Differential Hepatic and Renal Cholesterol Levels in Diabetes-Prone BHE/cdb Rats Fed Menhaden Oil or Beef Tallow (44102)

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Abstract. **A series of experiments were conducted to determine whether the feeding of beef tallow compared with menhaden oil would affect renal cortex membrane composition, Na,K-ATPase activity, renal cholesterol uptake, and plasma lipoprotein cholesterol profile. BHE/cdb rats were used because they carry a genetic trait for non–insulin-dependent diabetes mellitus and are prone to develop diabetic nephropathy. Beef tallow feeding resulted in an increase in HDL cholesterol and an increase in Na,K-ATPase activity. The different fats also affected the arachidonic acid content of the membrane but not the membrane cholesterol content. These diet effects may explain why the development of renal disease in beef tallow–fed rats is delayed when compared with rats fed an equivalent amount of menhaden oil.** [P.S.E.B.M. 1997, Vol 214]

Animals of the BHE/cdb strain have a point mutation in the mitochondrial genome in the region of F_0ATPase 6,8 (1). These rats mimic humans with non–insulin-dependent diabetes mellitus (NIDDM) in that their impaired glucose tolerance and pancreatic β-cell insulin depletion is age and diet dependent (2–6). Among the many diabetic features of these rats is the development of diabetic nephropathy.

Earlier, we reported that BHE/cdb rats fed a 10% fat diet developed renal lesions that involved the glomerulus and which were characterized by a thickened mesangial membrane as well as other lesions characteristic of rodents that are prone to diabetic nephropathy. The age at which these lesions developed was dependent on the source of fat (5). Those rats that consumed a diet containing 1% corn oil plus 9% menhaden oil developed more severe lesions, sooner and died sooner than rats fed 1% corn oil and 9% beef tallow. A subsequent study (6), examining similarly fed rats at 250 days of age showed greater lesion development in the menhaden oil (MO) fed rats than in the beef tallow (BT) fed rats. This lesion development was accompanied by mild albuminuria, decreased creatinine clearance, and renal hypertrophy. Tissue and blood analysis revealed that the fatty acid profile of the blood, kidney, and liver was similar to that of the dietary fat. Further, those rats fed BT had more cholesterol in their renal tissues, yet their plasma and hepatic cholesterol levels were lower than those in rats fed MO.

Reyes *et al.* (7) reported that streptozotocin-diabetic rats, when fed a cholesterol enriched diet, had less severe renal lesions than those diabetic rats fed a control diet. Reyes *et al.* suggested that the dietary cholesterol ameliorated the development of diabetic nephropathy. Our studies seemed to suggest the same thing. Those rats fed BT had less severe renal lesions and lived longer than rats fed MO (5). The BT contains 10 times more cholesterol than MO. The results of our long-term studies thus suggested that the cholesterol in the BT might ameliorate the renal lesions in the same way as suggested by Reyes *et al.* (7). How this lesion development was ameliorated was not shown. Hence, the present work was conducted. We needed to know whether the source of dietary fat had its effects on plasma cholesterol transport and renal tissue uptake and whether increases in tissue uptake would result in functional changes in that tissue. Several short-term experiments were designed to test the following hypotheses: dietary beef

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tallow compared with menhaden oil (i) would affect the lipoprotein profile of the blood; (ii) would result in an increase in renal tissue cholesterol uptake; and (iii), due to its influence on membrane lipid composition, would affect the activity of the renal plasma membrane Na,K-ATPase, an important enzyme for the exchange of sodium and potassium.

Materials and Methods

Three experiments were conducted to collect data needed to support or deny the above hypotheses. In each, weanling animals were segregated into two groups. One group was fed a 1% corn oil–9% MO diet, while the other group was fed a 1% corn oil–9% BT diet. The composition of the diets was similar to those used previously (5, 6) and contained 0.018 mg cholesterol or 0.945 mg cholesterol/g MO or BT diet, respectively. Group size varied in each experiment to accommodate the variability associated with each of the analytical measurements. Extra vitamin E (0.04%) in addition to that provided by the AIN vitamin mix was provided in the diet to assure adequacy of intake. Because fat sources can vary, the fatty acid profile of the diet fat mixtures (corn oil plus MO or BT) was determined using gas chromatography (Table I). Great care was taken to minimize the auto-oxidation of the MO. The MO was added to the dry ingredients just before the animals were fed. Feeding commenced just before the onset of the dark period of the lighting cycle, fresh food was provided daily, and the MO was kept under a layer of nitrogen and stored at −20°C. Each experiment was of 8 weeks' duration. The animals were housed individually in hanging wire mesh cages in a room controlled for temperature (21° ± 1°C), humidity (40%–50%), and light (lights on 0600–1800 hr). The animals were cared for according to the standards of care put forth by the American Association for Laboratory Animal Care as described in the NIH Publication 88-23, *NIH Guide for the Care and Use of Laboratory Animals.* The rats were weighed weekly, and food intake (g food consumed over a 24-hr period) determined at the same time. At the end of eight weeks, the animals were sacrificed, and the appropriate tissues harvested.

