

Lipid fluidity of triacylglycerol-rich lipoproteins isolated from copper-deficient rats*

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Triacylglycerol-rich lipoproteins (TGRLP) were isolated from Cu-deficient and control rats. TGRLP from Cu-deficient rats appeared more fluid than those from controls as sensed by the fluorescence anisotropy of 1,6-diphenyl-1-3,5-hexatriene (DPH). This high fluidity was related to a low cholesterol:phospholipid ratio and high triacylglycerol content in these lipoproteins. TGRLP from Cu-deficient rats were more susceptible to *in vitro* peroxidation than lipoproteins from control rats as shown by the rate of diene conjugation. The damage induced by the peroxidation resulted in a more ordered state of the lipid fraction especially in lipoproteins from Cu-deficient rats. Thus, after *in vitro* peroxidation, TGRLP from Cu-deficient rats were more rigid than those from controls. These results suggest that Cu deficiency induces modifications in physicochemical properties of TGRLP which could affect their metabolism.

Copper: Lipoproteins: Peroxidation: Lipid fluidity

For many years, nutrition researchers have attempted to define the links between dietary trace elements and atherosclerotic cardiovascular disease. Severe Cu deficiency in rats results in a number of unfavourable changes that might contribute to cardiovascular risk and affect lipid metabolism (Lei, 1990). Hypercholesterolaemia is associated with an increase in apolipoprotein B plasma concentration (Nassir *et al.* 1993). A corresponding increase in plasma triacylglycerol-rich lipoproteins (TGRLP) containing apolipoprotein B (VLDL+LDL) occurs in Cu-deficient animals and marked alterations in TGRLP composition have been described (Rayssiguier *et al.* 1993). Moreover, Cu deficiency increases the susceptibility of lipoproteins to *in vitro* peroxidation (Rayssiguier *et al.* 1993). Both chemical composition (Sola *et al.* 1990) and oxidative processes (Borchman *et al.* 1992) may affect the bulk fluidity of lipoproteins. This is of special importance because the metabolic fate of plasma lipoproteins is profoundly influenced by their physical properties (Deckelbaum *et al.* 1977). The present report describes the effects of Cu deficiency in rats on lipid fluidity of TGRLP and the influence of *in vitro*-induced oxidative modification of lipoproteins on their physical properties.

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MATERIALS AND METHODS

Animals and diets

The Institute's guide for the care and use of laboratory animals was followed. Twenty-four weanling male Wistar rats (IFFA-CREDO, L'Arbresle, France) weighing 60 (SE 3) g were divided at random into Cu-deficient and control groups (twelve rats per group). Rats were housed in wire-bottomed cages in a temperature-controlled room (22°) with a 12 h light-dark cycle and pair-fed on the appropriate diets for 6 weeks. The semi-purified diets contained (g/kg): casein 200, sucrose 650, maize oil 50, alphacel 50, DL-methionine 3, choline bitartrate 2, modified AIN-76 mineral mix 35, AIN-76A vitamin mix 10 (ICN Biomedicals, Orsay, France). Cupric carbonate was omitted from the AIN-76 mineral mix. The Cu concentrations of the diets were 0.6 mg/kg (deficient) diet and 7.5 mg/kg (control) diet. Fed animals were killed after being anaesthetized with sodium pentobarbital (40 mg/kg body weight given intraperitoneally). Blood was collected into heparinized tubes and plasma was obtained by low-speed centrifugation (2000 g). Packed cell volume was determined by centrifugation in a capillary tube system.

Lipoprotein separation

Equal volumes of plasma samples from two animals were pooled for lipoprotein separation. Ultracentrifugation was performed at 15° in a Beckman L-5-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA) with a Ti 50 rotor (Havel *et al.* 1955). EDTA (1 g/l) and butylated hydroxytoluene (BHT) (4.4 mg/l) were added immediately before lipoprotein separation. Samples were overlaid with 0.15 M-NaCl (density 1.006 kg/l) and chylomicrons were discarded following two centrifugations for 30 min at 12000 g. To isolate the VLDL + LDL fraction, the plasma was adjusted to density 1.050 kg/l with solid KBr. Centrifugation was at 100000 g for 20 h. The VLDL + LDL fraction was then washed by a further period of ultracentrifugation at the same density.

