Copper deficiency increases hepatic parenchymal cell's maximal binding capacity and impairs Kupffer cell's internalization of apolipoprotein E-free high density lipoprotein in rats

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The binding and internalization of apolipoprotein (apo) E-free high density lipoprotein (HDL) by hepatic parenchymal and Kupffer cells were examined with cells and HDL derived from rats fed copper (Cu)-deficient (11 nmol/g) and -adequate (126 nmol/g) diets. After 8 weeks of dietary treatment, plasma apo E-free HDL was isolated by a combination of ultracentrifugation, gel filtration, and heparin-Sepharose affinity chromatography. Liver parenchymal and Kupffer cells were obtained by collagenase perfusion and purified by centrifugal elutriation. Freshly isolated cells were incubated with ^{125}I-apo E-free HDL, either from the same treatment group or in a crossover design, to establish if treatment differences were associated with cells, HDL, or both. Binding studies performed at 0° C with increasing apo E-free HDL concentrations demonstrated increases in specific binding and maximum binding capacity (B_{max}) in parenchymal cells from Cu-deficient rats. In addition, cell association studies at 37° C indicated that the amount of apo E-free HDL bound to the cell surface (trypsin releasable) was greater, but the amount internalized (trypsin resistant) was not altered in parenchymal cells from Cu-deficient rats. In contrast, the amount of apo E-free HDL internalized was reduced and that bound to the cell surface was unaltered in Kupffer cells from Cu-deficient rats. Thus Cu deficiency may exert different effects on HDL metabolism in hepatic parenchymal and Kupffer cells. Furthermore, the crossover design demonstrated for the first time that the source of cells from Cu-deficient rats, not HDL, was responsible for the enhanced B_{max} and altered internalization. The results of the present study also support the contention that the hypercholesterolemia associated with the copper-deficient rat model is not the result of decreased HDL uptake by the liver.

Keywords: copper deficiency; apo E-free HDL; liver parenchymal cells; Kupffer cells

Introduction

The hypercholesterolemia induced by copper (Cu) deficiency has been observed in many species including humans.[1] In rats, numerous studies have been performed to examine the possible mechanisms responsible for the hypercholesterolemia in Cu deficiency. In Cu-deficient rats, elevations in protein and cholesterol contents of high (HDL) and low density lipoproteins (LDL), as well as triglyceride level of LDL[2,3] were observed in the enlarged plasma volume.[3,4] Because in the rat about 85% of plasma cholesterol is normally transported in HDL, most of the increase in plasma cholesterol was associated with HDL, in particular the apolipoprotein E (apo E)-rich HDL of Cu-deficient rats.[5,6] Within the apo E-rich subclass of HDL, the level

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of apolipoproteins AI and E (apo A-I, apo E) were also elevated in Cu deficiency.[5,6] In addition, Cu deficiency exerted little or no influence on the rate of bile acid synthesis[7] and biliary sterol excretion.[7,8] However, increases in hepatic 3-hydroxy-3-methylglutaryl (HMG) CoA reductase activity[9] and de novo cholesterol synthesis[10] were established in Cu-deficient rats. Furthermore, a two-fold increase in hepatic uptake of cholesteryl ester from plasma HDL, accompanied by only a small increase in hepatic uptake of HDL apolipoproteins, was observed in Cu-deficient rats.[4] In spite of these increases in cholesterol supply, the hepatic cholesterol content of Cu-deficient rats remained depressed.[4,7,9] This depression may have resulted from a prolonged small net efflux of cholesterol, secreted mainly as very low density lipoprotein (VLDL) from the liver to the plasma, resulting in hypercholesterolemia. Indeed, a greatly accelerated secretion of nascent cholesteryl ester, newly synthesized from mevalonate, was observed from the liver to plasma in Cu-deficient rats.[11] Although previous studies have established that hypercholesterolemia in Cu-deficient rats appears to be sustained by an enhanced hepatic cholesterol synthesis and secretion into the plasma, the mechanism responsible for the increase in hepatic uptake of plasma HDL cholesteryl ester has not been examined. The present study is designed to establish that liver cells derived from Cu-deficient rats may exhibit increased maximal binding capacity for HDL, which would provide the opportunity for an enhanced hepatic uptake of plasma HDL cholesteryl ester.

Available data indicate that the liver is a major site of uptake and catabolism of HDL cholesteryl ester and apolipoproteins, probably in the liver parenchymal cells.[12–15] A greater uptake of HDL cholesteryl ester than apolipoproteins has been observed in vivo by the liver and a variety of other tissues[15] as well as in vitro by rat hepatocytes,[15–17] human Hep G2 hepatoma cells,[16,18] and other cell types.[15–17,19] Liver perfusion studies also demonstrate a four- to six-fold higher uptake of HDL cholesteryl ester than that of the protein moiety and further suggest that a fraction of the initially bound HDL is eventually released as smaller particles depleted of core cholesteryl ester.[20,21] In view of the enhancement of in vivo uptake of HDL cholesteryl ester by the liver[4] and in vitro apo E-rich HDL binding to liver plasma membranes[22] as a result of Cu deficiency, the present study was designed to examine the influence of Cu deficiency on the processes of binding and internalization of apo E-free HDL by isolated liver parenchymal and Kupffer cells. Because apo E-containing HDL can bind to a chylomicron remnant or apo E receptor[23] as well as the LDL or apo B, E receptor,[24] apo E-free HDL was selected to eliminate the contribution of these receptors to the overall HDL binding provided solely by the HDL high affinity sites. The present study demonstrated for the first time an increased B_{max} and impaired internalization in parenchymal and Kupffer cells, respectively, as a result of Cu deficiency. In addition, the crossover design also established that the source of cells, but not HDL, was responsible for these changes.