The effects of dietary fat on plasma fatty acid profiles, renal plasma membrane fatty acid and cholesterol profiles, and the activity of the Na,K-ATPase were determined in Experiment 1. The plasma fatty acid profiles were determined in rats (6/group) that were anesthetized with 0.15 mg/100 g body wt sodium pentobarbital. Blood was collected by heart puncture using heparinized vacutainer tubes. Plasma was harvested after centrifugation in the cold (20 min, 0°–4°C, 3000*g*), and used for the determination of fatty acid profile using gas chromatography (courtesy of R. Etheridge, UGA Joint Nutrition Laboratory). For the other measurements, rats (12/group) were sacrificed by decapitation, and the kidneys were excised, chilled, and weighed, and the cortex carefully separated from the medulla. The plasma membranes were isolated using the ultracentrifugation techniques of Touster *et al.* (8). The kidneys of two animals were pooled to give an *n* of 6/treatment group. These membranes were then used for the determination of their fatty acid profile and for their Na,K-ATPase activity using methods previously described (9–11). ATPase activity was expressed in units. A unit is the amount of enzyme that catalyzes the release of one millimole

Table I. Fatty Acid Composition of the Diet, Plasma, and Kidney as Affected by Dietary Fat Source

Fatty acid[a] (mole %)	Diet		Plasma		Kidney cortex plasma membrane	
	BT	MO	BT	MO	BT	MO
12:0	—	—	0.4 ± 0.1[b]	2.0 ± 0.8[c]	0.7 ± 0.3	1.3 ± 0.5
14:0	2.3	8.5	1.6 ± 5	2.8 ± 0.5[c]	1.5 ± 0.2	1.7 ± 0.3
16:0	20.7	23.9	23.2 ± 0.4	26.2 ± 1.2[c]	24.3 ± 0.6	30.5 ± 0.4[c]
16:1	2.8	14.9	4.0 ± 1.1	3.7 ± 1.0	1.2 ± 0.3	1.74 ± 0.3
18:0	15.5	6.1	15.2 ± 1.2	16.7 ± 1.5	18.1 ± 1.2	21.7 ± 0.8[c]
18:1	46.5	21.5	38.7 ± 1.8	34.1 ± 2.9	24.4 ± 4	16.9 ± 1.4[c]
18:2 (n-6)	9.1	0.4	6.5 ± 0.6	7.7 ± 0.7	6.8 ± 0.5	7.1 ± 0.4
(n-3)	—	6.2	—	t	—	t
18:3 (n-3)	—	1.3	—	—	1.5 ± 0.3	1.8 ± 0.2
20:4	t	0.3	10.6 ± 1.0	3.7 ± 0.6[c]	18.4 ± 0.2	10.2 ± 0.6[c]
20:5 (n-3)	—	4.2	—	0.3 ± 0.1	—	t
22:6 (n-3)	—	7.3	—	3.7 ± 0.6	—	5.6 ± 1.0

[a] Fatty acids having less than 0.3% of the area are not shown. The BT diet had 99.42% of the total area matched to the standard fatty acid profile, while the MO diet had 99.57% of its fatty acids matched. The diet contained 1% corn oil (CO) and 9% beef tallow (BT) or menhaden oil (MO). t = trace.
[b] Mean ± SEM; $n = 6$.
[c] Diet effect is significant ($P < 0.05$)

of inorganic phosphorous (Pi) per hour corrected for the protein in the tissue homogenate.

