# Specific binding and uptake of apolipoprotein E-free high density lipoproteins by cultured liver parenchymal cells of copper-deficient rats

**J.J. Zhang and K.Y. Lei**

*Department of Nutrition and Food Science and Nutritional Sciences Program, University of Arizona, Tucson, AZ, USA*

*The influence of copper deficiency on the binding and uptake of apolipoprotein E-free high density lipoprotein (apo E-free HDL) in cultured rat hepatic parenchymal cells was examined in this study. Male weanling Sprague-Dawley rats were randomly divided into two treatments, a Cu-adequate (7.33 mg Cu/kg diet) or a Cu-deficient (1.04 mg Cu/kg diet) group. After 7 weeks, plasma apo E-free HDL were isolated by a combination of ultracentrifugation, gel filtration, and heparin-Sepharose affinity chromatography. Parenchymal cells were isolated from collagenase perfused liver of Cu-deficient and adequate rats and cultured for 16 hours at 37°C prior to incubation with iodinated apo E-free HDL from the same treatment group. Cells were incubated with 5 μg/ml $^{125}$I-apo E-free HDL for 2, 6, or 12 hours in the presence or absence of 200 μg/ml (40-fold) excess unlabeled apo E-free HDL. Increases in specific binding at 4°C and specific cell-associated uptake at 37°C as a function of time were observed with cells and HDL from Cu-deficient rats. Cells were also incubated for 6 hours with 8 concentrations of $^{125}$I-apo E-free HDL in the presence or absence of excess unlabeled HDL. Although no significant increase in specific binding was detected at 4°C as a function of ligand concentration, the response tended to be higher at 5 to 15 μg HDL/ml for the Cu-deficient treatment. However, at 37°C the specific cell-associated uptake was increased markedly with cells and HDL from Cu-deficient rats. The observed increases in HDL binding and uptake indicate that these processes may be enhanced in Cu-deficient rats. These data are also consistent with recent in vivo results which indicate that plasma clearance and tissue uptake of HDL are increased in Cu-deficient rats.*

**Keywords:** apo E-free HDL; copper-deficiency; liver parenchymal cells; HDL binding and uptake

## Introduction

Hypercholesterolemia has been observed by numerous investigators in copper (Cu)-deficient rats as well as in other species, including humans.[1] A number of studies have been performed to examine the possible mechanisms responsible for the hypercholesterolemia observed in Cu deficiency. The hypercholesterolemia in Cu-deficient rats is characterized by elevations in protein and cholesterol contents of high (HDL) and low density lipoprotein (LDL), and in triglyceride level of LDL[2,3] within an enlarged plasma pool.[2,4] The increase in plasma cholesterol resides mostly in HDL, since about 85% of plasma cholesterol in the rat is transported normally by HDL.[5] Increases in the contents of protein and cholesterol as well as apolipoprotein AI and E were observed in an apo E-rich subclass of HDL in Cu-deficient rats.[6,7] In addition, Cu deficiency was found to have little or no influence on the

Address reprint requests to K.Y. Lei, Department of Nutrition and Food Science, 309 Shantz Building, University of Arizona, Tucson, AZ 85721, USA.
Supported by USDA Human Nutrition Competitive Grants Program 86-CRCR-1-1925 and Arizona Agricultural Experiment Station.
Received October 11, 1989; accepted January 17, 1990.

rate of bile acid synthesis[8] and production[9] as well as biliary sterol excretion.[8,9] In contrast, an increase in hepatic HMG CoA reductase activity[10] and de novo cholesterol synthesis[11] was established in Cu-deficient rats. Furthermore, the supply of cholesterol to the liver was also enhanced by a 2-fold increase in hepatic uptake of cholesterol ester from plasma HDL, whereas the hepatic uptake of HDL protein was increased only slightly in Cu-deficient rats.[4] However, the hepatic cholesterol content of Cu-deficient rats still remained depressed. Since a much accelerated clearance of nascent cholesterol ester newly synthesized from mevalonate was observed from the liver to the plasma,[12] a small prolonged net efflux of cholesterol from the liver to the plasma may have resulted in the depressed hepatic cholesterol content and the hypercholesterolemia.

Previous studies have demonstrated that the liver can accumulate injected HDL,[13-16] and the HDL taken up are associated primarily with parenchymal cells.[17] In addition, it has been estimated that 20 to 30% of the overall HDL apolipoprotein catabolism may occur in the liver, probably in the liver parenchymal cells.[18-20] Furthermore, the liver is the major site of HDL cholesteryl ester uptake.[19,21] In view of the enhanced in vivo uptake of HDL cholesteryl ester by the liver[4] and the increased in vitro HDL binding to liver plasma membranes[22] as a result of Cu deficiency, the present study was designed to investigate the influence of Cu status on the binding and uptake of apo E-free HDL by the rat liver parenchymal cells in primary culture.