Methods and materials

Materials

Albumin (Fraction V, fatty acid free), collagenase type I, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO USA). Heparin-Sepharose CL 4B was obtained from Pharmacia (Woerden, The Netherlands). ^{125}I (carrier-free) in NaOH was purchased from Amersham (Arlington Heights, IL USA). Ham's F-10 medium was obtained from Gibco-Europe (Breda, The Netherlands).

Animals and diets

Thirty weanling male Sprague-Dawley rats were randomly allotted into two dietary treatments (Cu-deficient and adequate). The basal diet (Cu-deficient) was prepared according to the American Institute of Nutrition specifications,[25] except glucose monohydrate was used as the carbohydrate source and no Cu supplement was included in the mineral mix. It contained 11 nmol of Cu/g of diet as measured by atomic absorption spectrophotometry[26] using certified reference standards (Fisher Scientific Co., Los Angeles, CA USA). The adequate diet was prepared by adding $CuCO_3$ to the basal diet to attain 126 nmol of Cu/g. Diet and distilled-demineralized water were provided ad libitum.

Rats were housed in pairs in plastic cages with raised stainless-steel wired floors in a laboratory maintained at 22° C with a 12-hr light cycle. After 8 weeks of dietary treatment, rats were fasted for 12 hr and used for lipoprotein or liver cell preparations. In addition, livers were excised from different groups of rats and liver Cu contents were determined by atomic absorption spectrophotometry.[26]

Experimental design for in vitro incubations

A 2 × 2 factorial design, involving four different combinations of cells and HDL from Cu-deficient and -adequate rats was used. The four types of incubation were: (1) cells and HDL from deficient rats, (2) cells from deficient rats and HDL from adequate rats, (3) cells from adequate rats and HDL from deficient rats, and (4) cells and HDL from adequate rats. This design was selected because it would identify whether the source of the cells or HDL contributed to the alterations in binding or uptake.

Cell-isolation procedure at 37° C

Liver cells, namely parenchymal and Kupffer cell preparations, were isolated by perfusion of the rat liver in situ with collagenase (0.5 g/L) and purified by centrifugal elutriation using the method of Nagelkerke et al.[27] Nycodenz was used instead of metrizamide in the density gradient centrifugal purification of Kupffer cells. Trypan blue exclusion tests indicated that viability of parenchymal and Kupffer cells was greater than 95%. Light and electron microscopy analyses[28] demonstrated that on average the purity of preparations for parenchymal and Kupffer cells was greater than 99% and 80%, respectively.

Lipoprotein isolation and iodination

Rats were exsanguinated under ether anesthesia. Blood was collected into EDTA (3 mmol/L final concentration) by heart puncture and plasma obtained by low-speed centrifugation. Plasma lipoprotein was obtained by ultracentrifugal flotation and HDL was separated and purified by agarose column chromatography as previously described[5] using the method of Rudel et al.[29] HDL obtained by this method was not subjected to repeated and prolonged ultracentrifugation and was not contaminated by albumin and other lipoprotein fractions. The apo E-free HDL was obtained by heparin-Sepharose affinity chromatography as previously described[22] using the method of Weisgraber and Mahley[30] without the use of $MnCl_2$ in the equilibration and elution buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis (SDS-PAGE)[31] indicated that apart from being free of apo B, as in the apo E-rich HDL, the apo E-free HDL was also devoid of apo E. Human LDL ($1.024 < d < 1.055$) was isolated by two repetitive differential ultracentrifugations using the method of Redgrave et al.[32] SDS-PAGE analysis indicated that the protein of LDL consisted only of apo B. These lipoproteins were filter-sterilized with 0.45 μm filters and stored at 4° C.

The apo E-free HDL isolated were radiolabeled by the iodine monocholoride method[33] as modified by Goldstein et al.,[34] filtered sterilized (0.45 μm), and stored at 4° C. Radiolabeled HDL preparations from each treatment were similar in specific activity and distribution of label among apolipoproteins, as well as containing <5% trichloroacetic acid (TCA)-soluble and lipid-associated radioactivity. The amount of ^{125}I-apo E-free HDL used, ranging from 2–50 μg/mL, was similar to that used by other investigators and was within the range found in the plasma (624 μg/mL).[5] Protein content of lipoprotein fractions were determined by the method of Lowry et al.[35] using bovine serum albumin as a standard. Plasma cholesterol level was determined by the methods of Braun et al.[36]

