Effect of experimental nephrosis on hepatic lipoprotein secretion and urinary lipoprotein excretion in rats expressing the human apolipoprotein A-I gene

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Abstract When human apolipoprotein A-I was expressed in transgenic rats, induction of the nephrotic syndrome resulted in plasma A-I levels exceeding 10 mg/ml. Plasma lipids were no higher than in non-transgenic nephrotic rats. To explain this, the livers from four groups of rats were perfused: wild-type controls (WC), high expressor human apoA-I transgenic controls (TrGC), wild-type nephrotics (WN), and high expressor transgenic nephrotics (TrGN). Compared to the WC group, TrGC rats secreted the same amount of d < 1.063 g/ml lipoproteins but 50% more high density lipoprotein (HDL), with a 5-fold increase in total apoA-I output due to human apoA-I. Compared to the WC group, nephrosis in the WN rats caused a 2-fold increase in both d < 1.063 g/ml lipoproteins and HDL secretion with a 4.6-fold increase in rat apoA-I output. Compared to the TrGC group, nephrosis in the TrGN rats did not increase d < 1.063 g/ml lipoprotein secretion, but caused a 50% increase in HDL secretion and a 6-fold increase in human apoA-I output. The hepatic levels of mRNA for apoB and for HMG-CoA reductase, as well as the degree of apoB mRNA editing, were unchanged. Examination of the perfusate HDL by electron microscopy revealed spherical particles averaging 30 nm in diameter in the WC and WN rats and 17 and 20 nm in the TrGC and TrGN rats. Urinary HDL particles from the TrGN rats did not contain rat apoA-I and averaged 8.2 nm versus 11 nm in the WN rats. We conclude that the size of the nascent HDL, and subsequently of the mature HDL, is determined by the primary structure of apoA-I. In the TrGN rats, the heterogeneous mature HDL has a population of smaller human HDL which is more readily lost in the urine, accounting for the failure of plasma HDL levels to rise above those in TrGC rats. The fact that plasma triglyceride levels in TrGN rats were also not increased may relate to the failure of hepatic apoB secretion to increase, which in turn may have been due to saturation of the protein synthetic capacity by human apoA-I production.—**Marsh, J. B., M. R. Diffenderfer, E. A. Fisher, M. Sowden, M. Dong, J. R. Paterniti, and B. F. Burkey.** Effect of experimental nephrosis on hepatic lipoprotein secretion and urinary lipoprotein excretion in rats expressing the human apolipoprotein A-I gene. *J. Lipid Res.* 1996. **37:** 1113–1124.

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In earlier work, we reported the effects of the nephrotic syndrome in rats transgenic for human apolipoprotein A-I on the levels of plasma lipids and of rat and human A-I (1). Human apoA-I plasma levels exceeded 10 mg/ml in the transgenic nephrotic (TrGN) rats. However, the transgenic-nephrotic rats had serum levels of triglycerides, phospholipids, and cholesterol each of which averaged only 44–52% of the levels found in non-transgenic nephrotic rats. The plasma lipoprotein profiles obtained by sizing column chromatography reflected these differences and were particularly revealing with respect to HDL. In spite of the very high plasma human A-I levels, the transgenic-nephrotic rats had no increase in HDL when compared to non-nephrotic rats and the HDL particles were larger (1). While these experiments established the responsiveness of the human transgene to the stimulus for increased hepatic production of apolipoproteins in the nephrotic syndrome, they did not answer the question of why plasma lipid levels in general, and HDL levels in particular, did not increase in comparison to non-transgenic (WN) rats. The present experiments were designed to investigate the nature of the hepatic lipoprotein and A-I secretion in four groups of animals (WC, TrGC, WN and TrGN) in a liver perfusion system.

In addition to determining the amount and the composition of the nascent secretory lipoproteins, we also

Abbreviations: AEBSF, aminoethylbenzene-sulfonyl fluoride; apoA-I, apolipoprotein A-I; EC, esterified cholesterol; EDTA, ethylenediamine tetraacetate; FC, free cholesterol; GLC, gas–liquid chromatography; PL, phospholipid; TC, total cholesterol; TG, triglyceride; TrGC, high-expressor transgenic control rats; TrGN, high-expressor transgenic nephrotic rats; WC, wild-type control rats; WN, wild-type nephrotic rats.

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investigated the nature of the urinary HDL as our earlier work (1) indicated increased urinary excretion of phospholipids and cholesterol in the TrGN vs WN rats. Because plasma total cholesterol levels were increased in the TrGC and TrGN rats, we also measured the hepatic cholesterol content and the levels of mRNA for HMG-CoA reductase and apoB, as well as the extent of editing of the apoB mRNA.

METHODS

Human apoA-I transgenic and transgenic-nephrotic rats

The high-expressor human apoA-I transgenic rats used in these experiments, and the production of nephrosis with puromycin aminonucleoside (PAN), have been described previously (1). The present experiments used male rats, 7–12 weeks old, at an average weight of 270 g, which did not differ significantly among the four groups studied. With the exception of three rats in the WC group, all controls were non-transgenic litter mates of the transgenic animals. Nephrotic animals were studied at 7–9 days after the first injection of PAN. All animal protocols were approved by the Animal Use Review Committees of the Medical College of Pennsylvania and Hahnemann University and Sandoz Research Institute.

Liver perfusion

Animals were fed ad lib on a standard laboratory chow diet and anesthetized by the intraperitoneal injection of 65 mg/kg of sodium pentobarbital. A blood sample was taken from the inferior vena cava and the livers were perfused in situ as previously described (2) using a Krebs-Ringer-bicarbonate buffer containing 0.1% glucose and essential amino acids equilibrated with 5% CO_2–95% O_2. The livers were perfused at a flow rate of 25–28 ml/min by the flow-through method for 20 min. Recirculation with 115 ± 10 ml of medium was then begun and continued for 1 h, after which the medium was collected and cooled on ice. Sodium azide, EDTA, and AEBSF (Calbiochem 101500) were added as previously described (2). Aliquots of the perfusion medium were used immediately for lipoprotein isolation. The remainder was stored at -20°C for later analysis. At the end of most perfusions, a sample of the liver was immediately placed in 10% trichloroacetic acid at 0°C for glycogen analysis and a sample of the median lobe was rapidly crushed between two blocks of dry ice and stored at -70°C for later analysis.

