform: methanol and the lipid phase was saved for FC and CE analysis by gas chromatography (GC), as previously described.² The FC and CE results were expressed as nmol/ μmol of phospholipid phosphate (Pi) in the recovered lipid fraction.²

PHN

Rats were anesthetized with ether and subjected to intraperitoneal injections of either sheep anti-Fx1A³¹ or nonimmune sheep serum in a dose of 5 ml/kg body weight. Anti-Fx1A was obtained from sheep that had been immunized with cortical tissue fraction Fx1A in incomplete Freund's adjuvant.³¹ The rats were studied at either 10 or 30 days after serum injection (control and PHN rats: $n = 6$ to 8 for each group at each of the two time points). The day before sacrifice a timed urine collection was obtained for determination of urine protein excretion rates, as noted above. The following day, blood and renal cortical tissue samples were obtained and processed, as noted above.

Isolated Glomeruli and Isolated Proximal Tubule Segment (PTS) Analysis

To assess whether changes in renal cortical cholesterol levels predominantly reflect changes in glomeruli and/or in proximal tubular epithelium, the 30-day PHN model, with an equal number of control rats ($n = 10$ each), was selected for further study. This particular subgroup of rats was selected because they expressed the greatest cholesterol increments (see Results), thereby facilitating cholesterol localization studies. At the 30-day time point, the rats were anesthetized and the kidneys were resected. They were then used for either glomerular isolation by a previously reported sieving technique ($n = 6$ PHN and 6

control rats),³⁰ or for isolated PTS collection ($n = 4$ PHN and 4 controls), as previously described in detail.^{35,36} The isolated tubules and glomeruli were subjected to lipid extraction, followed by FC and CE analysis, as noted above.

Western Blot Analysis

Selected renal cortical tissue samples were probed by Western blot for the following proteins: HMGCR, HSP-72, SR-B1, and ABCA1. The general methodologies used for Western blotting were as previously described.^{5,37} Probing for HMGCR and HSP-72 used methodologies and reagents as previously reported.^{5,36,37} In the case of SR-B1, 7 μ g of protein extract were electrophoresed into a 12% Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA) and probed with rabbit anti-SR-B1 antibody (catalogue number NB-400-104; Novus Biologicals, Littleton, CO). For ABCA1 detection, 30 μ g of protein was electrophoresed into a 4 to 12% gradient Bis-Tris acrylamide gel (Invitrogen). Rabbit anti-ABCA1 (catalog number NB 400-15; Novus Biologicals) was used as the primary antibody according to the manufacturer's instructions. Secondary detection was performed with horseradish peroxidase-labeled donkey anti-rabbit IgG (Amersham-Pharmacia, Piscataway, NJ) and enhanced chemiluminescence (ECL kit; Amersham-Pharmacia). Nonspecific secondary antibody staining had previously been ruled out by the fact that the secondary antibody, in the absence of the primary antibody, did not identify the relevant protein band(s). Blot semiquantitative analysis was performed by band optical density scanning.

HK-2 Proximal Tubular Cell Experiments

The following experiments were conducted to test the hypothesis that the SR-B1 and ABCA1 transporters within proximal tubular cells are responsive to changes in cell cholesterol levels. If so, then this would support the hypothesis that any changes in the expression of these proteins after induction of experimental glomerulonephritis could reflect adaptive responses to changes in cholesterol homeostasis.

SR-B1

Six T-75 flasks of near confluent HK-2 cells, a proximal tubule cell line established from normal human kidney,³⁸ were grown either in keratinocyte serum-free medium (K-SFM; $n = 3$) or in the same medium to which was added 15% complement-inactivated (56°C for 20 minutes) normal mouse serum ($n = 3$; Gemini Bio-Products, Woodland, CA). After completing 2-day incubations, the cells were washed with Hanks' balanced salt solution, they were recovered with a cell scraper, and proteins were extracted for Western blotting, as previously described.³⁹ [Note: addition of normal mouse serum to HK-2 cells causes an approximate 25% in cellular cholesterol levels via increased import (unpublished obser-

vations; RZ). Hence, feedback inhibition of the SR-B1 importer might be expected].

