Annu. Rev. Nutr. 1991. 11:265–83

DIETARY COPPER: Cholesterol and Lipoprotein Metabolism

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KEY WORDS: cholesterol synthesis and degradation, lipoprotein composition, high density lipoproteins, apolipoproteins, plasma pool size

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0199-9885/91/0715-0265$02.00

INTRODUCTION

In 1973, Klevay (23) demonstrated that an increased dietary zinc-to-copper (Cu) ratio may lead to hypercholesterolemia in rats. He subsequently hypothesized that a relative or an absolute deficiency of Cu, characterized by a high ratio of dietary zinc to Cu, results in hypercholesterolemia and is a major factor in the etiology of coronary heart disease (24). Thereafter, numerous studies have been performed, mainly in rats, because of the ease and rapidity in inducing Cu deficiency in this animal model. Hence, this review is based mostly on data derived from the rat. Results from 12 independent laboratories (summarized in Ref. 35) have firmly established that Cu deficiency induces hypercholesterolemia in rats. Most of these studies reported increases of 30 to 40%, but increases as high as 185% have been reported for plasma cholesterol levels in Cu-deficient (CD) rats. In addition, studies performed in other species, including humans and nonhuman primates, suggest that hypercholesterolemia induced by Cu deficiency may eventually be established as a universal phenomena regardless of species. Although the rat may be useful for studying the influence of Cu deficiency on certain aspects of cholesterol and HDL metabolism, a more suitable animal model needs to be established to examine the effect of Cu deficiency on LDL metabolism. Unlike a human, who has a high level of LDL protein and carries a substantial amount of plasma cholesterol in LDL, the rat is a high density lipoprotein (HDL) animal that has a high level of HDL protein and carries about 60% of the plasma cholesterol in HDL. Thus, care must be exercised in the extrapolation of data derived from the rat or other animal models to humans. Ideally, alterations in lipoprotein metabolism observed in CD animal models will have to be substantiated in humans.

TISSUE CHOLESTEROL LEVELS AND PLASMA LIPOPROTEIN COMPOSITION

Plasma and Tissue Cholesterol Concentrations

Apart from the established increase in plasma total cholesterol level (35), increases in both free (39–57%) and esterified (34–58%) cholesterol levels have been observed (17, 34, 48). The free-to-ester cholesterol ratio appeared to remain relatively constant, within individual studies, and did not indicate a disproportional increase in either of the cholesterol components. Since plasma volume is increased by Cu deficiency, plasma cholesterol pool size was found to increase twofold when plasma volume enlargement was taken into consideration (8, 9). Increases in plasma volume as well as in plasma cholesterol pool size were detected prior to the actual increase in plasma concentration in

a recent study designed to examine the developmental sequence of hypercholesterolemia and anemia in CD rats (4).

A small reduction in hepatic total cholesterol concentration (11–26%) in CD rats has been repeatedly observed in our laboratory (8, 9, 19, 20, 34, 48) and confirmed by other researchers (18, 31, 42). Reductions in both hepatic free (14%) and esterified (21–29%) cholesterol concentrations, as a result of Cu deficiency, have been demonstrated by us (34) and others (17, 31). A relatively constant ratio of free-to-ester cholesterol, indicative of no disproportional reduction in either component, was observed within individual studies. Unlike the liver and plasma cholesterol, no consistent change in the cholesterol concentrations of kidney (19, 20) heart (19, 20), and aorta (22) of CD rats was found.

Plasma Lipoprotein Composition

A detailed review of plasma lipoproteins and apolipoproteins in Cu deficiency has been published recently by Lei & Carr (36). Using precipitation methods, Allen & Klevay (3) and Lei & Lin (39) respectively indicated that plasma high density lipoprotein (HDL) cholesterol levels were markedly elevated by 85% and 33% in CD rats. In contrast, a 13 to 17% reduction was later reported by Harvey & Allen (18). However, all subsequent studies, using ultracentrifugation and gel permeation column chromatography to partition lipoprotein fractions (46), have consistently demonstrated increases in HDL cholesterol levels in the CD rat model (5, 8, 11, 19, 20, 32, 37). This method was selected by our laboratory because it is gentler than sequential ultracentrifugation, which subjects lipoproteins to high gravitational force for prolonged periods and may lead to loss of apolipoproteins (13). In addition, cross-contamination between lipoprotein fractions, as judged by apolipoprotein composition and the absence of albumin, is much smaller than that arising from gradient or sequential ultracentrifugation.

