

Effect of magnesium deficiency on triacylglycerol-rich lipoprotein and tissue susceptibility to peroxidation in relation to vitamin E content

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Given the current interest in the cardiovascular complications of Mg deficiency, the aim of the present experiment was to investigate the effect of Mg deficiency on the time-course of lipoprotein oxidation and to assess whether short-term Mg deficiency results in vitamin E depletion that predisposes lipoproteins and tissues to subsequent oxidation. Weanling rats were pair-fed for 8 d with control and Mg-deficient diets respectively. Plasma triacylglycerol and α -tocopherol levels were significantly greater in Mg-deficient rats compared with control animals. The increase in plasma apolipoprotein B concentration indicated that a corresponding increase in plasma triacylglycerol-rich lipoproteins (TGRLP) occurred in Mg-deficient animals. Hyperlipaemia was associated with modifications in the composition of TGRLP. The proportion of triacylglycerols was elevated whereas that of cholesterol and protein was reduced, and Mg deficiency resulted in a slight significant reduction in α -tocopherol content. When the TGRLP fractions were subjected to *in vitro* Cu-induced oxidation the lipoprotein fractions from Mg-deficient rats were more susceptible to oxidative damage than lipoprotein fractions from control rats. Mg deficiency did not modify the α -tocopherol content of liver, heart and skeletal muscle. However, after exposure of tissue homogenates to Fe-induced lipid peroxidation, thiobarbituric acid-reactive substances were significantly higher in tissues from Mg-deficient rats compared with those from control rats. These results complement previous findings, showing that Mg deficiency increases the susceptibility of TGRLP and tissues to peroxidation and suggest that oxidative damage is not the result of a decrease in vitamin E antioxidant status.

Magnesium: Vitamin E: Peroxidation

Insufficient Mg intake can result in serious cardiovascular complications. Young animals fed on diets deficient in Mg frequently develop cardiomyopathic lesions and several experiments support the role of experimental Mg deficiency in the pathogenesis of atherosclerosis (Rayssiguier & Gueux, 1986; Freedman *et al.* 1990; Bussièrre *et al.* 1994). Recently, we have shown that short-term Mg deficiency in rats increases tissue susceptibility to peroxidation (Rayssiguier *et al.* 1993*b*). The mechanisms responsible for the atherogenicity characteristic of Mg deficiency may be mediated by an increased susceptibility of lipoproteins to peroxidation (Gueux *et al.* 1993). However, the precise mechanism by which Mg deficiency can potentiate oxidative injury remains to be determined.

The possibility exists that dietary Mg deficiency results in a deficiency of antioxidant capacity that predisposes to oxidative stress. Vitamin E is one of the most effective

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scavengers of free radicals (Chow, 1991), and recent studies indicate that vitamin E administration offers significant protection against the pro-oxidant influence of Mg deficiency. Vitamin E is very effective in preventing the Mg deficiency-induced cardiomyopathy (Freedman *et al.* 1990) and prevents the occurrence of the enhanced heart post-ischæmic injury following Mg deficiency (Kramer *et al.* 1994). Moreover, a decrease in tissue vitamin E content following long-term Mg deficiency in rats has been reported (Günther *et al.* 1992). However, the vitamin E depletion may be either the cause or the consequence of increased oxidative stress following Mg deficiency. Currently, the role of lipoprotein oxidation and the vitamin E protection in the pathogenesis of atherosclerosis is attracting a great deal of interest (Berliner & Haberland, 1993). Thus, the aim of the present study was to investigate the effect of Mg deficiency by studying in detail the time-course of lipoprotein oxidation and to assess whether short-term Mg deficiency results in vitamin E depletion that predisposes lipoproteins and tissues to subsequent oxidative stress.

MATERIALS AND METHODS

Animals and diets

The institution's guide for the care and use of laboratory animals was followed.

Thirty-six weanling male Wistar rats (IFFA-CREDO, L'Arbresle, France), 3 weeks old, weighing 60 (SE 2) g were divided randomly into Mg-deficient and control groups (eighteen animals per group). The animals were pair-fed with the appropriate diets for 8 d using an automatic feeding apparatus. Distilled water was provided *ad lib*. The semi-purified diets contained the following (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). MgO was omitted from the AIN-76 mineral mix or added to the control diet. Mg levels determined by flame atomic absorption spectrophotometric analysis (model 560; Perkin Elmer, Norwalk, CT, USA) were 30 (deficient) and 960 (control) mg/kg. 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). The heart and liver (entire organs) and skeletal muscle (gastrocnemius) from six animals per group were rapidly removed, washed in ice-cold saline (9 g NaCl/l), placed in liquid N₂ and stored at -80°.