HDL binding assay

Freshly isolated parenchymal liver cells were suspended in Ham's F-10 medium, containing 0.3 mmol/L bovine serum albumin buffered with 6.7 mmol/L Hepes to pH 7.5 (buffer A). Binding incubations were performed in plastic centrifuge tubes in a total volume of 0.2 mL of buffer A, containing 2×10^6 cells, and with various amounts of ^{125}I-apo E-free HDL in the absence or presence of a 40-fold excess of unlabeled ligand. The mixture was gently mixed on a vortex mixer every 15 min during the 90 min incubation on ice. One mL of ice-cold buffer A was added to each tube at the end of incubation. Cells were pelleted by centrifugation and washed three times with buffer A prior to counting for cell associated (surface bound) radioactivity. Corresponding blanks were established with the incubation of HDL in the absence of cells. Total and nonspecific binding curves were determined in the absence or presence of 40-fold excess unlabeled apo E-free HDL. The difference between total and nonspecific binding provided the specific binding. Scatchard analyses[37] of the specific binding curves provided the binding constants K_d (binding affinity) and B_{max} (maximum binding capacity).

Trypsin treatment

Liver parenchymal and Kupffer cells (15×10^6) were incubated at 37° C with ^{125}I-apo E-free HDL (50 μg as protein) in 2.5 mL of buffer A. HDL was incubated in the absence of cells to provide the corresponding blanks. Two 200 μL aliquots were removed at each time interval. Cells were washed three times with 1 mL of ice cold Hanks' solution to remove unbound ligands. The washed cell pellet from one aliquot was used to determine total cell-associated radioactivity. The cell pellet from the other aliquot was resuspended and treated with 0.5 mL of trypsin (4 g/L) for 45 min on ice by the method of Oram et al.[38] Thereafter, cells were pelleted by centrifugation, resuspended, and washed three times in 0.8 mL of ice-cold buffer A. The supernatants were combined and radioactivity counted to provide the trypsin releasable or surface bound ^{125}I-apo E-free HDL. The radioactivity retained by the cells after trypsin treatment and repeated washing provided the trypsin-resistant component or the amount of HDL internalized.

Table 1 Body and tissue measurement of copper-deficient and adequate rats

Measurement	Control	Cu-deficient	*P* value
Body weight (g)	280 ± 4	267 ± 4	<0.05
Hematocrit (vol. fraction)	0.49 ± 0.07	0.42 ± 0.01	<0.001
Plasma cholesterol (mmol/L)	2.61 ± 0.03	2.92 ± 0.10	<0.005
Liver Cu (nmol/g wet weight)	70.2 ± 3.3	35.0 ± 4.3	<0.01

Weaning male rats were fed either the Cu-deficient or adequate diet for 8 weeks. Data are presented as mean ± SEM and analyzed by Student's two-tailed *t* test. With the exception of liver Cu when $n = 5$, all other measurements are $n = 18$.

Statistical analyses

Body and tissue data were analyzed by Student's two-tailed *t* test. Treatment differences for the rest of the results were tested by analysis of variance.[39]

Results

Body and tissue measurements

Reductions in body weight, hematocrit, and liver Cu concentration, as well as an increase in plasma cholesterol levels were observed in rats fed the Cu-deficient diet *(Table 1)*. Because these are well-established observations of Cu deficiency, the rats fed the Cu-deficient diet in the present study were indeed Cu deficient.

Binding of apo E-free HDL to liver parenchymal cells

A representative binding curve performed at 0° C as a function of extracellular ^{125}I-apo E-free HDL concentration with HDL and liver parenchymal cells derived from Cu-adequate rats is depicted in *Figure 1*. The overall total binding response was depressed about 55% in the presence of a 40-fold excess unlabeled apo E-free HDL, as demonstrated in the nonspecific binding curve. The specific binding, derived from the difference between total and nonspecific binding, demonstrated a typical saturation curve indicating the presence of a binding site specific for apo E-free HDL *(Figure 1)*. Analysis of the specific binding curves obtained from cells and HDL derived from Cu-adequate rats by Scatchard plot provided straight line response, indicating the presence of a binding site or groups of binding sites with a similar affinity.

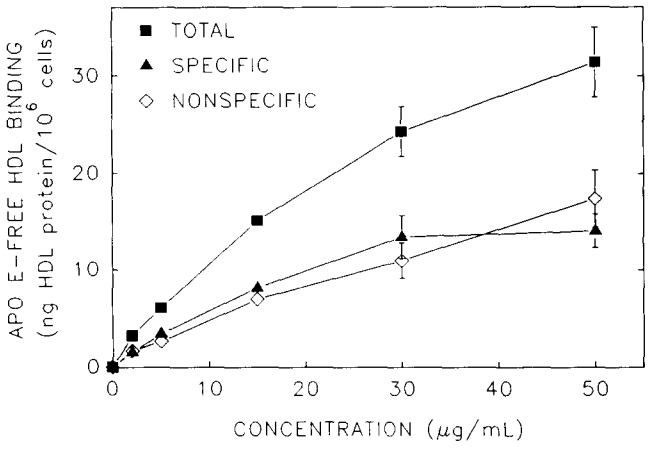

Figure 1 A representative binding curve of ^{125}I-apo E-free HDL to Cu-adequate liver parenchymal cells as a function of concentration at 0° C. Cells (2 × 10^6) and increasing amounts of ^{125}I-apo E-free HDL (2–50 μg/mL, specific activity, 1.92 × 10^5 cpm/μg protein) in 0.1 mL of buffer A were incubated for 90 min to provide total binding curve. Parallel incubation in the presence of 40 × excess unlabeled apo E-free HDL provided the nonspecific binding curve. The specific binding curve was derived as the difference between total and nonspecific binding. Three experiments were performed to construct the average total, nonspecific, and specific binding curves. Standard error of the mean larger than the treatment symbol was represented as vertical bars at each data point.