An extensive study on the influence of Cu deficiency on plasma lipoprotein concentration, composition, and pool size was recently reported by Al-Othman & Lei (5). Increases in the concentration of HDL cholesterol, triglycerides, phospholipids, and protein were observed in the CD rats (Table 1). The concentration of protein was not altered, but elevations in the concentration of cholesterol, triglycerides, and phospholipids were observed in the low density lipoprotein (LDL) in CD rats. However, changes in the concentration of very low density lipoprotein (VLDL) components were more diverse. An increase in triglyceride, an unaltered protein, as well as reductions in cholesterol and phospholipid concentrations of the VLDL fraction were observed in Cu deficiency. Similar increases in the lipid and protein levels of LDL and HDL have been reported (8, 9, 11, 30, 32, 37) by numerous

Table 1 The influence of copper deficiency on the concentration (mg/dl) of plasma lipoprotein components in rats

	Control	Cu-deficient	
	Concentration (mg/dl)		Change %
HDL			
Cholesterol	44.1	66.8	+52
Triglycerides	16.0	27.4	+71
Phospholipids	94.5	126.8	+34
Protein	91.8	118.4	+29
LDL			
Cholesterol	24.2	47.9	+98
Triglycerides	7.1	23.6	+332
Phospholipids	6.2	14.8	+139
Protein	6.5	7.2	NS
VLDL			
Cholesterol	6.82	3.04	−55
Triglycerides	7.45	36.80	+394
Phospholipids	7.25	3.55	−51
Protein	3.59	3.20	NS[a]

[a] NS = nonsignificant; $n = 5$.

investigators. A reduction in hematocrit was consistently observed in our CD rat model. Subsequently, an enlargement of plasma volume by 55% was established by Evan's blue dye dilution method (37) as well as by isotope dilution method (8). Thus the increases in lipoprotein levels observed were magnified when expressed in terms of pool size (5). The plasma pool size of cholesterol, triglycerides, phospholipids, and protein of LDL and HDL was increased two- or more fold by Cu deficiency (5). A sixfold increase in triglyceride, a 36% reduction in cholesterol, and no change in phospholipid and protein pool size were observed in VLDL of CD rats (5). A twofold increase in plasma HDL cholesterol pool size was also established in a previous study when plasma volume enlargement was taken into consideration (8, 9). Given that the increases in HDL protein and lipid components are relatively uniform, the HDL data indicate that the HDL particle number, but not particle size, may be increased. In the CD rats, the plasma pool sizes of LDL protein, cholesterol, phospholipids, and triglycerides increased 1.7, 2.8, 3.6, and 4.9-fold, respectively. Therefore, while the increase in LDL protein pool size indicates an increase in particle number, the disproportionate increase in lipids suggests that particles may be ladened with lipids, thus resulting in an enlarged particle size. Since the plasma pool size of VLDL

triglycerides was increased almost sixfold, whereas the protein and phospholipids remained unaltered by Cu deficiency, the VLDL particle number appears to be unchanged but the particle size may be enlarged. The markedly elevated triglyceride contents of VLDL and LDL may have resulted from a reduction in lipoprotein lipase (LPL) activity. Indeed, a depressed post-heparin LPL activity has been reported in CD rats (26, 29). Alternatively, an increased production of VLDL may have contributed to the observed changes in VLDL and LDL.

The influence of dietary Cu status on the apolipoprotein profiles of the total HDL and HDL subfractions has been examined by three research groups (11, 30, 31, 37). Apolipoproteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27, 53), stained with Coomassie blue, scanned densitometrically, and quantitated with an integrator. Differences in chromogenicity of stained apoprotein bands were not corrected. Increases in apolipoprotein E (apo E) (11, 30, 31) and in apolipoprotein A-I (apo A-I) (30, 31) were observed in the total HDL fraction of CD rats. Among the three research groups, the overall apolipoprotein profile appeared to be similar in terms of the direction and magnitude of changes induced by Cu deficiency. Heparin-Sepharose affinity column chromatography was used to partition HDL into apo E–rich and apo E–poor subfractions. Croswell & Lei (11) reported that the concentrations of cholesterol and protein for the apo E–rich HDL were elevated more than twofold by Cu deficiency and accounted for most of the increases observed in the total HDL fraction. Data from the study of Lefevre et al (31) also confirmed that the apo E–rich HDL fraction was increased in CD rats. Furthermore, their nondenaturing gradient gel data indicated that the apo E–rich HDL represented HDL_1 and was selectively increased by Cu deficiency. In contrast, Lee & Koo (30) indicated that cholesterol content was not elevated in their apo E–rich and very rich subfractions but was markedly increased in the apo E–free subfraction, which accounted for 72% of the increase in cholesterol content of the entire HDL fraction in CD rats.

In the study of Croswell & Lei (11), apo E and apo A-I concentrations were elevated 2.4 to 2.6-fold only in the apo E–rich HDL of CD rats. This marked increase in apo E accounted for most of the increase in the entire HDL fraction. When the apolipoprotein data from the study of Lefevre et al (32) were expressed as mg/dl, the magnitude of the increase in apo E and protein content of the apo E–rich subfraction, as a result of Cu deficiency, appeared to be in close agreement with the results reported by Croswell & Lei (11). In contrast, Lee & Koo (30) reported an increase of apo A-I content (twofold) only in the apo E–free HDL from CD rats. An increase in apo E content of the apo E–rich subfraction in CD rats was also reported, but the actual amount of apo E was three- and sixteenfold lower in their CD and Cu-adequate rats,

respectively, than the amount of apo E reported in other studies (11, 32). The observed differences in the distribution of cholesterol and apolipoproteins among the HDL subfractions may have resulted from the use of 1% cholesterol diets by Lee & Koo (30), and diets with no added cholesterol were used by Lei and coworkers (37) and by Lefevre et al (31, 32).

