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The influence of copper deficiency on the binding and uptake of high density lipoprotein by rat hepatic parenchymal cells

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THE INFLUENCE OF COPPER DEFICIENCY ON THE BINDING AND UPTAKE OF HIGH DENSITY LIPOPROTEIN BY RAT HEPATIC PARENCHYMAL CELLS

by

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ABSTRACT

This study was designed to examine the influence of Cu deficiency on the binding, uptake, and degradation of apolipoprotein E-free high density lipoproteins (apo E-free HDL) in cultured rat hepatic parenchymal cells. The binding of apo E-free HDL during time course studies was slightly but significantly increased in cells derived from Cu-deficient rats. In saturation studies, the amount of surface-bound apo E-free HDL appeared to be saturable, although no difference was observed between Cu-deficient and adequate animals. The amount of total and specific cell-associated uptake of apo E-free HDL was significantly increased in hepatic parenchymal cells of Cu-deficient animals. The present data suggest that hepatic uptake of the HDL protein moiety may be increased in rats fed a diet deficient in copper.

INTRODUCTION

Plasma lipoprotein are particles comprised of various lipids and protein complexes which function to transport cholesterol, triacylyglycerols and phospholipids by receptor-mediated endocytosis and receptor-independent process through the blood to various tissues of the body.

The liver plays a key role in cholesterol and lipoprotein metabolism. High density lipoproteins (HDL) are thought to mediate reverse cholesterol transport, the process by which excess cholesterol from peripheral tissues is transported in blood and lymph to the liver for excretion. Therefore, considerable attention has been focused on the processes that take place within the liver.

For the study of intact liver functions associated with lipoproteins under controlled conditions the isolated, perfused rat liver has been extensively used. This experimental system is excellent for many purposes, but has several major disadvantages (Seglen, 1976): (1) The liver as an organ is not completely homogeneous, containing up to 40% nonparenchymal cells; (2) the viability of an isolated liver can be maintained only for a limited period of time (8 to 10 hours); and (3) it is difficult to obtain many identical samples from one liver. Development of methods for the

isolation and culture of hepatic parenchymal cells has greatly facilitated elucidation of the characteristics and regulation on lipoprotein metabolism.

Alterations in the distribution of cholesterol and lipids among the various lipoprotein fractions have been observed in copper-deficient rats. Previous work from our laboratory as well as from other investigators' laboratories indicate that dietary deficiency of copper (Cu), a trace element necessary for animal growth and development, can increase plasma cholesterol levels in rats.

A number of studies have been performed to elucidate the mechanisms responsible for the hypercholesterolemia and hyperlipoproteinemia observed in Cu-deficient rats (Lei, 1977, 1978; Shao and Lei, 1980; Lin and Lei, 1981; Allen and Klevay, 1980). The goal of this study was to investigate the influence of Cu status on the binding, uptake, and degradation of apo E-free HDL mediated by the HDL receptor in the primary cultured rat parenchymal cell. The increased uptake observed in the present studies provided vital information on the mechanisms responsible for the hyperchoesterolemia as well as hyperlipoproteinemia associated with Cu deficiency.

LITERATURE REVIEW

Plasma Lipoproteins

The plasma lipoproteins are large spherical macromolecular structures containing the most hydrophobic lipids, triacylglycerols and cholesterol esters in the central core of these particles, and hydrophilic lipids, cholesterol, phospholipids and specific proteins (apolipoproteins) on the surface. They are biosynthesized by the intestine and liver. Their major function is to transport dietary or endogenously synthesized cholesterol, triglycerides and phospholipids to those tissues which utilize exogenous lipids for oxidative metabolism, steroid hormone biosynthesis and maintenance of their membrane integrity. According to the density, size, and net surface charge, there are five major classes of lipoproteins (Table-1): Chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL) (Oncley, 1956; Gofman, 1954; and Fredrickson, 1974).

Lipoprotein Metabolism

Since the major functions of plasma lipoproteins are to transport cholesterol, triglycerides and phospholipids via

Table-1 Major classes of the plasma lipoproteins

Name	Density range	Diameters	Molecular Weight
	(g/ml)	(nm)	(dalton x 10^6)
Chylomicron	< 0.95	> 100	400
VLDL	0.950-1.006	30-80	18-80
IDL	1.006-1.019	25-35	5-10
LDL	1.019-1.063	18-25	2.5
HDL	1.063-1.210	6-12	0.2-0.4

Table from Gotto *et al.* (1986)

the bloodstream to various tissues in the animal body, they are metabolized by different lipoprotein metabolic pathways (Goldstein and Brown, 1985).

The exogenous pathway has been postulated to be involved in the absorption of dietary cholesterol and triglycerides from the intestine by chylomicrons. Chylomicrons are the least dense and largest particles which are synthesized by the intestine in response to dietary fat (Gotto *et al*, 1986). Dietary fat and cholesterol are absorbed through the wall of the intestine and are packaged along with triglycerides into lipoprotein complex called chylomicrons in bloodstream. The triglyceride ester bond is cleaved by lipoprotein lipase on the surface of endothelial cells of extrahepatic tissues, and the fatty acids are used by adipose tissue for storage and by muscle for oxidation to supply energy (Cryer, 1981). The remnants of the chylomicrons, high in cholesterol ester, are cleared rapidly by the liver through receptor-mediated endocytosis (Brown and Goldstein, 1974). Their cholesterol is either secreted into the intestine as bile acids or packaged with triglycerides to form VLDL in the liver (Havel, Goldstein and Brown, 1980). VLDL are rich in triglycerides and contain a smaller amount of cholesterol ester particles. Three predominant proteins, apo B-100, E, and C are present on their surface. VLDL are secreted from the liver. Endogenous lipids are transported by VLDL, along

with IDL and LDL, through circulation in the so-called endogenous pathway (Havel *et al*, 1980). The triglycerides of VLDL are hydrolyzed to free fatty acids by endothelial lipoprotein lipase of the capillaries of adipose tissue or muscle and resulting in the removal of apo C. Thus a series of IDL particles, which are smaller in size, enriched in cholesterol esters but retaining their two surface apolipoproteins (apo B-100 and apo E), are formed (Brown *et al*, 1981). They are rapidly removed from the circulation by hepatic LDL receptors (apo B and E receptors) to make new VLDL and bile acids. Those IDL particles that escape hepatic clearance will remain in the circulation for much longer period of time. They will finally lose their apo E and triglycerides and are converted into LDL, with apo B-100 as their only surface protein (Havel *et al*, 1980). LDL are the major cholesterol transport proteins in human plasma. Elevated levels of plasma LDL are correlated with increased risk of atherosclerosis (Goldstein and Brown, 1983). LDL receptors (apo B, E receptors) on the liver and other tissues, which recognize apo B-100 of LDL are responsible for metabolizing about two-thirds of the LDL. The rest are metabolized by the receptor-independent process (Goldstein and Brown, 1983).

High density lipoproteins are synthesized by both the intestine and the liver. It has been postulated that HDL are

involved in a process of reverse cholesterol transport. According to this hypothesis, HDL remove cholesterol from peripheral tissues and transport this cholesterol to the liver for excretion from the body (Mahley *et al*, 1981, 1983). HDL may be used for the maintenance of cell membrane phospholipids or they may also transfer cholesterol to other lipoproteins which are later degraded by the liver. Therefore, high plasma levels of HDL are correlated with decreased risk of atherosclerosis.

Apolipoprotein B (apo B) is an obligatory structural component of chylomicrons, VLDL, IDL, and LDL. There are two kinds of apo B according to their molecular weight (Kane *et al*, 1980, Krishnaiah *et al*, 1980). The lower molecular weight apo B-48 is synthesized by the intestine and is associated primarily with chylomicrons and chylomicron remnants. The higher molecular weight apo B-100 is synthesized by the liver and is associated with VLDL and LDL.

Apolipoprotein E (apo E) is present in chylomicrons, VLDL and HDL. HDL are a heterogenous group of proteins that can be subfractionated into several subclasses. They can be separated into HDL-with apo E and HDL-without apo E subclasses by heparin-Sepharose affinity chromatography (Mahley *et al*, 1981). Apo E-containing HDL can be taken up by the LDL (apo B,E) receptors and apo E receptors. HDL that lack apo E can not interact with apo B, E or apo E receptors. However, these

apo E-free HDL can interact with apo A-I binding sites. Different types of apolipoproteins, as well as their molecular weights, major biosynthetic sites, plasma distributions, and functions are listed in Table-2.

Lipoprotein and Atherosclerosis

High percentage of death in Western countries is caused by atherosclerosis. This disease is initiated by the accumulation of cholesterol in the wall of arteries and eventually obstructing an artery and causing a heart attack (Ross, 1979). All animals require cholesterol for their plasma membrane, but the body can not tolerate excessive cholesterol in blood. Excessive cholesterol will deposit in the artery walls (Mahley, 1985). Since two-thirds of the cholesterol in human plasma are carried by LDL, the cholesterol that accumulates in the arterial wall is derived from LDL. High level of LDL in blood will accelerate atherosclerosis. High level of LDL receptor and high level of HDL will decrease the risk of this disease. Various studies with animals are providing clues in understanding the biology of human atherosclerosis.

Feeding experimental animal with high fat and cholesterol diet induce changes of lipoproteins such as: the appearance of cholesterol-enriched β-VLDL; the increase in the size, cholesteryl ester content, and level of plasma LDL; and

Table-2 The Different Types of Apolipoproteins

	MW (daltons)	Biosynthetic site	Distribution	Functions
A-I	28,000	Intestine, Liver	HDL_3,CM	LCAT activation, receptor binding
A-II	17,000	Intestine, Liver	HDL_3,CM	Hepatic lipase activator
A-IV	46,000	Intestine,	HDL,CM	LCAT activation, receptor binding
B-100	549,000	Liver	VLDL,LDL	Lipoprotein biosynthesis and secretion
B-48	264,000	Intestine	CM, and CM remnant	Lipoprotein biosynthesis and secretion
E	34,145	Liver	CM,VLDL, HDL_2	Receptor binding
C-I	6,550	Liver	CM,VLDL,HDL	LCAT activator
C-II	8,837	Liver	CM,VLDL,HDL	Lipoprotein lipase activator
C-III	8,240	Liver	CM,VLDL,HDL	Inhibition of LPL and premature remnant clearance

Table from Dolphin (1985)

also the elevation of apo E-rich HDL. In most animals these diets are associated with accelerated atherosclerosis (Mahley, 1979, 1981, 1983). Familial hypercholesterolemia (FH) is caused by extremely high LDL level (twice the normal level) in the plasma. The mechanism responsible for the hypercholesterolemia in FH is revealed by the LDL receptor research work which indicated a mutation in the LDL receptor gene, leading to a defective LDL receptor (Goldstein, Kita and Brown, 1983).

Lipoprotein Receptors

On the cell surface there are a lot of proteins, some of them can function as receptors which can bind with specific molecules. The first lipoprotein receptor which was studied extensively is the LDL receptor. It was discovered by studying tissue cultures of human skin cells called fibroblast (Goldstein and Brown, 1973). During the last decade, evidence has been accumulated to describe a number of receptor systems that may be responsible for the interaction of lipoproteins and cells. The concept of receptor-mediated endocytosis was developed to explain the regulation of cellular cholesterol metabolism (Brown and Goldstein, 1985). Most lipoprotein receptors function to provide a mechanism for receptor-mediated endocytosis. Some receptors apparently have additional functions. The most established receptor-mediated

endocytosis was the LDL receptor-mediated endocytosis (Brown and Goldstein, 1985). The circulating LDL bind to the LDL receptors on the cell surface where the cell membrane is indented to form coated pits. The pits pouch rapidly inward into the cell and pinch off from the surface to form coated endocytic vesicles. The LDL are then separated from the receptors and are delivered to the lysosome full of digestive enzymes. In this organelle LDL apo B-100 are broken down into amino acids and the cholesteryl ester bond is cleaved to yield unesterified cholesterol for membrane synthesis and other cellular needs, while the receptor cycles back to the cell surface to start a new cycle (Brown and Goldstein, 1985).