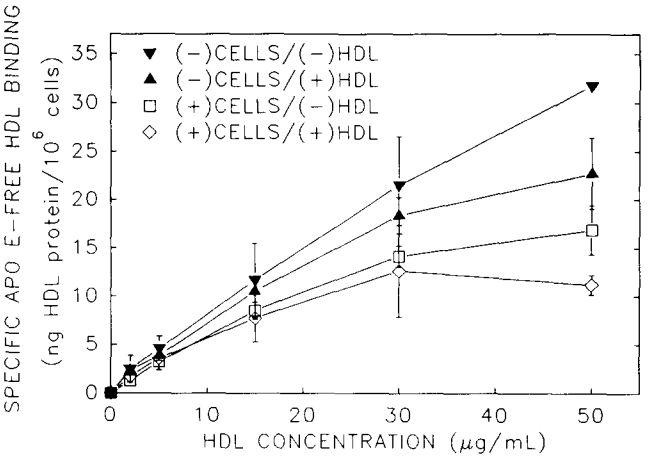

Figure 2 Comparison of specific binding curves derived from a crossover design with parenchymal cells and ^{125}I-apo E-free HDL obtained from Cu-deficient (−) and adequate (+) rats. Cells (2 × 10^6) and increasing amount of ^{125}I-apo E-free HDL (2–50 μg/mL, specific activity, 1.92 × 10^5 cpm/μg adequate HDL protein and 2.49 × 10^5 cpm/μg deficient HDL protein) in 0.1 mL of buffer A were incubated for 90 min at 0° C, with or without 40 × excess unlabeled apo E-free HDL, to provide the total and nonspecific binding. Specific binding was derived as the difference between total and nonspecific binding. Three experiments were performed to generate the average specific binding curve for each treatment. Vertical bars represent ± standard error of the mean at each data point. ANOVA was performed as a 2 × 2 × 3 completely randomized design with two levels of cell, two levels of HDL, and five levels of concentration (cell effect, df = 1, $P < 0.05$).

The average specific binding curves of the four types of incubation obtained from a crossover design involving four combinations of cells and HDL derived from Cu-deficient and adequate rats are depicted in *Figure 2*. The specific binding curves obtained from the cells derived from adequate rats regardless of the source of HDL appeared to be similar and saturable within the HDL concentration range. In contrast, they are lower than those derived from Cu-deficient cells. Because the nonspecific binding response curves were not different among the four treatments, the elevated specific binding responses associated with Cu-deficient cells are attributed solely to the higher total binding observed.

The best overall response appeared to be provided by cells and HDL derived from the Cu-deficient rats followed by that obtained from deficient cells and adequate HDL. Analysis of variance indicated that specific binding curves were significantly higher ($P < 0.05$) in cells from Cu-deficient than from Cu-adequate rats. However, these specific binding curves derived from Cu-deficient cells appeared not to be fully saturable at the highest HDL concentration used in the present study. Nevertheless, Scatchard analysis provided straight-line response for all individual specific binding curves regardless of the source of cells and HDL. In addition, similar to the specific binding data, the highest B_{max} (maximal binding capacity) appeared to be derived from cells and HDL from Cu-deficient rats *(Table 2)*. Analysis of variance also indicated that B_{max} was significantly higher ($P < 0.05$) in cells from Cu-deficient than from Cu-adequate rats. Furthermore, no treatment difference was observed for K_d (binding affinity, *Table 2*).

Table 2 Maximal binding capacity (B_{max}) and binding affinity (K_d) derived from a crossover design with parenchymal cells and apo E-free HDL obtained from copper-deficient and adequate rats

Apo E-free HDL	Liver parenchymal cells: Cu-adequate	Liver parenchymal cells: Cu-deficient	ANOVA
B_{max}	ng protein bound/10^6 cells		
Cu-adequate	25.4 ± 9.1	31.6 ± 4.1	Cell effect, $P < 0.05$ HDL effect, NS
Cu-deficient	27.7 ± 8.3	43.9 ± 7.3	Cell × HDL, NS
K_d	ng protein bound/10^6 cells		
Cu-adequate	1.40 ± 0.54	1.47 ± 0.34	Cell effect, NS HDL effect, NS
Cu-deficient	1.87 ± 1.04	2.48 ± 0.49	Cell × HDL, NS

After 8 weeks of dietary treatments, apo E-free HDL and cells were obtained for HDL binding assay. Scatchard analyses[36] of the specific binding data obtained at 0° C *(Figure 2)* provided the B_{max} and K_d. Values are presented as mean ± SEM and each mean is derived from three specific binding curves. Data were analyzed by two-way analysis of variance, for cell effect (df = 1), HDL effect (df = 1), and cell × HDL interaction (df = 1). A significant $P < 0.05$ cell effect was observed for B_{max}; NS = nonsignificant.