Lipoprotein isolation

Fifty-four ml of perfusion medium was subjected to sequential density ultracentrifugation at 5°C and d 1.063 g/ml for 18 h at 47,000 rpm, followed by 24 h at d 1.21 g/ml and 49,000 rpm in a Beckman Ti50.2 rotor as previously described (2). Isolated lipoproteins were dialyzed against 0.02 M sodium phosphate (pH 7.4)–2 mM EDTA before analysis.

For the isolation of HDL from the urine, collected as described (1), sequential density ultracentrifugation was carried out at 5°C at a density of 1.063 g/ml at 47,000 rpm, and for 24 h at densities of 1.21 and 1.25 g/ml at 49,000 rpm in a 50Ti rotor.

Apolipoprotein analysis

Human and rat A-I were measured by an ELISA immunoassay (1). Apolipoproteins were analyzed by gel scanning after isolation by SDS-PAGE, staining with Coomassie blue R250, and correction for differential dye-binding (2). ApoB in aliquots of the d < 1.063 g/ml fraction of the liver perfusates was measured by the method of Egusa et al. (3) after extraction with ether to remove a portion of the neutral lipid, which allowed pelleting of the precipitate.

Lipid class analysis

Lipids were extracted from the isolated lipoprotein fractions, or from the frozen livers, with 10 volumes of chloroform–methanol 1:1 for 30 min at room temperature. After centrifugation to remove protein, one-half volume of chloroform was added and water-soluble compounds were removed by the procedure of Folch, Lees, and Sloane Stanley (4). Triglycerides were determined enzymatically using the kit supplied by Boehringer-Mannheim Diagnostics (816370). Phospholipids were determined as inorganic phosphate by the sulfuric-perchloric acid procedure (5). Free and esterified cholesterol were determined by GLC analysis (6). Plasma total cholesterol was measured by the Sigma cholesterol reagent no. 352-1000. Liver total cholesterol was measured by the method of Zlatkis, Zak, and Boyle (7).

Protein and glycogen analysis

Total protein output of liver perfusates or urine was determined by precipitation of aliquots or samples with 10% trichloroacetic acid (TCA) followed by extraction with 1% TCA in ethanol. The insoluble precipitate was dissolved in 0.1 N NaOH for protein determination by the micro biuret reaction (8), using bovine serum albumin as the standard. The acid alcohol-soluble protein was precipitated with 3 volumes of ether and the precipitate was dissolved in 0.1 N NaOH followed by protein estimation. The sum of these two fractions was taken as the total protein output. In the isolated lipoprotein fractions, total protein was measured directly by the Morton and Evans (9) modification of the bicinchoninic

TABLE 1. Plasma cholesterol and apoA-I concentrations

Rat Group	Cholesterol	Rat ApoA-I	Human ApoA-I
	mg/dl		
WC (3)	116 ± 28.9	76.6 ± 24.1	-
TrGC (12)	304 ± 103[a]	50.0 ± 37.9	239 ± 236
WN (12)	442 ± 110[a]	172 ± 32.3[a,b]	-
TrGN (11)	312 ± 115[a,c]	28.9 ± 13.7[b,c]	1,212 ± 512[b]

Results shown represent the mean values ± SD. The numbers in parentheses represent the number of observations. The values shown include five animals in each group reported previously (1). In this table and in all subsequent tables, the *P* values for statistical significance represent the highest number for all comparisons having the same superscript letter. Statistical significance was determined using a two-tailed Student's *t*-test.

[a]Significantly different from the WC mean, $P < 0.0001$.
[b]Significantly different from the TrGC mean, $P < 0.001$.
[c]Significantly different from the WN mean, $P < 0.012$.

acid method. Total liver protein was determined in the precipitate remaining after delipidation with 10 volumes of chloroform–methanol as described below. Liver glycogen was determined by carbohydrate assay (10) after extraction of samples with 10% TCA and precipitation of glycogen from aliquots of the supernatant fluid with 2 volumes of ethanol.

Albumin

Plasma albumin determination involved the quantitative precipitin reaction (11) using a rabbit polyclonal antibody against rat albumin, with whole rat plasma as a secondary standard. The washed immune precipitates were dissolved in 0.1 N NaOH, and protein was measured by the micro biuret method. The standard curves were linear between 10 and 100 μg of rat albumin. The albumin content of the plasma standard was determined by the method of Doumas, Watson, and Briggs (12).

Electron microscopy

Negative staining electron microscopy of the perfusate and urinary HDL particles followed the procedure of Forte and Nordhausen (13).

Liver mRNA

RNA isolation from rat liver and the determination of the abundances of mRNA for apoB and β-actin, as well as the percentage of apoB mRNA that was edited, was performed as previously described (14). The abundance of HMG-CoA reductase mRNA was determined by the same general procedure, but using a cDNA probe (pHRED-102) obtained from the American Type Culture Collection (Rockville, MD).

RESULTS

Plasma levels of apoA-I and cholesterol

The elevated plasma levels of human A-I in the TrGC rats, the augmentation of these levels in the TrGN rats, and the increased concentration of rat A-I in the WN rats are shown in **Table 1.** Rat A-I levels in the TrGN rats were 35% lower than those in the TrGC rats but this difference was of borderline statistical significance ($P = 0.052$). The expression of the human transgene raised plasma cholesterol levels by 162% in the TrGC group, compared to the WC group. When nephrosis was induced, plasma cholesterol was increased by 281% in the WN rats and by 168% in the TrGN rats.