The hypothesis that the fatty acid and cholesterol profile of the diet would change the lipoprotein profile of rats fed these diets was tested in Experiment 2. Seven rats per group were sacrificed by exsanguination *via* heart puncture. The blood was separated into the different lipoprotein fractions using ultracentrifugation techniques of Warnick and Albers (12). In this method, two lipoprotein-containing fractions are prepared, and their cholesterol concentrations are determined. The first, containing high-density lipoprotein (HDL) is prepared by precipitating apo B containing very low density lipoproteins (VLDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL), and chylomicrons with heparin and $MnCl_2 \cdot 4H_2O$. HDL cholesterol is quantified as the cholesterol remaining in the supernatant. The second fraction, containing LDL and HDL, is prepared by ultracentrifugation of plasma at d 1.006 g/ml. In this separation, VLDL Cholesterol = (Total Plasma Cholesterol) − (d 1.006 Infranatant Cholesterol) and LDL Cholesterol = (d 1.006 Infranatant Cholesterol) − (HDL Cholesterol). The plasma triglycerides were determined using the methods of Fletcher (13), while cholesterol content of each of the lipoprotein classes was determined enzymatically (Sigma kit #354; Sigma Chemical Co., St. Louis, MO). The liver and kidneys of these rats were used for cholesterol analysis after extraction with isopropanol.

Finding that the source of dietary fat affected the lipoprotein cholesterol profile, we then determined the uptake of cholesterol by liver and kidneys. A pulse of radiolabeled cholesterol (4 μCi [cholesteryl-1,2,6,7-^{3}H(N)]-linoleate/100 g body wt) was injected ip. The labeled cholesterol ester was obtained from New England Nuclear (Boston, MA) with a specific activity of 2701 GBq/mmol and was checked for purity using thin-layer chromatography. Thirty minutes later, the animals were anesthetized with 0.15 mg/100 g body wt sodium pentobarbital, blood withdrawn by heart puncture, and kidneys and liver excised, chilled, and weighed. The lipids were extracted using the Dole and Meinertz technique (14) and were saponified. The radiolabeled cholesterol, separated from the saponifiable fraction, was counted in a liquid scintillation counter. Results were expressed as Bq tritiated cholesterol/g tissue. The means for the two diet groups were compared for each of the different measurements using a Student's *t* test. Probabilities of less than 0.05 were considered significant.

Results

Diet had no effect on the food intakes of the animals in any of the experiments. Both groups of rats grew at a normal rate, and both consumed about the same amount of food (data not shown). In general, those rats fed the MO diet gained more weight than those fed the BT diet. This is consistent with previous reports (5, 6, 15) showing an increase in feed efficiency in rats fed MO. This is probably due to a diet fat effect on mitochondrial efficiency, as reported earlier (16). The fatty acid profiles of the diet, plasma, and the renal cortex plasma membranes are shown in Table I (Experiment 1). Less than 100% of the total fatty acids extracted from the plasma and kidney cortex membranes are shown. Omitted are those fatty acids that individually comprised less than 0.3% of the total. In the plasma, the MO rats had more 12:0, 14:0, and 16:0, and less 20:4 fatty acids than did the BT rats. In addition, the MO rats had measurable amounts of 22:4, while the BT rats had none of this fatty acid in either their blood plasma or their renal membranes. In the renal cortex membranes, rats fed MO had more 16:0 and 18:0, and less 18:1 and 20:4 fatty acids than rats fed BT.

Table II. Plasma Lipoprotein Profiles in Fasting BHE/cdb Rats Fed Either a Beef Tallow or Menhaden Oil Diet

	Diet	
	Beef tallow	Menhaden oil
Triglycerides (m*M*)	0.59 ± 0.01[a]	0.47 ± 0.01[b]
Cholesterol (m*M*)	1.17 ± 0.05	1.01 ± 0.05[b]
HDL (m*M*)	0.58 ± 0.03	0.31 ± 0.05[b]
LDL (m*M*)	0.09 ± 0.02	0.09 ± 0.01
VLDL (m*M*)	0.50 ± 0.08	0.62 ± 0.09

[a] Mean ± SEM; $n = 7$.
[b] Diet effect is significant ($P > 0.05$).