Apo B, E and apo E receptors have been extensively studied by many investigators to elucidate the mechanism responsible for the regulation of lipoprotein metabolism. A genetic deficiency of LDL (apo B,E) receptor is found in familial hypercholesterolemic (FH) patients and in Watanabe Heritable Hyperlipidemic rabbits (WHHL) (Goldstein *et al*, 1983). The cells of FH homozygote, having inherited two defective receptor genes, can not synthesize any normal receptors. Heterozygotes have one normal receptor gene and one mutant gene. They synthesize half the normal number of receptors and can therefore bind, internalize and degrade LDL at half the normal rate. This results in high levels of LDL in plasma and ultimately leads to the development of

atherosclerosis (Brown and Goldstein 1985). Both apo B, E and apo E receptors are found on liver membranes of young growing dogs. However, only the apo E receptors are present in liver membranes of adult dogs. In humans, the apo E receptors and small amount of apo B, E receptors are present on liver membranes (Mahley and Innerarity, 1983). The expression of apo B,E receptors in adult human liver membranes can be induced by prolonged fasting, cholesterylamine treatment and pharmacologic doses of estrogen. In contrast, they can be reduced by high cholesterol diets and increasing ages.

Lipoprotein Metabolism in Isolated and Cultured Hepatic Parenchymal Cell

It is well known that the liver plays an important role in lipoprotein metabolism and as such has received an increasing amount of interest over the past decades. The liver is not an homogeneous tissue and contains parenchymal cells and non-parenchymal cells (NPC), consisting of endothelial, Kupffer, fat storing and pit cells. The initial lipolysis of chylomicron and VLDL triglycerides by the extrahepatic lipoprotein lipase (LPL) is essential for their efficient catabolism in isolated rat liver cells (Felts *et al*, 1970). Cholesterol ester labeled chylomicron remnants are efficiently taken up and degraded by monolayers of rat parenchymal cells (Floren and Nilsson, 1977). An initial

hydrolysis of 10% triglycerides by LPL is sufficient to produce VLDL-remnants which are taken up at the same rate by the perfused liver as smaller particles obtained after 80% hydrolysis (Windler *et al*, 1980). Studies in several species have demonstrated that the liver is responsible for about 60% of LDL turnover (Pittman *et al*, 1979). About two-thirds of the hepatic uptake of human LDL are performed by receptor-mediated process (Carew *et al*, 1982) and the remaining LDL are catabolized by a receptor-independent process by different types of hepatic cells in rats. The tissues responsible for HDL metabolism are not well established yet. *In vitro* studies with perfused livers, freshly isolated or cultured liver parenchymal cells have provided a comprehensive view of hepatic lipoprotein metabolism. These studies have confirmed that the liver is an important site of HDL catabolism and suggested that 20% to 30% of HDL catabolism may occur in the liver, probably primarily in the parenchymal cells (Glass *et al*, 1983). The study by Nakai *et al*, (1976) demonstrated that the binding of rat HDL to isolated liver cells in suspension was saturable and could be displaced by an excess of unlabeled HDL. They suggested that there might be a specific HDL receptor. Other studies using isolated liver parenchymal cells suggested that hepatic HDL uptake is a receptor-mediated process (Ose *et al*, 1981, Van Berkel *et al*, 1981), but there has been disagreement as to the specificity

of the binding site for HDL. Receptor-mediated uptake of HDL has also been shown in studies using rat cells (Ghiselli *et al*, 1981). The HDL degradation process was very slow (Nakai *et al*, 1976). HDL binding studies indicated that this process was not Ca^{++}-dependent or competitively inhibited by VLDL and LDL (Ose *et al*, 1981). It was also demonstrated that apo E did not contribute to the binding of HDL, confirming that the binding site was different from the LDL (apo B, E) and the remnant (apo E) receptors. Following uptake of HDL containing both cholesterol-ester and an apo A-I with covalently linked ^{125}I-labeled tyramine-cellobiose, Glass *et al* (1983) showed that cholesteryl ester accumulated predominantly in the liver but that apo A-I was trapped less extensively. The data suggested that perhaps more cholesterol molecules than the whole HDL particles were taken up. A selective uptake of the cholesterol moiety compared to the protein has also been observed in monolayer of rabbit and rat liver parenchymal cells incubated with homologous HDL (O'Malley *et al*, 1981). Apo E-free HDL have been reported to bind to a receptor different from that of the apo E-containing lipoproteins. Thus, apo E seems not to be involved in the HDL uptake process. Actually, apo A-I has been proposed to be the ligand responsible for the binding of rat HDL_2 (Tamai *et al*, 1983) and HDL_3 (Nakai 1976) to HDL binding sites. In order to evaluate HDL binding sites distinct from the LDL receptor, the

apo E-(deficient or free) HDL were used in many studies *in vivo*, since LDL receptor recognizes apo E as well as apo B, the major apolipoprotein of LDL. Studies with rat hepatoma cells (Tamai *et al*, 1983), isolated rat liver parenchymal cells (Rifici *et al*, 1984; Glass *et al*, 1985), and cultured pig liver parenchymal cells (Bachorik *et al*, 1982) suggested that apo E-free HDL bind specifically to these cells and are degraded by them. Experiments performed with human liver plasma membranes and with the human hepatoma cell line, HEPG 2, also indicated that a specific, saturable receptor for apo E-free HDL is present in the human liver (Hoeg *et al*, 1985). In addition apo A-IV has also been speculated to mediate the binding and uptake of HDL by liver parenchymal cells (Dvorin *et al*, 1986). The high affinity uptake of ^{125}I-apo E-free HDL (mediated by apo A receptor) has a reversible component in cultured pig liver parenchymal cells (Bachorik *et al*, 1985). Thus HDL can alter parenchymal cholesterol homeostasis, resulting in changes in cholesterol synthesis and esterification, primarily the latter, in response to high affinity HDL binding.

Copper

Copper has been considered as an essential trace element since Cu, in addition to iron, is necessary for hemoglobin formation (Hart *et al*, 1928). The healthy adult

human body has been estimated to contain 80 mg of total Cu (Cartwright and Wintrobe, 1964). Newborn and very young animals contain higher Cu per unit of body weight than adults of the same species (Spray and Widdowson, 1951). The Cu level of a newborn is decreased during growth until the adult value is reached. The distribution of total body Cu among the tissues also varies with the species, ages, and Cu status of the animal (Underwood, 1977). The liver, brain, kidneys, heart, and hair contain relatively high Cu concentration in animal body. The pancreas, skin, muscle, spleen, and bones represent tissues of intermediate Cu concentration. The pituitary, thyroid, thymus and prostate glands, and the ovary and testis are examples of organs low in Cu (Carlton and Henderson, 1963).

Liver Cu concentrations vary with the species and age of the animal, the chemical concentration of the diet and in various disease conditions (Underwood, 1977). There is no effect of sex on liver Cu concentration except that the female Australian salmon carries higher levels than the male (Beck, 1961). Different animal species have different hepatic Cu concentrations. The liver contains 10 to 50 ppm Cu (dry basis) in most normal adult species (Beck, 1956; Lorenzen and Smith, 1947). Sheep, cattle, ducks, frogs, and certain fish exhibit consistently higher liver Cu levels, with normal range of 100 to 400 ppm. Liver Cu concentrations in rats increase

rapidly when dietary Cu levels reach 200 ppm. Sheep and cattle probably have a superior capacity to bind Cu in liver since blood Cu levels do not rise in these species with increased Cu intake (Milne and Weswig, 1968). Liver Cu concentrations are higher in newborn than in adults in many species. Cu distribution among the subcellular fractions changes as the rat matures (Greogoriadis and Sourkes, 1967). At birth over 80% of total hepatic Cu content is associated with the nuclear and mitochondrial fractions, while in the adult rat the supernatant contains about one-half the total Cu content of the liver. Evans (1973) observed that the major portion of the total hepatic Cu is located in the cytosol, where Cu is bound to the enzyme superoxide dismutase and metallothionein. Liver Cu levels are sensitive to low-Cu intake and are affected by other dietary factors such as zinc, cadmium, iron, and calcium carbonate that influence Cu retention in the body through their effects on Cu absorption and excretion (Underwood, 1977).

Both erythrocytes and plasma contain Cu. About 60% of total red blood cell Cu is bound to a protein called erythrocuprein which functions as a superoxide dismutase (McCord and Fridovich, 1969). Holmberg and Laurell (1951) observed that the Cu in plasma is firmly bound to blue Cu protein ceruloplasmin which is a true oxidase (ferroxidase) involved in iron utilization and in promoting the rate of iron

saturation of transferrin in the plasma (Osaki and Frieden, 1966). About 90% of plasma Cu exists as ceruloplasmin in normal mammals (Cartwright, 1950). In addition to ceruloplasmin, a small proportion of the plasma Cu also exists in combination with albumin (Gubler *et al*, 1953) and with amino acids (Neuman *et al*, 1967). The Cu concentration is usually higher in the plasma than that in the erythrocytes in mammals (Lahey *et al*, 1952; Cartwright *et al*, 1950; Bingley *et al*, 1969). The normal range of Cu concentration in the blood of healthy animal is 0.5 to 1.5 μg/ml. Plasma Cu does not increase after meals or decrease during fasting (Cartwright, 1950). In most species whole blood and plasma Cu levels are similar in males and females. However, plasma Cu is slightly higher in human females than in males. In humans, the normal range of Cu in the blood has been reported to be 105.5 ± 5.03 μg Cu/100 ml for men and 114 ± 4.67 μg Cu/100 ml for women (Cartwright, 1950). Serum Cu levels are significantly increased in women taking oral contraceptives. Administration of estrogen or stilbestrol can greatly elevate plasma Cu levels in men (Johnson *et al*, 1959) and in rats respectively (Turpin *et al*, 1952).

Elevated plasma Cu levels during pregnancy have been reported in women (Hambidge and Droegemueller, 1974). The levels returned to normal in the first few weeks postpartum. Ewes maintained on a constant diet also exhibited a decline

in whole blood, plasma and ceruloplasmin Cu during pregnancy (Howell *et al*, 1968). Newborn human infant serum Cu and ceruloplasmin levels are about one-third of the normal adult range (Fay *et al*, 1949; Scheinberg *et al*, 1954; Henkin *et al*, 1973). Whole blood and plasma Cu levels are lower, while erythrocyte Cu levels are higher in newborn calves than in their mothers (Bingley *et al*, 1969).

Blood Cu concentration can be affected by the status of diet. Cu deficiency can result from low-Cu intake. Additions of Cu to normal adequate diets exert little effect on blood Cu levels. No increase in plasma Cu was observed in rats when the Cu content of the diet was raised from 10 to 50 ppm. However, the plasma Cu concentration was increased two-fold from 1.13 to 2.34 μg Cu/ml when Cu intake was increased to 100 ppm (Milne and Weswig, 1968). Extremely high Cu intake can cause toxic symptoms in many species. A severe hypercupremia can be caused by a toxic intake of 750 ppm Cu in pigs. On the other hand, this hypercupremia can largely be prevented by the concurrent administration of 500 ppm zinc (Suttle *et al*, 1966). Elements such as zinc, cadmium, and iron that depress Cu absorption can reduce plasma Cu concentrations by high dietary levels. The effect of molybdenum and sulfate depends on the status of the animal with respect to these

nutrients and Cu (Gray and Daniel, 1964). In addition, plasma Cu levels can be affected by many diseases (Underwood, 1977).

Copper Metabolism

Dietary Cu is absorbed in the stomach and portions of the small intestine, particularly the upper small intestine, although the mechanisms are not yet understood. In most species dietary Cu is poorly absorbed. The extent of Cu absorption is influenced by the amount and chemical form of the Cu intake, by the dietary level of several other metal ions and organic substances and by the age of the animal. Cu absorption from the intestine is regulated according to the body Cu needs. In rats and mice, higher intestinal absorption of Cu is observed when Cu was administered at low dosages. Intestinal Cu uptake and transfer to the serosal solution are elevated in Cu deficiency (Schwarz and Kirchgessner, 1974).