Lipoprotein separation

Equal volumes of plasma samples from two animals were pooled for lipoprotein separation. Ultracentrifugation was performed at 15° in a Beckman model L-5-50B ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA) 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 removed after centrifuging twice for 30 min at 12000 g. To isolate the triacylglycerol-rich lipoprotein (TGRLP) fraction (VLDL + LDL) the infranatant was adjusted to a density of 1.050 kg/l with solid KBr. Centrifugation was carried out 100000 g for 20 h. The TGRLP 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 (PBS) that was made O₂-free by vacuum degassing followed by purging with N₂. Suitable volumes from the dialysed solutions were diluted with 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 the addition of freshly prepared CuSO_4 solution ($5 \mu\text{M}$ final concentration) at 37° . The kinetics of the oxidation of lipoproteins was determined by monitoring continuously the change in the 234 nm absorbance at 37° using a spectrophotometer (Uvikon 820; Kontron, St-Quentin en Yvelines, France). The initial absorbance at 234 nm was set to zero and the increase in absorbance was recorded. The values for conjugated dienes at 4 h following *in vitro* incubation were compared with those of thiobarbituric acid-reactive substances (TBARS) and of lipid hydroperoxides (Fox-reactive substances). TBARS were determined as previously reported but with slight modifications (Dousset *et al.* 1990). A mixture (thiobarbituric acid (8 g/l)–perchloric acid (70 g/l) 2:1, v/v; 750 μl) was added to 100 μl lipoprotein. After agitation, the mixture was placed in a water-bath at 95° for 60 min and then cooled in an ice-bath. The fluorescing compound was extracted by mixing with *n*-butanol for 2 min. The TBARS content of the *n*-butanol layer was determined using a spectrofluorometer (LS 5; Perkin Elmer, Norwalk, CT, USA), excitation wavelength 532 nm, emission 553 nm. Lipid hydroperoxides (Fox-reactive substances) were determined as recently reported (Jiang *et al.* 1992). Portions (100 μl) of incubated VLDL + LDL samples were added to 900 μl of the following reaction mixtures and incubated for 30 min at room temperature before measurement at 560 nm: 100 mM-xylene orange, 250 mM- Fe^{2+} , 25 mM- H_2SO_4 and 4 mM-BHT in methanol (900 ml/l; calibration with H_2O_2). For lipid peroxidation studies of tissues, homogenates were prepared on ice in the proportion 1 g wet tissue:9 ml 150 mM-KCl using a Polytron homogenizer. TBARS were measured in tissue homogenates after lipid peroxidation induced with FeSO_4 (10 μM)–ascorbate (250 mM) for 30 min in a water-bath at 37° in an O_2 -free medium, using a standard of 1,1,3,3-tetraethoxypropane, as previously described (Ohkawa *et al.* 1979).

Plasma and tissues analysis

Triacylglycerols (Biotrol, Paris, France), cholesterol and phospholipids (Biomérieux, Charbonnières-les-Bains, France) were determined in plasma and lipoprotein fractions by enzymic procedures. Plasma apolipoprotein B (apo B) was determined by radial immunodiffusion using sheep anti-rat apo B antiserum, as previously described (Felgines *et al.* 1994). The protein concentration of the TGRLP fraction was determined by a modified Lowry method (Markwell *et al.* 1978), using bovine serum albumin (fraction V; Sigma, l'Isle d'Abeau, France) as a standard. Mg in plasma was determined with a Perkin Elmer 560 atomic absorption spectrophotometer. Vitamin E was assayed by reverse-phase HPLC (HPLC apparatus; Kontron serie 400; Kontron, St-Quentin-en-Yvelines, France) using a hexane extract. Briefly, α -tocopheryl acetate (Sigma) was added to samples as an internal standard; then they were extracted twice with hexane, after ethanol precipitation of the proteins. This extract was evaporated to dryness under N_2 , dissolved in methanol–methylene chloride (65:35, v/v) and injected onto a C_{18} column (Nucleosil; 250 mm long, i.d. 4.6 mm, 5 μm particles). Pure methanol, at a flow-rate of 2 ml/min eluted α -tocopherol in 5.0 min and tocopheryl acetate in 6.3 min. The compounds were detected by u.v. (292 nm), then quantified by internal and external calibration using daily-controlled standard solutions.