Oxidation experiments

Before oxidation experiments the purified lipoprotein fractions were dialysed against 0.01 M-phosphate buffer (pH 7.4) containing 0.15 M-NaCl, which was made O₂-free by vacuum-degassing followed by purging with N₂. Suitable volumes from the dialysed solutions were diluted with phosphate-buffered saline (PBS) to obtain a final concentration of 0.5 g/l. Lipoprotein concentration was calculated from the mass of protein plus individual lipids. Oxidation experiments were performed as previously described (Esterbauer *et al.* 1989). Oxidation was initiated by addition of freshly prepared CuSO₄ solution (5 μM final concentration) at 37°. Oxidation of lipoproteins *in vitro* is accompanied by characteristic changes in chemical properties. Conjugated dienes develop in lipoproteins through the oxidation of polyunsaturated fatty acids (PUFA) with isolated double bonds to PUFA-hydroperoxides with conjugated double bonds (dienes) with a u.v. absorption maximum at 234 nm. The kinetics of the oxidation of lipoproteins were monitored by the change in the 234 nm absorbance at 37° (Esterbauer *et al.* 1989) on a spectrophotometer (Uvikon 820, Kontron, St-Quentin-en-Yvelines, France) and conjugated diene formation was expressed as the difference between the absorbance 5 h after and before *in vitro* oxidation.

Polarization fluorescence studies

Lipoprotein fluidity was assessed at 25° by determining the anisotropy of fluorescence from the probe 1,6-diphenyl-1-3,5-hexatriene (DPH) (Ben Yashar & Barenholz, 1991). Probe incorporation was accomplished by diluting 2 mM-DPH in tetrahydrofuran 1000 times into the aqueous lipoprotein solution before and 5 h after *in vitro* oxidation and incubating with

Table 1. *Body and tissue weights (g), packed cell volume (volume fraction), plasma copper ($\mu\text{mol/l}$) and lipid (mmol/l) concentrations in control and copper-deficient rats†*

(Mean values with their standard errors for twelve rats per group)

Diet group	Body weight		Heart weight		Packed cell volume		Copper		Triacylglycerols		Total cholesterol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	279	4	0.96	0.06	0.42	0.02	14.50	0.61	0.39	0.06	1.50	0.06
Copper-deficient	246***	7	1.74**	0.20	0.15***	0.02	0.80***	0.12	0.71**	0.09	1.96***	0.08

Mean values were significantly different from those of the control, ** $P < 0.01$, *** $P < 0.001$.
 † For details of diets see p. 768.

Table 2. Proportions of protein, triacylglycerols, cholesterol and phospholipids in VLDL+LDL fractions (mg/g) in rats fed on a control or copper-deficient diet†
(Mean values with their standard errors for six samples per group)

Diet group	Protein		Triacylglycerols		Total cholesterol		Phospholipids	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	114	6	518	17	186	9	181	7
Copper-deficient	102	4	619***	12	104***	4	174	5

Mean values were significantly different from those of the control, *** $P < 0.001$.

† For details of diets see p. 768.

agitation at 25° for 1 h. Fluorescence polarization intensity was measured with a SEFAM spectrofluorometer (Vandoeuvre-lès-Nancy, France). In order to avoid any scattering problems, labelled and non-labelled samples were studied and the corrected anisotropy calculated (Gruneberger *et al.* 1982). With the amount of lipoproteins used, no correction was necessary. Moreover, the dilution method with the extrapolated final anisotropy led to the same conclusion (Lentz *et al.* 1979).

Plasma and lipoprotein analysis

Triacylglycerols (Biotrol, Paris, France), cholesterol and phospholipids (Biomérieux, Charbonnières-les-Bains, France) were determined in plasma and lipoprotein fractions by enzymic procedures. The protein concentration of the VLDL+LDL fraction was determined by a modified Lowry method using bovine serum albumin, fraction V (Sigma, L'Isle d'Abeau, France) as a standard (Markwell *et al.* 1978). Cu in plasma and diets was determined with a Perkin Elmer 560 atomic absorption spectrophotometer (Norwalk, CT, USA).