Alterations in plasma lipoprotein profile as a result of Cu deficiency have been established in humans and rabbits. Klevay et al (25) reported a 32% increase in plasma total cholesterol level (from 202 to 234 mg/100 ml) in a young male subject after 15 weeks of Cu depletion with a diet providing 0.8 mg Cu/day. An increase in LDL cholesterol was mentioned, but the magnitude of the increment was not given. After 39 days of repletion with 2 mg of Cu given twice daily, Cu status promptly reverted toward the normal range and plasma total cholesterol returned to normal level (198 mg/100 ml), but the fall of LDL cholesterol did not reach the control value. In another study, total plasma cholesterol level remained constant, but a 14% increase in LDL cholesterol and a 20% reduction in HDL cholesterol level were observed in 24 male subjects following 11 weeks of depletion with a diet supplying 1.02 mg Cu per day (44). In addition, twofold elevations in LDL protein, cholesterol, triglyceride, and apo B concentrations were observed in young rabbits made Cu deficient by 10 days of 2, 3, 2 tetramine HCl(TETA) treatment while receiving a marginal Cu diet (2.3 mg Cu/kg). Plasma LDL protein level was found to remain elevated, and plasma Cu level was still reduced 45% in rabbits treated with TETA for 12 days and maintained for an additional 10 weeks on the same marginal diet (21). If one compares humans and rabbits with rats, differences in the distribution of the elevated plasma cholesterol among HDL and LDL as a result of Cu deficiency may be explained by the apolipoprotein synthetic capacity of the animal model.

CHOLESTEROL SYNTHESIS

Several lines of evidence from in vivo and in vitro studies indicate that an increase in hepatic cholesterol synthesis leads to an increased net efflux of cholesterol from liver to plasma, followed by the hypercholesterolemia observed in Cu deficiency.

Hepatic Cholesterogenesis and Lipogenesis from [^{14}C]acetate

The influence of Cu deficiency on the rates of cholesterol and fatty acid synthesis was examined in an in vitro incubation system using freshly prepared liver slices (33). A factorial design consisting of 4 treatments, with 2 levels of dietary Cu (<2 and 18 mg/kg) and 2 levels of dietary cholesterol (0 and 20 g of cholesterol per kg) was used. Weanling male Sprague-Dawley rats were fed their respective diets ad libitum for 9 weeks. Thereafter, liver slices

prepared by a microtome were incubated for 3 h at 37°C in 3 ml Krebs-Ringer bicarbonate buffer containing per ml: 0.2 μCi [1-^{14}C]acetate, 10 μmol acetate, 5 μmol glucose, and 0.1 U porcine insulin. Incorporation of [1-^{14}C]acetate into fatty acids and into digitonin precipitable sterols (DPS) was used to represent the capacity of tissues to synthesize fatty acids and cholesterol, respectively. Cardiac hypertrophy, growth depression, reductions in plasma and hepatic Cu levels, as well as reductions in hepatic lipid and cholesterol levels were observed in rats fed CD diet. When the data were expressed as synthetic rate per 100 mg liver tissue (nanomoles of [^{14}C]acetate incorporated into DPS and fatty acids per 100 mg wet liver tissue per 3 h), a significant increase in the rate of synthesis of fatty acids but not of cholesterol was observed in CD rats, regardless of cholesterol supplementation. Since marked differences in body and liver weights existed between treatments, the synthetic rates were recalculated as nanomoles of [^{14}C]acetate incorporated per liver organ per 100 g body weight. When the synthetic rates were corrected for organ and body weight differences, a small but significant increase in cholesterol synthesis and a twofold increase in fatty acid synthesis were established in CD rats not supplemented with cholesterol. Thus, the liver of CD rats appeared to have an increased synthetic capacity for providing lipids to sustain and enhance lipoprotein production.

Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

Recently, Yount et al (54) examined the influence of Cu deficiency on hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity in rats. This highly regulated enzyme catalyzes the formation of mevalonate from HMG CoA, which is the rate-limiting reaction in cholesterol biosynthesis (45). The enzyme activity is down regulated by cholesterol feeding (45) but is enhanced to replace bile acids depleted by cholestyramine (7).

Male weanling Sprague-Dawley rats were fed either a semipurified CD diet with 0.9 μg Cu/g or a Cu-adequate diet with 6.5 μg Cu/g for 7 weeks. Consistent with previous findings observed in CD rats, hypercholesterolemia and reductions in hepatic cholesterol and Cu levels were detected at the end of the study. Activity of HMG CoA reductase was assayed by the method of Shapiro et al (49). In the liver of adequate rats, the active form of HMG CoA reductase accounted for 30% of total activity in the nonfasted state. Both total and active forms of the hepatic reductase were elevated about twofold in the nonfasted CD rats. Increases in reductase activity imply that the hepatic cholesterogenesis capacity is enhanced by Cu deficiency, which corresponds well with the expected induction response to the reduction in hepatic cholesterol concentration found in CD rats. These findings support the hypothesis that the hypercholesterolemia and hyperlipoproteinemia associated with Cu deficiency result from increased cholesterol and lipoprotein production.