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

The mean final body weight (g) of the Mg-deficient and control rats were 88 (SE 2) v. 93 (SE 1) g respectively (n 18, $P < 0.05$) at the end of the experimental period. The results of

Table 1. Effect of dietary magnesium on concentrations of plasma Mg (mmol/l), lipids (mmol/l), apolipoprotein B (apo B; mg/l) and α -tocopherol (μ g/ml) in rats†
(Mean values with their standard errors for six rats per group)

Diet group	Mg		Triacylglycerols		Cholesterol		Phospholipids		Apo B		α -Tocopherol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	0.80	0.02	0.63	0.10	1.19	0.05	1.31	0.08	190	22	3.61	0.19
Mg-deficient	0.12***	0.01	3.55***	0.25	1.47*	0.10	1.74**	0.08	351***	28	5.93***	0.34

Mean values were significantly different from control values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† For details of diets and procedures, see pp. 850–851.

Table 2. Proportion of protein, triacylglycerols, cholesterol and phospholipids (g/kg) and α -tocopherol content (μ g/mg) in VLDL+LDL fractions in rats fed on a control or Mg-deficient diet†

(Mean values with their standard errors for six values per group)

Diet group	Protein		Triacylglycerols		Cholesterol		Phospholipids		α -Tocopherol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	118	2	530	7	166	5	185	1	1.8	0.1
Mg-deficient	85***	1	628***	7	104***	6	182	3	1.4*	0.1

Mean values were significantly different from control values: * $P < 0.05$, *** $P < 0.001$.

† For details of diets and procedures, see pp. 850–851.

plasma analysis showed the hypomagnesaemia usually observed in Mg-deficient rats. Plasma triacylglycerol concentrations were significantly higher in Mg-deficient rats than in controls, whereas plasma cholesterol was only slightly elevated. The hypertriacylglycerolaemia was associated with a significant increase in plasma apolipoprotein B concentrations, and plasma α -tocopherol levels were significantly greater in Mg-deficient rats compared with those of control animals (Table 1). Hyperlipaemia was associated with modifications in the composition of TGRLP. The proportion of triacylglycerols was elevated, whereas that of cholesterol and protein was reduced and Mg deficiency resulted in a slight but significant reduction in the α -tocopherol content of TGRLP (Table 2).

When the TGRLP fractions from the two experimental groups were subjected to *in vitro* Cu-induced oxidation, marked differences were observed in the susceptibility to lipid peroxidation, as indicated by the rate of diene conjugation (absorption at 234 nm). The lipoprotein fraction from the Mg-deficient rats was readily oxidized, as indicated by the short lag phase and the rapid increase in the rate of formation of conjugated dienes. The lipoprotein fraction from rats fed on the control diet was more resistant to lipid peroxidation, as indicated by the length of the lag phase (Fig. 1).

Lipid hydroperoxides and TBARS measurements performed 4 h after the induction of peroxidation were in agreement with the greater formation of conjugated dienes (Table 3). Mg deficiency did not modify the vitamin E content of liver, heart and skeletal muscle. However, after exposure of tissue homogenates to Fe-induced lipid peroxidation, TBARS were significantly higher in tissues from Mg-deficient rats compared with those from control rats (Table 4).