ABCA1

Six flasks of HK-2 cells, maintained as noted above, were incubated either under control conditions ($n = 3$) or in the presence of 10 μ mol/L of mevastatin ($n = 3$), which causes significant reductions in HK-2 cell cholesterol levels.^{2,39} After a 2-day incubation, the cell proteins were recovered and probed for ABCA1 levels, as noted above. A reduction in ABCA1 would be expected, assuming that a decrease in cell cholesterol causes feedback inhibition of this FC exporter.

HMGCR mRNA Assessment by Reverse Transcriptase-Polymerase Chain Reaction

To complement the results of the HMGCR Western blots, renal cortical samples from 10-day and 30-day PHN rats were analyzed by competitive reverse transcriptase-polymerase chain reaction for HMGCR mRNA levels, using GADPH as a reference housekeeping gene, as previously described in detail.³⁹ The PHN model was selected for analysis because it, unlike the NTS model, was associated with progressive proteinuria and cholesterol accumulation. cDNA bands were visualized and quantified by densitometry.³⁹ HMGCR cDNA bands were expressed as a ratio to the simultaneously obtained GADPH cDNA bands. RNA samples obtained from a total of four controls/five PHN kidneys and nine controls/eight PHN kidneys were probed at the 10- and 30-day time points, respectively.

Statistics

All results are presented as means $\pm$ 1 SEM. Results were contrasted by either paired or unpaired Student's *t*-test, as required by the experimental protocol. If multiple group comparisons were made, the Bonferroni correction was applied. For nonparametric data, comparisons were made by the Wilcoxon rank sum test. Statistical significance was judged by a *P* value of <0.05 .

Results

Renal Functional Assessments after Induction of Glomerulonephritis

NTS Nephritis

Rats injected with anti-glomerular basement membrane serum became significantly azotemic by 48 hours after serum injection (Figure 1, left). Massive proteinuria also resulted (Figure 1, right). However, this injury rapidly resolved, with BUNs returning to control values at the 1-week time point. Proteinuria also improved, falling by ~85% from the values observed at 2 days after disease

Figure 1. Renal injury after induction of NTS nephritis as assessed by BUN concentrations (**left**) and proteinuria (**right**). Marked BUN elevations were apparent at 2 days after NTS induction (**, $P < 0.001$), which completely resolved by the 7-day time point. Similarly, marked proteinuria, apparent at 2 days, in large part resolved by 7 days ($\Delta\Delta = P < 0.025$) ($n = 6$ experimental and control animals at each of the two time points).

induction. Thus, NTS induced a severe, but rapidly resolving, nephropathy.

PHN

PHN also induced significant renal functional impairment (Figure 2). At 10 days, both substantial azotemia and proteinuria were present. By 30 days, azotemia had almost completely remitted. Nevertheless, progressive proteinuria was apparent, with urine protein excretion nearly doubling throughout the 10-day values. In summary, in contrast to NTS, PHN was associated with progressive proteinuria that was expressed at a time of improving GFR.

Renal Cortical FC and CE Analyses

NTS Model

As shown in Figure 3, left, the NTS model induced either no, or trivial, increases in renal cortical FC content. However, dramatic CE elevations were apparent. At either the 2- or 7-day time point, the CE increase in each NTS kidney strikingly correlated with the degree of proteinuria that was observed (CE *versus* protein excretion for each rat: day 2: $r = 0.94$; $P < 0.001$; day 7: $r = 0.95$,

Figure 2. Renal injury after induction of PHN as assessed by BUN concentrations (**left**) and proteinuria (**right**). Significant azotemia (**, $P < 0.0001$) and marked proteinuria (*, $P < 0.001$) were seen 10 days after PHN induction. At 30 days, azotemia had primarily resolved (Δ , $P < 0.05$). However, proteinuria had almost doubled in amount, compared to the values observed at the 10-day time point ($n = 6$ to 8 animals for control and experimental groups at each time point).