Cholesterogenesis from [^{14}C]mevalonate

The influence of Cu deficiency on in vivo cholesterogenesis was determined by using [^{14}C]mevalonate, a more immediate precursor of cholesterol than acetate (48). Because of the extremely small tissue concentration of mevalonate, an estimate of this precursor pool is often not possible. Hence, the mevalonate pool was assumed to remain constant between treatments in this study. Weanling male Sprague-Dawley rats were fed either a CD diet containing 2 mg Cu and 120 mg Zn/kg or a Cu-adequate diet supplemented with Cu to 18 mg/kg for eight weeks. Five fasted rats from each treatment were killed at 40, 80, and 120 min after injection of [2-^{14}C]mevalonate (0.5 μCi/100 g body weight, 46 μCi/μmol) via the femoral vein. Lipids from serum and tissue samples were extracted with chloroform-methanol (2/1; vol/vol) and partitioned by thin layer chromatography (TLC).

Among the two treatments, no difference in the serum and liver-free cholesterol specific activity (SA) response curves was detected. The serum SA curves appeared to increase rapidly from 0 to 40 min, gradually slow down from 40 to 120 min, and approach a peak around 120 min. In contrast, the hepatic SA curve seemed to have peaked prior to 40 min and then declined progressively thereafter. Although no difference in SA curves was detected, the changes in tissue-free cholesterol level observed in other studies (31, 34) as well as the present study indicate that the metabolism of free cholesterol may also be altered by Cu deficiency.

In the CD rats, the serum cholesteryl ester SA response curve was consistently elevated and paralleled the gradual increase observed for the Cu-adequate rats. In addition, the liver cholesteryl ester SA of CD rats appeared to have peaked at or before 40 min, but that of Cu-adequate rats was increasing progressively throughout the study and attained the same peak value of CD rats only after 120 min. These data suggest that the turnover rate of liver cholesteryl ester pool may be accelerated in Cu deficiency. Furthermore, cholesteryl ester newly synthesized by liver and extra hepatic tissues may be entering the vascular pool at an increased rate and contributing to the consistently elevated serum cholesteryl ester SA response curve.

In a similar study, Allen & Klevay (1) reported a reduction of both plasma and liver cholesteryl ester SA in CD rats at 4 h after a subcutaneous injection of 3 μCi of [5-^{3}H]mevalonate per 100 g body weight. Their control rats demonstrated increases in weight gain and feed efficiency even though the feed intake was limited by pair feeding. When feed intake is restricted in meal-fed rats, enhanced efficiency in nutrient absorption and utilization as well as hepatic cholesterogenesis have been established (12, 40). Thus, pair-fed controls may have adapted to a meal-feeding pattern in response to feed restriction that results in enhanced cholesterogenesis. This may account

for the elevated liver and serum cholesteryl ester SA observed in the pair-fed Cu-adequate rats.

Cholesterogenesis from [^{3}H]water

Cholesterol synthesis was recently estimated by determining the rate of [^{3}H]water incorporation into DPS (55). A semipurified diet, with or without added Cu (0.62 or 7.13 μg/g diet), was fed to weanling male Sprague-Dawley rats for five weeks. Thereafter, [^{3}H]water incorporation in DPS present within various organs was determined in tissues excised 1 h after injection of labeled substrates. Since this approach does not account for newly synthesized sterols secreted from the tissues, plasma DPS represent newly synthesized cholesterol cleared into the plasma pool in 1 h. A twofold increase in the rate of accumulation of [^{3}H]DPS was observed in the plasma of CD rats, which implies an increase flux of newly synthesized cholesterol into the plasma pool. However, the amount of [^{3}H]DPS present in the kidney, small intestine, and liver were not significantly altered by Cu deficiency. Nevertheless, the amount of [^{3}H]DPS present in the carcass (including muscle but not the other organs sampled) was elevated 1.9 fold in CD rats. Although the amount of [^{3}H]DPS present in the liver was not elevated at 1 h post injection, hepatic sterol synthesis may be markedly elevated in CD rats for the following reasons. Previous studies have shown a strong correlation between [^{3}H]DPS levels in the liver and blood of rats and other species (50). When animals are fed increasing amounts of cholesterol, which reduces hepatic cholesterol synthesis to a minimal level, a reciprocal decline in [^{3}H]DPS is found in blood. Unlike the liver, other organs such as the intestine, skin, and carcass demonstrate little correlation between organ and blood [^{3}H]DPS content (50). Thus, data derived from studies on cholesterol synthesis suggest that Cu deficiency induced increases in cholesterol synthesis and clearance into the plasma. In addition, these results support the hypothesis that the elevated level of hepatic HMG CoA reductase observed in Cu deficiency is associated with an increased rate of hepatic cholesterol synthesis.