Copper occurs in food in many chemical forms which can affect the absorption. Various compounds labeled with ^{64}Cu has been used for testing the Cu uptake in many species. Chapman and Bell (1963) reported that the relative rate of appearance of ^{64}Cu in the blood of beef cattle is in the following $CuCO_3$ > $Cu(NO_3)_2$ > $CuSO_4$ > $CuCl_2$ > Cu_2O > CuO (powder) > CuO (needles) > Cu (wire). Many dietary factors influence the uptake of Cu by the gastrointestinal tract. The acidic environment may promote Cu solubility and enhance its transport across the

gastric mucosa (Marceau *et al*, 1970). Amino acids (Kirchgessner and Grassman, 1970) and high protein diets (Davis *et al*, 1962) increase copper absorption. Dietary phytate (Davis *et al*, 1962), ascorbic acid (Van Campen *et al*, 1968), thiomolybdate (Suttle 1974), and fiber (Kelsay *et al*, 1979) appear to complex with Cu and limit its absorption. Dietary fructose also reduces Cu uptake. Bile reduces the reabsorption of secreted Cu (Gollan *et al*, 1973). Several dietary metals also affect Cu absorption. These include particularly calcium, cadmium, zinc, iron, lead, silver, and molybdenum plus sulfur. Zinc reduces Cu absorption (Van Campen and Scaife, 1967). Van Campen demonstrated that high zinc intakes depressed Cu uptake and provided the first direct evidence that Cu competes with zinc for absorption in rat duodenal segments. Perfusion of the isolated intestine with solution of high zinc content caused some reduction in Cu uptake in the mucosal cells (Oestreicher *et al*, 1985). However, it is not clear whether excess zinc competes for a specific Cu receptor on the brush border membrane or at intracellular sites or competes for absorbable binding ligands. A reduction in dietary zinc supply has been found to enhance uptake and absorption of Cu as well as zinc (Schwarz *et al*, 1974). This suggested that a common site may be involved in the uptake of these metals at the brush border membrane. In mice, a high-Ca diet can reduce Cu absorption,

compare to a low-Ca diet, due to an increase in intestinal pH (Thompsett *et al*, 1940). However, diets high in calcium are capable of enhancing Cu toxicity in pigs, presumably due to a lowering of zinc availability (Suttle *et al*, 1966). Iron also interferes with Cu absorption through competition for absorption binding sites. Mo and sulfate can either increase or decrease the Cu status of an animal, depending on their intakes relative to that of Cu. High dietary levels of cadmium depress Cu uptake, and even a relatively small increase in cadmium intake can adversely affect Cu metabolism when Cu intakes are marginal. The regulatory mechanism for Cu absorption is not well established. At present it seems clear that metal-binding components are involved and that the inhibition of Cu absorption brought about by various metals results from competition for protein metal-binding sites. Probably there are two possible mechanisms, one involving Cu transport from the intestinal lumen into the mucosal cells and one from the mucosal cells to the plasma. Little is known about mechanisms responsible for hepatic uptake of Cu.

After absorption, Cu is immediately attached to albumin or to plasma amino acids and the complex is rapidly transferred to the liver. Henkin (1974) indicated that the equilibrium established between amino acid-bound Cu and albumin-bound Cu, may influence how much copper is accumulated by hepatocyte. The amount of Cu absorbed is far in excess of

metabolic needs and most Cu is returned to the intestine in the form of bile. In this manner the liver provides the major pathway of Cu excretion via the bile. Cu that is retained by the liver may be stored, presumably bound to metallothionein or used for the synthesis of various Cu-containing proteins.

Cartwright and Wintrobe (1964) reported that out of the 2 to 5 mg Cu ingested daily by man, 0.6 to 1.6 mg (32%) is absorbed, 0.5 to 1.3 mg is excreted in the bile, 0.1 to 0.3 mg passes directly through the bowel, and 0.01 to 0.06 mg appears in the urine. In many species the biliary route is the major pathway of Cu excretion.

Copper Deficiency

Copper deficiency has been observed in a number of animal species. The symptoms of Cu deficiency are influenced by the species, age, and sex of the animal, as well as the severity and duration of deficiency. However, they include anemia, bone abnormalities, demyelination and degeneration of the nervous system leading to neonatal ataxia, impaired reproductive performance, lesions in cardiovascular system and depigmentation of hair, wool, or skin (Underwood, 1977). Anemia has been reported as a common symptom of Cu deficiency in all species. Hyperchromic and microcytic anemia has been observed in many Cu-deficient species, but hypochromic and macrocytic anemia has also been observed in Cu-deficient ewes

and cattle (Cunningham, 1946). In Cu-deficient chicks (Matrone, 1960) and dogs (Baxter *et al*, 1953) the anemia is normocytic and normochromic. Defective maturation of the erythrocytes has been observed in the Cu-deficient pig. In pigs and dogs with Cu deficiency anemia, the percentage of reticulocyte in blood is lower than those with Fe deficiency anemia (Lahey *et al*, 1952). Reduction in ceruloplasmin activity has been reported in Cu deficiency. This enzyme is required for the oxidation of ferrous to ferric iron, a necessary step in the transport of iron from tissue to plasma (Osaki *et al*, 1966). Thus Cu plays an important role in the metabolism of iron through ceruloplasmin, in mobilizing absorbed iron for hemoglobin synthesis (Evans and Abraham, 1973).

Copper deficiency can cause skeletal abnormalities in many species. In severely Cu-deficient young dogs, a gross bone disorder develops with fractures and severe deformities (Baxter *et al* 1953). The fragile bones from Cu-deficient chicks fracture with less deformation and torque than bone from controls. This may have resulted from a reduction in bone collagen cross-linking (Rucker *et al*, 1975), since a marked reduction in amine oxidase activity occurs in the bones of Cu-deficient chicks, and collagen extracted from such bones is more easily solubilized than collagen from control bones.

Copper deficient animals also have a nervous disorder characteristic in many species. Bennetts and Chapman (1937) reported that the ataxia of lambs occurring in Western Australia was associated with subnormal levels of Cu in pastures and in the blood and tissues of both ewes and affected lambs. The ataxia could be prevented by Cu supplementation of the ewe during pregnancy. Neonatal ataxia was also observed in Cu-deficient goats, pigs and rats (Underwood, 1977). In addition, Gallagher *et al* (1956) demonstrated that Cu deficiency causes a depression in cytochrome oxidase activity resulting in a failure of synthesis of its prosthetic group heme and inhibition of phospholipid synthesis. Swayback is a nervous disorder observed in lambs. The disease is characterized by uncoordinated movements (Underwood, 1977). The molybdenum-induced hypocuprosis has been speculated as a contributing factor in the incidence of this disease (Alloway, 1973).

Achromotrichia is one of the manifestations of Cu deficiency in rats, rabbits, guinea pigs, cats, dogs, cattle, and sheep. It describes the reduction in growth and appearance of hair, fur and wool. The associated depigmentation is caused by a reduction of tyrosinase required to convert tyrosine to melanin (O'Dell, 1976). Impaired keratinization, characterized by the appearance of abnormally

straight, "stringy" hair, has been observed in many copper-deficient species. A reduction in the formation of disulfide groups in keratin synthesis may be responsible for the impaired keratinization (Underwood, 1977). The first evidence of cardiovascular disorders was found in Cu-deficient cattle occurring in Western Australia called "falling disease" (Bennetts and Hall, 1939). The essential lesion of this disease is a degeneration of the myocardium with replacement fibrosis. Sudden cardiac failure associated with cardiac hypertrophy has been reported in Cu-deficient rats and pigs (Grubler *et al*, 1957). Cardiac hypertrophy, and a marked increase in the mitochondrial area of the heart muscles has also been observed in Cu-deficient rats (Goodman *et al*, 1970). O'Dell (1961) demonstrated a derangement in the elastic tissue of the aortas of Cu-deficient chicks. The mortality in these animals appeared to be caused by a rupture of the major blood vessels. Cu deficiency can also induce ruptures of major blood vessels and death in pigs (Coulson *et al*, 1963). Subsequent studies demonstrated that aortic rupture with degeneration of the elastic membrane exist in Cu-deficient chicks (Carlton *et al*, 1963; Simpson *et al*, 1964). Extensive internal hemorrhage with high incidence of aortic aneurysms was also observed in young Cu deficiency guinea pigs (Everson *et al*, 1967).

The most significant findings were obtained by studying the role of Cu in elastin (and collagen) biosynthesis (Carnes, 1971). For example, the elastin content of the aortas of Cu-deficient pigs (Weismann *et al*, 1963) and chicks (Starcher *et al*, 1964) is decreased. The elastin from Cu--deficient animals contains an elevated content of lysine and decreased levels of desmosine and isodesmosine. These alterations may have resulted from a reduction of lysyl oxidase activity (Miller *et al*, 1965; O'Dell *et al*, 1966). Both desmosine and isodesmosine are the key cross-linkage groups in elastin (Partridge *et al*, 1964). The condensation of lysine residues is catalyzed by lysyl oxidase, which is a Cu-containing enzyme (Hill *et al*, 1962). Hill *et al* (1968) demonstrated that Cu deficiency causes a reduction of lysyl oxidase activity. Thus less lysine is being converted to desmosine, which is the cross-linkage group of elastin. This results in fewer cross-linkage to be present in the connective tissue and subsequently yielding less elasticity in the aorta.

Copper deficiency in human adults is rare in the United States. Severe Cu deficiency has been reported in the human infant and children with protein-energy malnutrition and in some individuals maintained on total parenteral alimentation (Mason, 1979). The common manifestations of Cu deficiency are severe anemia, leukopenia, neutropenia and occasionally demineralization of bone, decreased pigmentation

of skin, and neurological abnormalities (Mason, 1979). Cartwright and Wintrobe (1964) estimated the dietary Cu requirements of infants and children to be 50 to 100 μg/kg body weight/day. The Food and Nutrition Board of the U.S. National Academy of Sciences recommends 2 to 3 mg Cu/day for adults. However, a number of studies have reported that various United States diets provided only slightly more than 1 mg Cu/day (Klevay, 1979; Wolf *et al*, 1977; Lei *et al*, 1980).

Copper Deficiency and Lipoprotein Metabolism

In 1972, Murthy *et al* demonstrated that there may be an important relationship between dietary zinc and Cu on lipid metabolism in rats. Hypercholesterolemia was observed in rats by increasing in the ratio of zinc to Cu ingested (Klevay, 1973). He postulated that the high ratio of dietary zinc to Cu is associated with hypercholesterolemia and further hypothesized that coronary heart disease is predominantly a disease of imbalance in regard to zinc and Cu metabolism. By using large scale factorial experiments, with various levels of dietary zinc and Cu, Petering *et al* (1977) and Murthy and Petering (1976) indicated that there is an inverse relationship between dietary Cu, serum Cu and levels of serum cholesterol, triglycerides and phospholipids. They suggested that dietary Cu governs the metabolic fate of cholesterol more than the dietary zinc to Cu ratio. Lei (1977) suggested that

the hypercholesterolemia could be partially caused by a shift of cholesterol from the liver pool to the plasma pool in Cu-deficient rats. Allen and Klevay (1978) also suggested that a more rapid clearance of cholesterol from the liver to the plasma pool, with this cholesterol being unavailable for excretion as biliary steroids, may be responsible for the hypercholesterolemia in Cu-deficient rats. Subsequent studies demonstrated that cholesterol ester, newly synthesized from [2-^{14}C]mevalonate, cleared the liver faster in Cu-deficient rats than controls (Shao and Lei, 1980). Lin and Lei (1981) observed marked increases in the size and half-life of the rapidly exchangeable cholesterol pool, which consisted of serum cholesterol and tissues, such as the red blood cell and liver, which equilibrated rapidly with serum cholesterol in Cu-deficient rats. Furthermore, a prolonged half-life of the free cholesterol and total cholesterol carried by the high density lipoprotein (HDL) was observed in Cu-deficient rats (Lin and Lei, 1981). The influence of Cu deficiency on cholesterol clearance from plasma and on half-life of HDL cholesterol suggest copper may play an important role in lipoprotein metabolism.

Alterations in the distribution of cholesterol and lipids among the various lipoprotein fractions have been observed in Cu-deficient rats. In rats, plasma HDL were found to carry about 85% of total plasma cholesterol in normal rats

and the percentage was decreased in Cu-deficient rats, but the absolute amount of cholesterol carried by HDL was markedly increased by Cu deficiency (Allen and Klevay, 1980; Lei and Lin, 1981). Lei *et al* (1983) observed similar results when the lipoprotein fractions were partitioned by ultracentrifugation and column chromatography into high, low, and very low density lipoproteins (HDL, LDL, VLDL). Elevations in protein and cholesterol contents of HDL and LDL and in triglyceride content of LDL were observed in Cu-deficient rats (Lei *et al*, 1983). An increase in apolipoprotein E (apo E) concentration of HDL was also observed in Cu-deficient rats. Since HDL is a heterogenous lipoprotein fraction containing several different subpopulations of particles, Croswell and Lei (1985) separated HDL by heparin-Sepharose columns into subclasses with or without apo E and demonstrated that the protein and cholesterol contents of apo E-rich HDL were elevated as a result of Cu deficiency.