Figure 3. Renal cortical FC and CE levels at 2 and 7 days after induction of NTS nephritis. Only trivial elevations in FC levels occurred after NTS, with a statistically significant increase (*, $P < 0.01$) being observed only at the 7-day time point (c, control tissues). In contrast, substantial increases in CE levels were observed at both time points (**, $P < 0.001$; $n = 6$ for each group at each time point).

$P < 0.002$). Interestingly, however, the CE elevations were greater at the 7-day *versus* the 2-day time point, despite the fact that, as shown in Figure 1, both proteinuria and azotemia were resolving.

PHN Model

As depicted in Figure 4, left, renal cortical FC accumulation was observed with the PHN model, with 15% and 17% increases being observed at the 10- and 30-day time points, respectively. In addition, dramatic CE increases were observed. The PHN-associated CE increases were approximately twofold to fourfold higher than those observed in the above-described NTS rats. Renal cortical CE increments for the PHN rats strongly correlated the degree of proteinuria (day 10: $r = 0.85$, $P < 0.001$; day 30: $r = 0.87$, $P < 0.001$). However, unlike the case with the NTS rats, the CE and protein excretion rates each increased throughout the course of the experiments.

FC and CE Levels in Isolated Glomeruli and Proximal Tubules

Because the most severe nephrotic syndrome was observed in the 30-day PHN rats (based on degrees of

Figure 4. Renal cortical FC and CE levels at 10 and 30 days after induction of PHN. Significant FC levels were observed at both time points, in comparison to control (c) tissue samples. Much more dramatic CE elevations were observed, particularly at the 30-day time point (**, $P < 0.001$; $n = 6$ to 8 animals for each group at each time point).

Figure 5. FC and CE levels in glomeruli and proximal tubule segments (PTS) isolated from 30-day PHN rats. Isolated proximal tubules, but not isolated glomeruli, demonstrated significant elevations in FC levels (**left**; Δ , $P < 0.05$). Both glomeruli ($*$, $P < 0.01$) and tubules had elevated CE levels, but the increase was approximately twice as great in the tubule *versus* the glomerular preparations ($n = 6$ for control and experimental glomeruli; $n = 4$ for control and experimental tubules). C, controls.

proteinuria), and because these samples demonstrated the greatest FC and CE increments (see above), kidneys from this experimental group were used for isolated glomerular and tubule cholesterol analysis.

FC Assessments

Proximal tubules (PTS) harvested from 30-day PHN rats manifested significant (30%) FC elevations, compared to their controls (Figure 5, left). The percent FC elevation was approximately two times greater than those observed in whole renal cortex (18%). In contrast, isolated glomeruli obtained from 30-day PHN rats showed no significant FC increases (Figure 5, left).

CE Assessments

Both isolated tubules and isolated glomeruli obtained from the 30-day PHN rats demonstrated significant elevations in CE content (Figure 5, right). However, the extent of increase was approximately two times greater in tubules *versus* glomeruli. The degree of proximal tubule CE elevations essentially reproduced the results obtained in whole renal cortex.

Western Blot Analysis

HMGCR Reductase (HMGCR)

As shown in Figure 6 (top two lanes), HMGCR appeared as two bands at ~120 kd (top) and ~94 kd (bottom), corresponding with inactive/uncleaved, and active/cleaved protein moieties respectively.^{5,37} In both the PHN and NTS models, the active HMGCR moiety (lower band, ~94 kd) appeared increased, a result that was confirmed by densitometric analysis (Figure 7, left). A trend toward a reciprocal decrease in the inactive (~120 kd) HMGCR protein band was also observed (Figure 6A and Figure 7, right). Although the inactive band changes were not statistically significant, when viewed in the context of an increase in the active band, the overall data suggest the possibility of increased HMGCR proteolytic

Figure 6. Western blotting for HMG CoA reductase (HMGCR; **top lanes**) and heat shock protein-72 (HSP-72; **bottom lanes**) in renal cortex harvested from PHN and NTS kidneys at the time points (d, days) shown, and in paired control (C) tissues. HMGCR appears as an ~120-kd band (**top**) and as an ~94-kd band (**bottom**) corresponding with inactive/uncleaved and active/cleaved bands, respectively. In both the NTS and PHN kidney samples, there was an increase in the active band. A trend toward a decrease in the inactive band was also apparent (see Figure 7 for quantitation). The diseased kidneys also showed an obvious increase in HSP-72 expression, consistent with a renal stress response (**bottom lanes**).

cleavage, producing increases in the active HMGCR moiety.