CHOLESTEROL ABSORPTION AND DEGRADATION

A number of studies have been performed to examine the influence of Cu status on the processes of cholesterol absorption and degradation. Although small treatment differences were detected, available data do not appear to support the contention that these alterations may contribute significantly to the observed hypercholesterolemia.

Lymphatic Absorption of Cholesterol

Koo et al (26) collected lymph from cannulated major mesenteric lymph ducts, following an intraduodenal load of 10 mg of [4-^{14}C]cholesterol, and

demonstrated that cholesterol absorption was slightly reduced in CD rats. A general reduction in absorption capacity, as a result of partial intestinal villi atrophy (14) and impairment of energy-requiring processes involved in chylomicron synthesis and secretion, was postulated as being responsible for the reduction in cholesterol absorption. In addition, a small transient increase in the plasma clearance rate of chylomicrons, labeled with [^{3}H]retinol, was observed in CD rats (26). Since the half-life of [^{3}H]chylomicrons and total ^{3}H-radioactivity taken up by the liver were not influenced by Cu deficiency, the small reduction in intestinal cholesterol absorption may have little or no biological significance.

Biliary Steroid Excretion

An impairment of biliary excretion of bile acids and cholesterol, the major pathway for cholesterol elimination, may contribute to the hypercholesterolemia observed in Cu deficiency. The reaction most likely to be affected by Cu deficiency is the hydroxylation of the steroid nucleus in bile acid synthesis, since the mixed-function oxygenase (mono oxygenase) involved shares certain similarities to other mono oxygenases requiring Cu (52). Thus, Allen & Klevay (2) quantitated bile acid and cholesterol collected from biliary cannulae to determine whether biliary steroid excretion is compromised by Cu deficiency. Male weanling Sprague-Dawley rats either were fed a CD diet (0.57 μg Cu/g) or were pair fed an adequate diet (5.57 μg Cu/g) for at least 36 days. Biliary excretions were collected at 0 to 8, 8 to 24, 24 to 48, and 48 to 72 h after bile duct cannulation. Total biliary cholesterol was determined with cholesterol esterase and oxidase. Ether extracts of the saponifiable fraction derived from bile samples were used for the determination of total bile acid with 3α-hydroxysteroid dehydrogenase (51).

During the first 24 h of collection, total biliary cholesterol and bile acid excretion as well as bile volume, for both treatments, tended to be lower than during later periods. A steady state was attained by the second and third day for both treatments. In the controls, these parameters were lower during the first day and were comparable to those of the CD rats by the second and third days. The initial depression appeared to reflect smaller volume of bile produced and may have resulted from a more intensive surgical trauma suffered by the controls. Alternatively, the transient increase in bile acids collected from CD rats may indicate the presence of an enlarged enterohepatic pool as a result of Cu deficiency. Nevertheless, subsequent collections on days 2 and 3 may be more meaningful because they represent steady-state production to replace the diverted bile acids. Since the steady state biliary steroid excretion was not influenced by Cu deficiency, cholesterol from the enlarged plasma cholesterol pool appeared to be unavailable for bile acid production and elimination in order to normalize the observed hypercholesterolemia.

Oxidation, Excretion, and Tissue Distribution of [26-^{14}C]cholesterol

In the CD rat model, Lei (34) labeled tissue cholesterol with [26-^{14}C]cholesterol and determined exhaled $^{14}CO_2$ as a measure of cholesterol oxidation and bile acid formation, since the three-carbon fragment of the cholesterol side chain is mainly oxidized to CO_2. Four dietary treatments in a factorial arrangement with two levels of Cu (<2 and 18 mg/kg of diet) and two levels of cholesterol (0 and 20 g/kg of diet) were used in the study. Following eight weeks of treatment, 2 μCi of [26-^{14}C]cholesterol per 300 g body weight were injected intraperitoneally into the eleven-week-old male Sprague Dawley rats. Ten days later, respiratory $^{14}CO_2$ was collected for 20 min and used as a measure of cholesterol catabolism. On days 7 to 11 postinjection, feces were collected to determine [26-^{14}C]cholesterol excretion as fecal lipids. Cholesterol oxidation and excretion rates were derived respectively from the serum total cholesterol SA and from the amounts of radioactivity exhaled in respiratory CO_2 or excreted in fecal lipids.