Apo E-rich HDL appear to be formed in the plasma of extracellular space by certain HDL becoming enriched in cholesterol and acquiring the apo E (Mahley, 1979). Cholesterol feeding also induces increased concentration of apo E-rich HDL, which are rapidly cleared by the liver through a high affinity, receptor-mediated process (Mahley, 1981).

MATERIALS AND METHODS

Experimental Animals

Male weanling Sprague-Dawley rats, weighing between 40 to 60 grams, were selected as the experimental animals. All rats were kept individually in suspended stainless steel wire cages in a laboratory with 12 hours light and 12 hours darkness and maintained at 20-22°C. Diets and distilled-demineralized H_2O were provided ad libitum. Body weights were recorded weekly throughout the experiments. After seven weeks of dietary treatment, the rats were anesthetized with diethyl ether and killed.

Experimental Diet

Rats were randomly divided into two dietary treatments, Cu-deficient and adequate. The basal diet was prepared according to the formulation of the American Institute of Nutrition (1977) except no Cu supplement was included in the mineral mix (Table 3). The Cu-deficient diet which contained 1.044 mg Cu/kg diet was measured by flame atomic absorption spectrophotometry using a NAS Hitachi model 180-70 polarized Zeeman atomic absorption spectrophotometer. The Cu-adequate diet was prepared by adding $CuCO_3$ to the basal diet to provide a final concentration of 7.330 mg Cu/kg diet.

Table-3 Diet Composition

Ingredient	Copper Deficient %	Copper Adequate %
Casein	20.0	20.0
DL-Methionine	0.3	0.3
Glucose Monohydrate	65.0	64.0
Fiber Cellulose	5.0	5.0
AIN Mineral Mix	3.5	3.5
Vitamin Mix (water soluble)	1.0	1.0
Vitamin Mix (fat soluble)	1.0	1.0
Corn Oil	4.0	4.0
Choline Bitartrate	0.2	0.2
Cupric Carbonate Mix (1.05 g/kg)	---	1.0
Dietary Copper (ppm)	1.044	7.330

Plasma Lipoprotein Preparation and Apo E-free HDL Isolation

Plasma lipoproteins were isolated and purified by ultracentrifugation as well as gel filtration chromatography according to Rudel *et al*, (1974). Blood was collected into a syringe with EDTA (1 mg/ml) and centrifuged at 1000 x g at 15°C for 20 min. Plasma was obtained and pooled from three rats to provide a 10 ml sample. The plasma density was adjusted to d 1.221 by adding KBr (0.3517 g KBr/ml plasma) and overlayered with 5 ml of d 1.225 buffered solution (EDTA 0.05 g, NaCl 5.71 g, KBr 177.20 g, and 500 ml H_2O, pH 7.4) in an ultracentrifuge tube. Plasma were centrifuged at 100,000 x g in Ti 70 rotor for 24 hours at 15°C in a Beckman Model L8-80M ultracentrifuge (Fullerton, CA). The top 2 ml containing the lipoproteins were collected and applied to agarose chromatography column. The lipoproteins were eluted at 20 ml/hour, with 0.15 M NaCl, 0.01% EDTA, 0.02% Na Azide at pH 7.4 through a single 6% agarose (A-5M Agarose, Bio-Rad Co., CA) column (25 cm x 90 cm) maintained in a cold room at 6°C. The eluate were collected at 4 ml/tube by a LKB model 2111 fraction collector (LKB Instruments Inc., Rockvill, MD) and protein concentrations were monitored at 280 nm. There were three major peaks representing VLDL, LDL, and HDL. Fraction numbers 81-90, corresponding to the apo E-poor HDL fraction, were collected and concentrated by ultrafiltration through Amicon CF-25 ultrafiltration membrane cones (Amicon

Corp. Danvers, MA 01923). The concentrated HDL were then subfractionated by using heparin-Sepharose affinity chromatography (Weisgraber and Mahley, 1980) with a modification that $MnCl_2$ was not included in the buffer. Two ml of HDL, about 10 mg lipoprotein (from 10 ml plasma) were loaded to a 1.0 x 30 cm column (Pharmacia), equilibrated overnight, and eluted with a step gradient buffer system of buffer A for fractions 1-11; buffer C for fractions 12-22; buffer D for fractions 23-35 and buffer A for fractions 36-60 at 24 ml/hour. Fractions were collected at 4 ml/tube and protein concentrations were monitored at 280 nm. Buffer A contained 0.05 M NaCl, 50 mM Tris-HCl and 0.02% Na Azde; buffer C, 0.29 M NaCl and 50 mM Tris-HCl; buffer D, 0.60 M NaCl and 50 mM Tris-HCl). Fractions 2-8 containing HDL with no detectable apo E were pooled and concentrated to 2 ml by Amicon CF-25 ultrafiltration cones. The protein concentration of apo E-free HDL was measured (Lowry *et al*, 1951). Identification of apolipoproteins was conducted by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods of Laemmli (1970). Aliquotes of 200 μg lipoproteins were brought to 200 μl and incubated with sample buffer which containing 0.05 M Tris-HCl, 1.0% SDS, 0.01% Bromphenol blue, 30.0% glycerol and 20 μl 2-mercaptoethanol per ml sample buffer for 4 hours at 37°C. The incubated samples (about 30 μg protein) and 30 μg standard

apolipoproteins were applied to a 7.5% to 20% gradient acrylamide gel. Electrophoresis was performed on a Hoefer model SE 400 vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA) with 20 mA for 5 hours. The gels were stained overnight with staining solution which contained 50% methanol, 10% acetic acid and 0.25% Coomassie brilliant blue R (Bio-Rad Laboratories, Richmond, CA) and then destained with 25% methanol, 10% acetic acid to visualize the protein bands. The lipoprotein bands were identified according to their molecular weights.

Lipoprotein Deficient Serum (LPDS) Preparation

Blood was collected by cardiac puncture from both treatments, and centrifuged at 1000 x g for 20 min to obtain plasma. The plasma was adjusted to the density 1.300 by adding KBr and capped with 5 ml of overlayering buffer (EDTA 0.05 g, NaCl 5.71 g, KBr 177.20 g, and 500 ml H_2O, pH 7.4, d 1.225). The plasma was centrifuged at 100,000 x g for 24 hours, at 15°C in a Ti 70 rotor. After that the top 3 ml containing lipoproteins were collected. The plasma was transferred to another clean tube, capped with 5 ml of overlayering buffer and centrifuged at same g force again. The lipoprotein deficient serum was transferred to a dialysis bag (VWR 25225-226) and dialyzed for two days against 0.9 g NaCl/100 ml distilled water (3 changes of 2 liters each). The

LPDS was sterilized by passing through a 0.45 μm Millipore filter (Millipore Corp., Bedford, MA) and stored in a glass bottle below -4°C until use.

Tissue Sampling and Analysis

Liver was perfused in situ with saline to remove all the blood. Liver and heart were then quickly excised and weighted. Duplicate one-gram samples from each liver and the entire heart were used for mineral analysis.

Liver and heart samples were placed into tared 18 ml Nelgene tubes and dried in an 80°C oven for three days. Five ml of concentrated HNO_3 were added to each dried sample and cooled on ice for half day. After the cold digestion, samples were vortex-mixed and placed in a hot water bath (95-100°C) for four hours. The samples were cooled and diluted to 10 ml for mineral analysis by flame atomic absorption spectrophotometry using a Hitachi model 180-70 polarized Zeeman atomic absorption spectrophotometer.

Iodination of Apo E-free HDL

Apo E-free HDL was radiolabeled with ^{125}I according to the iodine monochloride method (McFarlane, 1958) which was modified by Goldstein, Basu, and Brown (1983). All steps were performed at 0-4°C. About 3.2 mg HDL protein were reacted with two mCi $Na^{125}I$ for each iodination. ICl stock solution

was diluted with 12.5 volumes of 2.0 M NaCl prior to iodination to make final concentration 2.64 mM ICl. HDL protein (3.2 mg) was mixed with 0.5 ml 1 M Glycine-NaOH, pH 10 and brought to 0.8 ml with solution A (150 mM NaCl and 0.24 mM EDTA, pH 7.4 with NaOH). This HDL solution was mixed with 2 mCi $Na^{125}I$ in 0.4 ml glycine-NaOH. Diluted ICl solution (0.42 ml) was then injected to the mixture and incubated on ice for 5 min. Following this, the iodination mixture was brought to 2.5 ml and the entire mixture was applied to a prepacked Sephadex G-25 column, preequilibrated with 20 ml buffer A, to separate the iodinated lipoprotein from the free ^{125}I. The column was eluted with 3.5 ml buffer A. The initial 2.5 ml were discarded and the following 3.5 ml eluate were collected. Iodinated protein was transferred to a boiled dialysis tubing and dialyzed against buffer A for 24 hours (3 changes of 4 liters each). The iodinated HDL was sterilized by passing through a 0.45 μm filter, stored at 4°C and used within two weeks.

Isolation of Rat Liver Parenchymal Cells

All material must be sterile and all steps were conducted under sterile environment as much as possible. After seven weeks of dietary treatment, average body weight of Cu-adequate rats was 303 g and average body weight of Cu-deficient was 268 g. The rats were anesthetized with

diethyl ether. Rats were laid on their back and abdominal surface was wet with 70% alcohol. The abdominal skin was then removed and muscle layer was cut. The intestines were moved to the right to locate the hepatic portal vein. The perfusion needle 20 G x 3.2 cm (Travenol, Quik-Cath) was inserted into the portal vein and two sutures were tied very tightly around the needle. Prior to perfusion, a cut was made in the lower vena cava to permit free outflow. The liver was first perfused in situ through the portal vein for 3 min at a rate of 10 ml/min with warm (37°C) perfusion buffer (pH 7.4, 8.3 g NaCl, 0.5 g KCl, 2.4 g Hepes, 10 ml 50 mM EGTA [ethylene glycol-bis-(B-aminoethyl ether)N-N'-tetracetic acid] and 10 ml 10% dextrose per 1000 ml). The flow rate was increased to 35 ml/min for 3 min with same solution. The liver was cut, placed on a sieve very carefully with vena cava (outflow) face down and covered with sterile aluminum foil to decrease probability of contamination. The liver was then perfused with chelator-free buffer containing the same component as above, except with no EGTA, for 2 min at 35 ml/min. Following this, a 0.05% solution of collagenase (class II, Cooper Biomedical) was perfused for 12-15 min at 35 ml/min with recirculation via the thoracic inferior vena cava return cannula. The enzyme solution contains 0.39 g NaCl, 0.05 g KCl, 0.07 g $CaCl_2 \cdot 2H_2O$, 2.4 g Hepes, 1 ml 10% dextrose and 0.05 g collagenase in 100 ml double-distilled H_2O, pH 7.4. All

perfusion solutions were filtered through 0.20 μm Nalge filter units (Nalgene 125-0020) and kept at 37°C during perfusion. The liver was excised, trimmed and placed in 25 ml of serum-free M199 with Hank's salts solution at 4°C (Sigma, Catalog No. M0393). The solution was made by adding 3.57 g Hepes and 0.35 g $NaHCO_3$ to 1000 ml medium solution, adjusted to pH 7.4 with 1 N NaOH and filtered sterile by 0.20 μm Nalge filter units (Nalgene 450-0020). The liver was held by forceps on the connective tissue attached to the portal vein. Liver capsule was detached by combing with a sterile plastic spatula. The crude cell suspension was filtered through 250 μm nylon mesh and the volume was adjusted to 50 ml with the same solution. This initial crude cell suspension was centrifuged at 30-40 g for 4 min. The supernatant was removed and the cell pellet was resuspended with suspension solution and the cells were centrifuged again. The pellet was suspended and adjusted to 5 x 10^6 cells/ml with suspension solution. In order to get higher viabilities of cell preparation, Percoll (Pharmacia Fine Chemicals) was used since Percoll can form a self-generated gradient during the centrifugation. One part (v/v) of 1.5 M NaCl was added to nine parts (v/v) of Percoll. This mixture was neutralized with 1 N HCl to pH 7.4. Twenty-five ml of cell suspension were added to 24 ml neutralized Percoll solution, mixed and centrifuged for 10 min at 40-50 g. The pellet which

contained viable cells was collected and resuspended in suspension solution and washed 2-3 times to remove Percoll. Finally, the cell pellet was suspended with M199 with Earle's salts solution (Sigma Catalog No. M5017). The solution was made by adding 0.055 g Na pyruvate and 2.2 g $NaHCO_3$ to 1000 ml medium solution and adjusted to pH 7.4 with 1 N NaOH. The solution contained penicillin 100 units/ml, streptomycin 100 mg/ml and 10% fetal calf serum (FCS). A drop of trypan blue stain (0.4%, GIBCO Control No. 16 N 7465) was added to 200 μl diluted cell suspension, and cell number and viability were assessed on a hemocytometer within 2 min. The average of viability of cell was 70% and no difference was found between Cu-adequate and Cu-deficient rats for viability determination. The average number of total viable cell was 2.5×10^8 cells per Cu-adequate rat and 4.2×10^8 cells per Cu-deficient rat.