## Materials and methods

### *Experimental animals*

Male weanling Sprague-Dawley rats, weighing between 40 to 60 g, 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 to 22°C. Diet and distilled-demineralized water were provided ad libitum. After 7 weeks of dietary treatment, the rats were anesthetized with 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[23] except no Cu supplement was included in the mineral mix (*Table 1*). The Cu-deficient diet contained 1.044 mg Cu/kg diet as measured by atomic absorption spectrophotometry. 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.

### *Tissue sampling and analysis*

Liver was perfused in situ with saline to remove all the blood. Liver and heart were then quickly excised and weighed. Duplicate 1-g samples from each liver and the entire heart were dried, digested in nitric acid at 95°C, diluted to 10 ml, and analyzed for Cu content by atomic absorption spectrophotometry.

**Table 1** 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 | 1.0 | 1.0 |
| Corn oil | 5.0 | 5.0 |
| Choline bitartrate | 0.2 | 0.2 |
| Cupric carbonate mix (1.05 g/kg) | — | 1.0 |
| Dietary copper (ppm) | 1.04 | 7.33 |

### *Plasma lipoprotein preparation and apo E-free HDL isolation*

Blood was collected into a syringe with EDTA (1 mg/ml) and plasma was obtained by low speed centrifugation. Plasma from three rats was pooled, adjusted to d 1.225 g/ml with solid KBr, and overlayered with 5 ml of d 1.225 buffered solution. Plasma was centrifuged at 100,000 × *g* in Ti 70 rotor for 24 hours at 15°C in a Beckman L8-80M ultracentrifuge (Beckman Instruments Inc., Fullerton, CA). The top 2 ml containing the lipoproteins were collected and applied to a single 6% agarose (A-5m Agarose, Bio-Rad Co., CA) column (2.5 cm × 90 cm) maintained in a cold room at 6°C. The sample was eluted with 0.15 M NaCl, 0.01% EDTA, 0.02% Na Azide, pH 7.4 at 20 ml/hour and was collected at 4 ml/fraction. Protein concentration was monitored at 280 nm. There were three major peaks representing VLDL, LDL, and HDL. Fractions corresponding to the apo E-poor HDL were collected and concentrated by ultrafiltration in Amicon CF-25 ultrafiltration membrane cones (Amicon Corp., Danvers, MA). The concentrated HDL were then subfractionated by using heparin-Sepharose affinity chromatography.[22,24] The HDL were equilibrated overnight in the heparin column (1.0 × 30 cm) at 6°C and eluted the following day using an increasing stepwise NaCl-gradient system at 24 ml/hr, and the protein concentrations were monitored at 280 nm. Fractions 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.[25] Identification of apolipoproteins was conducted by 7.5 to 20% gradient SDS-polyacrylamide gel electrophoresis.[26]

### *Iodination of apo E-free HDL*

The apo E-free HDL were radiolabeled using the iodine monochloride method[27] as modified by Goldstein et al.[28] Iodinated HDL were passed through a

Sephadex G-25 column and then dialyzed at pH 7.4 against 150 mM NaCl containing 0.24 mM EDTA for 24 hours (3 changes of 4 liters each). The iodinated HDL were sterilized by passing through a 0.45 μm filter (Millipore Corp., Bedford, MA), stored at 4°C, and used within 2 weeks. All radiolabeled HDL preparations contained < 2% TCA-soluble radioactivity and < 5% lipid-associated radioactivity.

### *Lipoprotein deficient serum (LPDS) preparation*

Blood was collected by cardiac puncture and the plasma was obtained by low-speed centrifugation. The plasma was adjusted to the density 1.300 g/ml by adding KBr and capped with 5 ml of overlayering buffer (d 1.225 g/ml). The plasma was centrifuged at 100,000 × *g* for 24 hours, at 15°C in a Ti 70 rotor. The top 3 ml containing lipoproteins were collected and discarded. The remaining plasma was transferred to another clean tube, capped with 5 ml of overlayering buffer, and centrifuged at the same *g* force again. The lipoprotein deficient serum was dialyzed for 2 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 filter and stored below −4°C until use. LPDS derived from each treatment was used with $^{125}$I-apo E-free HDL of the same treatment in incubation studies.

