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INCREASED HEPATIC CHOLESTEROL PRODUCTION DUE TO LIVER HYPERTROPHY IN RAT EXPERIMENTAL NEPHROSIS

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Control and nephrotic rats were compared as to the liver contents of cholesterol, phospholipid and the activity of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Whole liver homogenates as well as endoplasmic reticulum membrane samples showed increased free cholesterol and phospholipid mass in the nephrotics. Correction of the values by the protein content indicated membrane expansion, i.e. liver hypertrophy. However, total hepatic cholesterol synthesis as measured by the reductase activity was increased in the nephrotic rat. These results are in accordance with previous studies showing enhanced cholesterol production in experimental nephrosis. In short, enhanced cholesterol mass in the liver coexists with increased hepatic synthesis in the experimental model used.

Introduction

Sterol balance measurements have demonstrated that the hypercholesterolemia in nephrotic rats is caused by enhanced cholesterol synthesis [1]. The nephrotic liver is the site of increased plasma protein synthesis [2,3], including lipoprotein apolipoproteins [2], and as such the source of enhanced whole body cholesterol production is presumed to be the liver. Other studies [4,5], though controversial [6,7], have shown increased acetate and mevalonate incorporation into cholesterol in liver slices from nephrotic rats.

Hepatic feedback control of cholesterol synthesis has been shown to be operative in nephrotic rats [8]. However, we have previously reported an increased hepatic cholesterol content in nephrotic animals as compared to controls [1]. Thus, in the nephrotic rat, increased production of cholesterol may coexist even in face of the greater cholesterol mass in the livers of these animals. In order to test this possibility, whole liver and hepatic cell fractionation studies of nephrotic and control animals were undertaken, aimed at determining the site of cholesterol storage and testing the feedback mechanism in experimental nephrosis by analysis of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A (hydroxymethylglutaryl-CoA) reductase (mevalonate : $NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34) activity.

Materials and Methods

Experimental nephrosis. During a 10 day period, male Wistar rats weighing 80–100 g were administered anti-kidney serum intravenously as previously described [9], to establish a proteinuria in excess of 7 mg/h, without accompanying diarrhea, ascites or apparent edema. Nephrotic and

control groups were pair-fed a sterol-free, linoleic acid-rich (sunflower oil, 3.7% mass) synthetic diet containing 40% protein by weight and 4 kcal/g. This diet was fed throughout the induction of nephrosis and the ensuing 3–4 week period needed to develop hypercholesterolemia and increase the hepatic cholesterol mass [1]. Final body weights of experimental and control groups did not differ in any of the experiments. Of the 60 animals studied, only four died during the pre-experimental period. Nephrosis was characterized by bi-weekly proteinuria analysis [10] and further measurements of serum protein [10], cholesterol [11] and triacylglycerols [12] upon death.

Whole liver experiment. After an overnight fasting period all rats were beheaded and blood collected for serum analyses. Livers were immediately excised, rinsed in phosphate buffer, weighed and minced. Samples were weighed for homogenization in isotonic sucrose (5 : 1, v/w) and for lyophilization. Liver water content was calculated by weight difference between fresh and dry (lyophilized) material. Homogenates were analyzed for protein [13], phospholipid [14], DNA [15], RNA [16] and cholesterol content. Free and esterified cholesterol were extracted [17] and separated on silica-gel H TLC (diethyl ether/heptane, 55 : 45). After reflux in 2 M NaOH, both fractions were purified again on silica-gel H TLC [18]. An internal radioactive standard of [^{14}C]cholesterol was used to correct for procedural losses during the isolation. Cholesterol mass was measured by gas-liquid chromatography [19].

Cell fractionation studies. After an overnight fasting period, the animals were beheaded at 10 a.m. in groups of three, on consecutive days. Livers were immediately excised, weighed, washed in phosphate buffer, minced and homogenized in 4 vol. 0.88 M sucrose in an Elvehem-Potter type homogenizer fitted with a tight pestle. Experimental rats were killed according to level of nephrosis. Smooth and rough endoplasmic reticulii were obtained by the modified method of Rotschild [20] and Fouts [21]. The homogenate was centrifuged twice at $9\,000 \times g$ for 25 min. The post-mitochondrial supernate was diluted with 5 vol. deionized water and layered over 2 ml of 1.31 M sucrose and centrifuged in a Spinco rotor 40 at $60\,000 \times g$ for 14 h (Beckman L3-50 ultracentrifuge). The rough endoplasmic reticulum pellets formed were washed twice with iced phosphate buffer and stored for further analysis. The supernate and smooth endoplasmic reticulum phase together were centrifuged in a Spinco 50Ti rotor at $140\,000 \times g$ for 1 h. The smooth endoplasmic reticulum pellet was washed and stored. Supernates were pooled and represented the cytosol fraction. Pellets were submitted to electron micrography to monitor the purity of separation. No plasma membranes and very few mitochondrion and lysosomes were found in these pellets; however, the smooth endoplasmic reticulum pellet contained RNA indicating about 15% cross-contamination of rough endoplasmic reticulum on smooth endoplasmic reticulum. DNA was essentially absent, excluding nuclear or mitochondrial contamination. Distended vesicles suggesting increased activity were present in nephrotic rough endoplasmic reticulum micrographs.