Approximately 1×10^7 viable parenchymal cells were added to each collagen coated well (35 mm diameter). The cells were incubated with culture medium at 37°C in a 5% CO_2 incubator (Water-Jacketed Incubator, from Forma Scientific) for 16 hours. Two sets of cells were prepared from each dietary treatment. After 16 hours of preculture, the medium was removed and the cells were transferred to the culture medium containing rat LPDS 10% v/v (LPDS from Cu-adequate rats was used for Cu-adequate cells and LPDS from Cu-deficient rats

was used for Cu-deficient cells). These cells were then used for the following studies.

Binding and Saturation Studies at 4°C

In binding studies, cells were incubated at 4°C with 5 μg/ml ^{125}I-apo E-free HDL, with or without 200 μg/ml (40-fold) excess of unlabeled apo E-free HDL for 2, 6, 12, or 24 hours. Cells were then washed four times with ice-cold phosphate-buffered saline (PBS), pH 7.4 and dissolved in 1.0 ml NaOH. The cell surface-bound radioactivity was measured by using an LKB Minigamma 1275 gamma counter (LKB Instruments, Rockville, MD). The counting efficiency of the gamma counter was 75.8% as measured by an ^{129}I standard. Four aliquotes of 10 μl of ^{125}I-labeled apo E-free HDL were measured to calculate the specific radioactivity. The cell protein was measured by Lowry assay.

In saturation studies at 4°C, various amounts of ^{125}I-labeled HDL from 1 μg/ml to 40 μg/ml were incubated with constant amount of cells (about 1 x 10^7 cells per well) for 6 hours. The cell surface-bound radioactivity and the cell protein were measured as in binding studies.

Uptake, Degradation, and Saturation Studies at 37°C

In uptake and degradation studies, cells were incubated at 37°C with 5 μg/ml ^{125}I-apo E-free HDL, with or

without excess of unlabeled apo E-free HDL (200 μg/ml) 40-fold, for 2, 6, 12, or 24 hours. The medium was removed, cells were then treated the same as in binding studies. The cell-associated radioactivity and the cell protein were measured. For the measurement of degradation, the medium (2 ml/well) was added to 0.5 ml trichloroacetic acid (50% wt/vol) and mixed well, and allowed to stand at 4°C for 30 min. The mixture was then centrifuged at 1,000 RPM for 15 min. The clear supernatant was collected and counted for radioactivity.

In uptake and saturation studies at 37°C, various amounts of ^{125}I-labeled HDL from 1 μg/ml to 40 μg/ml were incubated with constant amount of cells (about 1 x 10^7 cells per well) for periods up to 6 hours. The cell-associated radioactivity and the cell protein were measured.

Reversibility of Iodinated HDL Binding Studies

Two parallel sets of cells were incubated with 5 μg/ml ^{125}I-apo E-free HDL, with or without 40-fold excess of unlabeled apo E-free HDL (200 μg/ml) at 4°C. The first set was incubated for periods up to 24 hours to follow the time course of binding. The second set was pulsed for 6 hours, during which time the binding reaction reached equilibrium, the cells were transferred to the chase medium (without any labeled or unlabeled apo E-free HDL), and maintained at 4°C for another 6 or 12 hours. The cell-associated

(surface-bound) radioactivity was readily released into the medium. The decrease in the surface-bound radioactivity was measured and the cell protein was also determined.

RESULTS

Body and Organ Weights, and Tissue Copper Contents

The body weight was significantly reduced in rats fed the Cu-deficiency diet (Table-4). The heart weight and the heart weight expressed as percentage of body weight were markedly increased in rats from Cu-deficient treatment. A reduction in liver weight was not observed in rats fed with Cu-deficient diet, but when the liver weight was expressed as percentage of body weight, it was significantly higher than the rats fed the control diet. Also, significantly lower cardiac and hepatic Cu contents were found in rats fed the Cu-deficient diet (Table-4).

Profiles of Plasma Lipoproteins

The typical lipoprotein profiles for normal rat plasma lipoprotein obtained from agarose column chromatography are shown in Figure-1. Three major regions, which represent VLDL, LDL, and HDL, were eluted. This figure also demonstrated the effect of dietary treatment on lipoprotein profiles. No alteration in VLDL and LDL levels was observed in rats fed the Cu-deficient diet. The HDL was significantly increased, as measured by absorbance at 280 nm, in rats fed the Cu-deficient diet. The HDL peak was partitioned into three parts according

Table-4 Effect of Dietary Copper Deficiency on Various Body Measurements[a]

Measurement	Cu-Adequate	Cu-Deficient	P-value[b]
Body wt (g)	303 ± 5	268 ± 5	<0.001
Heart wt (g)	1.07 ± 0.02	1.30 ± 0.04	<0.001
Liver wt (g)	11.50 ± 0.10	11.10 ± 0.20	NS
Heart wt (g/100 g BW)	0.35 ± 0.01	0.49 ± 0.01	<0.0001
Liver wt (g/100 g BW)	3.82 ± 0.08	4.16 ± 0.11	<0.02
Heart Cu (ug/g wet wt)	6.12 ± 0.21	2.24 ± 0.18	<0.0001
Liver Cu (ug/g wet wt)	5.47 ± 0.10	1.63 ± 0.09	<0.0001

a: Mean ± SEM; n = 10

b: Student's two-tailed t-test; NS = not significant.

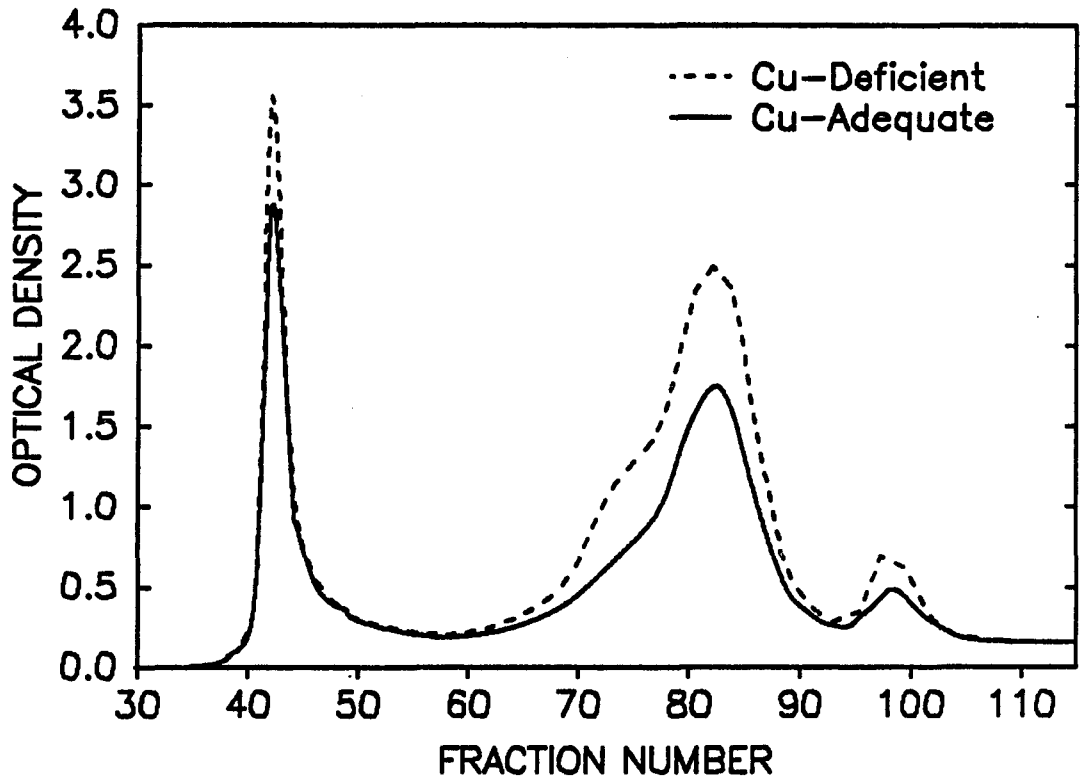

Fig.1. Separation of rat plasma lipoproteins by agarose column chromatography. Rat lipoproteins (d<1.225) from 10 ml plasma were floated by ultracentrifugation and applied to a single agarose column system (25 cm x 90 cm). The elution was conducted at 6°C and the lipoproteins were eluted at 20 ml/hour, 4 ml/tube with 0.15 M NaCl, 0.01% EDTA, 0.02% NaAzide at pH 7.4. The protein concentrations for Cu-adequate (——) and Cu-deficient (----) were monitored at 280 nm.

to our previous studies (Hassel, 1987). The first part (HDL_1) contains much apo E, and the third part (HDL_3), fraction numbers 81-90, contains less apo E. Comparison of the apolipoprotein profile of HDL demonstrated that there was higher percentage of apo E in HDL from Cu-deficient rats than from Cu-adequate rats (Hassel, 1987). Since only apo E-free HDL will be used in all experiments, the HDL_3 was collected and concentrated. The subfractionation of HDL_3 by heparin-Sepharose affinity chromatography is illustrated in Figure-2. Three HDL subfractions differing in their apo E content were obtained. Lipoproteins from the first subfraction (I), fraction numbers 2-8, which contained no detectable traces of apo E as analyzed by SDS-PAGE, were used as apo E-free HDL in all experiments.

Binding Studies at 4°C

At 4°C, the amount of cell-associated apo E-free HDL would mainly represent the amount of apo E-free HDL bound to the cell surface only (Brown *et al*, 1979). Cells were incubated with 5 μg/ml of labeled apo E-free HDL for different time periods 2, 6, 12, and 24 hours. In addition to ^{125}I-apo E-free HDL, 40-fold excess unlabeled apo E-free HDL were added to the medium to determine nonspecific binding. The specific binding data were obtained by subtracting the nonspecific from

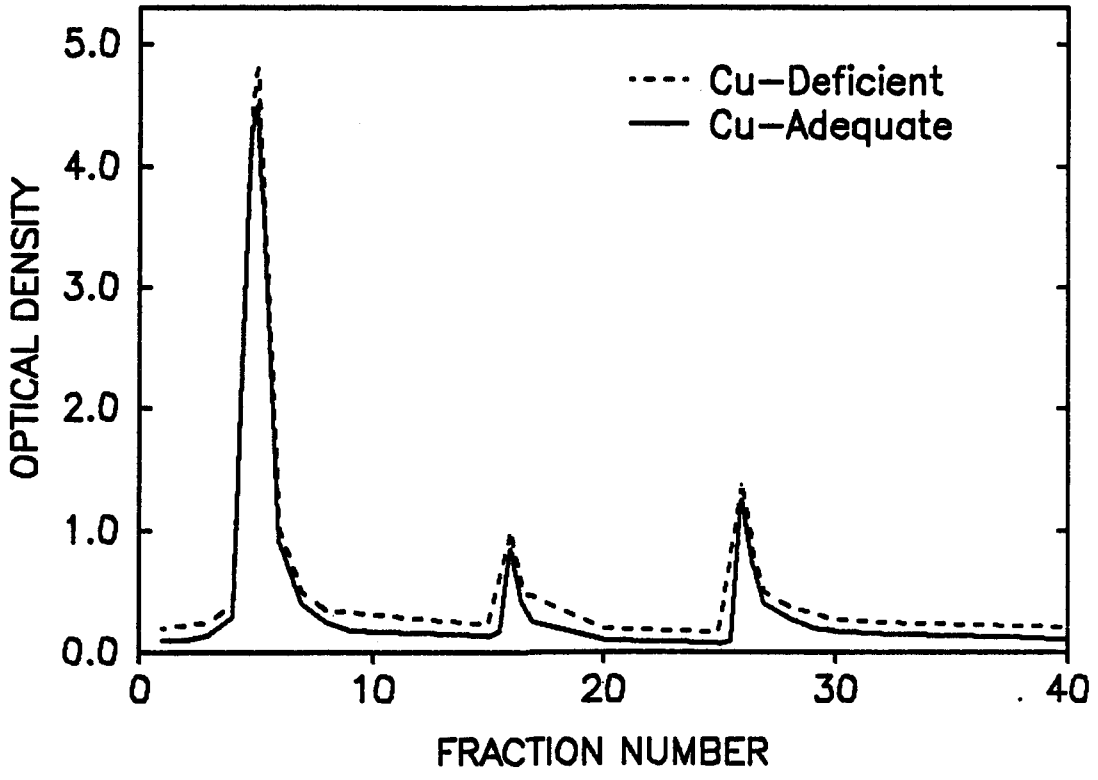

Fig.2. Subfractionation of HDL_3 by Heparin-Sepharose affinity chromatography. The HDL_3 isolated from 10 ml plasma was equilibrated in the heparin column (1.0 cm x 30 cm) overnight and eluted the following day at 24 ml/hour, 4 ml/tube using a step gradient of NaCl buffer system at 6°C. The apo E-free fractions (peak I) were eluted by 0.05 M NaCl; apo E-rich HDL (peak II) was eluted by 0.29 M NaCl; and Peak III was eluted by 0.6 M NaCl. The protein concentrations for Cu-adequate (——) and Cu-deficient (----) were monitored at 280 nm. The apolipoprotein composition was determined by 7.5-20% gradient SDS polyacrylamide gel electrophoresis.