In rats not supplemented with cholesterol, elevations in the concentration of serum-free and ester cholesterol (39%, 34%) and reductions in SA (14%, 9%) of serum-free and ester cholesterol were observed as a result of Cu deficiency. These alterations may have resulted from an increased pool size. In addition, reductions in liver-free cholesterol level (14%) as well as in cholesterol specific activity (24%) were also observed in CD rats not supplemented with cholesterol. Thus, Cu deficiency may have induced an accelerated turnover and efflux of liver cholesterol that contributed to the observed hypercholesterolemia. In both cholesterol-supplemented CD and Cu-adequate rats, the hepatic cholesteryl ester was markedly elevated (elevenfold) and its SA depressed (46%) when compared to that of CD rats receiving no cholesterol. In addition, cholesterol feeding also increased the concentration and reduced the SA of serum-free and esterified cholesterol. Furthermore, cholesterol supplementation appeared to have partially or completely masked the observed changes in serum and liver cholesterol parameters induced by Cu deficiency alone.

As expected, cholesterol supplementation markedly increased the rates of cholesterol oxidation (3.25-fold) and excretion (1.9-fold) in both CD and Cu-adequate rats. In contrast, Cu deficiency exerted no effect on cholesterol oxidation and excretion in rats not supplemented with cholesterol (34). Since the CD rats receiving cholesterol supplement were capable of increasing the rates of cholesterol oxidation and excretion, the CD rats receiving no cholesterol supplement must also possess the capacity to increase cholesterol oxidation and excretion. However, this mechanism was not utilized by the rats fed the CD diet with no added cholesterol as a means to normalize the

hypercholesterolemia. The possibility that an impairment in cholesterol degradation and elimination is responsible for the hypercholesterolemia associated with Cu deficiency appears to be unlikely.

LIPOPROTEIN METABOLISM

In view of the marked elevation of HDL in CD rats and its abundance for purification and radiolabeling, a number of studies have been designed to examine the metabolism of HDL in this animal model.

High Density Lipoprotein Apolipoprotein and Cholesteryl Ester Catabolism

The plasma clearance rates and tissue sites of uptake of HDL cholesteryl ester and protein have been determined in the CD rat model by using doubly labeled HDL particles as a tracer (9). CD and adequate rats were injected intravenously with a single dose of labeled HDL previously isolated from rats subjected to the same dietary treatment. Cholesteryl esters were labeled by incorporating [^{3}H]cholesteryl linoleyl ether (CLE) into the particle core, which served as a nondegraded marker of tissue uptake. The protein moiety was radiolabeled with ^{125}I. Kinetic analysis of the plasma clearance data for the doubly labeled HDL indicated that [^{3}H]CLE was cleared from the plasma faster than ^{125}I-protein in both CD and Cu-adequate rats. Since CD rats exhibited increases in HDL concentration and plasma volume, which contributed to marked increases in the plasma pool size of HDL components, absolute mass clearance and subsequent tissue uptake were determined. The absolute catabolic rate (ACR) of HDL protein removal from the plasma was 369 ± 22 and 278 ± 12 μg/h in CD and Cu-adequate rats, respectively. The ACR of HDL cholesteryl ester was 647 ± 47 μg/h in CD rats and 321 ± 13 μg/h in controls. In CD rats, the magnitude of increase in cholesteryl ester mass removal (102% above controls) was much greater than the increase in protein removal (33% above controls), indicating that selective clearance of HDL cholesteryl ester was preferentially increased threefold in Cu deficiency. When expressed as μg/h per organ per 100 g body weight, the liver was clearly the most important organ for [^{3}H]CLE uptake in both treatment groups. Moreover, the [^{3}H]CLE uptake by the liver of CD rats was 2.1-fold higher than in controls and accounted for virtually all of the increased removal of plasma HDL cholesteryl ester in Cu deficiency. The skin and muscle were the major sites of ^{125}I-protein uptake in both treatment groups; however, an increased uptake was detected only for the skin of CD rats.

Since ^{125}I-HDL uptake by the liver was not altered by Cu deficiency, the increase in hepatic uptake of HDL cholesteryl ester appeared not to be accompanied by a concurrent increase in protein moiety of HDL. Given that

HDL cholesteryl ester transport back to the liver was increased in Cu deficiency and in view of previous studies (34) demonstrating no increase in cholesterol excretion and degradation, these data suggest that the net flux of HDL cholesterol may be increased and that hepatic HDL production may also be enhanced to sustain the hypercholesterolemia in this model.

High Density Lipoprotein Binding to Liver Plasma Membranes

Three studies have applied the lipoprotein-binding techniques to examine the interaction of HDL with lipoprotein receptors in the CD rat model. First, Lefevre et al (32) compared the binding responses of ^{125}I-HDL ($d < 1.21$, total HDL fraction) and crude liver membrane, derived from CD and adequate rats, using only one level of radioligand concentration. No treatment differences were detected when binding activity was expressed on a traditional membrane protein basis. Nevertheless, the binding response in the CD group was reduced when expressed on the unusual basis of plasma membrane marker enzyme (5'-nucleotidase) activity. However, the validity of expressing data on this basis has not been justified by establishing that enzyme activity accurately reflects plasma membrane content of crude liver homogenate.