The source of the dietary fat affected the lipoprotein profiles of these rats (Table II). Rats fed BT had higher levels of triglycerides and cholesterol than rats fed the MO diet (Table II). The BT rats had more HDL than did the rats fed the MO diet. The density of the VLDL fraction was less than 1.006 g/ml^{-1}, while LDL density ranged from 1.006 to 1.063, and the HDL density ranged from 1.063 to 1.210 g/ml^{-1}. When hepatic tissues were compared, the BT rats had 53.6 ± 2.3 mg cholesterol/g liver, whereas the MO rats had 95.1 ± 4.7 mg/g liver (Table III). This difference was significant. The difference in hepatic lipid values probably related to decreased hepatic lipid output that characterizes the MO fed rat (17, 18). The source of fat in the diet affected Na,K-ATPase activity (Fig. 1). Rats fed the BT diet had significantly ($P < 0.05$) higher enzyme activity (5.84 ± 0.41 units/mg protein) than rats fed the MO diet (4.75 ± 0.40 units/mg protein). No difference in renal cortex plasma membrane cholesterol were found. The BT rats had 24.0 ± 0.7 mg/100 g membrane, whereas the MO rats had 23.5 ± 1.3 mg/100 g membrane (Table III). No differences due to diet were observed in the total

Table III. Influence of Dietary Fat on Total and Radiolabeled Cholesterol in Hepatic and Renal Tissues of BHE/cdb Rats

	Beef tallow	Menhaden oil
Body weight (g)	319 ± 8[a]	352 ± 15[b]
Liver weight (g)	12.3 ± 0.5	15.4 ± 0.9[b]
Hepatic cholesterol (mmoles/g)	139 ± 6	246 ± 12[b]
Hepatic ^{3}H cholesterol (Bq/g)	374 ± 1 × 10^7	134 ± 2 × 10^7
Renal weight (g)	3.47 ± 0.20	3.88 ± 0.21
Renal cholesterol (mmoles/g)	216 ± 6	206 ± 4
Renal ^{3}H cholesterol (Bq/g)	67 ± 0.6 × 10^7	55 ± 0.5 × $10^{7\,b}$
Renal plasma membrane cholesterol (mg/100 g membrane)	24.0 ± 0.7	23.5 ± 1.3

[a] Mean ± SEM; $n = 5$.
[b] Significant diet differences ($P < 0.05$).

cholesterol content of the renal tissue. Lastly, the rats fed the BT diet had more labeled cholesterol in their hepatic and renal tissue compared with the MO rats. The diet-induced difference in the renal tissue radiolabeled cholesterol was far smaller than that observed in the hepatic tissue.

Discussion

This work focused on three hypotheses, all of which were conceived as a way of understanding why diabetic nephropathy develops more rapidly in the NIDDM-prone BHE/cdb rat fed MO than in that fed BT. The first hypothesis was that the type of diet fat would affect the lipoprotein profile with respect to the cholesterol carried in each of the major lipid carrying classes. In the human population, diet can affect this profile; however, Kris-Etherton *et al.* (19) have reported that in rats the degree of fatty acid saturation is without effect on the lipoprotein profile. However, in that study only vegetable fats were used. In the present study, fats of animal origin were used, and there were not only differences in fatty acid saturation but also a difference in cholesterol

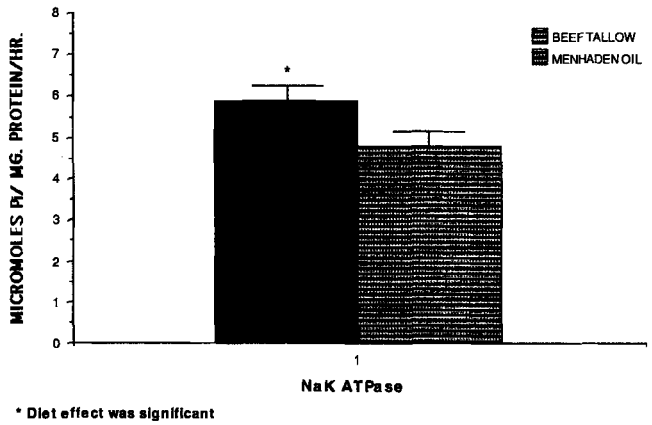

Figure 1. Effects of dietary fat on the activity of the renal cortex plasma membrane Na,K-ATPase.

content: BT contained more cholesterol than did MO. The cholesterol content of the diet most likely explains the difference in HDL reported in Table II. Thus, as has been reported many times in the human studies literature, the lipoprotein profile was affected. The HDL increase in the BT rats probably was related to an increase in blood lipid clearance that was needed by these rats. The MO rats likely had an accumulation of lipid in the liver due to a decreased lipid output, which in turn would have affected their HDL levels.