the total binding data. The time course of specific cell-surface binding at 4°C is shown in Figure-3. Significant difference in specific binding was observed between the two treatments. At two hours, both specific binding curves appeared to have reached a plateau. The amount of surface-bound apo E-free HDL was about 55 ng/mg cell protein, which was very close to the specific surface binding of apo E-free HDL at 4°C (52 ng/mg cell protein) and trypsin-releasable apo E-free HDL at 37°C (51 ng/mg cell protein), reported by Bachorick *et al* (1985). Trypsin-releasable radioactivity has been assumed to provide a reasonably accurate measure of surface-bound lipoproteins (Bachorik *et al*, 1985).

Total Surface-Bound Saturation Studies at 4°C

Cells were incubated with increasing amounts of labeled apo E-free HDL (from 1 μg/ml to 20 μg/ml) for 6 hours and total surface-bound apo E-free HDL radioactivity was measured. The surface-bound apo E-free HDL for both treatments appeared to be saturable. No difference was observed between the two treatments (Figure-4).

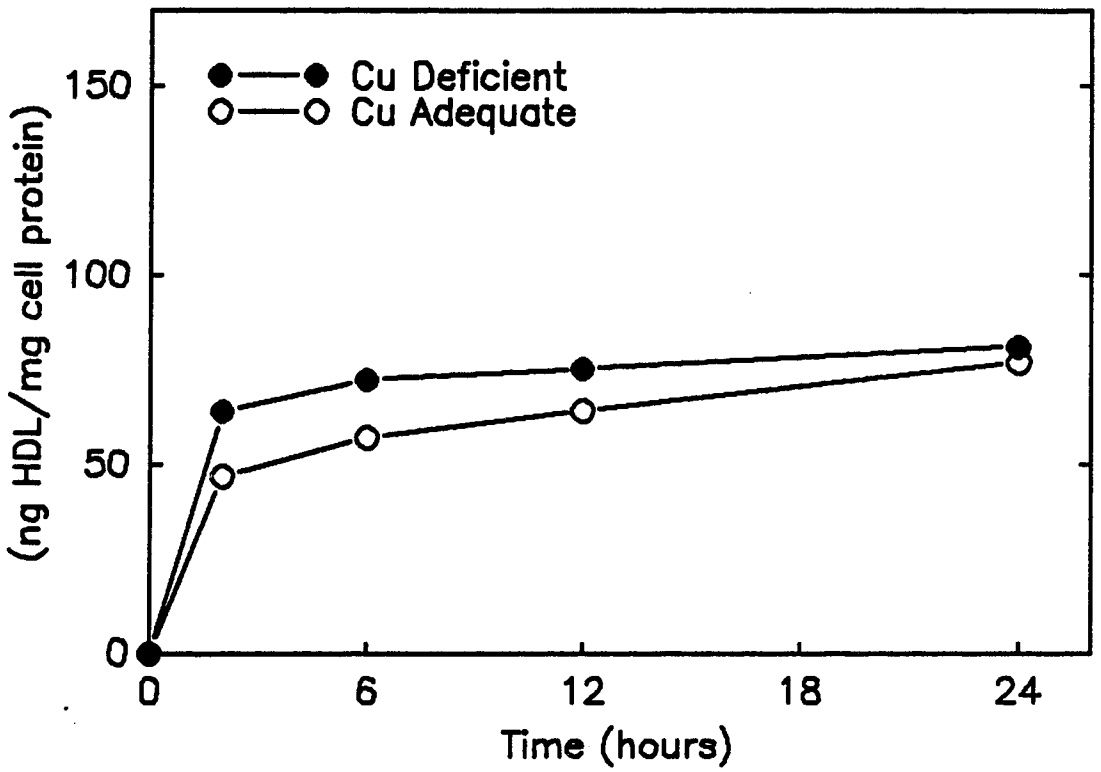

Fig.3. Time course of specific cell-surface binding of ^{125}I-apo E-free HDL in cultured rat hepatic parechymal cells at 4°C. Cells were incubated with 5 μg/ml of ^{125}I-apo E-free HDL, with or without 200 μg/ml (40-fold) excess of unlabeled apo E-free HDL for the time isolated. The specific cell surface binding was obtained by subtracting non-specific component from the total. Curve averages (n = 3 for each treatment) are shown for the Cu-adequate (O) and Cu-deficient (●) assays. Curves were analyzed by two-way analysis of variance.

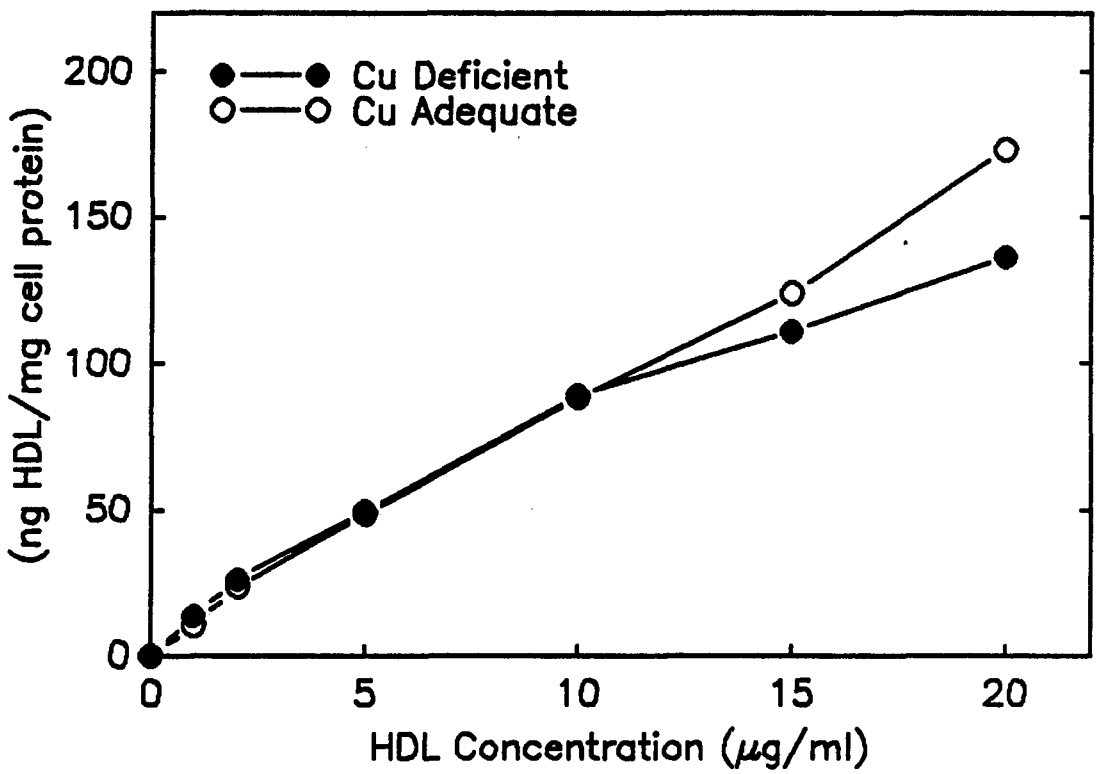

Fig.4. Total surface-bound of ^{125}I-apo E-free HDL at 4°C. Cells were incubated with increasing amounts of labeled apo E-free HDL (from 1 to 20 μg/ml) for 6 hours. Curve average (n = 3 for each treatment) are shown for Cu-adequate (O) and Cu-deficient (●) assays. Curves were analyzed by two-way analysis of variance.

Uptake and Degradation Studies at 37°C

The total cell-associated uptake of apo E-free HDL at 37°C would represent both surface-bound HDL and that which had been internalized (Figure-5). The cells were incubated with 5 μg/ml labeled apo E-free HDL and incubation was terminated at various intervals 2, 6, 12, and 24 hours. The cell-associated radioactivities were measured. A significant elevation of total uptake of apo E-free HDL was observed in cells derived from Cu-deficient rats. By 6 hours, these curves have almost reached a steady state. In addition to ^{125}I-apo E-free HDL, 40-fold excess unlabeled apo E-free HDL were added to the culture medium to provide the nonspecific uptake curves. No difference in the nonspecific curves were observed between treatments. The difference in uptake of apo E-free HDL can be seen more clearly when the nonspecific component is subtracted from the total to provide the specific uptake of apo E-free HDL (Figure-6). At six hours, the specific uptake appeared to have reached steady state (150 ng/mg cell protein). This value agreed well with equilibrium cell-associated data obtained at 37°C (120 ng/mg cell protein) by Bachorik and co-workers (1985). A significantly higher specific uptake of apo E-free HDL was observed in cells from Cu-deficient than from Cu-adequate rats. The difference was most prominent at or after 6 hours.

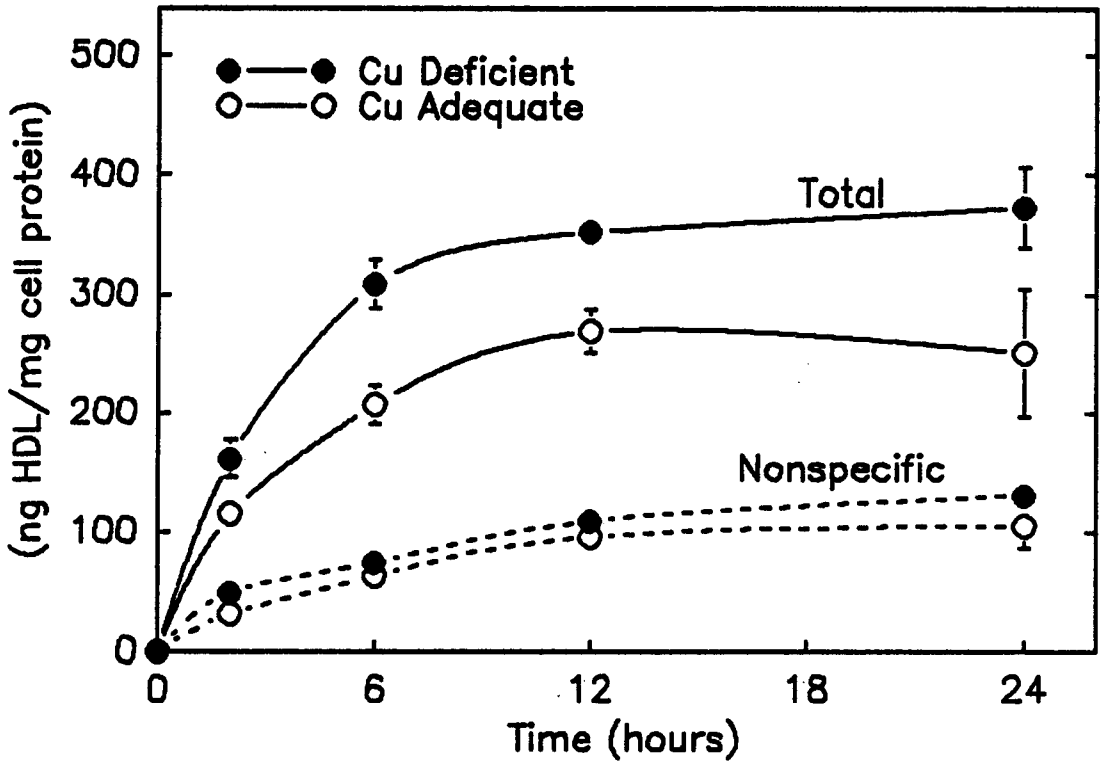

Fig.5. Time course of ^{125}I-apo E-free HDL of total uptake at 37°C. Cells were incubated with 5 μg/ml of ^{125}I-apo E-free HDL with or without 200 μg/ml (40-fold) excess of unlabeled apo E-free HDL for the time indicated. Curve averages (n = 3 for each treatment) are shown for the Cu-adequate (O) and Cu-deficient (●) assays. Curves were analyzed by two-way analysis of variance.