HSP-72 Expression

Mirroring the changes in HMGCR, both the NTS (days 2 and 7) and PHN (days 10 and 30) models demon-

Figure 7. Quantitation of HMGCR Western blots by densitometry in tissues harvested from control (c), PHN, and NTS nephritis rats. For these analyses, data obtained at each time point for the PHN (10 + 30 days) or NTS (2 + 7 days) rats were combined and compared *versus* their simultaneous controls. The **left panel** compares expression of the active 94-kd band, and the **right panel** compares the 120-kd inactive band. Significant increases in the active band were observed for both experimental groups ($\Delta\Delta$, $P < 0.025$). Although there was a trend toward a decrease in the inactive band in the PHN and NTS groups (suggesting increased cleavage), these differences did not achieve statistical significance ($n = 4$ to 5 at 10 days; $n = 8$ to 9 at 30 days, respectively).

Figure 8. Western blots of SR-B1 and ABCA1 in renal cortical tissues. SR-B1 was detected at 82 kD. A modest decrease in expression was observed in NTS and PHN kidneys, compared to their controls (C). The most prominent reductions were at 30 days after PHN induction, consistent with the fact that the nephrotic syndrome and renal cortical cholesterol overload were most fully expressed in this group (see Figure 9 for quantitation). ABCA1 blotting of 30-day PHN kidneys are shown at the **bottom**. It is observed as a triplet at ~220 kD (consistent with differing degrees of glycosylation; see text). In contrast, normal renal cortex had minimal ABCA1 expression (as is typical for unstimulated tissue; see text). When subjected to densitometric analysis, a greater than 2× increase in ABCA1 expression was observed in PHN tissue samples.

strated clear and significant increases in HSP-72 expression (Figure 6, bottom two lanes; $P < 0.05$ for each disease *versus* control by densitometry).

SR-B1 Expression

Probing NTS kidney samples demonstrated modest reductions in SR-B1, particularly in the 7-day samples. Reductions in SR-B1 were also apparent in the PHN model, particularly in the 30-day samples (Figures 8 and 9).

Figure 9. SR-B1 densitometric analysis in NTS (**left**) and in PHN (**right**) tissue samples *versus* their simultaneous controls (c). Overall analysis of NTS *versus* control samples and PHN *versus* control samples demonstrated statistically significant reductions in SR-B1 expression in each disease model (*, $P < 0.01$; $n = 4$ to 5 determinations per group).

Figure 10. SR-B1 (**top**) and ABCA1 (**bottom**) in cultured HK-2 cells: sensitivity to cell cholesterol manipulation. SR-B1 was reduced in HK-2 cells by incubation with serum (which increases cell cholesterol levels; S, serum; C, controls). This is consistent with cholesterol loading causing physiological suppression of SR-B1 protein mass. ABCA1 levels were suppressed by incubation with mevastatin, consistent with suppression of this cholesterol exporter by reductions in cell cholesterol levels (M, mevastatin; C, controls).

ABCA1 Expression

As shown at the bottom of Figure 8, probing control kidney samples with anti-ABCA1 demonstrated virtually no discernible expression in the relevant molecular weight range (~220 kD). This is consistent with very low levels of ABCA1 in most cell types in an unstimulated state (as per Novus Biologicals; source of ABCA1 antibody). In contrast, the 30-day PHN kidneys demonstrated the presence of a triad of bands in the relevant molecular weight range. [Note: the presence of three bands, the expected result, is thought to represent differing degrees of ABCA1 glycosylation; as per Novus Biologicals). When the blots were subjected to densitometric analysis, a significant ABCA1 increase in PHN kidneys was found (80 ± 11 *versus* 182 ± 13 , $P = 0.001$).]

