Effects of copper deficiency on hepatic and cardiac antioxidant enzyme activities in lactose- and sucrose-fed rats

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1. A number of dietary sugars are known to mediate the effects of copper deficiency. The effects of lactose (compared with sucrose) and a dietary Cu deficiency on hepatic and cardiac antioxidant enzyme activities and tissue mineral element status were investigated in the rat.

2. Groups (n 6) of male weanling Wistar rats were provided *ad lib.* with deionized water and diets containing sucrose (580 g/kg) or sucrose and lactose (387 g/kg and 193 g/kg respectively) with either control (12·0 mg/kg) or deficient (1·5 mg/kg) quantities of Cu for 77 d.

3. Animals consuming the low-Cu diets exhibited significantly decreased tissue Cu levels ($P < 0·01$), hepatic and cardiac cytochrome *c* oxidase (*EC* 1.9.3.1, CCO) activities ($P < 0·01$ and $P < 0·001$ respectively) and hepatic Cu–zinc superoxide dismutase (*EC* 1.15.1.1, CuZnSOD) activity ($P < 0·05$). The low-Cu diets also significantly decreased cardiac manganese superoxide dismutase (*EC* 1.15.1.1, MnSOD), catalase (*EC* 1.11.1.6) and glutathione peroxidase (*EC* 1.11.1.9, GSH-Px) activities ($P < 0·01$, $P < 0·05$ and $P < 0·001$ respectively).

4. Hepatic Mn was significantly increased in both lactose-fed ($P < 0·001$) and Cu-deficient ($P < 0·01$) animals. These increases were unrelated to hepatic MnSOD activity. Cardiac Zn was significantly ($P < 0·01$) increased in Cu-deficient animals.

5. Lactose feeding resulted in significantly increased cardiac CCO activity ($P < 0·001$) but significantly decreased hepatic CuZnSOD ($P < 0·05$), catalase ($P < 0·01$) and GSH-Px ($P < 0·001$) activities.

6. The activities of lactose dehydrogenase (*EC* 1.1.1.27, LDH) and glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49, G6PDH) were found to be significantly ($P < 0·05$ and $P < 0·01$ respectively) increased in Cu-deficient animals and G6PDH activity was significantly ($P < 0·01$) decreased as a result of lactose consumption.

7. The observed changes in antioxidant enzyme activities associated with both Cu deficieny and lactose consumption may have important implications for the development of free radical mediated cell damage. However, no significant differences in either hepatic or cardiac levels of thiobarbituric acid reactive substances, a measure of lipid peroxidation, were found.

The involvement of copper in lipid and glucose metabolism and cardiac function in man has prompted Klevay (1983) to propose that a dietary Cu deficiency may be of major importance in the aetiology of ischaemic heart disease (IHD). Cu is also an essential component of several enzymes including the Cu–zinc form of the antioxidant enzyme superoxide dismutase (*EC* 1.15.1.1; CuZnSOD) (Fridovich, 1975). This enzyme and the other cellular antioxidant enzymes, catalase (*EC* 1.11.1.6), the selenoenzyme glutathione peroxidase (*EC* 1.11.1.9; GSH-Px) and manganese superoxide dismutase (*EC* 1.15.1.1; MnSOD), perform an important function in protecting the cell from the potentially harmful effects of oxygen free-radicals (Blake *et al.* 1987). A link between IHD and chronic inflammation has been recognized (Majno *et al.* 1985), and since O_2 free-radicals are important in the development of inflammation and peroxidative damage (Blake *et al.* 1987), these cellular antioxidant enzymes may play an important protective role against the development of IHD.

The consumption of milk has been identified in several epidemiological studies as correlating with the incidence of IHD (see Strain, 1988). The high fat content of milk was initially proposed (Briggs *et al.* 1960) as a putative causal factor, but more recently attention has focused on both the protein (Beynen *et al.* 1983; Stemmer *et al.* 1985) and carbohydrate (Segall, 1980; Pearce, 1984; Lember & Tamm, 1988) fractions.