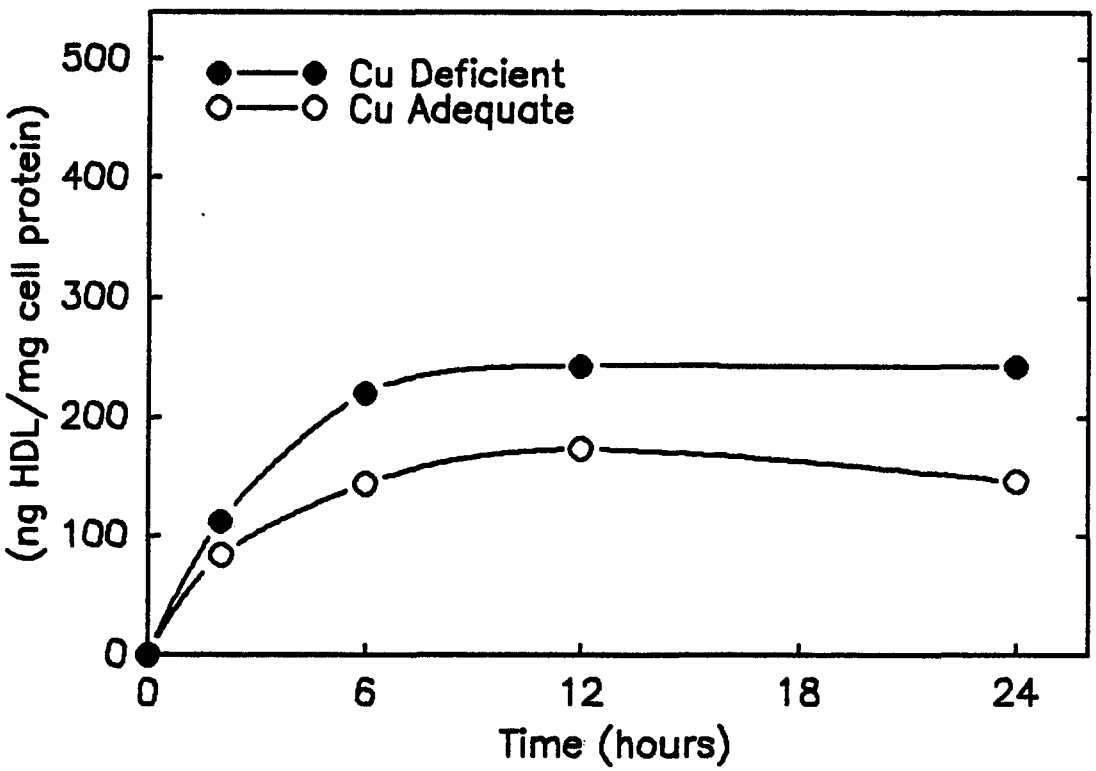

Fig.6. Time course of specific cell-associated uptake of ^{125}I-apo E-free HDL in cultured rat hepatic parenchymal cells at 37°C. Cells were treated exactly the same as in Fig. 5. The specific uptake of ^{125}I-apo E-free HDL were obtained by subtracting the non-specific component from the total. Curve averages (n = 3 for each treatment) are shown for the Cu-adequate (O) and Cu-deficient (●) assays. Curves were analyzed by two-way analysis of variance.

After the incubation for the varying time periods, the medium was added to 0.5 ml trichloroacetic acid (50 % wt/vol) and allowed to stand at 2 to 4°C for 30 min. Degradation was calculated from the measurements of acid-soluble radioactivity. Degradation from both treatment medium appeared to be linear with time (Figure-7). At 6 or 12 hour incubation time point, the degradation of ^{125}I-apo E-free HDL determined from Cu-deficient cell medium appeared to be slightly higher than from Cu-adequate cell medium. However, no significant difference in degradation was observed between the two treatments.

Total Uptake Saturation Studies at 37°C

The results of total uptake saturation study performed at 37°C are shown in Figure-8. Cells from deficient and adequate rats were incubated with increasing amounts of labeled apo E-free HDL (from 1 μg/ml to 40 μg/ml) for 6 hours and total cell-associated apo E-free HDL radioactivity was measured. The apo E-free HDL uptake for both treatments appeared to be saturable. The saturation curve is significantly elevated in the Cu-deficient than in the adequate treatment. The differences were most prominent from 2 μg/ml labeled HDL on.

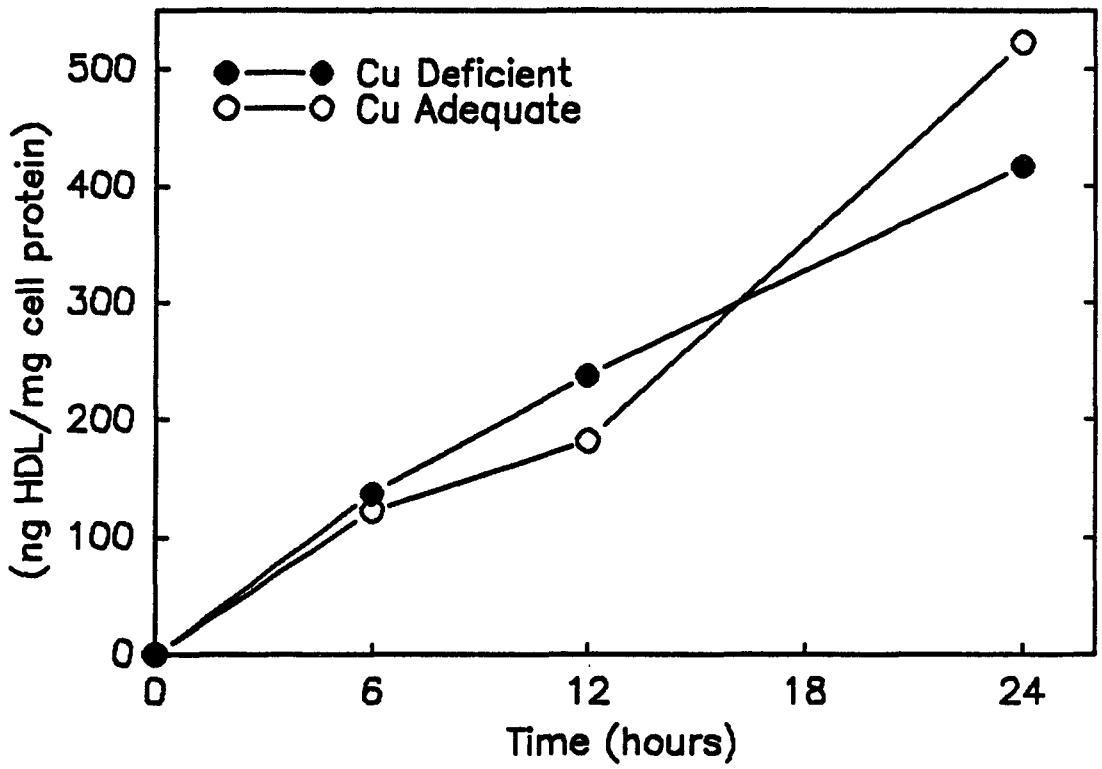

Fig.7. Time course of ^{125}I-apo E-free HDL of specific degradation at 37°C. Cells were treated exactly the same as in Fig. 5. The medium was added to 0.5 ml TCA (50% w/v) and allowed to stand at 2 to 4°C for 30 min. Degradation was calculated from the measurements of acid-soluble radioactivity.

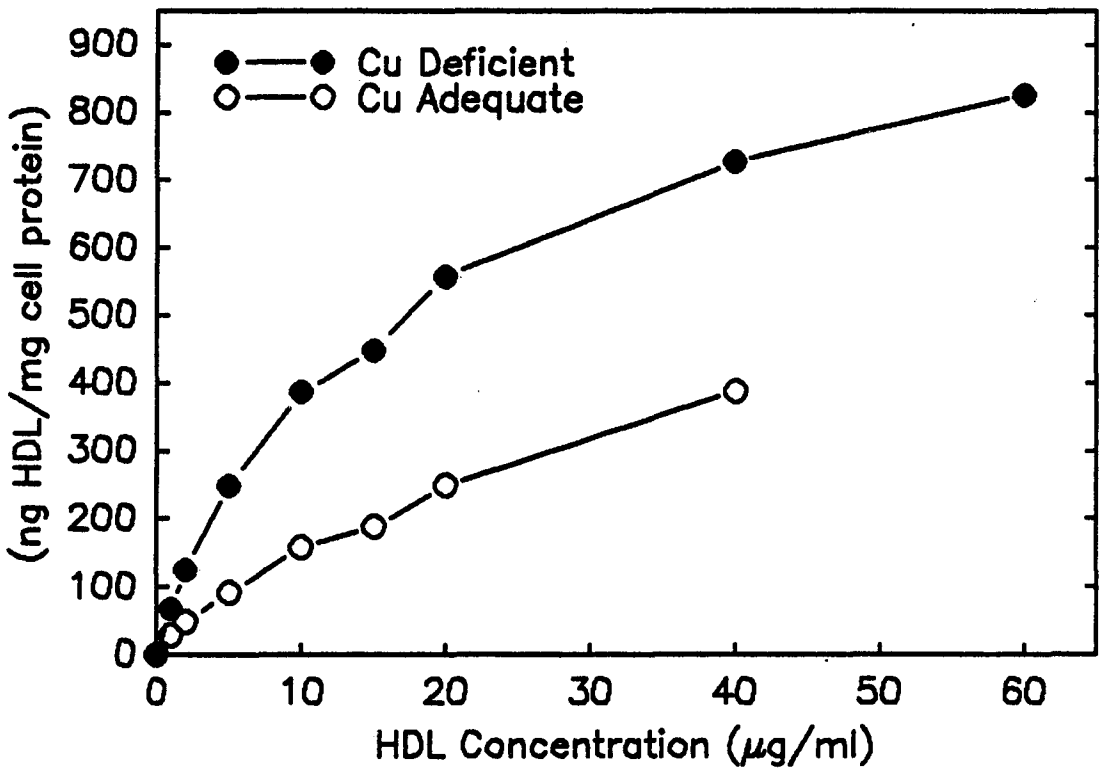

Fig.8. Total uptake of ^{125}I-apo E-free HDL at 37°C. Cells were incubated with increasing amount of labeled apo E-free HDL (from 1 to 40 μg/ml) for 6 hours. Curve averages (n = 3 for each treatment) are shown for Cu-adequate (O) and Cu-deficient (●) assays. Curves were analyzed by two-way analysis of variance.

Reversibility of ^{125}I-Apo E-free HDL Binding at 4°C

The reversible binding of apo E-free HDL at 4°C was shown in Figure-9. Since both the pulse and the chase were conducted at 4°C, the uptake and release of ^{125}I-apo E-free HDL was presumed to be associated with the cell surface. The amount ^{125}I-apo E-free HDL released from the cell surface after an 18 hours chase accounted for almost one-half of that observed when chase was initiated. No difference in reversibility of HDL binding was observed between the two treatments.