### *Isolation of rat liver parenchymal cells*

After 7 weeks of dietary treatment, rats were fasted for 12 hours and hepatic parenchymal cells were isolated by the method of Berry and Friend,[29] using collagenase in an in situ recirculating liver system. Rats were anesthetized with diethyl ether. The abdomen was opened and the perfusion needle 20 gauge × 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 minutes 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, and 10 ml 10% dextrose per 1000 ml).The flow rate was increased to 35 ml/min for 3 minutes with the same solution. The liver was cut and placed on a sieve very carefully with vena cava (outflow) face down. The liver was then perfused with chelator-free buffer containing the same component as above, except with no EGTA, for 2 minutes at 35 ml/min. Following this, a 0.05% solution of collagenase (class II, Cooper Biomedical) was perfused for 12 to 15 minutes 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 of double-distilled $H_2O$, pH 7.4. At the end of perfusion, the liver became markedly soft. The liver was excised, trimmed, and placed in 25 ml of serum-free M199 with Hank's salts solution (Sigma, Catalog No. M0393) at 4°C. The liver capsule was detached by using 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 to 40 × *g* for 4 minutes. The supernatant was removed, the cell pellet was resuspended, and the cells were centrifuged again. The pellet was suspended and adjusted to $5 \times 10^6$ cells/ml. In order to get higher viabilities of cell preparation, Percoll (Pharmacia Fine Chemicals) was used to form a self-generated gradient during the centrifugation. One part of 1.5 M NaCl was added to nine parts (v/v) of Percoll. Twenty-five ml of cell suspension were added to 24 ml of neutralized Percoll solution, mixed, and centrifuged for 10 minutes at 40 to 50 × *g*. The pellet which contained viable cells was collected and resuspended and washed 2 to 3 times to remove Percoll. Finally, the cell pellet was suspended with M199 culture medium with Earle's salts solution (Sigma Catalog No. M5017) containing penicillin 100 units/ml, streptomycin 100 μg/ml, and 10% fetal calf serum (FCS). A drop of trypan blue stain (0.4%, GIBCO) was added to 200 μl of diluted cell suspension, and the cell number and viability were assessed on a hemocytometer within 2 minutes. The average viability was 90%, and no difference was found between Cu-adequate and Cu-deficient rats in viability determination. The average number of total viable cells was $3.4 \times 10^8$ cells per rat liver. Approximately $1 \times 10^7$ 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. After 16 hours of pre-culture, the medium was removed and fresh culture medium containing rat LPDS 10% v/v was added; LPDS from Cu-adequate or deficient rats was used for Cu-adequate or deficient cells, respectively. Cells were immediately used in two types of incubation. First, deficient cells were incubated with deficient HDL; and second, adequate cells with adequate HDL.

### *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, or 12 hours. Cells were then washed 4 times with ice-cold phosphate-buffered saline (PBS), pH 7.4 and dissolved in 1.0 ml 1N NaOH. The cell surface-bound radioactivity was measured by using a LKB Minigamma 1275 gamma counter (LKB Instruments, Rockville, MD). The cell protein was measured by the method of Lowry et al.[25] The response curve for each treatment represents the average of 4 experiments. Each experiment involved cells prepared from a separate rat. Duplicate observations were performed at each time point for each experiment.

In saturation studies performed at 4°C, various amounts of $^{125}$I-labeled HDL from 1 μg/ml to 60 μg/ml were incubated with a constant amount of cells ($1 \times 10^7$ cells per well) in the presence or absence of 40-fold excess unlabeled apo E-free HDL for 6 hours. The cell surface-bound radioactivity and the cell protein were

measured as described above. The response curve for each treatment represents the average of 3 experiments. Each experiment involved cells prepared from a separate rat.

### *Uptake and saturation studies at 37°C*

In uptake 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, or 12 hours. The medium was removed, and cells were then treated the same as in binding studies. The cell-associated radioactivity and the cell protein were measured. The response curve for each treatment represents the average of 4 experiments. Each experiment involved cells prepared from a separate rat. Duplicate observations were performed at each time point for each experiment.

In saturation studies performed at 37°C, various amounts of $^{125}$I-labeled HDL from 1 μg/ml to 60 μg/ml were incubated with constant amounts of cells ($1 \times 10^7$ cells per well) in the presence or absence of 40-fold excess unlabeled apo E-free HDL for 6 hours. The cell-associated radioactivity and the cell protein were measured. The response curve for each treatment represents the average of 2 experiments. Each experiment involved cells prepared from a separate rat.

### *Statistical analyses*

Treatment comparisons for body and tissue parameters were determined by the Student's two-tailed t-test, and the HDL binding and uptake response curves were determined by the two-way analysis of variance.[30]

## Results

### *Body and organ weights, and tissue copper contents*

The body weight was significantly reduced in rats fed the Cu-deficiency diet (*Table 2*). The heart weight and the heart-to-body-weight ratio were markedly increased in rats from Cu-deficient treatment ($P < 0.001$ and $P < 0.0001$). 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 ($P < 0.02$). These are observations usually associated with the Cu-deficient rat model used in our laboratory. In addition, significantly reduced cardiac and hepatic Cu contents ($P < 0.0001$) confirm that the rats fed the deficient diet were indeed Cu-deficient.