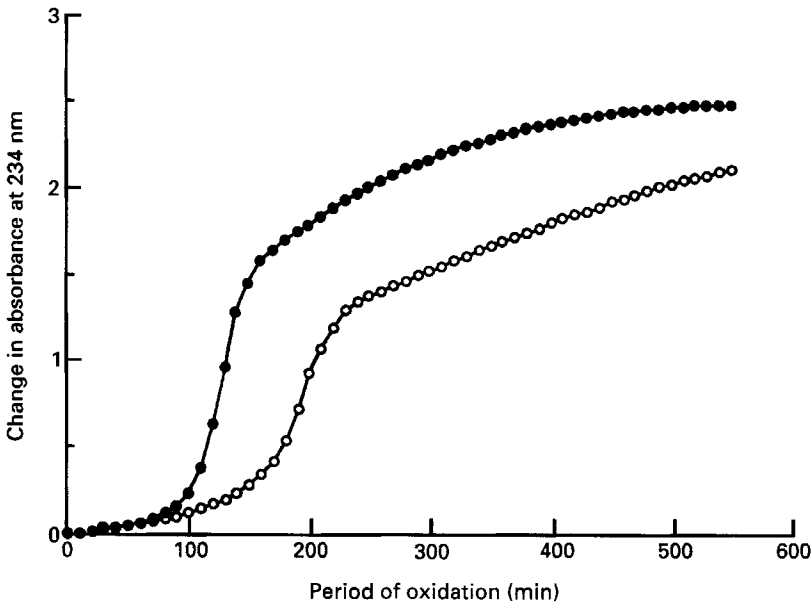


Fig. 1. Rate of formation of conjugated dienes in VLDL+LDL fractions from control (○) and magnesium-deficient (●) rats. Values are means of six samples of VLDL+LDL fractions for each dietary group. Fractions were oxidized in presence of 5 μmol Cu²⁺/l at 37°; absorbance was measured at 234 nm. The zero-time levels were subtracted from the values shown. For details of diets and procedures, see pp. 850–851.

Table 3. Effect of dietary magnesium on lipid peroxidation of VLDL+LDL fractions from rats after 4 h in vitro incubation†‡
(Mean values with their standard errors for six values per group)

Diet group	Conjugated dienes (Δ absorbance at 234 nm)		FOXRS (nmol/mg lipoprotein)		TBARS (nmol/mg lipoprotein)	
	Mean	SE	Mean	SE	Mean	SE
Control	1.334	0.055	264	27	26.2	7
Mg-deficient	1.964***	0.062	1304**	250	136**	27.8

TBARS, thiobarbituric acid-reactive substances; FOXRS, Fox-reactive substances; Δ, change.

Mean values were significantly different from control values: **P < 0.01, ***P < 0.001.

† For details of diets and procedures, see pp. 850–851.

‡ The values of conjugated dienes were compared with those of TBARS and of lipid hydroperoxides (FOXRS).

DISCUSSION

We previously reported that Mg deficiency in rats induces hyperlipaemia and affects plasma lipoprotein distribution and composition (Rayssiguier *et al.* 1981; Gueux *et al.* 1991). The most obvious effect of Mg deficiency on plasma lipids is a marked increase in triacylglycerol concentration. The increase in apo B plasma concentration, as shown in the present study, indicated that a corresponding increase in plasma TGRLP containing apo B (VLDL+LDL fraction) occurs in Mg-deficient animals. Whereas total cholesterol concentration in plasma was slightly modified, previous studies indicate that Mg deficiency produced a marked effect on the cholesterol distribution among the lipoprotein fractions, as shown by the

Table 4. *Effect of magnesium deficiency on susceptibility of tissues to peroxidation (thiobarbituric acid-reactive substances (TBARS) nmol/g wet weight) and on tissue α -tocopherol contents (μ g/g wet weight) of rats†*

(Mean values with their standard errors for six values per group)

Diet group...	Control				Mg-deficient			
	TBARS		α -Tocopherol		TBARS		α -Tocopherol	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Liver	43.8	1.2	29.7	1.8	97.2***	8.8	32.8 NS	4.1
Heart	212	21	63.3	4.7	314**	18	69.1 NS	6.9
Skeletal muscle	369	36	13.3	1.6	481*	31	21.0 NS	3.4

Mean values were significantly different from control values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NS, not significant.

Mean values were significantly different from control values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† For details of diets and procedures, see pp. 850–851.

increased cholesterol concentrations in LDL fractions and the decreased cholesterol concentrations in HDL fractions (Rayssiguier *et al.* 1981).

Unlike other fat-soluble vitamins, vitamin E has no specific plasma transport protein but rather is transported in plasma lipoproteins. α -Tocopherol is first secreted from the intestine in chylomicrons, then is secreted from the liver in VLDL and appears in the plasma simultaneously in LDL and HDL (Kayden & Traber, 1993). It is known that vitamin E distribution parallels that of total plasma lipids. Thus, it is not surprising that, because of elevated TGRLP levels, Mg-deficient rats had significantly higher plasma α -tocopherol concentrations than control rats.