Protein and lipid secretion

Table 2 gives the output of total protein, albumin, triglyceride, phospholipid, and cholesterol by the perfused livers. Expression of the transgene (TrGC vs. WC rats) had no significant effect on these parameters except for a 79% increase in cholesterol output. After the induction of nephrosis, total protein output was increased to approximately the same extent in both wild-type (WN) and transgenic (TrGN) rats, but in the latter group, albumin secretion was significantly decreased by 39%. Also in the TrGN rats, the output of triglyceride was decreased by 37% and that of cholesterol was increased by 54%; there was no change in phospholipid. Total lipid output was unchanged by transgene expression whether in normal or nephrotic rats, while nephrosis itself resulted in a 1.5- to 2.0-fold increase in lipid output and in all other secretory parameters measured except for albumin.

TABLE 2. Protein and lipid output from perfused livers

Rat Group	Total Protein	Albumin	Total Lipid	Triglyceride	Phospholipid	Cholesterol
	mg/g/h			*μg/g/h*		
WC (9)	1.35 ± 0.37	1.02 ± 0.31	476 ± 153	282 ± 125	109 ± 44	56.4 ± 22
TrGC (8)	1.64 ± 0.56	0.85 ± 0.28	499 ± 142	257 ± 87	111 ± 47	101 ± 41[a]
WN (10)	2.25 ± 0.56[a]	0.76 ± 0.32	855 ± 250[a]	520 ± 180[a]	214 ± 54[a,b]	117 ± 31[a,b]
TrGN (9)	2.43 ± 0.57[a]	0.46 ± 0.17[a,b,c]	717 ± 160[a,b]	329 ± 116[c]	208 ± 43[a,b]	180 ± 32[a,b,c]

[a]Significantly different from the WC mean, $P < 0.02$.
[b]Significantly different from the TrGC mean, $P < 0.03$.
[c]Significantly different from the WN mean, $P < 0.03$.

TABLE 3. ApoA-I secretion by perfused livers

Rat Group	Total Rat A-I	Total Human A-I	HDL Rat A-I	HDL Human A-I
		μg/g/h		
WC (9)	6.1 ± 2.2	-	2.1 ± 1.2	-
TrGC (8)	4.6 ± 1.4	25.4 ± 28	0.28 ± 0.56[a]	5.3 ± 3.1
WN (10)	27.9 ± 12[a]	-	9.9 ± 5.1[a,b]	-
TrGN (9)	12.6 ± 2.6[a,b,c]	178 ± 61[b]	0.03 ± 0.02[a,b,c]	21.4 ± 4.6[b]
WN/WC	4.57		4.71	
TrGN/TrGC	2.74	7.01		4.04

[a]Significantly different from the WC mean, $P < 0.001$.
[b]Significantly different from the TrGC mean, $P < 0.005$.
[c]Significantly different from the WN mean, $P < 0.0001$.

ApoA-I secretion

The output of rat and human A-I in these experiments is shown in **Table 3.** In the wild-type rats, nephrosis produced a 4.6-fold increase in rat A-I secretion. A similar increase was observed in the HDL-associated apoA-I. One-third of the total A-I was isolated with HDL. Expression of the human transgene decreased the total rat A-I secretion in the TrGC group by 25%, which was not statistically significant, but only 6% was associated with HDL. In these transgenic rats, human A-I secretion was 5.5 times that of rat A-I (25.4/4.6, Table 3). When nephrosis was induced, the TrGN rats increased rat A-I secretion 2.7-fold but this was less than the 4.6-fold increase seen in the WN group. However, human A-I secretion was increased 7-fold. In the HDL recovered from the TrGN rats, the increase in human A-I was 4-fold, virtually the same increase found with rat HDL A-I in the WN group. The percent of total human A-I secreted as HDL decreased from 21% in the TrGC group to 12% in the TrGN group. Thus, there appears to be a limit to the amount of A-I that can be incorporated into HDL under these conditions.

d < 1.063 g/ml Lipoprotein secretion and composition

The output of protein and lipid at d < 1.063 g/ml is shown in **Table 4(A).** The effect of nephrosis (WN vs. WC rats) in increasing the secretion of lipoproteins is in accord with earlier work (15). The transgene itself (TrGC rats) had little effect on the lower density lipoprotein secretion, except for an increase in cholesterol at the expense of phospholipid (Table 4A). In the TrGN rats, compared with WN rats, 34% less d < 1.063 g/ml lipoproteins were secreted, as shown in the last column of Table 4(A). All components of this lipoprotein fraction in the TrGN rats were significantly decreased except for cholesterol, most of which was unesterified. The composition of the d < 1.063 g/ml particles is shown in **Table 5(A).** In the TrGC and WN rats, this lipoprotein fraction was enriched in esterified cholesterol, compared to the WC rats, while the TrGN rats showed a marked increase in free cholesterol compared to all other groups.

The apolipoprotein composition of the d < 1.063 g/ml lipoproteins is shown in **Table 6(A).** The TrGC and WC

TABLE 4. Lipoprotein secretion by perfused livers

Rat Group	Protein	ApoB	TG	PL	FC	EC	Total LP
			μg/g/h				
A. d < 1.063 g/ml lipoproteins							
WC (9)	44.5 ± 18.9	11.7 ± 2.4	255 ± 122	83.5 ± 37.5	24.3 ± 10.2	12.1 ± 6.1	419 ± 168
TrGC (7)	49 ± 13.7	12.5 ± 1.5	230 ± 79	67.3 ± 28.7	37 ± 21.4	26.8[a] ± 15.5	404 ± 150
WN (10)	110[a,b] ± 24	21.3[a,b] ± 3.9	477[a,b] ± 107	147[a,b] ± 35	39.7[a] ± 21.9	46.4[a] ± 6.2	820[a,b] ± 145
TrGN (9)	66.5[c] ± 24	11.7[c] ± 3.9	275[c] ± 107	100[c] ± 35	77.8[a,b,c] ± 21.9	21.9[a,c] ± 6.2	535[c] ± 145
B. HDL (1.063 < d < 1.21 g/ml lipoproteins)							
WC (10)	32 ± 16.1	-	30.6 ± 15	25.6 ± 9.4	5.2 ± 3.8	10.7 ± 5.1	104 ± 29.9
TrGC (7)	64.2[a] ± 17.3	-	27.4 ± 10.3	43.5[a] ± 17.1	14.7[a] ± 4.0	16.9[a] ± 4.5	155[a] ± 60.7
WN (10)	64.7[a] ± 27.3	-	42.5 ± 15.4	67.2[a] ± 20.6	164 ± 17.4	18.5[a] ± 6.0	209[a] ± 703
TrGN (9)	117[a,b,c] ± 22.0	-	53.3[a,b] ± 20.1	107[a,b,c] ± 22.7	62.9[a,b,c] ± 11.0	17.6[a] ± 3.1	345[a,b,c] ± 69.9