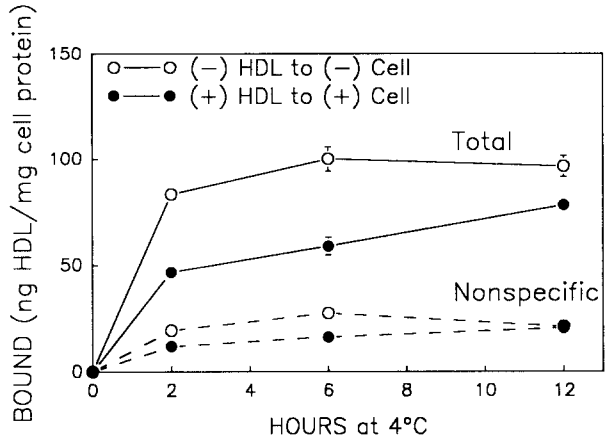


**Figure 1** Time course of total and nonspecific cell-surface binding of $^{125}$I-apo E-free HDL in cultured rat hepatic parenchymal cells at 4°C. Cells were incubated with 5 μg/ml of $^{125}$I-apo E-free HDL, with or without 200 μg/ml (40-fold) excess of unlabeled apo E-free HDL to provide total or nonspecific binding for the time indicated. HDL and cells represented by (−) and (+) were derived from Cu-deficient and adequate rats, respectively. The curve for each treatment represents the average of 4 experiments. Each experiment involved cells derived from a separate rat. Duplicate observations were performed at each time point for each experiment.

### *Binding studies performed as a function of time at 4°C*

At 4°C, the amount of cell-associated apo E-free HDL would represent mainly the amount of apo E-free HDL bound to the cell surface only.[31] Cells from both treatment groups were incubated at 4°C with labeled apo E-free HDL from the same treatment group to provide the total binding as a function of time (*Figure 1*). In addition to labeled apo E-free HDL, excess unlabeled apo E-free HDL were added simultaneously to the medium to determine nonspecific binding as a function of time (*Figure 1*). The specific binding data were obtained by subtracting the nonspecific from the total binding data (*Figure 2*). No difference in nonspecific

**Table 2** Effect of dietary copper deficiency on various body and tissue 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 body wt) | 0.35 ± 0.01 | 0.49 ± 0.01 | <0.001 |
| Liver wt (g/100 g body wt) | 3.82 ± 0.08 | 4.16 ± 0.11 | <0.02 |
| Heart Cu (μg/g wet wt) | 6.12 ± 0.21 | 2.24 ± 0.18 | <0.001 |
| Liver Cu (μg/g wet wt) | 5.47 ± 0.10 | 1.63 ± 0.09 | <0.001 |

[a] Mean ±SEM; $n = 10$.
[b] Student's two-tailed $t$ test; NS = not significant.

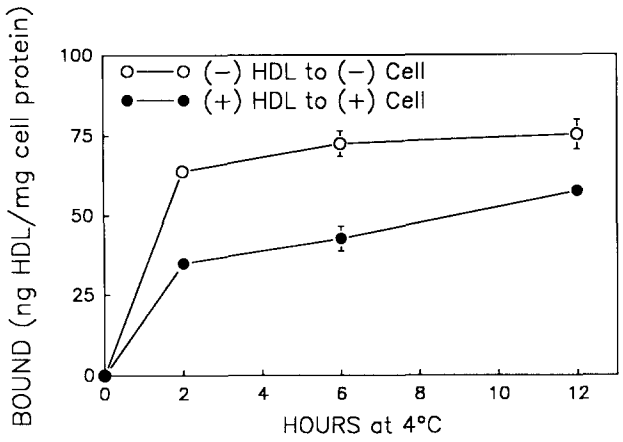


**Figure 2** Time course of specific cell-surface binding of $^{125}$I-HDL apo E-free HDL in cultured rat hepatic parenchymal cells at 4°C. Cells were incubated with 5 μg/ml of $^{125}$I-apo E-free HDL, with or without 200 μg/ml (40-fold) excess of unlabeled apo E-free HDL for the time indicated. The specific cell-surface binding was obtained by subtracting nonspecific component from the total. HDL and cells represented by (−) and (+) were derived from Cu-deficient and adequate rats, respectively. The curve for each treatment represents the average of 4 experiments. Each experiment involved cells derived from a separate rat. Duplicate observations were performed at each time point for each experiment. The data were analyzed by analysis of variance. The interaction of Cu × time (d.f. = 3) was significant ($P < .001$).