Specificity of apo E-free HDL binding to parenchymal cells evaluated by competition with excess unlabeled lipoproteins

Results of a typical competition experiment involving ^{125}I-apo E-free HDL and liver parenchymal cells from

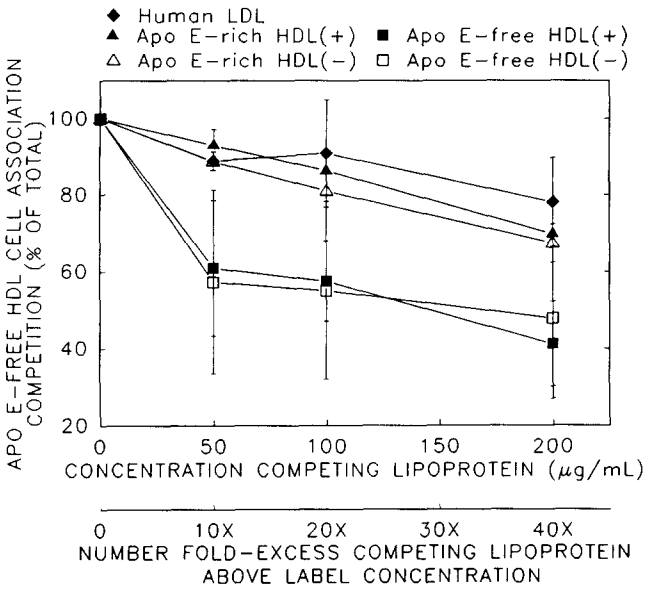

Figure 3 Competition of unlabeled lipoproteins with ^{125}I-apo E-free HDL bound to liver parenchymal cells at 0° C. Each incubation was performed in a total volume of 0.2 mL of buffer A with 2 × 10^6 cells, 1.0 μg of ^{125}I-apo E-free HDL (specific activity, 1.92 × 10^5 cpm/μg protein) and one of the following unlabeled completing ligands: apo E-free HDL from adequate (○) and deficient (●) rats; apo E-rich HDL from adequate (▲) and deficient (△) rats; and human LDL (◆). Following a 90-min incubation at 0° C, the radioactivity associated with the cells was measured and considered to be surface bound. The binding data were expressed as a percentage of the amount of ^{125}I-apo E-free HDL bound in the absence of any competing ligands. The averages of two competition studies performed with cells and ^{125}I-apo E-free HDL from adequate rats are depicted here.

adequate rats are depicted in *Figure 3*. Regardless of the source of unlabeled apo E-free HDL, a 40-fold excess lipoprotein depressed the amount of cell-bound ^{125}I-apo E-free HDL by about 60%. However, the amount of cell-bound ^{125}I-apo E-free HDL was only mildly depressed about 30% by a 40-fold excess unlabeled apo E-rich HDL derived from either Cu-deficient or -adequate rats. Furthermore, the unlabeled human LDL was the least effective, depressing the amount of cell-bound ^{125}I-apo E-free HDL by less than 20%. Similar data were obtained for competition studies using cells and ^{125}I-apo E-free HDL from Cu-deficient rats. These studies demonstrated the presence of binding sites on parenchymal cells that are specific for certain ligands in the apo E-free HDL fraction.

Apo E-free HDL surface binding and endocytotic uptake by parenchymal cells at 37° C

To examine a possible treatment difference in endocytic uptake of apo E-free HDL, a process occurring after the initial binding, cells were incubated with ^{125}I-apo E-free HDL at 37° C. At the end of incubation cells were treated with trypsin to partition the portion of ^{125}I-apo E-free HDL, which was surface bound (trypsin releasable) and internalized (trypsin resistant). *Figure 4* depicts a representative set of total trypsin releasable and trypsin resistant cell-associated apo E-free HDL response curves as a function of time of incubation with cells and HDL derived from Cu-adequate rats. Total cell association proceeded rapidly and reached a plateau at about 90 min. This was established by earlier studies performed for up to 2 hours in our laboratories, as well as by Schouten et al.[40] Most of the total cell-associated ligand is accounted for by surface-bound HDL. The trypsin-resistant response curve was relatively low throughout the incubation. This equilibrium may be explained by relatively slow processes of internalization and retroendocytosis observed in the unpublished pulse-chase studies cited by Schouten et al.[40] In addition, a slow rate of degradation and excretion may also have contributed to the low response. In the present study the quantities of HDL internalized by the adequate cells amounted to only 0.75% of the I^{125}-HDL in the medium.

Among the four treatments, the response curves for intracellular HDL accumulation were not different. However, the response curves for surface-bound HDL were significantly elevated (Cu effect, $P < 0.05$) in cells isolated from deficient rats as compared with those from adequate rats, regardless of the source of HDL *(Figure 5)*. In addition, the amount of HDL bound to the surface of cells obtained from Cu-deficient rats appeared not to have reached a plateau in 90 min (at the end of incubation) in contrast to Cu-adequate cells. A similar pattern was also obtained for total cell association among the four types of incubation.