[a]Significantly different from the WC mean, $P < 0.03$.
[b]Significantly different from the TrGC mean, $P < 0.01$.
[c]Significantly different from the WN mean, $P < 0.02$.

TABLE 5. Percent composition of perfusate lipoproteins

Rat Group	Protein	TG	PL	FC	EC
			%		
A. d < 1.063 g/ml					
WC (8)	11.1 ± 2.7	59.2 ± 12.7	21.3 ± 12.3	6.0 ± 1.0	2.9 ± 0.9
TrGC (7)	11.9 ± 4.7	58.0 ± 5.6	16.5 ± 2.2	8.8 ± 2.3[a]	6.4 ± 1.7[a]
WN (10)	13.7 ± 2.6	57.6 ± 4.2	18.1 ± 3.6	5.0 ± 1.6[b]	5.7 ± 1.3[a]
TrGN (9)	12.5 ± 3.1	50.3 ± 9.0	19.5 ± 7.4	14.8 ± 3.4[a,b]	4.2 ± 1.0[a,b,c]
B. HDL (1.063 < d < 1.21 g/ml)					
WC (10)	30.2 ± 9.7	29.9 ± 12.7	25.0 ± 6.3	4.8 ± 2.7	10.1 ± 3.1
TrGC (7)	37.2 ± 5.1	20.8 ± 11.2	30.7 ± 10.5	8.5 ± 1.2[a]	9.8 ± 1.4
WN (10)	30.4 ± 4.7	21.2 ± 8.6	32.4 ± 2.5[a]	6.9 ± 4.1	9.1 ± 2.0
TrGN (9)	32.7 ± 3.0	15.6 ± 5.8[a]	31.6 ± 7.4	18.5 ± 3.3[a,b]	5.2 ± 0.9[a,b,c]

[a]Significantly different from the WC mean, $P < 0.01$.
[b]Significantly different from the TrGC mean, $P < 0.002$.
[c]Significantly different from the WN mean, $P < 0.02$.

rats had similar apolipoprotein compositions. The WN rats had twice as much A-I in this fraction and the TrGN rats had four times as much, compared to the WC rats. Both WN and TrGN rats had less apoB-48 than their corresponding WC or TrGC controls. However, as shown in **Table 7,** no change in the extent of the editing of the apoB mRNA was found in the TrGN livers compared to those from the TrGC rats. No significant change in the amount of apoB or HMG-CoA reductase mRNA was noted (Table 7).

Nascent HDL secretion and composition

Table 4(B) shows that in comparison to the WC group, the presence of the transgene doubled total HDL output in the TrGC rats and tripled it in the TrGN rats. Although there were differences in lipid secretion, the lipid compositions were similar (Table 5B), but as in the d < 1.063 g/ml fraction, much more of the cholesterol was unesterified in the TrGN rats. The apolipoprotein compositions are shown in Table 6(B). In all groups, A-I was the dominant apolipoprotein and more of it was present in the three experimental groups compared to the WC rats. The apoA-I of the nascent HDL in the TrGC and TrGN rats was almost entirely human A-I (Table 3).

Electron microscopy of nascent HDL particles

Based on the EM images of the nascent HDL the size distribution of the particles is shown in **Fig. 1.** The mean particle diameters averaged 30.4, 17.5, 29.3, and 20.3 nm for the WC, TrGC, WN, and TrGN rats, respectively (measured on a pooled sample from each group).

TABLE 6. Percent composition of the apolipoproteins of perfusate lipoproteins

Rat Group	A-I	C	E	B-48	B-100
			%		
A. d < 1.063 g/ml					
WC (4)	12.3 ± 3.4	42.3 ± 2.6	13.8 ± 2.3	21.3 ± 1.2	10.3 ± 3.9
TrGC (6)	14.6 ± 5.6	46.6 ± 4.6	12.4 ± 2.6	19.6 ± 1.5	6.8 ± 1.7
WN (3)	25.9 ± 3.1[a,b]	28.5 ± 1.9[a,b]	19.3 ± 1.7[a,b]	12.8 ± 1.1[a]	13.5 ± 3.0[b]
TrGN (6)	50.1 ± 7.5[a,b,c]	10.3 ± 4.1[a,b,c]	11.7 ± 2.0[c]	12.1 ± 2.2[a,b]	14.9 ± 2.5[b]

	A-I	C	E	A-IV	B-48	B-100
B. HDL (1.063 < d < 1.21 g/ml)						
WC (4)	52.6 ± 3.8	13.0 ± 3.5	18.0 ± 5.2	3.3 ± 3.2	8.0 ± 2.8	5.2 ± 2.1
TrGC (5)	68.7 ± 9.6	10.6 ± 4.9	11.1 ± 3.3	1.1 ± 1.9	5.6 ± 2.0	2.9 ± 1.2
WN (10)[d]	62.7[e]	12.9	12.1	4.9	5.3	2.1
TrGN (6)[d]	83.1[e]	6.5	3.3[e]	0	4.2	2.8

[a]Significantly different from the WC mean, $P < 0.02$.
[b]Significantly different from the TrGC mean, $P < 0.05$.
[c]Significantly different from the WN mean, $P < 0.03$.
[d]These analyses were carried out in duplicate on a pooled sample.
[e]This value is more than two standard deviations from the WC mean.