Our previous studies, based on several different time-dependent analyses, demonstrated that Mg deficiency affects the susceptibility of TGRLP to *in vitro* peroxidation (Gueux *et al.* 1993). The results of the present experiment expressed in terms of conjugated dienes, lipid hydroperoxides and TBARS contents showed that VLDL+LDL particles from deficient animals are less protected against oxidative modification than those of control rats. The oxidation of lipoproteins *in vitro* can be roughly divided into three consecutive phases: lag phase, propagation phase and a decomposition period (Esterbauer *et al.* 1992). Mg deficiency resulted in a short lag phase and a rapid increase in the rate of formation of conjugated dienes. During the lag phase, vitamin E and other antioxidants present in lipoproteins are consumed and non-significant oxidation of fatty acids occurs. When lipoproteins are depleted of antioxidants, unsaturated fatty acids are rapidly oxidized to lipid hydroperoxides (propagation period) which are then converted into a variety of other products including reactive aldehyde (decomposition period) (Esterbauer *et al.* 1992). Most biological studies of modified lipoproteins were performed with lipoproteins oxidized by Fe or Cu ions (Rayssiguier *et al.* 1993*b*); it would be interesting to see whether other oxidizing agents may produce similar results to those obtained with Cu.

Antioxidants such as vitamin E can protect lipoproteins by preventing the propagation initiated by free-radical attack. However, once the antioxidants are consumed, peroxidation can go on unabated. Vitamin E, whether added *in vitro* to LDL or administered orally *in vivo*, can prolong the lag phase before oxidation of other molecules (Esterbauer *et al.* 1992). However, even if our findings showed a slight decrease in vitamin E content of TGRLP in Mg-deficient rats, its relationship with the increased peroxidability of lipoproteins should be interpreted with caution. Several studies do not indicate clear correlations between the

vitamin content of lipoproteins and their oxidizability and suggest that oxidation resistance depends on more than one (i.e. vitamin E content) variable (Esterbauer *et al.* 1992). The possibility exists that a slowed catabolism of TGRLP, as reported in Mg-deficient rats (Rayssiguier *et al.* 1991), may reduce their α -tocopherol content by increased utilization. Moreover, peroxidation of lipids in isolated lipoproteins by Cu^{2+} requires the presence of preformed lipid peroxides (Parthasarathy & Steinberg, 1992). Recent studies indicate that Mg deficiency induces an inflammatory state and elevates circulating levels of inflammatory cytokines (Weglicki *et al.* 1992; Rayssiguier *et al.* 1994). During an inflammatory process the phagocytic cells are metabolically stimulated and a marked rise in oxidative metabolism occurs. Thus, *in vivo* lipoprotein oxidation in Mg-deficient rats may be related to the presence of inflammatory cells in the arterial wall (Rayssiguier *et al.* 1994). Alterations in the lipid composition of TGRLP occurs in Mg-deficient rats, as shown by the present experiment, and changes in the fatty acid composition of lipoproteins have been reported in an earlier study (Gueux *et al.* 1991). Therefore, these changes may also affect the susceptibility of lipoproteins to peroxidation.

Mg deficiency increased tissue susceptibility to peroxidation but did not result in vitamin E deficiency, as shown by the α -tocopherol content of liver, heart and skeletal muscles. Recent results suggest a defect in the catabolism of TGRLP as the major factor underlying the altered plasma lipoprotein profile in Mg-deficient rats in relation to a decrease in lipoprotein lipase (EC 3.1.1.34) activity (Rayssiguier *et al.* 1991). Tissues that receive most of their lipids during the delipidation cascade probably obtain α -tocopherol as a result of lipoprotein lipase activity. However, in patients with lipoprotein lipase deficiency, who also have a markedly slowed catabolism of TGRLP and high plasma α -tocopherol concentrations, normal tissue α -tocopherol concentrations have also been reported (Kayden & Traber, 1993). The oxidative stress in Mg-deficient animals could be due to an imbalance between inflammation-induced free-radical production and existing antioxidant capacity (Weglicki *et al.* 1994). In addition, membrane permeability and intracellular Ca content are increased in Mg deficiency. An increased concentration of intracellular Ca could act to enhance lipid peroxidation further and produce cell injury (Rayssiguier *et al.* 1993a). The possibility exists that Mg deficiency during a long experimental period (Günther *et al.* 1992) may accelerate vitamin E utilization, in relation to increased formation of free radicals. However, increased tissue peroxidation in Mg-deficient rats is not the result of a decrease in vitamin E antioxidant status.

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