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Table 1. *Composition (g/kg) of diets*

Component	Diets			
	Sucrose		Lactose	
	Control	Cu-deficient	Control	Cu-deficient
Sucrose*	580	580	387	387
Lactose†	—	—	193	193
Fibrous cellulose powder†	30	30	30	30
Casein†	200	200	200	200
Maize oil‡	100	100	100	100
Rat vitamin mix§	10	10	10	10
Vitamin A, D, E, K mix‖	30	30	30	30
Mineral mix¶	50	50	50	50
$CuSo_4.5H_2O$	0·06	—	0·06	—

* William McKinney Ltd, Belfast.
† Sigma Chemical Co., Poole, Dorset.
‡ Mazola Corn Oil, Corn Products Co.
§ Contained in sucrose (mg/kg diet): choline chloride 1000, thiamin 16, riboflavin 16, pyridoxal hydrochloride 16, calcium pantothenate 40, biotin 0·52, cyanocobalamin 0·06, pteroylmonoglutamic acid 5·25, nicotinamide 15.
‖ Contained in maize oil (mg/kg diet): retinol acetate 36, cholecalciferol 0·07, α-tocopheryl acetate 20, menadione 10.
¶ Contained (g/kg diet): $CaCO_3$ 10·56, $CaHPO_4.2H_2O$ 16·45, $ZnSO_4$ 0·21, $MgCO_3$ 0·98, $MgSO_4.7H_2O$ 1·18, NaCl 5·02, KCl 0·83, $FePO_4$ 1·58, KH_2PO_4 12·46, $MnSO_4.H_2O$ 0·75, $AlK(SO_4)_2.12H_2O$ 0·0095, KIO_3 0·03, $CoCl_2.6H_2O$ 0·0024, NaF 0·04, $Na_2MoO_4.4H_2O$ 0·0057, Tris-CrCl (157 g/kg) 0·8 mg, $Na_2SeO_3.5H_2O$ 0·5 mg.

Common dietary sugars, e.g. sucrose and fructose, have been shown to affect Cu status adversely in experimental animals and human beings (e.g., Holbrook *et al.* 1986; Johnson & Hove, 1986), and milk-based diets have been widely used to induce Cu deficiency in experimental animals (e.g. Kincaid & Carlton, 1982; Stemmer *et al.* 1985). Although lactose is one of the main dietary sugars in those countries experiencing a high incidence of IHD (Gray, 1971), few studies have examined the potential effects of this sugar on Cu status. The aim of the present study was to investigate possible effects of lactose consumption on cellular antioxidant and related enzyme activities and on Cu and selected trace element status in animals receiving control and Cu-deficient diets.

EXPERIMENTAL

Animals, diets and tissue preparation

Groups (*n* 6) of male, weanling, Wistar rats with mean initial body-weights matched to within 0·5 g (mean initial weight (*n* 24), 60·8 (SE 2·8) g) were used. The animals were housed individually in stainless-steel and polypropylene cages and maintained at 25° with a 12 h light–12 h dark cycle. Diets (Table 1), containing sucrose or sucrose and lactose with either control (12·0 mg/kg) or deficient (1·5 mg/kg) quantities of Cu, were provided *ad lib.* with deionized water for 77 d.

After this period the animals were anaesthetized with diethyl ether and killed by exsanguination. The liver and heart were removed immediately and placed in ice-cold 0·25 M-sucrose, pH 7·4. After weighing these organs a representative portion of the liver and the entire heart were stored at −20°. The remainder of the liver (approximately 9 g) was finely minced and homogenized in 0·25 M-sucrose buffer, pH 7·4 (20 ml). The homogenate was centrifuged (800 ***g***, 5 min) after which the supernatant fraction was

removed and the pellet resuspended in fresh sucrose buffer (20 ml). The resuspended pellet was centrifuged (800 *g*, 5 min), the supernatant fraction pooled with that from the initial centrifugation and this solution was diluted to 90 ml with sucrose buffer. This supernatant solution was stored at −20° in 3–4 ml portions. Heart homogenates (100 mg/ml) were prepared as described previously from the frozen hearts but without the centrifugation steps. Appropriate dilutions of the heart and liver homogenates were used for the various enzyme assays.