BHE/cdb rats, like humans with NIDDM, show an age-related increase in serum cholesterol (2, 5) and like humans also develop the secondary complication of NIDDM, glomerulosclerosis. Is this glomerulosclerosis and impaired renal function a response to hypercholesterolemia? Reyes *et al.* (7) have reported that streptozotocin-diabetic rats fed a cholesterol enriched diet had less severe renal tubule lesions. In our earlier study (5) and in a subsequent one (6), we also showed that rats fed BT had less severe lesions at each age than rats fed MO.

Was this due to a diet fat effect on renal cholesterol uptake? In the present work, we tested this hypothesis using labeled cholesterol. We found that there were no diet-related differences in renal cholesterol level or renal plasma membrane cholesterol levels, but there was a small increase in radiolabeled cholesterol. There might have been an increase in cholesterol turnover in the renal tissue (note a significant diet effect on radiolabeled cholesterol), which might be related to the observed increase in HDL. But, is this related to the increase in the Na,K-ATpase activity found in the BT rats? Probably not. The greater ATPase activity in the BT rats could explain our previously reported diet differences in renal electrolyte exchange (6), but the difference in ATPase activity was not related to differences in the cholesterol content alone. Likely, both the cholesterol and the fatty acid profile contributed to the difference in ATPase activity.

Many membrane-embedded enzymes have their activity modified by the lipid milieu in which they are

embedded. These enzymes are those whose activity depends on their mobility within the membrane structure (20–22). The ATPase is one of these. The Na,K-ATPase is an important component in the energy-driven exchange of the sodium and potassium ions, and, as such, helps maintain electrolyte balance. In other tissues, notably the liver, ATPase activity is affected by the lipid composition of the membrane that surrounds it. While the amount of cholesterol in the renal tissue and the plasma membrane did not differ between the diet groups, the fatty acid profile did. The BT rats had less 16:0 and 18:0, and more 18:1 and 20:4 fatty acids in their renal cortex membranes than did the MO rats. This meant that these membranes had more double bonds and thus were more unsaturated (with respect to the fatty acid ratio), even allowing for the fact that the membranes from the MO rats had a small but measurable amount of 22:6. The membranes from the BT rats were probably more fluid. Fluidity is determined not only by the ratio of unsaturated to saturated fatty acids but also by the ratio of the fatty acids to cholesterol (21, 22). Since the cholesterol in the membranes was equivalent, the differences in the ratios of the fatty acids could have been the determining factor with respect to ATPase activity. Regional differences in fluidity (especially around the ATPase) could explain the diet effects on ATPase activity, and the difference in ATPase activity could explain the previously reported (6) diet differences in electrolytes in the blood and urine. How the maintenance of this electrolyte exchange pertains to the pathophysiology of renal disease is far from clear. Nonetheless, the previous and present results do suggest that electrolyte exchange is more active in the BT rats than in the MO rats, and these results relate to our earlier report (6) of differences in urine and blood sodium and potassium levels in rats that had early renal lesions. Those with lesions (the MO rats) were less able to regulate their electrolyte exchange than those animals without lesions (the BT rats).

It is also pertinent to point out that the tissue fatty acid profiles are quite different from those of the diet. It suggests that in these rats there is a very active fatty acid remodeling process that works to provide an "optimal" fatty acid composition. Chain elongation as well as desaturation has occurred, and these processes are influenced not only by the diet but also by the hormonal status of the animal (22–24). We have reported previously on some of these adaptations in the BHE/cdb rat (6, 23, 24).

There may be another explanation for these results having nothing to do with the dynamics of cholesterol in the kidney. This explanation relates to our earlier report of diet fat effects on renal peroxide levels (15). Rats fed MO have a greater potential to form highly reactive free radicals, which can target membranes and affect their function. If this has occurred, then one might anticipate a decline due to free radical–induced damage to the plasma membrane and the ATPase, with the result of less enzyme activity. Indeed, following this line of reasoning one might anticipate an increase in membrane leakiness, which would then explain the previously reported differences in electrolyte exchange. In any event, clearly there is a diet-fat effect on lipoprotein profile, renal cholesterol turnover, and renal ATPase activity that seems to be related to a diet fat effect on the progression of diabetic nephropathy in NIDDM-prone BHE/cdb rats.

In summary, NIDDM-prone BHE/cdb rats responded differently to dietary menhaden oil than to dietary beef tallow with respect to cholesterol metabolism by the liver and kidney. This diet-related difference was not due to diet-induced changes in renal cholesterol levels but was probably due to other attributes of the dietary fat that had both direct and indirect effects on the renal tissue, which in turn affected the previously reported time course for the development of diabetic nephropathy.

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