TABLE 7. Liver mRNA

Rat Group	ApoB	% ApoB Editing	HMGCoA Reductase
TrGC	1.07 ± 0.08 (5)	42 ± 7.0 (3)	1.26 ± 0.59 (4)
TrGN	1.07 ± 0.12 (4)	51 ± 8.3 (3)	1.17 ± 0.45 (4)

The livers used for these analyses were frozen and stored at -70°C but were not perfused prior to analysis. The values shown were derived by densitometric signals from autoradiograms and are relative to β-actin mRNA (14). ApoB mRNA editing assays were carried out as described (14).

Liver composition

The livers of the four groups of rats that were the subjects of the perfusion studies were analyzed for protein, lipid, and glycogen. As shown in **Table 8,** the TrGC and WC rat livers did not differ significantly in protein and lipid class composition. Induction of nephrosis in the WN rats likewise did not significantly alter the composition except for a lower triglyceride content. The livers of the TrGN rats, in comparison to the WN rats, had 13% less total protein, 25% more cholesterol, and 4.8 times as much triglyceride. Only four of the eight TrGN livers, however, showed elevated triglycerides. Acid-soluble glycogen was similar in all four groups.

Urinary HDL

The urinary excretion of HDL and apoA-I was measured prior to liver perfusion in some of the WN and TrGN rats, as shown in **Table 9.** The total daily urinary protein loss of 440 mg, much of which was albumin (1), was the same in the WN and TrGN rats, although we previously reported greater proteinuria in two TrGN rats (1). Isolation of HDL from the WN urine at d 1.21 and d 1.25 g/ml recovered a total of 2.25 mg of protein, whereas in the TrGN urine 10.3 mg was recovered. Virtually all (99.9%) of the apoA-I in HDL from the TrGN urine was human A-I, as judged by the ELISA assay. With respect to the additional protein recovered between d 1.21 and d 1.25 g/ml (the VHDL fraction), it should be noted that this was not a pure fraction; part of it represents incomplete recovery of HDL after the first 24-h ultracentrifugation procedure. We carried out the isolation in this fashion to minimize losses of A-I during ultracentrifugation, and to determine whether significant amounts of denser particles might have been present. This appears to have been the case in the TrGN rat urine since, as shown in **Table 10,** the VHDL fraction was significantly enriched in protein and contained relatively less phospholipid and more cholesterol than that from the WN rats. Table 10 also shows that the urinary HDL of the TrGN rats was enriched in phospholipid at the expense of cholesterol and that more of the cholesterol was unesterified.

In a pooled sample of WN urinary HDL ($1.063 < d < 1.21$ g/ml), the apolipoprotein composition, as judged by SDS-PAGE, was as follows: apoE, 3%; apoA-IV, 3%; apoC plus apoA-II, 38%; and apoA-I, 56%. In the TrGN rats, the apoCs constituted 13%, apoA-I 87%, and no bands corresponding to those of apoE or apoA-IV were seen, probably due to displacement by A-I.

Negative staining electron microscopy of the urinary HDL is shown in **Fig. 2.** All of the particles were spherical. Many more smaller particles were seen in the TrGN group, with an average diameter of 8.2 nm, whereas the average diameter was 11 nm in the WN group.

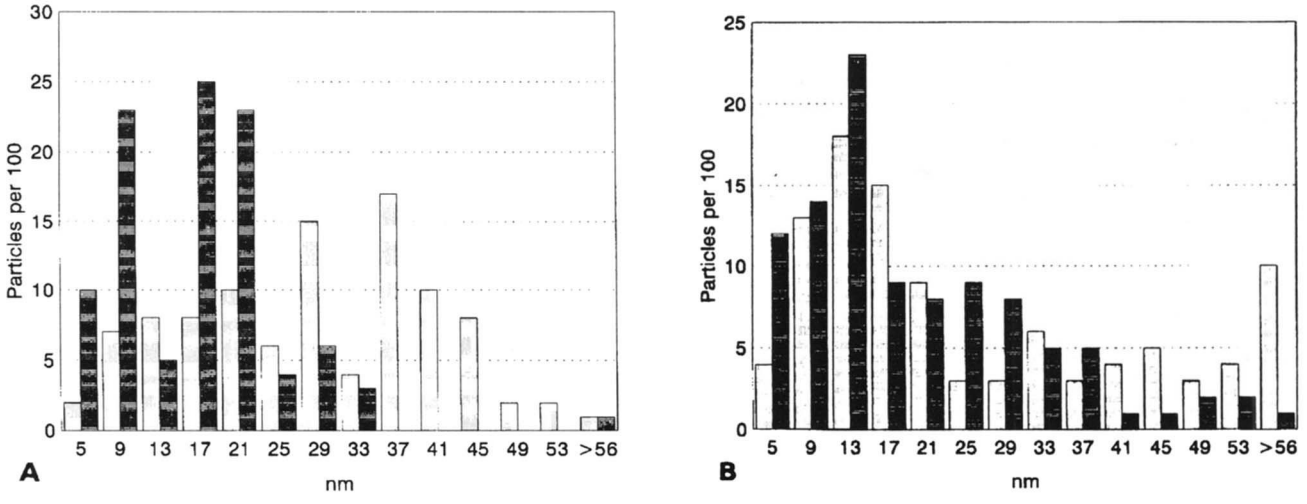

Fig. 1. Particle size distribution of liver perfusate nascent HDL, based on negative staining electron microscopy. The numbers on the abscissa represent the beginning of each size range (i.e., from 5 to 9 nm for the first pair of bars). A: Nascent HDL from WC (light bars) and TrGC rats (dark bars). B: Nascent HDL from WN (light bars) and TrGN rats (dark bars).