Statistical methods

Results are expressed as means with their standard errors. The statistical significance of differences between means was assessed by Student's *t* test.

RESULTS

Rats fed on the Cu-deficient diet had a lower body weight and lower plasma Cu concentration than those fed on the Cu-adequate diet. In addition, Cu-deficient rats were characterized by reduced packed cell volumes and enlarged hearts. Plasma cholesterol and triacylglycerol concentrations were significantly greater in Cu-deficient rats than control animals (Table 1).

Marked alterations in the lipid composition of lipoproteins occurred in the Cu-deficient rats. The proportion of triacylglycerols in the VLDL+LDL fraction was elevated. The proportion of cholesterol was markedly reduced whereas that of phospholipids was not significantly modified (Table 2). Consequently, Cu-deficiency resulted in a decrease in the cholesterol:phospholipid ratio.

Cu deficiency substantially affected lipoprotein fluidity. VLDL+LDL anisotropy values were significantly lower in Cu-deficient rats than in controls. The lipoproteins from the deficient rats were more susceptible to *in vitro* lipid peroxidation as shown by the rate of diene conjugation (absorption at 234 nm). Lipid oxidation resulted in an increased

Table 3. Effect of dietary copper on conjugated diene production (Δ absorbance at 234 nm) and anisotropy (r) of VLDL + LDL fractions†
(Mean values with their standard errors for six samples per group)

Dietary group	Conjugated dienes‡		Anisotropy§			
			Unincubated		Incubated	
	Mean	SE	Mean	SE	Mean	SE
Control	1.285	0.048	0.125	0.003	0.143	0.007
Copper-deficient	1.851**	0.124	0.098***	0.002	0.175**	0.004

Mean values were significantly different from those of the control, ** $P < 0.01$, *** $P < 0.001$.

† For details of diets and procedures, see pp. 768–770.

‡ Conjugated diene formation was expressed as the difference between the absorbance at 234 nm 5 h after and before *in vitro* oxidation.

§ Anisotropy values were determined before incubation and 5 h after *in vitro* oxidation.

anisotropy of lipoproteins ($P < 0.05$ for lipoproteins from control rats and $P < 0.001$ from Cu-deficient rats). However, after *in vitro* peroxidation the lipoproteins from Cu-deficient rats exhibited significantly higher anisotropy values compared with those obtained under the same conditions with lipoproteins from control animals (Table 3).

DISCUSSION

Several measurements, such as decreased body weight, increased heart weight and lower packed cell volume (Prohaska, 1990), are indicators of dietary Cu deficiency in addition to the reduced plasma Cu concentration. The mechanism by which Cu deficiency induces hyperlipaemia is not understood completely. Studies on hepatic apolipoprotein synthesis indicate that apolipoprotein B100 synthesis is increased in Cu-deficient rats (Nassir *et al.* 1993). Thus, the hyperlipaemia observed appears to be sustained by an increase in hepatic lipoprotein synthesis (Al-Othman *et al.* 1993). However, the possible contribution of defective uptake of plasma lipoproteins may also be involved in hyperlipaemia (Lei, 1990). Our results confirm previous observations that the lipid composition of VLDL + LDL from Cu-deficient rats differs markedly from that of control rats (Rayssiguier *et al.* 1993). Results of the present experiment indicate that the overall fluidity of the lipoproteins as sensed by the fluorescence anisotropy of DPH is markedly enhanced in deficient animals. Well-established lipid modulators of the fluidity are known and especially the cholesterol:phospholipid ratio (C:PL) (Shinitzky, 1982). In Cu-deficient rats the C:PL ratio is drastically reduced and the triacylglycerol content increased, which strongly favours a high fluidity. In fact, cholesterol acts as an ordering molecule for the acyl chains of phospholipids and this results in restriction of movements of these chains; the low C:PL observed is consistent with the consequent effect on the bulk lipid fluidity (Shinitzky, 1982). Moreover, an increased content of triacylglycerols results in a more fluid and dynamic state of the core of lipoproteins (Berlin & Young, 1983; Ben Yashar & Barenholz, 1991). In the present experiment DPH was used to determine the core properties of lipoproteins. To determine if peroxidation affects the lipoprotein envelopes it would be useful to use trimethyl ammonium (TMA)-DPH (Ben Yashar & Barenholz, 1991). Under normal conditions Cu does not occur *in vivo* in free form, being tightly bound to proteins (Parthasarathy & Steinberg, 1992), whereas oxidation of lipoproteins *in vitro* proceeds at a high rate when the