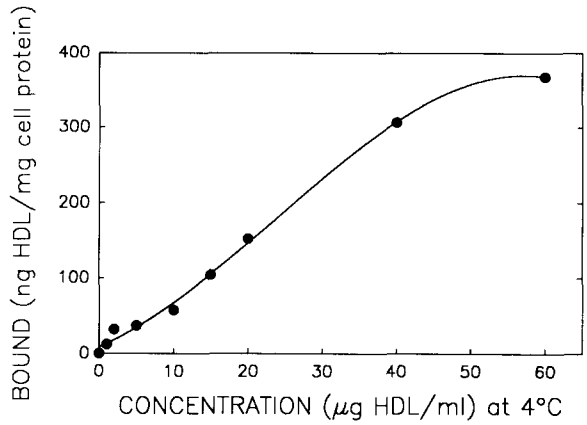


**Figure 3** Specific surface-bound of $^{125}$I-apo E-free HDL at 4°C. Cells were incubated with increasing amounts of labeled apo E-free HDL (from 1 to 60 μg/ml), with or without 40-fold excess of unlabeled apo E-free HDL, for 6 hours. The specific surface-bound value was obtained by subtracting the nonspecific component from the total. HDL and cells represented by (−) and (+) were derived from Cu-deficient and adequate rats, respectively. Since no treatment difference in the overall specific binding response was detected by analysis of variance, a single curve of the average data of two treatments is depicted. The curve represents data of 3 experiments from both treatments. Each experiment involved cells derived from a separate rat.

binding was observed among the two treatment groups. Significantly elevated total binding as well as specific binding were observed in the incubations with cells and HDL derived from Cu-deficient rats, mainly in the first 12 hours of incubation. At 2 hours, the binding curves appeared to be approaching a plateau. The amount of specific surface-bound apo E-free HDL at 2 hours was 35 and 64 ng/mg cell protein, for the cells and HDL derived from Cu-adequate and deficient rats, respectively.

### *Binding studies performed as a function of ligand concentration at 4°C*

Experiments were performed to determine the surface-bound apo E-free HDL by using eight different labeled ligand concentrations. Although the specific binding response tended to be elevated at 5 to 15 μg/ml for the Cu-deficient treatment, no significant increase in the overall response curve was observed. Since no overall treatment difference was detected, an average specific surface-bound saturation curve is presented for both treatments in *Figure 3*. Nevertheless, the specific surface-bound apo E-free HDL appeared to be saturable.

### *Uptake studies performed as a function of time at 37°C*

The total cell-associated uptake of apo E-free HDL at 37°C would represent the sum of both surface-bound HDL and that which had been internalized. A significant elevation of total uptake of apo E-free HDL as a function of time was observed in incubations with cells and HDL derived from Cu-deficient rats (data not shown). By 6 hours, these curves had almost reached a steady state. In addition to labeled HDL, 40-fold excess unlabeled HDL were added to the medium to provide the nonspecific uptake curves. No difference in the nonspecific curves was observed in two types of incubation (data not shown). 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 4*). At 6 hours, the specific uptake appeared to have

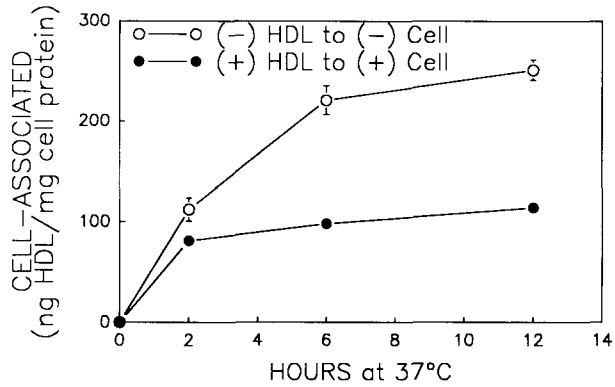


**Figure 4** Time course of specific cell-associated uptake of $^{125}$I-apo E-free HDL in cultured rat hepatic parenchymal cells at 37°C. Cells were incubated with 5 μg/ml of $^{125}$I-apo E-free HDL with or without 200 μg/ml (40-fold) excess of unlabeled apo E-free HDL for the time indicated. The specific uptake of $^{125}$I-apo E-free HDL were obtained by subtracting the nonspecific component from the total. HDL and cells represented by (−) and (+) were derived from Cu-deficient and adequate rats, respectively. The curve for each treatment represents the average of 4 experiments. Each experiment involved cells derived from a separate rat. Duplicate observations were performed at each time point for each experiment. Data were analyzed by analysis of variance. The interaction of Cu × time (d.f. = 3) was significant ($P < .01$).

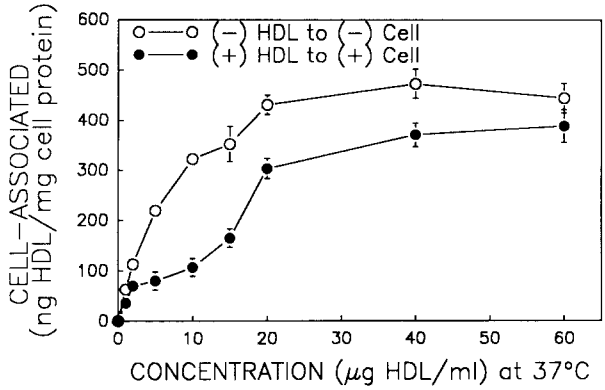


**Figure 5** Specific uptake of $^{125}$I-apo E-free HDL at 37°C. Cells were incubated with increasing amounts of labeled apo E-free HDL (from 1 to 60 μg/ml), with or without 40-fold excess of unlabeled apo E-free HDL, for 6 hours. The specific uptake was obtained by subtracting the nonspecific component from the total. HDL and cells represented by (−) and (+) were derived from Cu-deficient and adequate rats, respectively. The curve for each treatment represents the average of 2 experiments. Each experiment involved cells derived from a separate rat. Data were analyzed by analysis of variance. The interaction of Cu × concentration (d.f. = 8) was significant ($P < .01$).