TABLE 8. Liver composition

Rat Group	Protein	PL	TC	TG	Glycogen
			mg/g		
WC (10)	158 ± 11.0	37.6 ± 2.36	3.97 ± 0.56	12.6 ± 2.88	24 ± 14 (7)
TrGC (8)	151 ± 13.7	35.2 ± 5.94	3.79 ± 1.26	8.7 ± 5.67	30 ± 3.8 (5)
WN (9)	155 ± 8.23	35.7 ± 4.61	3.49 ± 0.63	5.1 ± 1.81[a]	22 ± 11 (5)
TrGN (9)	135 ± 10.5[b]	34.0 ± 4.80	4.37 ± 1.03[c]	24.3 ± 18.7[d]	23 ± 4.8 (6)

[a]Significantly different from the WC mean, $P < 0.01$.
[b]Significantly different from all other means, $P < 0.05$.
[c]Significantly different from the WN mean, $P < 0.05$.
[d]Significantly different from the WN mean, $P < 0.03$ (non-parametric test).

DISCUSSION

Human apoA-I transgenic rats

The overexpression of human apoA-I in transgenic animals provides unique models that help to clarify many aspects of A-I and HDL metabolism, including the anti-atherogenic effect of elevated HDL levels (16). In a similar way, the experimental nephrotic syndrome, reflecting an endogenous hyperlipidemia, has been a powerful tool that has helped elucidate many aspects of lipoprotein metabolism (15), including regulation of apolipoprotein gene expression (17, 18).

The nucleotide sequence of the gene for both human and rat apoA-I have been reported (19, 20). Recent studies have shown that the -256 to -41 DNA region upstream from the transcription site (+1) of the human A-I gene contains the regulatory elements that are necessary and sufficient for expression in HepG2 cells (21–23) and in the liver of transgenic mice (24, 25). This element contains at least three distinct nuclear protein binding sites (23) designated as A (-214 to -192), B (-169 to -146), and C (-134 to -119). These sites bind nuclear proteins from HepG2 cells (22), rat liver (26), and rabbit liver (27). Several transcription factors have been shown in vitro to bind to these sites, including members of the steroid/thyroid receptors superfamily of ligand-inducible transcription factors (23, 28). Nucleotide sequence analysis revealed that the human and rat A-I genes are highly conserved between positions -240 to -15 (22). Chan, Nakabayashi, and Wong (29) identified four *cis*-acting DNA elements in the rat A-I promoter designated A (-232 to -187), B (-185 to -146), C (-141 to -102) and D (-79 to -48), which indicates a similar organization of the rat and human apoA-I promoters.

The molecular mechanisms responsible for regulating the expression of the A-I gene in experimental nephrosis are only partially known at present. In PAN nephrosis (18), and in the Heymann nephritis or in the Nagase analbuminemic rat models (30), increased hepatic mRNA for apoA-I was associated with increased transcriptional activity. To compare the responses of the human and rat A-I genes to the induction of nephrosis, we have studied transgenic rats expressing human A-I (1). These rats were bred with a 13 kilobase pair (kbp) DNA construct containing the human A-I gene plus 10 kbp of 5′ flanking sequences which include the *cis*-acting DNA sequences controlling liver-specific expression. We found an increase of both human and rat apoA-I mRNA after induction of nephrosis with PAN. This induction could be due to interaction of known transcription factors with the A-I regulatory elements at sites A, B, or C, or there may be new factors produced in the nephrotic syndrome. The mapping and localization of the liver elements controlling the nephrosis-inducible expression of the human A-I gene are currently under investigation.

Lipoprotein secretion by the liver

The expression of the human apoA-I gene in vivo in rats resulted in elevated plasma levels of HDL (1). As the construct was expressed only in the liver, increased hepatic production of human A-I must have been re-

TABLE 9. Urinary excretion of HDL, VHDL, and apoA-I

Rat Group	Total Protein	HDL Protein	VHDL Protein	Rat ApoA-I	Human ApoA-I
			mg/day		
WN (3)	437 ± 6.1	1.68 ± 0.24	0.57 ± 0.09	1.3 ± 0.18	-
TrGN (4)	443 ± 143	7.35 ± 2.41[a]	2.99 ± 0.99[a]	-	9.0 ± 3.0

The values for apoA-I are based on the sum of the HDL and VHDL ($1.21 < d < 1.25$ g/ml) protein shown in this table, multiplied by the average percentage of apoA-I based on SDS-PAGE analysis.
[a]Significantly different from the WN mean, $P < 0.006$.

TABLE 10. Percent composition of urinary HDL and VHDL

Rat Group	Protein	PL	TC	% TC as EC
		%		
A. HDL (1.063 < d < 1.21 g/ml)				
WN (3)	47.8 ± 4.90	26.2 ± 2.82	29.9 ± 3.38	85.9 ± 1.77
TrGN (4)	47.2 ± 2.26	32.0 ± 2.97[a]	20.6 ± 1.35[a]	73.1 ± 3.44[a]
B. VHDL (1.21 < d < 1.25)				
WN (3)	44.0 ± 7.49	41.7 ± 4.85[b]	19.9 ± 8.67	n.d.
TrGN (4)	57.1 ± 7.16[a,b]	34.3 ± 4.07[a]	26.9 ± 7.05	n.d.