medium contains transition metal ions (Esterbauer *et al.* 1991). Our experimental model used the Cu oxidation process for lipoproteins. The conjugated diene values 5 h after *in vitro* oxidation confirmed that VLDL + LDL particles from deficient animals are more susceptible to oxidative damage than those of control rats. The aim of a previous study was to determine in more detail the time-course of lipoprotein oxidation (Rayssiguier *et al.* 1993). The lipoprotein fraction from Cu-deficient rats was readily oxidized as indicated by the short lag phase and the rapid increase in the rate of conjugated diene formation. In this previous experiment (Rayssiguier *et al.* 1993) lipid hydroperoxide and thiobarbituric-acid-reactive substance measurements after the induction of peroxidation were in agreement with the greater formation of conjugated dienes. It is well known that lipid oxidation results in an apparently more ordered lipid organization (Richter, 1987). In fact, the oxidative process, throughout its development, reaches a final point leading to linkage between fatty acids, reducing the mobility of the chains (Richter, 1987). This reduction of acyl-chain movement affects the bulk fluidity of the particles. Our results from fluorescence polarization are fully in agreement with these kinds of oxidative damage. All the lipid organization results in an important rigidification of the lipid fraction as indicated by the DPH movements. However, the same oxidative process affects lipoproteins from control and deficient rats differently. The differences observed between anisotropy values for incubated and unincubated particles are markedly enhanced in Cu-deficient rats compared with control ones. A more ordered state of lipoproteins from Cu-deficient rats may depend on the more severe degree of oxidation of these lipoproteins (Richter, 1987). Major disturbances of antioxidant status occur in Cu deficiency and the role of several alterations in the composition of the TGRLP that may affect lipoprotein oxidation has been previously discussed (Rayssiguier *et al.* 1993). Moreover, changes in fatty acid metabolism have been described with the fatty acid unsaturation index usually being higher in Cu-deficient rats (Cunnane, 1990). These changes would help to explain the increase in fluidity and peroxidation of TGRLP. The present study also suggests that the state of lipid order before oxidation may influence the susceptibility. A highly fluid lipid organization, as observed in the VLDL + LDL particles from Cu-deficient rats, could be more affected by an oxidative process and the lack of cholesterol content in TGRLP from Cu-deficient rats could favour more damaging oxidative processes. Increased levels of cholesterol in synthetic liposomes have a stabilizing effect which may in turn decrease the rate of peroxidation providing protection against lipid peroxidation (Smith, 1991; McLean & Hagamen, 1992). Thus, the less ordered state of lipid particles, as shown in Cu-deficient rats, may allow better access of free radicals to peroxidation sites. However, results from the present experiments indicate that lipoproteins from Cu-deficient rats are more fluid than those from controls following 6 weeks on experimental diets. So, it would be of interest to investigate if a long-term Cu deficiency results in decreased lipid fluidity related to enhanced lipid peroxidation *in vivo*. Modifications of lipoproteins in Cu deficiency as shown by increased susceptibility to peroxidation, and the resulting rigidification, may interact with the ability of receptors to recognize such modified lipoproteins (Parthasarathy & Steinberg, 1992). The relationship between lipoprotein fluidity and atherogenicity remains to be elucidated. However, it has been suggested that the atherogenicity of lipoproteins increases as they become less fluid (Souter, 1978). The role of Cu as an antioxidant nutrient for cardiovascular health has recently been emphasized (Allen & Klevay, 1994). Dietary Cu is low in the Western diet and low intake may affect several stages of atherosclerosis adversely (Allen & Klevay, 1994). The present study suggests that the alterations of the physicochemical properties of lipoproteins in Cu deficiency may play an important role in their metabolic fate.

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