reached a steady state. A significantly higher specific uptake response curve was observed for cells and HDL from Cu-deficient than from Cu-adequate rats. The difference was most prominent at or after the first 6 hours of incubation.

### *Uptake studies performed as a function of ligand concentration at 37°C*

The specific apo E-free HDL uptake as a function of labeled ligand concentration for both treatments appeared to be saturable (*Figure 5*). The specific uptake curve was significantly elevated in incubations using cells and HDL derived from Cu-deficient rats than from Cu-adequate rats. The differences were most prominent from 2 to 40 μg/ml of labeled HDL.

## Discussion

Depressed growth,[12,32] cardiac hypertrophy,[33,34] and decreased hepatic Cu content[35] are well established observations of Cu deficiency in various animal species. Since these symptoms were observed also in the present study (*Table 2*), the rats fed the Cu-deficient diet were indeed Cu deficient.

The binding study performed at 4°C, as a function of time, provided significantly higher total and specific cell-surface binding response curves for incubations involving cells and HDL from Cu-deficient than from Cu-adequate rats (*Figures 1* and *2*). The increase was most prominent at 2, 6, and 12 hours. At 4°C, the amount of cell-associated apo E-free HDL was assumed to represent solely the cell-surface bound HDL. The enhanced binding data suggest that Cu-deficient cells are capable of binding more HDL than adequate cells at the ligand concentration of 5 μg/ml. The maximum amount of specific apo E-free HDL bound by adequate cells was approximately 58 ng HDL/mg cell protein. A similar value of 52 ng of apo E-free HDL/mg cell protein was obtained at 4°C with the same labeled ligand concentration for pig hepatic parenchymal cells.[36] In order to partition the cell-associated $^{125}$I-apo E-free HDL into cell surface bound and internalized HDL, Bachorik et al.[36] incubated cells with $^{125}$I-apo E-free HDL in the presence or absence of excess unlabeled HDL at 4°C or 37°C and then treated the cells with trypsin for 10 minutes at 37°C to liberate the surface bound lipoproteins. 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 in the present study the amount of specific surface bound HDL at a steady state was also similar to the amount of trypsin-releasable HDL reported by Bachorik.[36]

Specific binding studies were also performed as a function of ligand concentration at 4°C to generate saturation response curves. Although no significant difference was detected between the two treatments, the specific binding curve tended to be higher, at 5, 10, and 15 μg of $^{125}$I-apo E-free HDL/ml, for incubations involving cells and HDL from deficient rats (data not shown). In addition, the average specific binding curve (*Figure 3*) appeared to be saturable, demonstrating the presence of binding sites specific for apo E-free HDL.

The uptake studies performed at 37°C, either as a function of time or of ligand concentration, provided cell association data which represented the sum of HDL bound to cell surface as well as HDL internalized. The specific cell-associated uptake of apo E-free HDL was found to reach a steady state or equilibrium by 6 hours for both Cu-deficient and adequate treatments (*Figure 4*). In the Cu-adequate treatment, the maximum amount of specific cell-associated uptake was about 114 ng HDL/mg cell protein. This value is very similar to the amount of specific cell associated apo E-free HDL (120 ng HDL/mg cell protein) at equilibrium observed in pig hepatic parenchymal cells incubated at 37°C with the same ligand concentration.[36] In the present study, the maximum binding at 4°C (*Figure 2*) was 51% of the steady state uptake at 37°C. The result is similar to that obtained in cultured pig parenchymal cells in which surface binding accounted for about 43% of the steady state uptake of HDL at 37°C.[36] Furthermore, Nakai[16] reported that the surface binding was about 60% of the steady state uptake of HDL in freshly isolated rat hepatic parenchymal cells. Since the increase in specific binding at 4°C was small in the Cu-deficient treatment, the marked increase in cell-associated uptake data at 37°C would suggest that the amount of HDL internalized, although not measured, may also be increased as a result of Cu deficiency.

Finally, the specific uptake response as a function of ligand concentration at 37°C was also elevated in incubations with cells and HDL from Cu-deficient rats (*Figure 5*). The magnitude of increase in HDL uptake was most prominent at lower ligand concentrations of

5 to 20 μg/ml. In both treatments, the response curves appeared to have reached a plateau around 20 μg/ml, indicating that the processes of binding and internalization are saturable.