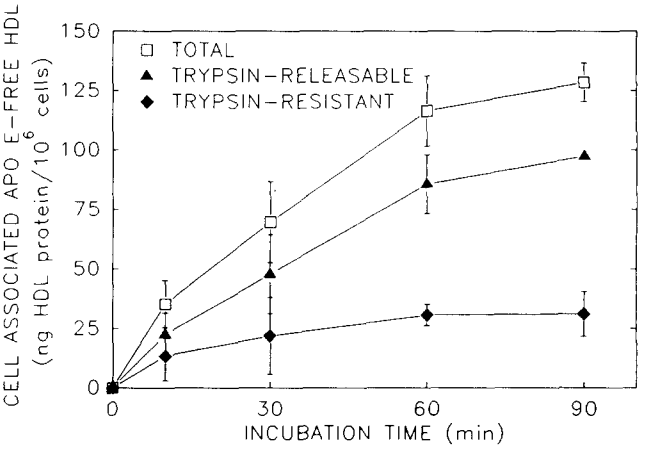

Figure 4 A representative response curve of trypsin-releasable and resistant components of Cu-adequate liver parenchymal cell-associated ^{125}I-apo E-free HDL. Cells (15 × 10^6) were incubated at 37° C with 20 μg of ^{125}I-apo E-free HDL/mL (specific activity, 1.92 × 10^5 cpm/μg protein) in 2.5 mL of buffer A. Two 200-μL aliquots were removed from the incubation mixture at each time interval. Unbound ligands were removed by repeated washings with ice-cold buffer A. In one aliquot, cells were used for total cell-associated ligand determination. Cells in the other aliquot were treated with trypsin to provide trypsin-releasable and -resistant component of cell-associated ligand. Three experiments were performed to generate the average total as well as trypsin-releasable and resistant curves. Vertical bars represent standard error of the mean associated with each data point.

Internalization and surface binding of apo E-free HDL by Kupffer cells at 37° C

In view of the small number of Kupffer cells isolated from each liver and the small amount of HDL associated with a given number of cells, all Kupffer cells obtained from the present isolations were solely used to provide internalization and binding data at 37° C. The total Kupffer cell-associated HDL also proceeded very rapidly and reached equilibrium at about 60 min, which was established in studies performed for up to 2 hours. Comparable results have been reported by Murakami et al.[41] for the interaction of apo E-free HDL with liver sinusoidal cells. Unlike parenchymal cells, the trypsin resistant curves for Kupffer cells, which are indicative of HDL internalized, were markedly depressed in Cu-deficient as compared with adequate cells (Cu effect, $P < 0.05$) regardless of the source of lipoproteins *(Figure 6)*. After 60 min of incubation, the amount of HDL internalized accounted for 50–51% and 35–40% of the total cell-associated HDL for the cells derived from Cu-adequate and -deficient rats, respectively. In the present study, the amount of HDL internalized is in the same order of magnitude as that reported by Murakami et al.[41] in a study on the interaction of apo E-free HDL with liver sinusoidal cells derived from rats fed a commercial stock diet. Furthermore, the present response curves for surface-bound HDL (trypsin releasable) were not different among the four treatments. These data suggest that the process of HDL internalization was depressed,

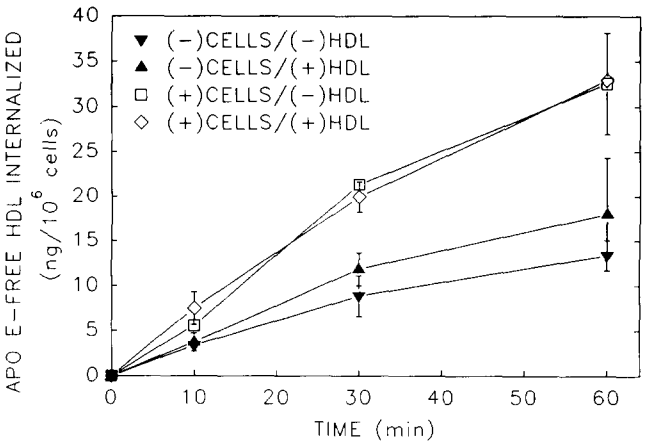

Figure 6 Comparison of apo E-free HDL internalized (trypsin resistant) by Kupffer cells in a crossover design with HDL and cells derived from Cu-deficient (−) and adequate (+) rats. Cells (6×10^6) were incubated at 37° C with 20 μg of ^{125}I-apo E-free HDL/mL (specific activity, 1.92×10^5 cpm/μg adequate HDL protein and 2.49×10^5 cpm/μg deficient HDL protein) in 2.5 mL of buffer A. At each time interval, a 200-μL aliquot was removed and washed free of unbound ligand. Following the incubation of the washed cells with 0.5 mL of trypsin (4 g/L) for 45 min at 0° C and the removal of the trypsin releasable component by repeated washing, the trypsin-resistant component retained by the cells provided the amount of HDL interanlized. Three experiments were performed to construct the average internalization curve for each treatment. Vertical bars represent standard error of the mean associated with each data point. ANOVA was performed as a $2 \times 2 \times 3$ completely randomized design with two levels of cell, two levels of HDL, and three levels of time (cell effect, df = 1, $P < 0.05$).