HK-2 Proximal Tubular Cell Experiments

Addition of serum caused modest suppression of SR-B1 (Figure 10, top). Because serum exposure increases HK-2 cell cholesterol levels, this result would be consistent with presumably normal feedback inhibition. Statin treatment essentially obliterated the ABCA1 signal in HK-2 cells (Figure 10, bottom). Again, this would be consistent with suppression of this cholesterol exporter in the presence of a cholesterol depletion state. Thus, these results provide support for the presence of these two transporters in proximal tubule cells and are consistent with the concept that they are subject to cholesterol-dependent regulation (to our knowledge, the first docu-

mentation of these results in proximal tubule cells). This lends credence to the concept that the SR-B1 and ABCA1 changes in diseased renal tissues stemmed from altered cholesterol homeostasis.

HMGCR mRNA Expression in PHN

At the 10-day time point, HMGCR/GADPH levels were slightly, but not significantly, higher in the PHN group (1.6 ± 0.2 versus control values of 1.3 ± 0.1 ; $P = 0.17$). By 30 days, a 2.5 $\times$ increase in HMGCR mRNA had developed in the PHN kidneys, compared to control values (4.8 ± 0.5 versus 1.9 ± 0.2 , respectively; $P < 0.0005$). Thus, the mRNA results are consistent with the HMGCR protein Western blots, suggesting up-regulation of the HMGCR pathway.

Discussion

The results of the present study confirm that experimental glomerulonephritis with concomitant massive proteinuria can cause an increase in renal cortical cholesterol content. It is noteworthy that this change predominantly reflects the CE, not the FC, pool. The results from the 30-day PHN rats illustrate this point: FC levels rose from ~ 260 to ~ 300 nmol/ μ mol Pi. In contrast, a CE increase from ~ 3 to ~ 135 nmol/ μ mol Pi was observed in the same cortical tissue samples. Thus, in absolute terms, CEs contributed more to the total renal cholesterol increases than did FCs. This seems surprising, given that under normal circumstances, CEs represent only $\sim 1\%$ of the total renal cortical cholesterol pool.

To gain insights into whether the cholesterol changes predominantly affected the glomerular versus the proximal tubular cell compartment, those rats that had the greatest renal cortical cholesterol increases (30-day PHN) were studied for isolated glomerular and proximal tubular cholesterol analysis. The results obtained indicate that proximal tubules, rather than glomeruli, are predominantly affected. Only the tubules manifested any significant FC increases. Whereas both tissue compartments had elevated CEs, the tubules, once again, were more prominently involved. It is noteworthy that the degree of renal cortical CE elevations correlated with the degree of proteinuria observed at any single point in time. This suggests that lipoprotein filtration, with subsequent reabsorption, is pathogenically involved in the proximal tubular/renal cortical cholesterol overload state. The isolated tubule results are also significant in one additional, and important, regard: given that isolated tubules are free of any contaminating blood, and hence, serum lipids, the finding of both FC and CE elevations in them confirms that the observed renal cortical cholesterol elevations cannot simply be explained by blood contamination. Further supporting this conclusion are unpublished data from this laboratory that indicate that marked hypercholesterolemia, as expressed in low-density lipoprotein knockout mice, have normal FC content within cortical tissues, using the isolation techniques used in the current experiments.

SR-B1 (or the high-density lipoprotein receptor) is thought to be a dominant pathway for cellular CE uptake.¹²⁻¹⁷ It can also impact FC levels, potentially via a direct action^{40,41} and/or by inhibiting the ABCA1 efflux pathway.¹² Given that CE overload was the dominant cholesterol change observed in these experiments, we hypothesized that this would induce a compensatory decrease in SR-B1 expression, because cells might attempt to limit the CE overload by specifically decreasing CE uptake. Indeed, this appeared to be the case: SR-B1 expression was reduced by ~ 40 to 50% in both the 10- and 30-day PHN tissue samples. That significant SR-B1 reductions were also documented in NTS kidneys supports the concept that SR-B1 down-regulation is likely a compensatory response to high CE levels, rather than simply being a disease-specific phenomenon. In this regard, it is notable that addition of serum to HK-2 cells, which raises cell cholesterol levels by $\sim 25\%$ (RAZ, unpublished data), also reduced SR-B1 expression. This further supports the hypothesis that SR-B1 can be suppressed after induction of a cellular cholesterol overload state. Finally, it has previously been reported that hepatic SR-B1 is down-regulated in the setting of experimental nephrotic syndrome, contributing to hypercholesterolemia via a reduction in hepatic cholesterol clearance.⁴² When our present findings in renal tissues are interpreted along with these hepatic results, it seems that alterations in SR-B1 expression have broad implications for the nephrotic state.