## Conclusion

In summary, the present findings of enhanced specific binding at 4°C and cell-associated uptake at 37°C as a function of time, as well as the increased specific cell-associated uptake at 37°C as a function of ligand concentration, suggest that the processes of lipoprotein binding and internalization are not impaired as a result of Cu deficiency. In fact, the present data, as well as the results of increased in vivo plasma clearance and tissue uptake of apolipoproteins and cholesteryl ester of HDL,[4] indicate that processes involved in HDL catabolism may be accelerated in Cu deficiency. However, in order to induce and sustain the enlarged HDL pool size in Cu deficiency,[37] the overall HDL production must be increased.

In Cu-deficient rats, the absolute amount of HDL cholesteryl ester cleared from the plasma pool was enhanced more than 2-fold, and virtually all of the increased removal of HDL cholesteryl ester was attributed to the liver.[4] In addition, data from the in vitro study of hepatic HMG CoA reductase activity[10] and in vivo study of cholesterol synthesis[11] indicate that hepatic cholesterol synthesis is enhanced in Cu-deficient rats. The inability of the liver, which is presented with an increased supply of cholesterol from plasma HDL and de novo synthesis, to normalize the depressed hepatic cholesterol store[37] suggests that the available cholesterol is excreted or secreted rapidly out of the liver, resulting in a net efflux of cholesterol from the liver. Since bile acid production and cholesterol elimination are not affected by Cu deficiency,[8] the increased supply of cholesterol available to the liver is not diverted to enhance the excretory process. Thus, the secretory process must be increased. Indeed, a markedly accelerated clearance of nascent cholesteryl ester, newly synthesized from mevalonate, observed from the liver to plasma[12] indicates that cholesterol derived from an increased hepatic synthesis is packaged into lipoproteins to facilitate an enhanced clearance of nascent cholesteryl ester from the liver to the plasma. A prolonged net efflux of cholesterol from the liver to plasma in time may result in a depressed liver cholesterol content as well as an increased plasma cholesterol concentration and an enlarged plasma cholesterol pool in the Cu deficient rats.

## References


1 Lei, K.Y. (1990). Plasma cholesterol response in copper deficiency. In *Roles of Copper in Lipid Metabolism* (K.Y. Lei and T.P. Carr, eds.), CRC Press, Inc., Boca Raton, FL (in press)

2 Lei, K.Y., Hassel, C.A., and Allen, D.K. (1983). Alterations in plasma lipid, lipoprotein and apolipoprotein concentrations in copper-deficient rats. *J. Nutr.* **113,** 2178–2183

3 Al-Othman, A.A., and Lei, K.Y. (1989). Time course development of enlarged plasma volume and cholesterol pool size in copper-deficient rats. *FASEB J.* **3,** A358

4 Carr, T.P., and Lei, K.Y. (1990). High density lipoprotein cholesteryl ester and protein catabolism in hypercholesterolemic rats induced by copper deficiency. *Metabolism* (in press)

5 Spady, D.K., Dietschy, J.M. (1983). Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. *J. Lipid Res.* **24,** 303–315

6 Croswell, S.C., and Lei, K.Y. (1985). Effect of copper deficiency on the apolipoprotein E-rich high density lipoproteins in rats. *J. Nutr.* **115,** 473–482

7 Lefevre, M., Keen, C.L., Lonnerdal, B., Hurley, L.S., and Schneeman, B.O. (1986). Copper deficiency-induced hypercholesterolemia: Effects on HDL subfractions and hepatic lipoprotein receptor activity in the rat. *J. Nutr.* **116,** 1735–1746

8 Lei, K.Y. (1978). Oxidation, excretion, and tissue distribution of [26-$^{14}$C]cholesterol in copper-deficient rats. *J. Nutr.* **108,** 232–237

9 Allen, K.G.D., and Klevay, L.M. (1978). Copper deficiency and cholesterol metabolism in the rat. *Atherosclerosis* **31,** 259–271

10 Yount, N.Y., McNamara, D.J., Al-Othman, A.A., and Lei, K.Y. (1990). The effect of copper deficiency on rat hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity. *J. Nutr. Biochem.* **1,** 27–33

11 Yount, N.Y., Carr, T.P., Lei, K.Y., and McNamara, D.J. (1990). Incorporation of tritiated water into sterols in copper deficient rats. *FASEB J.* A509

12 Shao, M.J.S., and Lei, K.Y. (1980). Conversion of [2-$^{14}$C]mevalonate into cholesterol, lanosterol and squalene in copper-deficient rats. *J. Nutr.* **110,** 859–867

13 Ose, L., Ose, T., Norum, K.R., and Berg, T. (1979). Uptake and degradation of [$^{125}$I] labelled high density lipoproteins in rat liver cells in vivo and in vitro. *Biochim. Biophys. Acta* **574,** 521–536