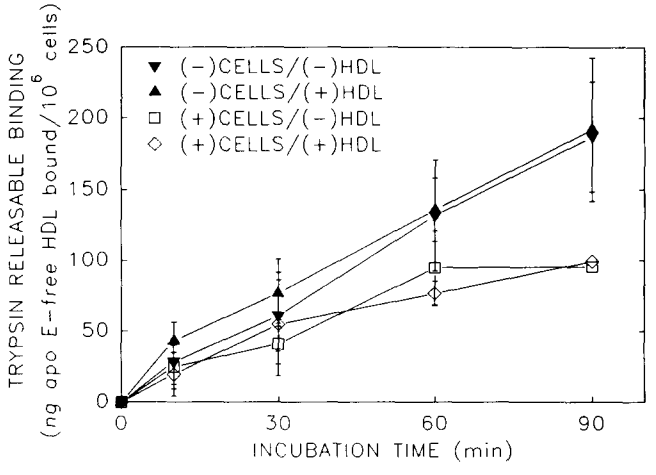

Figure 5 Comparison of trypsin releasable binding derived from a crossover design with parenchymal cells and ^{125}I-apo E-free HDL obtained from Cu-deficient (−) and adequate (+) rats. Cells (6×10^6) were incubated at 37° C with 20 μg of ^{125}I-apo E-free HDL/mL (specific activity, 1.92×10^5 cpm/μg adequate HDL protein and 2.49×10^5 cpm/μg deficient HDL protein) in 2.5 mL of buffer A. At each time interval, a 200-μL aliquot was removed and washed free of unbound ligand. Binding of ^{125}I-apo E-free HDL was determined by incubating the washed cells with 0.5 mL of trypsin (4 mg/mL) for 45 min at 0° C. Three experiments were performed to construct the average trypsin releasable binding curve for each treatment. Vertical bars represent standard error of the mean associated with each data point. ANOVA was performed as a $2 \times 2 \times 4$ completely randomized design with two levels of cell, two levels of HDL, and four levels of time (cell effect, df = 1, $P < 0.05$).

but surface binding was unaffected in Kupffer cells as a result of Cu deficiency.

Discussion

In a recent in vitro study the specific binding of HDL at 37° C by liver cells (derived from rats fed a commercial stock diet) as a function of ligand concentration was determined from the trypsin-releasable cell-associated HDL by Schouten et al.[40] Their specific binding data demonstrated the presence of a high affinity binding site for apo E-free HDL on three liver cell types (parenchymal, Kupffer, and endothelial cells) and provided values for K_d of 10–20 mg HDL/L and B_{max} of 25–50 ng HDL/mg of cell protein. In the present study, the K_d and B_{max} obtained at 0° C are expected to be lower than those reported by Schouten et al.[40] at 37° C, because the HDL binding process is temperature dependent. Nevertheless, the K_d and B_{max} obtained in the present study appeared to be in the same order of magnitude as those observed by Schouten et al.[40] In addition, Schouten et al.[40] also performed competition studies at 37° C for 10 min to evaluate apolipoprotein specificity for the high-affinity sites and reported that LDL was much less effective than HDL and VLDL in competing for the bound ^{125}I-apo E-free HDL in all three liver cell types. Comparable competition characteristics were also observed by Hassel et al.[42] in the binding of apo E-free HDL to liver plasma membranes. The present

competition studies performed at 0° C for 90 min also demonstrate that LDL was much less effective than HDL in competing for bound ^{125}I-apo E-free HDL. Thus, the findings of the present studies are in agreement with those of other studies showing that specific binding sites exist on parenchymal cells, which are specific for certain ligands in the apo E-free HDL fraction.

In the present studies the interaction of freshly isolated parenchymal cells and apo E-free HDL derived from Cu-deficient rats demonstrated for the first time: (a) an increase in B_{max} at 0° C, and (b) an increase in trypsin-releasable or specific surface binding accompanied by no change in internalization at 37° C. A similar enhancement of the binding process was also observed by Zhang and Lei[43] in cultured parenchymal cells from Cu-deficient rats. Their preliminary study involved only two treatments: incubations of both HDL and cells in primary culture, derived from adequate or deficient rats. An enhanced binding of HDL was demonstrated as a function of time with only one HDL concentration (5 μg/mL). Specific binding response, as a function of ligand concentration, tended to be elevated at 5–15 μg/mL for the Cu-deficient treatment; but no significant increase in the overall response curve was detected. This may have resulted from the primary culture system used in that preliminary study. As such, the data were not further characterized and processed for B_{max} and K_d. To firmly establish that binding and B_{max} are affected by Cu deficiency, the freshly isolated cell system was selected for the present studies. This system provided a large number of viable cells not influenced by culture conditions (i.e., not exposed to culture media containing Cu for 2–3 days), which may be more representative of the nutritional status of the intact animal or organ. The enhanced maximum binding capacity, observed in the present study, may facilitate an accelerated uptake of HDL cholesteryl ester into the liver. In vivo plasma clearance studies utilizing doubly labeled HDL particles have established that the absolute amount of HDL cholesteryl ester cleared from the plasma occurred at a faster rate than HDL protein in control rats.[4] In Cu-deficient rats, the magnitude of this disproportionate clearance of cholesteryl ester was enhanced, and virtually all of the increased removal of HDL cholesteryl ester was attributed to the liver. The absolute amount of HDL protein cleared was only slightly increased by Cu deficiency and this was mostly attributed to increased uptake by peripheral bulk tissues.[4] In view of these findings and the fact that the in vitro internalization of HDL apolipoproteins by parenchymal cells remained unchanged, the enhanced binding observed appeared to have a small effect on the process of whole particle uptake by the liver. Nevertheless, the increased binding may have facilitated the accelerated hepatic uptake of HDL cholesteryl ester.