A second pathway by which cells could limit cholesterol overload could be an increase in the ABCA1 cholesterol efflux pathway. ABCA1, expressed in the plasma membrane and the Golgi apparatus, mediates apo-A1-associated cholesterol (and phospholipid) efflux from cells.¹⁸⁻²⁰ Our finding of relatively small changes in FC levels in renal cortex in nephrotic animals suggested the possibility that a secondary increase in renal ABCA1 protein mass/activity might occur. To gain initial insights into this issue, ABCA1 expression was probed in PHN kidneys after 30 days, and a $>2\times$ increase in protein mass was observed. Finally, it is notable that statin therapy caused a marked reduction in HK-2 cell ABCA1 levels. This further supports the concept that ABCA1 within kidney is, indeed, responsive to changes in cellular cholesterol content.

The above-discussed up- and down-regulation of ABCA1 and SR-B1, respectively, seem to be physiologically appropriate in the setting of a renal cholesterol overload state. This is because these changes theoretically should limit further cholesterol accumulation (assuming that protein mass, as assessed by Western blot, correlates with functional activity; an issue that has not been directly addressed). By analogy with the SR-B1 and ABCA1 changes, marked suppression of renal cholesterol synthesis, via a down-regulation of HMGCR enzyme, would also be expected.^{37,39} Hence, the final goal of this study was to seek confirmation for this last assumption. Paradoxically, results opposite to those that were predicted were obtained: HMGCR protein mass, as assessed by Western blot, was elevated in the setting of both NTS and PHN. Because this implies dysregulation of

the HMGCR axis, confirmatory data were sought by HMGCR mRNA analysis. Consistent with the Western blot results, a trend toward HMGCR mRNA elevations was seen at 10 days after PHN, and by 30 days, a 250% HMGCR mRNA increase was observed ($P < 0.0005$). These findings raise the intriguing possibility that cholesterol accumulation within renal cortex in experimental glomerulopathy is not simply because of increased uptake of circulating or filtered lipids. Rather, increased synthesis, or at least a failure of physiologically appropriate HMGCR suppression, might also be involved. The underlying stimulus for this apparent dysregulation of the HMGCR/mevalonate axis in two disparate models of experimental nephropathy remains to be defined. However, potentially noteworthy in this regard are our previous observations that HMGCR up-regulation can be a component of a tissue stress response (as denoted by increased heat shock protein expression).⁵ That the stress protein HSP-72 was increased in both the NTS and PHN models (which we believe to be a novel finding) lends credence to the possibility that tissue stress triggered the observed increases in HMGCR mRNA/protein mass. How tissue stress might evoke these results remains unknown at this time. Finally, as alluded to above, increases in protein mass and protein activity are not necessarily linked. To make this case, renal-specific inhibitors of HMGCR, SR-B1, and ABCA1 would be needed to more fully assess protein contribution to renal cholesterol levels. Unfortunately, however, no such inhibitors currently exist.

In conclusion, the present study indicates that experimental glomerulonephritis with concomitant massive proteinuria can increase both free, and in particular, esterified cholesterol content within renal cortex. These changes seem to be most prominently expressed within proximal tubular cells. An apparent compensatory down-regulation of SR-B1 (a CE importer) and up-regulation of ABCA1 (a FC exporter) result. Both of these would seem to represent appropriate homeostatic responses to a cholesterol overload state. However, increases in HMGCR protein and its mRNA can also develop, indicating a dysregulation of the HMGCR/mevalonate pathway. This raises the possibility that an inappropriate increase in HMGCR, or at least a failure of its down-regulation, could potentially contribute a glomerulopathy-associated renal cholesterol overload state.

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