Cholesterol and phospholipid were extracted from resuspended pellets with 10 vol. chloroform/methanol (2 : 1) followed by two additional extractions with 10 vol. of chloroform [17], and submitted to the same procedure described above. Cholesterol samples were measured by the enzymatic cholesterol oxidase method from Abbott laboratories. Triacylglycerol concentration in smooth endoplasmic reticulum, rough endoplasmic reticulum and cytosol were extremely low and were not included in this study.

Hydroxymethylglutaryl-CoA reductase assay. Non-fasted animals were killed at 10 a.m., and livers homogenized in 2.5 vol. of mercaptoethanol buffer as previously described [22]. After centrifuging twice at $12\,000 \times g$ for 15 min, the resulting supernate was centrifuged at $48\,000 \times g$ in the Spinco rotor 40 for 1 h to obtain the microsomal fraction. Samples were assayed for hydroxymethylglutaryl-CoA reductase activity [22], and their protein, phospholipid and cholesterol content measured as described above.

Results

Whole liver experiments were conducted in order to determine total liver water in the nephrotic as compared to normal controls and to distinguish hyperplasia from hypertrophy. Increased

TABLE I

CHARACTERIZATION OF LIVER HYPERTROPHY IN THE CHRONIC NEPHROTIC SYNDROME

Samples for these measurements were obtained from liver homogenates. Water content was measured as the fresh/dry weight ratio of liver samples. Figures in brackets indicate number of animals analyzed. Values represent mean ± S.D. n.s., not significant. Significance of differences between control and nephrotic groups was analysed by the Wilcoxon statistics.

	Control (6)	Nephrotic (6)	*P*
Fresh liver weight (g)	6.6 ± 0.6	8.8 ± 1.5	0.002
Protein (mg/whole liver)	782.9 ± 112.0	965.8 ± 134.1	0.013
Protein (mg/g of liver)	117.7 ± 9.5	109.0 ± 11.1	n.s.
DNA (mg/whole liver)	5.0 ± 1.3	5.3 ± 2.1	n.s.
RNA (mg/whole liver)	58.6 ± 6.7	90.2 ± 21.3	0.008
% water content/liver	71.3 ± 1.0	72.3 ± 1.0	n.s.

water content was ruled out as the cause for the heavier nephrotic livers. Hyperplasia was not involved since the DNA content was equal in both groups. However, total hepatic protein and RNA were higher in the nephrotic group, indicating the presence of hypertrophy. These results are shown on Table I.

Table II shows that, when whole liver was analyzed, cholesterol and phospholipid values were significantly increased in nephrotics. However, calculation of cholesterol and phospholipid concentrations per unit of liver weight or protein mass would abolish the difference between the experimental and control groups. The increased sterol mass occurred entirely as free cholesterol and, furthermore, cholesterol ester fraction was significantly lower in the nephrotic livers.

Hypertrophy associated with increased phospholipid and free cholesterol contents suggested that enhanced total liver sterol may have been due to membrane expansion. Thus, cell fractionation studies were undertaken to test this hypothesis.

We chose to work on rough and smooth endoplasmic reticulum for several reasons: (a) the increase of RNA but not of DNA indicated microsomal membrane expansion; (b) the former is the site of the heightened plasma protein synthesis, which is known to occur in nephrosis [2]; and (c) the microsomes contain hydroxymethylglutaryl-CoA reductase and thus constitute the site of regulation of cholesterol synthesis.

Our initial experiments done in nephrotic and control groups showed that more than 90% of all liver cholesterol, including that of smooth endoplasmic reticulum and rough endoplasmic reticulum samples, was in free form. Thus, for the final

TABLE II

LIVER CHOLESTEROL AND PHOSPHOLIPID ANALYSES IN NEPHROTIC (N) AND CONTROL (C) ANIMALS

For homogenates liver samples were homogenized in 5 vol. isotonic sucrose, extracted, and purified on silica-gel H TLC and cholesterol was analyzed by GLC. Losses were corrected by internal radioactive standards. In this experiment the animals studied were six controls and six nephrotics. For microsomes mitochondrial supernates were centrifuged at 48000 × *g* for 1 h. Microsomal pellets obtained were extracted, purified on silica-gel H TLC, and cholesterol was measured by the oxidase enzymatic method. Losses were corrected by internal radioactive standards. In this experiment the animals studied were seven controls and eight nephrotics. Values represent mean ± S.D. n.s., not significant.