Analytical methods

Superoxide dismutase was assayed by the method of Oberley & Spitz (1984), using the modification suggested by Flohe & Otting (1984) in order to minimize interferences. Catalase was assayed by the method of Aebi (1974), and GSH-Px by that of Paglia & Valentine (1967) using 1·5 mM-hydrogen peroxide as substrate in an assay mixture containing 1·0 mM-sodium azide to inhibit catalase. Cytochrome *c* oxidase (*EC* 1.9.3.1; CCO), glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49; G6PDH) and lactate dehydrogenase (*EC* 1.1.1.27; LDH) activities were measured by the methods of Cooperstein & Lazarow (1951), Kornberg *et al.* (1955) and Wroblewski & LaDue (1955) respectively. Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Samples of liver and heart which had been stored at −20° were thawed and dried to constant weight in an oven at 80°. The dried liver and heart samples were digested in nitric acid at 80° until a clear solution was obtained. After dilution with deionized water, atomic absorption spectrophotometry (AAS) was performed to determine mineral levels using suitable control samples.

Hepatic and cardiac peroxidative damage was estimated by measuring the levels of thiobarbituric acid reactive substances (TBARS) present in uncentrifuged tissue homogenates (100 mg/ml) prepared from samples which had been stored at −20°, using the method of Ohkawa *et al.* (1979) with 1,1,3,3-tetramethoxypropane (TMP) as an external standard.

Statistical analysis

Results were analysed for significance by two-way analysis of variance (ANOVA) in order to investigate the independent effects of Cu deficiency and lactose consumption and to establish the influence on cellular processes of interactions between these dietary factors. Results are given as means with their standard errors.

RESULTS

Lactose consumption resulted in significant ($P < 0.05$) growth retardation (final body-weights: sucrose-fed animals 391·1 (SE 7·4) g, lactose-fed animals 347·8 (SE 14·4) g), while Cu deficiency had no significant effect on growth. Neither relative liver nor heart sizes (g/kg body-weight) were significantly influenced by dietary lactose or Cu deficiency.

Two-way ANOVA of the results obtained for the hepatic and cardiac indices of Cu status (Table 2) showed that animals consuming the Cu-deficient diet, when compared with those on control diet, had significantly decreased hepatic and cardiac Cu ($P < 0.01$) and hepatic and cardiac CCO activities ($P < 0.01$ and $P < 0.001$ respectively). These animals also exhibited significantly decreased hepatic CuZnSOD activity ($P < 0.05$). Cardiac Cu and CCO activity were both significantly ($P < 0.05$ and $P < 0.001$) higher in the lactose-fed animals compared with those fed only sucrose, while hepatic CuZnSOD activity was significantly ($P < 0.05$) lowered. Significant carbohydrate and Cu interactions were observed for hepatic CuZnSOD ($P < 0.01$) and cardiac CCO ($P < 0.05$) activities. Compared with controls the activity of the former enzyme increased with consumption of

Table 2. *Hepatic and cardiac indices of copper status in rats fed on control and Cu-deficient diets containing sucrose or lactose*
(Mean values with their standard errors)

Dietary group...	Sucrose				Lactose				Statistical significance of		
	Control		Cu-deficient		Control		Cu-deficient		Main effects		Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Cu effect	CHO effect	Cu × CHO
Hepatic											
Cu (μg/g)*	9·22	2·51	2·41	0·66	10·45	0·89	4·94	2·02	$P < 0·01$	NS	NS
CuZnSOD (U/mg)†	129·1	8·0	70·4	10·9	73·9	10·2	84·7	9·0	$P < 0·05$	$P < 0·05$	$P < 0·01$
CCO (U/mg)†	2·78	0·38	1·23	0·50	4·10	0·47	2·14	0·87	$P < 0·01$	NS	NS
Cardiac											
Cu (μg/g)*	22·50	5·52	8·33	2·38	33·75	5·76	20·83	2·56	$P < 0·01$	$P < 0·05$	NS
CuZnSOD (U/mg)†	7·35	2·49	6·70	2·66	6·28	1·73	1·48	0·65	NS	NS	NS
CCO (U/mg)†	5·73	0·80	2·36	0·57	11·06	1·21	4·08	0·75	$P < 0·001$	$P < 0·001$	$P < 0·05$

CHO, carbohydrate; NS, not significant; CuZnSOD, Cu–Zn superoxide dismutase (*EC* 1.15.1.1); CCO, cytochrome *c* oxidase (*EC* 1.9.3.1).
* Dry weight basis.
† Units are expressed on a per mg protein basis.