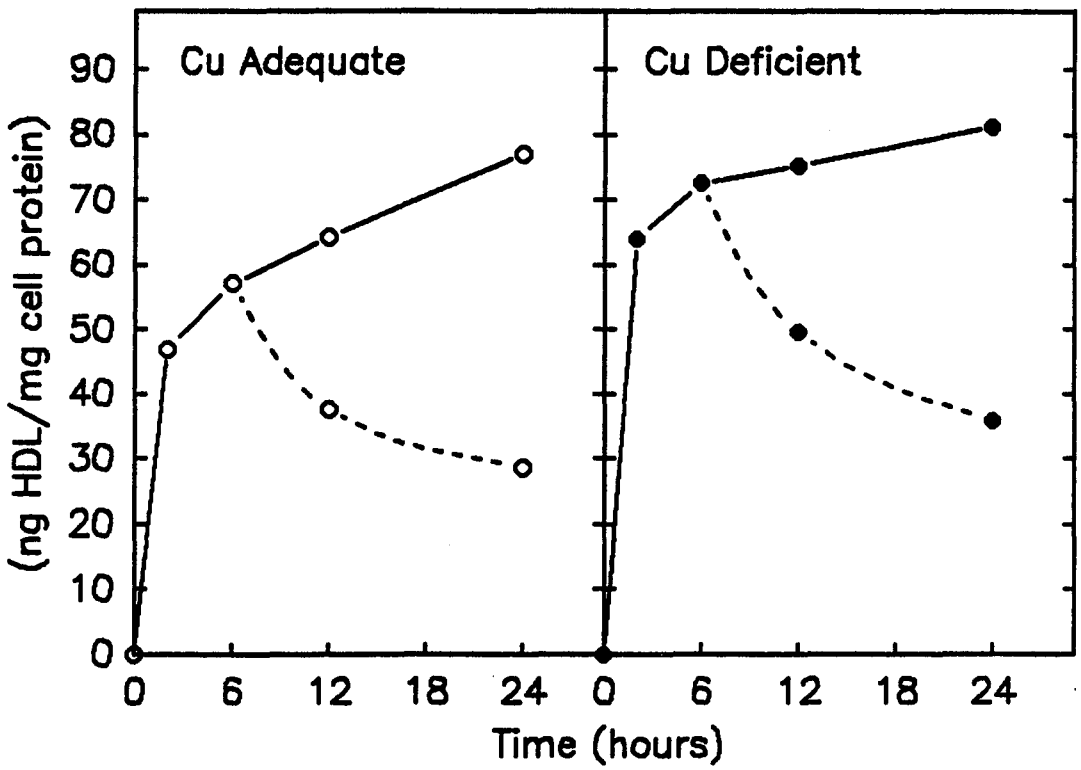

Fig.9. Reversibility of ^{125}I-apo E-free HDL binding at 4°C. Two parallel sets of cells were incubated with 5 μg/ml of ^{125}I-apo E-free HDL, with or without 200 μg/ml (40-fold) excess of unlabeled apoE-free HDL. The first set was incubated for up to 24 hours to provide the time course of binding. The second one was pulsed for 6 hours, then transfered to the chases medium (without any labeled or unlabeled HDL). The decrease in the surface-bound radioactivity was measured.

DISCUSSION

Depressed growth (Shao and Lei, 1980), decreased body weight gain (Underwood, 1977), cardiac hypertrophy (Hill, 1969), increased heart weight and heart to body weight ratio (Hill, 1969), as well as liver to body weight and decreased liver copper content (Lei, 1977) are well established observations of Cu deficiency in various animal species. All of these symptoms of Cu deficiency were present in rats fed the Cu-deficient diet in all experiments.

Low density lipoprotein (LDL) metabolism has been studied in various types of cultured cells (Goldstein *et al*, 1983). A receptor-mediated pathway was elucidated in human fibroblasts. In this pathway LDL is bound to a specific cell surface receptor, internalized, transported to lysosome, and degraded (Goldstein and Brown, 1977; Brown *et al*, 1979). Less is known about the metabolism of HDL. HDL participates in the esterification of cholesterol during the initial stages of the catabolism of triglyceride-rich lipoprotein (Brown *et al*, 1981) and may also function in part to transport cholesterol from peripheral tissues back to the liver. In some cultured cells, HDL has been found to stimulate the removal of cholesterol

(Glomset, 1968; Stein *et al*, 1975; Ho *et al*, 1981; O'Mally *et al*, 1981).

The liver is very important in the metabolism of LDL. High affinity uptake and degradation of LDL have been observed in freshly isolated rat (Van Berkel *et al*, 1981) and rabbit hepatic parenchymal cells (Soltys and Portman, 1979). The degradation of ^{125}I-LDL by an LDL receptor-mediated mechanism was observed in cultured swine hepatic parenchymal cells (Pangburn *et al*, 1981). The liver is also a major site of HDL metabolism. Several studies suggested that most of the catabolism of HDL apolipoproteins occurs extrahepatically (Van Tol *et al*, 1986). However, other studies indicate that the liver may contribute significantly to the catabolism of HDL. The liver accumulates significant amount of HDL (Eisenberg *et al*, 1984), and they are associated primarily with parenchymal cells (Rachmilewitz *et al*, 1972). HDL is bound to high-affinity sites on the plasma membrane of suspended rat parenchymal and nonparenchymal cells, the bound HDL will be subsequently processed by lysosomal degradation (Ose *et al*, 1981; Nakai *et al*, 1976; Van Berkel *et al*, 1980). High affinity uptake and degradation of HDL apolipoprotein have been observed in rat (Nakai *et al*, 1976; Van Berkel *et al*, 1980) and pig hepatic parenchymal cells (Bachorik *et al*, 1982). Since the low density lipoprotein (LDL) receptor recognizes apo E as well as apo B (Goldstein and Brown, 1977; Innerarity *et al*,

1980), but not apo A-I or apo A-II (Mahley and Innerarity, 1983), apo E-free HDL apolipoprotein was used to examine the high affinity uptake and lysosomal degradation of HDL in cultured hepatic parenchymal cells. The degradation was found to be mediated by a high affinity site with properties different than either the LDL receptor or the apo E-receptor (Bachorik *et al*, 1982). The present study was designed to investigate the binding, uptake, and degradation of HDL in Cu deficient rats by using apo E-free HDL to exclude the contribution of other receptors which interact with HDL containing apo E.

The HDL of Cu-deficient rats were reported to be enriched with apo E (Lei, 1983; Croswell and Lei, 1985). Apo E enrichment in HDL was also established by SDS-PAGE analyses of apolipoproteins in this study.

The binding study performed at 4°C, (Figure-3) provided the specific cell-surface binding data for the two treatments. At 4°C, the amount of cell-associated apo E-free HDL was assumed to represent solely the cell-surface bound HDL. Significant difference was observed in cells derived from Cu-deficient and Cu-adequate rats. The maximum amount of specific binding of HDL in this study was about 55 ng HDL/mg cell protein. Similar binding data were obtained at 4°C, which was 52 ng/mg cell protein in pig hepatic parenchymal cells (Bachorik *et al*, 1985). To distinguish cell

surface bound from internalized ^{125}I-apo E-free HDL, Bachorik *et al* (1985) studied cells incubated with ^{125}I-HDL in the presence or absence of excess unlabeled HDL at 4°C or 37°C and then cells were treated with trypsin for 10 min at 37°C (trypsin was assumed to digest surface bound lipoprotein). Their results indicated that at equilibrium, similar amounts of ^{125}I-apo E-free HDL were bound at both temperatures. It is interesting to note that the amount of specific surface bound HDL at steady state at 4°C observed in this study was also similar to the amount of trypsin-releasable ^{125}I-HDL reported by Bachorik (1985).

The total surface-bound saturation study was also performed at 4°C (Figure-4). Increasing amounts of ^{125}I-apo E-free HDL (1 to 20 μg/ml) were incubated with hepatic parenchymal cells from the both treatments. Total surface-bound HDL was measured in the absence of unlabeled HDL. Bachorik *et al* (1982) used higher concentration range of ^{125}I-apo E-free HDL (up to 80 μg/ml) in the saturation study and demonstrated binding saturability at 4°C. However, the lower levels of ^{125}I-apo E-free HDL used in this study did not demonstrate complete saturability. The total surface-bound curve of Cu-deficient cells appeared to approach saturability but the curve of the adequate cells appeared to be linear within the range of iodinated lipoprotein concentration used. Thus saturability was not fully apparent in the lower HDL

concentration range used in the present study. Furthermore, no difference was observed between the two treatments.

The uptake and degradation studies performed at 37°C (Figure-5) provided cell association data which represented the sum of HDL bound to cell surface as well as HDL internalized. The total cell-associated uptake of HDL had almost reached a steady state by 6 hours for both treatments. The maximum amount of total uptake of labeled apo E-free HDL was about 240 ng HDL/mg cell protein in the Cu adequate cells. This observation is very similar to the uptake of ^{125}I-apo E-fre HDL observed in pig hepatic parenchymal cells at 37°C (Bachorik *et al*, 1982). In addition, the maximum amount of specific cell-associated uptake was about 150 ng HDL/mg cell protein in Cu-adequate cells. This value is very similar to the amount of specific cell-associated apo E-free HDL (120 ng HDL/mg cell protein) in equilibrium observed in pig hepatic parenchymal cells at 37°C (Bachorik *et al* 1985). By comparing the specific binding data at 4°C (Figure-3) and the specific uptake data at 37°C (Figure-6), the results indicated that the maximum binding at 4°C was about 37% of the steady state uptake at 37°C. This result is similar to those in cultured pig hepatic parenchymal cells in which surface binding accounted for about 43% of the steady state uptake of HDL (Bachorik, 1985). However, Nakai (1976) reported that the

surface binding was about 60% of the steady state uptake of HDL in freshly isolated rat hepatic parenchymal cells.

In the present study, significant increases in total and specific uptake of HDL were observed in hepatic parenchymal cells from Cu-deficient rats. Furthermore, a marked elevation in the total uptake saturable curve was also obtained in hepatic parenchymal cells from Cu-deficient rats. Therefore, under these *in vitro* conditions, total and specific cell-associated uptake as well as saturable uptake were found to be significantly increased in the hepatic parenchymal cells from Cu-deficient rats. Although the cell-associated data obtained at 37°C represents the sum of surface-bound and internalized lipoproteins, the stable binding data observed at 4°C for both treatments would indicate that the increased total and specific cell-associated uptake of HDL observed in Cu-deficient cells was due mainly to an increase in the amount of HDL internalized. In view of the amount of HDL internalized was increased and the amount bound was relatively unaltered, the hyperlipoproteinemia associated with Cu deficiency appeared not to be caused by an impairment of the hepatic HDL degradation process.

In the study of reversibility of ^{125}I-apo E-free HDL binding at 4°C, the uptake and release of ^{125}I-HDL was presumed to be from the cell surface. In view of the absence of unlabeled HDL in the chase medium, the loss of radioactivity

from the cells would represent the actual release of ^{125}I-HDL, rather than the exchange of labeled apo A-I with unlabeled HDL. In the present study, the binding of ^{125}I-HDL to rat liver parenchymal cell surface appeared to be mostly reversible. Similar observations were reported by Bachorik *et al* (1985) for pig hepatic parenchymal cells.

SUMMARY

Binding, uptake, and degradation studies were performed to examine whether hyperlipoproteinemia associated with Cu deficiency was resulted from a alteration in the hepatic uptake of HDL. Heparin-Sepharose affinity chromatography was used to isolate apo E-free HDL to exclusively study the interaction of HDL and HDL receptors on the hepatic parenchymal cell surface. Hepatic parenchymal cells were isolated from Cu-deficient and Cu-adequate rats and cultured for 16 hours at 37°C prior to incubation with iodinated apo E-free HDL. In binding studies performed at 4°C and uptake studies carried out at 37°C, the cells were incubated with 5 μg ^{125}I-apo E-free HDL, in the presence or absence of 40-fold unlabeled apo E-free HDL, for 2, 6, 12, and 24 hours. After incubation, cell-associated radioactivity was measured and the medium was removed and treated with trichloroacetic acid (TCA). Degradation was calculated from the radioactivity of the TCA soluble fraction. In saturation studies performed at 4°C or 37°C, the cells were incubated with various amount of labeled ^{125}I-apo E-free HDL (from 1 μg/ml to 40 μg/ml) for 6 hours. The results of four separate binding and uptake studies are as follows:

(1) A slight, but statistically significant, increase in surface-bound apo E-free HDL was observed in cultured hepatic parenchymal cells from Cu-deficient rats.

(2) A significant increase in total or specific cell-associated uptake of apo E-free HDL was observed in cultured hepatic parenchymal cells from Cu-deficient rats.

(3) The increase in total or specific cell-associated uptake of HDL observed in Cu-deficient treatment appeared to be due to an increase amount of HDL internalized.

(4) The cell-surface binding appeared to be mostly reversible and the amount released was similar for both treatments.

Additional studies are currently in progress to further characterize the hepatic uptake and degradation of HDL subfractions in Cu deficienct rats.

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