Saturation experiments have been performed by Hassel et al (19) to examine the binding response of crude liver membrane to a wide range of ^{125}I-apo E–free HDL concentration. A 2 × 2 factorial design, involving four different combinations of membrane and HDL from CD and Cu-adequate rats, was used to identify whether the source of membrane or HDL contributed to changes in binding. No differences in the equilibrium dissociation constant (K_d) were observed, which indicates that the affinity of apo E–free HDL for binding sites was unaffected by Cu status. A significant lipoprotein effect was observed for maximal binding (B_{max}), thus demonstrating that binding was reduced when HDL from CD rats was used, regardless of the source of membrane. Hence, the reduction in binding was associated with apo E–free HDL from CD rats and was not due to a reduction in binding site numbers. In these two early studies, the use of crude liver membrane preparations may have contributed to the inconsistent binding results.

The most recent report addressed the influence of Cu deficiency on the binding of apo E–rich HDL to hepatic plasma membranes, purified by sucrose density gradient ultracentrifugation. In general, the majority of the binding appeared to be attributed to a high capacity, relatively nonspecific binding site observed for apo E–free HDL. Binding specificity was examined by displacement studies, using increasing concentration of unlabeled lipoprotein competitors. These studies indicated that in both treatments apo E appeared not to be a primary requisite for recognition by the binding site. Analysis of total and

specific binding data over a wide range of ligand concentration demonstrated elevated binding when HDL and membranes were derived from CD rats. The elevated binding may be due to an increased expression of this nonspecific, high capacity site or to subtle changes in lipoprotein structure, leading to enhanced recognition by this binding site, that result from Cu deficiency.

Specific Binding and Uptake of High Density Lipoprotein by Liver Cells

The binding and internalization of apo E–free HDL by hepatic parenchymal and Kupffer cells was examined with cells and HDL derived from rats fed Cu-deficient (0.7 μg/g) and Cu-adequate (7 μg/g) diets (38). After eight weeks of dietary treatment, liver cells were obtained by collagenase perfusion and were purified by centrifugal elutriation. Freshly isolated cells were incubated with ^{125}I-apo E–free HDL from either the same treatment or from other treatment in a crossover design, in order to establish if treatment differences were associated with hepatic cells or HDL or both. Binding studies performed at 0°C for 90 min, as a function of HDL concentration, demonstrated increases in specific binding and maximal binding capacity (B_{max}) in parenchymal cells from CD rats (38). In addition, cell association studies performed at 37°C with 20 μg/ml of ^{125}I-apo E–free HDL, as a function of time, were followed by a cold trypsin digestion to provide measurements of trypsin releasable or surface bound HDL and of trypsin resistant or internalized HDL. ^{125}I-apo E–free HDL bound to cell surface was increased, but the amount of HDL internalized was not altered in parenchymal cells from CD rats. In contrast, the amount of ^{125}I-apo E–free HDL internalized was reduced and that bound to cell surface was unaltered in Kupffer cells from CD rats. Furthermore, the source of HDL appeared to have little or no influence on the binding and uptake by these liver cells. Thus, Cu deficiency may exert different effects on HDL metabolism in these liver cells. Quantitatively, the parenchymal cells account for most of the overall uptake of HDL in vivo (47). In CD rats, the in vivo uptake of HDL by parenchymal cells may have masked the reduced contribution by Kupffer cells and maintained the normal overall level of in vivo hepatic uptake of ^{125}I-HDL observed by Carr & Lei (9). A similar enhancement of the binding process was also observed by Zhang & Lei (56) in cultured parenchymal cells from CD rats. Given that in vitro binding of HDL to isolated and cultured parenchymal cells was increased in Cu deficiency, and in view of in vivo plasma clearance studies demonstrating enhanced clearance of plasma HDL cholesterol ester and attributing to the liver virtually all of the increased removal (9), these data suggest that the enhanced binding may have facilitated the accelerated hepatic uptake of HDL cholesterol ester. However, the process of whole particle uptake by the liver appeared not to be enhanced, since no alterations were

detected for the in vitro internalization of ^{125}I-apo E–free HDL by parenchymal cells (38) nor for the in vivo uptake of ^{125}I-HDL by the liver (9) of CD rats.

Lipoprotein and Hepatic Lipases

Lipoprotein lipase (LPL) is responsible for hydrolyzing triglycerides of chylomicrons or VLDL, which leads to the formation of smaller chylomicron remnants or intermediate density lipoproteins. Lau & Klevay (29) reported a reduction (40 to 47%) in post-heparin plasma LPL activity in CD rats and postulated that such a reduction may lead to delayed formation and clearance of these smaller particles and subsequently may contribute to hypertriglyceridemia and hypercholesterolemia. Koo et al (26) also demonstrated reductions in post-heparin plasma LPL (28.5%) as well as in hepatic lipase (HL, 26.6%) activities in CD rats. Since HL is capable of hydrolyzing HDL phospholipids at the surface of hepatic endothelial cells (6), the reduction may hinder HDL cholesterol delivery to and uptake by parenchymal cells and subsequently may result in hypercholesterolemia; however, this is unlikely because of the increased in vivo hepatic uptake of HDL cholesteryl ester observed in CD rats (9). At present, the significance of the reductions in HL and LPL activities is uncertain, since the data were not corrected for the expanded plasma volume associated with Cu deficiency.