The values shown are averages of the individual samples analyzed and therefore do not add up to exactly 100%.
[a]Significantly different from the WN mean, $P < 0.05$.
[b]Significantly different from the corresponding mean for HDL, $P < 0.05$.

sponsible, and the present experiments provide direct evidence. In the transgenic control rats, the secretion of HDL was doubled whereas total A-I secretion was increased 5-fold. The marked increase in total A-I production was entirely due to human A-I (Table 3). These TrGC rats also had a 25% reduction in the secretion of rat A-I that was not statistically significant. In human A-I transgenic mice, Rubin et al. (16) and Chajek-Shaul et al. (31) have reported decreased amounts of murine A-I in plasma and decreased amounts of its mRNA in liver. In our TrGC rats, only 6% of the secreted rat A-I was associated with HDL, whose A-I was 95% human A-I. In the WC rats, 34% of the secreted rat A-I was associated with HDL, in agreement with a value of 30% reported in HepG2 cells (32). The A-I of this HDL was 95% human A-I. One may conclude that human A-I either displaces rat A-I from HDL or it competes successfully with rat A-I for nascent HDL formation. Marshall et al. (18) added 0.4 mg/ml of human apoA-I to rat plasma and found that it displaced apoE and enlarged the particles, which would suggest that, in the present experiments, competition for nascent HDL formation may be the more likely mechanism. The presence of more apoA-I in the d < 1.063 g/ml lipoproteins of plasma has previously been noted in non-transgenic nephrotic rats (33). In the present experiments, we do not know whether this enrichment occurred intracellularly, or after secretion in the space of Disse, or by interaction with free A-I during the recirculation of the perfusion medium.

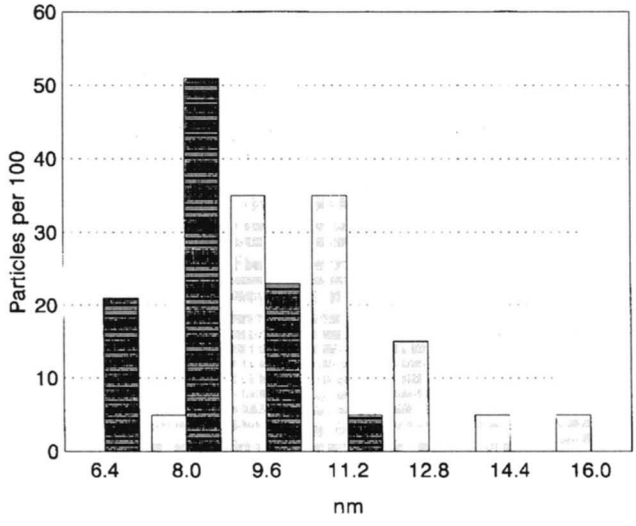

Fig. 2. Particle size distribution of urinary HDL (1.063 < d < 1.21 g/ml). The numbers on the abscissa represent each size range averaged over 1.6 nm. Light bars, WN rats; dark bars, TrGN rats.

As HDL particles have not been found by electron microscopy in rat hepatocyte Golgi fractions, nascent HDL formation probably occurs either at the cell membrane at the time of secretion or in the space of Disse after secretion of A-I. Nascent HDL found in rat liver perfusates after short periods of perfusion is a spherical particle containing up to 44% of its lipid as triglyceride (2, 34) which is rapidly hydrolyzed after secretion in vivo (35). The lipid compositions of the d < 1.063 g/ml and HDL perfusate lipoproteins were similar in all four groups of rats, except for the increased amount of free cholesterol in the transgenic rats, especially the TrGN rats (Table 5). Because the secreted lipoproteins were enriched in A-I, this observation may be related to the known ability of apoA-I, perhaps in the form of pre-beta HDL, to bind free cholesterol from cells (36). We do not know how much cholesterol may have been associated with the free A-I, but it was quantitatively not very large in comparison with lipoprotein-associated cholesterol as the recovery of cholesterol from the isolated lipoproteins was greater than 85% of that obtained after TCA precipitation of aliquots of whole perfusion medium (data not shown).

The induction of nephrosis in the transgenic rats increased human A-I production 7-fold over that seen in the TrGC rats, which clearly accounts for the very high plasma concentrations of human A-I (Table 1). However, the output of HDL was only doubled, indicating that the availability of lipid for HDL formation was a limiting factor. Enrichment of the apoB-containing lipoproteins by A-I occurred, but these particles accommodated only a small fraction of the secreted A-I.

The nascent HDL particles formed in the TrGC and

TrGN rat livers were smaller, averaging 17 and 20 nm, respectively; whereas in the non-transgenic WC or WN rats, they averaged 30 nm. By way of comparison, in an in vitro system, Lamon-Fava et al. (37) have reported that transfection of human apoA-I into 3T3 cells resulted in the secretion of spherical particles, 10–21 nm in diameter.

Quantitatively, the values for total A-I secretion in the perfusates from the WC rats are in reasonable agreement with values of 3–8 μg/g per h previously reported (38). However, the values for HDL-associated A-I in the present study obtained by immunoassay were lower than those based on HDL protein and SDS-PAGE apolipoprotein analysis. This may reflect the sensitivity of the ELISA assay to the conformation of apoA-I, but the results should not affect interpretation of the comparative differences among the four experimental groups reported here as they were all assayed under similar conditions.

Urinary lipoprotein excretion

It is clear from the liver perfusion data that the failure of plasma HDL levels to increase significantly in the TrGN versus TrGC rats (1) cannot be accounted for by failure of HDL formation from the human A-I secreted by the liver. Increased urinary loss of HDL, suggested in our earlier report of increased urinary lipid excretion (1), was probably responsible. Strong support for this hypothesis was obtained by the analysis of urinary HDL. The size and composition of the urinary HDL particles from the TrGN and WN rats differed significantly. The smaller particle size (8.2 nm for TrGN vs. 11 nm for WN) provides an explanation for the greater urinary loss of lipids, entirely accounted for by HDL, in the TrGN rats. This, in turn, explains the lower plasma levels of HDL. The differential loss of these small HDL particles in the TrGN rats explains why we observed larger particles in the plasma of these animals in our earlier report (1). In mice expressing the human A-I gene, two main classes of plasma HDL particles, 10.5 and 8.5 nm, have been reported (31). The 8.5 nm mouse particles containing human A-I are very similar to the 8.2 nm urinary HDL particles reported here. In a single human subject with the nephrotic syndrome, Shore, Forte, and Lewis (39) reported HDL particles present with an average diameter of 9.9 ± 1.1 nm, somewhat larger than the human-type HDL in the TrGN urine in the present experiments but still smaller than rat HDL. Human HDL_3 particles are in the 7.5–8.5 nm range (39), which would correspond to those of the urinary HDL in the TrGN rats.