14 Eisenberg, S., Windmueller, H.G., and Levy, R.I. (1973). Metabolic fate of rat and human lipoprotein apoproteins in the rat. *J. Lipid Res.* **14,** 446–458

15 Roheim, P.S., Rachmilewitz, D., Stein, O., and Stein, Y. (1971). Metabolism of iodinated high density lipoproteins in the rat. I. Half-life in the circulation and uptake by organs. *Biochim. Biophys. Acta* **248,** 315–329

16 Nakai, T., Otto, P.S., Kennedy, D.L., and Whayne, T.F., Jr. (1976). Rat high density lipoprotein subfraction ($HDL_3$) uptake and catabolism by isolated rat liver parenchymal cells. *J. Biol. Chem.* **251,** 4914–4921

17 Rachmilewitz, D., Stein, O., Roheim, P.S., and Stein, Y. (1972). Metabolism of iodinated high density lipoproteins in the rat. II. Autoradiographic localization in the liver. *Biochim. Biophys. Acta* **270,** 414–425

18 Glass, C., Pittman, R.C., Keller, G.A., and Steinberg, D. (1983). Tissue site of degradation of apoprotein apo A-I in the rat. *J. Biol. Chem.* **257,** 7161–7167

19 Glass, C., Pittman, R.C., Weinstein, D.B., and Steinberg, D. (1983). Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: Selective delivery of cholesterol ester to liver, adrenal, and gonad. *Proc. Natl. Acad. Sci. USA* **80,** 5435–5439

20 Koelz, H.R., Sherrill, B.C., Turley, S.D., and Dietschy, J.M. (1982). Correlation of low and high density lipoprotein binding in vivo with rates of lipoprotein degradation in the rat. *J. Biol. Chem.* **257,** 8061–8072

21 Stein, Y., Dabach, Y., Hollander, G., Halperin, G., and Stein, O. (1983). Metabolism of HDL-cholesteryl ester in the rat, studied with a nonhydrolyzable analog, cholesteryl linoleyl ether. *Biochim. Biophys. Acta* **752,** 98–105

22 Hassel, C.A., Carr, T.P., Marchello, J.A., and Lei, K.Y. (1988). Apolipoprotein E-rich high density lipoprotein binding to liver plasma membranes in copper-deficient rats. *Proc. Soc. Exp. Biol. Med.* **187,** 296–308

23 American Institute of Nutrition. (1976). Report of the AIN Ad Hoc Committee on standards for nutritional studies. *J. Nutr.* **107,** 1340–1348

24 Weisgraber, K.H., and Mahley, R.W. (1980). Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21,** 316–325

25 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275
26 Laemmli, U.K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage $T_4$. *Nature* **227,** 680–685
27 McFarlane, A.S. (1958). Efficient trace-labelling of proteins with iodine. *Nature* **182,** 53
28 Goldstein, J.L., Basu, S.K., and Brown, M.S. (1983). Receptor mediated endocytosis of low density lipoprotein in cultured cells. In *Methods in Enzymology*, vol. 98. (S. Fleischer and B. Fleischer, eds.), pp 241–260, Academic Press, Orlando, FL
29 Berry, M.N., and Friend, D.S. (1969). High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* **43,** 506–520
30 Winer, B.J. (1971). *Statistical Principles in Experimental Design*, pp 431–513, 2nd ed., McGraw-Hill, New York
31 Brown, M.S., and Goldstein, J.L. (1979). Receptor-mediated endocytosis: insight from the lipoprotein receptor system. *Proc. Natl. Acad. Sci. USA* **76,** 3330–3337
32 Underwood, E.J. (1977). Copper. In *Trace Elements in Human and Animal Nutrition*, 4th ed., pp 56–108, Academic Press, New York
33 Hill, C.H. (1969). A role of copper in elastin formation. *Nutr. Rev.* **27,** 99–100
34 Goodman, J.R., Warshaw, J.B., and Dallman, P.R. (1970). Cardiac hypertrophy in rats with iron and copper deficiency: Quantitative contribution of mitochondrial enlargement. *Pediatr. Res.* **4,** 244–256
35 Lei, K.Y. (1977). Cholesterol metabolism in copper deficient rats. *Nutr. Rep. Int.* **15,** 597–605
36 Bachorik, P.S., Franklin, F.A., Virgil, D.G., and Kwiterovich, P.O., Jr. (1985). Reversible high affinity uptake of apo E-free high density lipoproteins in cultured pig hepatocytes. *Arteriosclerosis* **5,** 142–152
37 Carr, T.P., and Lei, K.Y. (1989). In vivo apoprotein catabolism of high density lipoprotein in copper-deficient, hypercholesterolemic rats. *Proc. Soc. Exp. Biol. Med.* **191,** 370–376