	Cholesterol		*P*	Phospholipid		*P*
	C	N		C	N	
Homogenates						
mg/whole liver	22.7 ± 5.3	30.1 ± 4.7	0.0021	120.3 ± 29.9	161.2 ± 31.3	0.0021
mg/g of liver	3.1 ± 0.4	3.2 ± 0.4	n.s.	18.0 ± 3.3	18.3 ± 2.6	n.s.
Ester (mg/g liver)	0.53 ± 0.17	0.20 ± 0.06	0.0013			
Microsomes						
mg/total microsomal protein	0.68 ± 0.16	1.11 ± 0.34	0.0010	13.1 ± 6.8	19.4 ± 5.5	0.0010
μg/mg protein	38.3 ± 4.9	36.9 ± 5.5	n.s.	652.2 ± 158.3	650.2 ± 129.5	n.s.

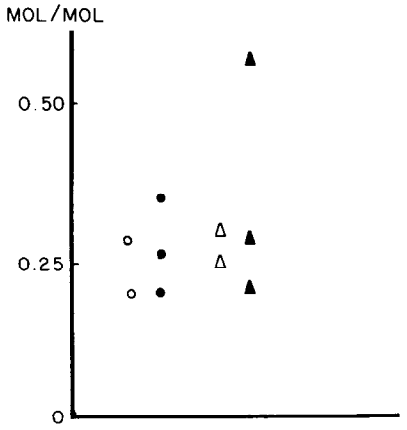

Fig. 1. Molar cholesterol/phospholipid fraction in endoplasmic reticulum membranes. Cholesterol and phospholipid values were obtained from rough and smooth endoplasmic reticulum samples isolated by discontinuous sucrose gradient ultracentrifugation. Molar fraction was calculated using cholesterol and phospholipid molecular weights of 386 and 760, respectively. Each value represents pooled liver endoplasmic reticulum from three animals. ○, smooth, control; ●, smooth, nephrotic; △, rough, control; ▲, rough, nephrotic.

part of the study only total cholesterol mass was determined. Again, total microsomal cholesterol and phospholipid mass was greater in nephrotics as compared to controls, but this difference disappeared when values were properly corrected for protein mass (see Table II). Molar cholesterol/phospholipid fractions measured in the smooth endoplasmic reticulum and rough endoplasmic reticulum membrane samples revealed that these were normal (see Fig. 1).

In order to substantiate this membrane expansion, and demonstrate that elevated cholesterol production may be achieved under conditions of increased hepatic cholesterol mass, hydroxymethylglutaryl-CoA reductase activity was measured in microsomal fractions. The enzymatic analyses obtained show a significant increase in the specific activity of the nephrotics (see Table III). Furthermore, when total hepatic hydroxymethylglutaryl-CoA reductase activity is taken into account, i.e., after correction for total microsomal protein content, values are considerably higher for the nephrotic animals.

TABLE III

3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE ACTIVITY IN CONTROL AND NEPHROTIC LIVER MICROSOMES

Figures in brackets indicate number of animals analyzed. Values represent mean ± S.D.

	Activity (pmol·min^{-1}·mg^{-1} protein)	Total liver activity (pmol·min^{-1})
Control (7)	11.5 ± 3.8	204.3 ± 90.3
Nephrotic (8)	16.8 ± 6.9	510.3 ± 253.5
P	0.060	0.003

Discussion

Our results demonstrate that cholesterol and phospholipid are increased in the livers of chronic nephrotic rats. Furthermore, this enlarged cholesterol store in free form has been, at least in part, located in the endoplasmic reticulum. Simultaneous enhanced cholesterol and phospholipid mass strongly suggests membrane expansion in the nephrotic liver and reflects the overall increase in the synthetic activity as a response to hypoalbuminemia [3]. In addition, hydroxymethylglutaryl-CoA reductase activity accompanies the nonspecific expansion in hepatic function, which could explain why nephrotic animals accumulate cholesterol in their bodies in spite of being fed a sterol-free diet [1]. In accordance, hepatic cholesterol ester level is actually reduced (see Table II).

Nevertheless, liver cholesterol synthesis is enhanced regardless of the elevated plasma low-density lipoprotein level seen in the nephrotic rat [1]. To this date, the presence of apolipoprotein B receptors in hepatocytes is questionable. Repeated experiments with hepatocyte monolayers [23], microsomal incubations [24] and liver perfusions [25,26] in the presence of low-density lipoprotein have failed to demonstrate uptake or feedback synthesis' inhibition of cholesterol, with the possible exception of apolipoprotein E carrying low-density lipoprotein [23,27]. Consequently, under circumstances of poor low-density lipoprotein uptake by the liver, in spite of the preserved feed-back regulation for cholesterol production by dietary cholesterol [8], the increased hydroxymethylglutaryl-CoA reductase activity persists in the nephrotic liver.

Finally, the finding of enlarged liver cholesterol mass, but at normal concentration, inherent to the microsomal membranes would explain why cholesterol and bile acid biliary output is not modified in the nephrotic syndrome [1], and further strengthens the notion of a functional intracellular compartmentation of newly synthesized cholesterol in the hepatocyte.

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