It is interesting that the urinary HDL from the TrGN rats had much more free cholesterol than that from the WN rats. One explanation might be that human A-I may not be as effective an activator of LCAT as rat A-I. However, Golder-Novoselsky et al. (40) reported that human A-I is a more efficient activator of mouse LCAT than mouse A-I, and one might expect the same to be true for the rat. Two other explanations are plausible, either the cholesteryl esters were hydrolyzed during passage through the nephron (41), or, as seems more likely to us, the urinary excretion was so rapid that LCAT simply did not have sufficient time to work.

Increased urinary loss of smaller human HDL_3-like particles in the TrGN rats in comparison with WN rats accounts for the lower plasma HDL levels, as well as the presence of larger plasma HDL particles (1). These observations may be relevant to the human nephrotic syndrome, where plasma HDL levels are generally normal. In humans, urinary loss of HDL has not been regarded as a satisfactory explanation because the losses are relatively small. In rats with Heymann nephritis, Kaysen, Hoye, and Jones (42) reported that total renal A-I catabolism did not differ from that of the controls and the urinary loss of A-I accounted for only 10% of the total labeled A-I disappearance rate. From their data, the total A-I catabolic rate in our rats would be approximately 28 mg/day. In the WN rats, urinary A-I loss (Table 9) would then account for 5% of A-I catabolism whereas in the TrGN rats it would account for 32%, assuming that all of the urinary apoA-I was in HDL (43). In two groups of subjects with the nephrotic syndrome, classified as mild or severe, Gherardi et al. (44) found that plasma HDL_3 levels were 18% above normal in mild nephrosis and 18% below normal in severe nephrosis. This suggests that in humans, the stimulus to increased apoA-I synthesis may be sufficient to overcome the urinary loss, but insufficient to raise HDL levels. Further work on A-I metabolism in the human nephrotic syndrome appears warranted.

Hepatic response to nephrosis

Increased urinary loss of HDL explains the lower plasma HDL levels in TrGN versus WN rats but does not explain the lower plasma triglyceride levels. We calculate from the data shown in Table 4 that 11% of the total triglyceride output was in the HDL fraction in the WC and TrGC rats, while 8.2% and 16% was in HDL in the WN and TrGN rats, respectively. In the TrGN rats, the percentage of TG was decreased, even though HDL output was increased, resulting in only a small change in TG output in this fraction. However, apoB or total triglyceride production was not higher in the TrGN rats compared to the TrGC controls. This can account for the failure of the plasma triglyceride levels to rise above the control levels in vivo, though the possibility of increased triglyceride lipolysis cannot be ruled out. The simplest explanation for the lack of increased hepatic triglyceride and apoB production may be that the secre-

tory protein synthetic machinery was overwhelmed by human A-I production in the TrGN rats. This suggestion is supported by the significant 39% decrease in albumin production. In the present experiments, we did not find an increase in albumin production in the WN rats, which has been consistently found in rats with antibody-induced nephrosis (11, 42). Although we have no explanation for this, it should be noted that PAN, or its active metabolites, may have effects on albumin synthesis independent of the nephrotic syndrome itself.

Overexpression of human A-I had little effect on the parameters we measured in the liver. The acid-soluble glycogen content, a measure of the nutritional state of the animal prior to liver perfusion, was the same in all four groups of rats and similar to previously reported values (45). Induction of nephrosis caused a slight (13%) but significant decrease in the measured liver total protein. This may have been due to an actual loss in total protein caused by the demands for amino acids for the synthesis of apoA-I, or it could be artifactual due to greater solubility of the protein, perhaps including cytoplasmic apoA-I, in the chloroform–methanol 1:1 used for the extraction of lipid. It is unlikely to be due to an increased water content of the tissue as the WN rats, with more edema, did not show a change in protein. The lipid class composition of the livers of the TrGC rats was not different from the WC or WN controls, but we did find, on average, decreased triglycerides in WN and increased triglycerides in TrGN rats. This may be related to the increased secretion of triglycerides in the WN rats and the relatively decreased secretion in the TrGN rats. However, we were not able to correlate the hepatic triglyceride content with its secretory rate, probably because only a small percentage of stored triglyceride is secreted.

The cholesterol content of the TrGC rat livers was normal. In the TrGN rats, the cholesterol content was higher than in the other groups, but the difference (25%) was significant only in comparison with the WN group. However, when the cholesterol content was expressed per mg of liver protein, it was significantly increased in the TrGN livers compared to all of the other groups. It is unlikely that increased cholesterol synthesis was responsible for the increase in hepatic cholesterol as hepatic HMG-CoA mRNA levels were unchanged. We think it more likely that the large amounts of A-I produced, containing some cholesterol derived in vivo from other lipoproteins or from peripheral tissue, may have been bound to cells in the space of Disse. Analysis of the cholesterol content of isolated hepatocytes would be necessary to support this hypothesis. Even though less apoB was secreted by the TrGN rats compared to the WN rats, no change in mRNA level was detected in the liver, supporting the idea that less was translated due to competition by A-I. While less of the apoB was secreted in the form of apoB-48 in the TrGN versus TrGC rats (Table 6), we did not detect comparable changes in the degree of hepatic mRNA editing, suggesting a translational or post-translational mechanism.

In summary, the induction of nephrosis in transgenic rats produces an amplication of the hepatic mRNA response to the stimulus for increased apoA-I synthesis and secretion (1). Because of the differences in particle sizes between human and rat HDL and the preferential urinary excretion of smaller human A-I-containing HDL, we now have additional evidence that the primary structure of apoA-I is a crucial determinant of the size of nascent hepatic HDL particles, as well as of mature HDL. The transgenic-nephrotic rat model, because of the amplification of the hepatic response to proteinuria, should prove useful in future studies of the nature of the stimulus to hepatic lipoprotein synthesis and the molecular mechanisms in both nucleus and cytoplasm responsible for the enhanced apolipoprotein gene expression.

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