A detailed pulse-chase study was performed by Bachorik et al.[44] to examine reversible surface binding of apo E-free HDL in cultured pig hepatocytes. Cells were pulsed at 37° C for 2.5 hr to provide a total cell association of HDL close to steady state level, and about half of this was trypsin removable (surface bound). Thereafter, cells were chased for 8 hr. Within 2 hr of chase most of the surface-bound HDL was released into the medium as denser lipoprotein particles. Thus, the surface-bound HDL resulted from an equilibrium rapidly established by the processes of binding and reversible release. Furthermore, this equilibrium is a function of the maximum binding capacity of cells. Indeed, the increased surface binding observed in the present trypsin-releasible study is confirmed by the enhanced B_{max} detected in parenchymal cells derived from Cu-deficient rats. In contrast, Bachorik et al.[44] reported that less than 16% of internalized HDL was released as acid-soluble radioactivity after 2 hr of chase. Similarly, in pulse-chase experiments cited by Schouten et al.[40] using isolated rat liver parenchymal cells, the rate of release of internalized label was reported as "slow." In view of the small amount of label being released into a large volume of medium, the extent of recycling of this label may be minute and should not confound the data observed in the present study. However, extensive pulse-chase studies should be performed to characterize the influence of Cu deficiency on rates of release of surface-bound HDL and internalized label into the medium. Furthermore, the contribution of recycling of label to the present incubation system should be examined.

In the present study the overall rate of internalization *(Figure 6)* was similar to that observed for parenchymal cells derived from adequate rats *(Figure 4)*. Although the internalization of HDL by Kupffer cells was depressed by Cu deficiency, the contribution of Kupffer cells to the overall uptake of HDL protein may be relatively small. The recent in vivo study of Schouten et al.[40] indicated that the liver parenchymal cells (derived from rats fed a nutritionally adequate diet), which contributed 92.5% of total liver mass, accounted for almost 78% of liver uptake of apo E-free HDL at 10 min after an intravenous administration of a tracer dose of ^{125}I-apo E-free HDL. Both endothelial and Kupffer cells, which contributed 3.3% and 2.5% of the total liver mass, respectively, amounted to only 11% of HDL uptake by each cell type. Quantitatively, the parenchymal cells are most important in HDL uptake in vivo. Thus, in Cu-deficient rats the in vivo uptake of HDL by parenchymal cells may have masked the contribution by Kupffer cells resulting in a small overall increase in hepatic uptake of ^{125}I-HDL observed by Carr and Lei.[4] At present, the significance of a depressed HDL internalization by Cu-deficient Kupffer cells is not clear.

A cohesive overview of the influence of Cu deficiency on lipoprotein metabolism can be derived from the currently available data. Three lines of evidence obtained from Cu-deficient rats indicate that the hyperlipoproteinemia does not result from an impaired degradation process. An increased in vivo plasma clearance and tissue uptake of cholesteryl ester and apolipoproteins of HDL,[4] an enhanced in vitro binding of HDL to purified liver plasma membranes,[22] as well as an elevated in vitro binding of HDL by freshly isolated liver parenchymal cells observed in the present studies could be considered as supportive evidence that hepatic uptake

of HDL cholesteryl ester may be accelerated in Cu deficiency. In addition, the following evidence derived from Cu-deficient rats: (a) an increase in hepatic HMG CoA reductase activity in vitro,[9] (b) an enhanced cholesterol synthesis by the liver in vivo,[10] and (c) a markedly accelerated transport of hepatic nascent cholesteryl ester, newly synthesized from mevalonate, into the plasma,[11] indicate that cholesterol derived from an increased hepatic synthesis is packaged into lipoproteins to facilitate an enhanced secretion of newly synthesized cholesteryl ester from the liver to the plasma. Furthermore, the depressed hepatic cholesterol store observed in Cu-deficient rats[4] indicates that the increase in cholesterol supplied by the enhanced de novo cholesterol synthesis[10] and hepatic uptake of plasma HDL cholesteryl ester[4] is excreted or secreted out of the liver. Because bile acid production and biliary cholesterol elimination are not affected by Cu deficiency,[7] the increased supply of cholesterol from de novo synthesis[10] and plasma HDL[4] is not diverted to the excretory route. Instead, all available hepatic cholesterol seemed to be used to sustain an accelerated secretion into the plasma, even to the extent of depressing the hepatic cholesterol store. Thus, the hypercholesterolemia in Cu-deficient rats appeared to be a new steady state, promoted mainly by an enhanced hepatic cholesterol synthesis and secretion. At present, studies are being conducted to examine hepatic lipoprotein synthesis, intracellular processing, and secretion to understand the mechanism(s) responsible for the hyperlipoproteinemia associated with Cu deficiency.

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