Plasma Lecithin: Cholesterol Acyltransferase

Lecithin: cholesterol acyltransferase (LCAT) catalyzes the esterification of free cholesterol to form cholesterol esters (15). Since free cholesterol of HDL is a preferential substrate (15) and the transfer of HDL cholesterol esters to LDL and VLDL is aided by LCAT, the subsequent hepatic uptake of these lower density lipoproteins represents an alternate route of removal other than the direct transfer of HDL cholesterol, derived from peripheral tissues, to the liver for degradation and elimination (16). A reduction in plasma LCAT activity was demonstrated in two earlier studies (17, 28), but a later study indicated that LCAT was not affected by Cu status (31). The possible influence of the enlarged plasma volume, associated with Cu deficiency, on LCAT activity was not considered in these studies. In addition, the ratio of plasma cholesteryl ester to free cholesterol or the percentage of HDL-free cholesterol was not altered by Cu deficiency. Thus, the inconsistent changes in LCAT activity may not contribute to hypercholesterolemia in CD rats.

Apolipoprotein Synthesis and mRNA

In Cu deficiency, increases in lipid and apolipoprotein contents reside mostly in HDL for the rat model, whereas in rabbits and humans increases are associated mainly with LDL. This may be explained by the apolipoprotein synthetic capacity of the animal models. At present, the hypercholesterolemia

observed in CD rats appears to have resulted from a net efflux of cholesterol, from the liver to the plasma, which is sustained by an enhanced hepatic cholesterol synthesis. Thus, an increased apolipoprotein synthesis may accompany the elevated cholesterol synthesis in order to maintain an enhanced lipoprotein secretion and accelerated clearance of newly synthesized cholesterol from the liver in Cu deficiency.

In the normal rat liver, the relatively high level of apo A-I mRNA (1% of total mRNA) (41) is capable of maintaining a high rate of synthesis and supplying about 50% of plasma apo A-I. Even a small increase in the expression of hepatic apo A-I mRNA by Cu deficiency may induce a substantial elevation in total plasma apo A-I and HDL levels. However, in rabbits, plasma apo A-I is mainly of intestinal origin, since their hepatic level of apo A-I mRNA is very low, amounting to only 0.002% of total mRNA (10). In addition, the rabbit's intestinal apo A-I mRNA is not responsive to dietary manipulations such as cholesterol feeding (10). Hence, a maximal expression of hepatic apo A-I mRNA activity may produce little or no appreciable increase in total plasma apo A-I and HDL levels in rabbits. Similarly, even an optimal induction of the normally low hepatic apo B synthesis capacity may only be able to produce the relatively small elevation in LDL protein and apo B levels observed in CD rats. Conversely, in Cu deficiency, the large response in LDL observed in humans and rabbits may be related to their inherently high hepatic capacity to synthesis apo B.

The contention that the elevated HDL apo E level observed in Cu deficiency is *not* the result of decreased catabolism is supported by findings of (*i*) enhanced in vivo clearance of plasma HDL, including apo E (8, 9), (*ii*) increased in vitro binding of apo E–rich HDL to hepatic plasma membranes (19), and (*iii*) a transient increase in vivo hepatic uptake of chylomicron remnants (26) in CD rats. Hence apo E synthesis may also be increased in Cu deficiency.

A preliminary report of reduced hepatic apo A-I mRNA levels in CD rats has been presented by Koo & coworkers (43). However, this study seemed to lack necessary controls to allow for appropriate detection of treatment differences. At present, direct evidence is not yet available to support the proposed induction of apolipoprotein synthesis by Cu deficiency. Additional research to study apolipoprotein mRNA expression and metabolism will be needed to fully understand the mechanism(s) regulating apolipoprotein synthesis in Cu deficiency.

CONCLUSIONS

Available data support the concept that in CD rats the cholesterol derived from an increased hepatic cholesterol synthesis is packaged into lipoproteins

to facilitate an enhanced clearance of newly synthesized cholesterol ester from the liver to plasma. In addition, an elevated amount of HDL cholesterol ester is recycled back to the liver of CD rats. However, the increased cholesterol supply from the enhanced hepatic synthesis and uptake of plasma HDL cholesterol ester is not routed for bile acid production and cholesterol elimination but is used to sustain a net efflux of hepatic cholesterol to the plasma, even to the extent of depressing the hepatic cholesterol store. The hypercholesterolemia in Cu deficiency appears to be a new steady state promoted mainly by an enhanced hepatic cholesterol synthesis. In CD rats, the observed increase in hepatic fatty acid synthesis and reduction in liver lipid content support the contention that an elevated amount of fatty acids is produced to sustain an enhanced lipoprotein synthesis and excretion, resulting in the enlarged plasma triglyceride pool. Future studies should be performed to elucidate the mechanisms responsible for the enhanced expression of lipid and lipoprotein synthesis induced by a depressed Cu status.

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