Table 3. *Hepatic and cardiac antioxidant and other enzyme activities in rats fed on control or copper-deficient diets containing sucrose or lactose*

(Mean values with their standard errors)

Dietary group...	Sucrose				Lactose				Statistical significance of		
	Control		Cu-deficient		Control		Cu-deficient		Main effects		Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Cu effect	CHO effect	Cu × CHO
Hepatic											
Catalase (U/mg)*	0·47	0·05	0·40	0·03	0·24	0·04	0·37	0·03	NS	$P < 0·01$	$P < 0·05$
GSH-Px (U/mg)*	0·64	0·06	0·53	0·03	0·36	0·02	0·43	0·04	NS	$P < 0·001$	$P < 0·05$
MnSOD (U/mg)*	54·3	11·1	89·2	21·4	52·8	5·3	89·7	25·3	NS	NS	NS
LDH (U/mg × 10^{-3})*	3·25	0·20	3·29	0·29	1·72	0·20	3·61	0·66	$P < 0·05$	NS	$P < 0·05$
G6PDH (mU/mg)*	38·1	3·3	59·6	9·1	21·6	4·7	35·4	4·6	$P < 0·01$	$P < 0·01$	NS
Cardiac											
Catalase (U/mg × 10)*	0·21	0·06	0·13	0·02	0·16	0·001	0·09	0·001	$P < 0·05$	NS	NS
GSH-Px (U/mg)*	0·53	0·04	0·48	0·06	0·75	0·09	0·35	0·02	$P < 0·001$	NS	$P < 0·01$
MnSOD (U/mg)*	194·6	21·8	165·8	34·4	225·1	27·6	132·2	12·6	$P < 0·01$	NS	NS

CHO, carbohydrate; NS, not significant; GSH-Px, glutathione peroxidase (*EC* 1.14.1.9); MnSOD, manganese superoxide dismutase (*EC* 1.15.1.1); LDH, lactate dehydrogenase (*EC* 1.1.1.27); G6PDH, glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49).

* Units are expressed on a per mg protein basis.

Table 4. *Hepatic and cardiac mineral element and malondialdehyde in rats fed on control or copper-deficient diets containing sucrose or lactose*

(Mean values with their standard errors)

Dietary group...	Sucrose				Lactose				Statistical significance of		
	Control		Cu-deficient		Control		Cu-deficient		Main effects		Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Cu effect	CHO effect	Cu × CHO
Hepatic											
Mn (μg/g)*	4·4	0·4	5·5	0·4	6·6	0·3	7·5	0·3	$P < 0·01$	$P < 0·001$	NS
Zn (μg/g)*	89·7	4·1	86·9	7·3	88·8	5·8	84·7	7·3	NS	NS	NS
Fe (μg/g)*	704·0	78·5	830·6	86·6	667·8	125·3	738·6	72·8	NS	NS	NS
TBARS (nmol/mg tissue × 10^2)	15·1	0·8	16·3	1·0	18·7	1·7	16·3	1·1	NS	NS	NS
Cardiac											
Zn (μg/g)*	70·8	6·7	95·8	4·0	70·0	8·2	88·8	7·8	$P < 0·01$	NS	NS
Fe (μg/g)*	395·7	29·6	516·7	80·6	406·5	83·1	417·5	25·9	NS	NS	NS
TBARS (nmol/mg tissue × 10^2)	12·7	1·1	10·0	1·9	15·1	2·0	12·6	1·2	NS	NS	NS

CHO, carbohydrate; NS, not significant; TBARS, thiobarbituric acid reactive substances.
* Dry weight basis.

the Cu-deficient lactose diet, in contrast to the marked decrease in activity with the Cu-deficient sucrose diet.

The hepatic and cardiac activities of the antioxidant enzymes MnSOD, GSH-Px and catalase are reported in Table 3. Lactose consumption resulted in significantly ($P < 0.01$ and $P < 0.001$ respectively) decreased hepatic catalase and GSH-Px activities. Except CuZnSOD, none of the hepatic antioxidant enzyme activities was significantly influenced by Cu deficiency. Cu deficiency, however, resulted in significantly decreased cardiac catalase, GSH-Px and MnSOD ($P < 0.05$, $P < 0.001$ and $P < 0.01$ respectively) activities. There were also significant carbohydrate and Cu interactions for hepatic and cardiac GSH-Px ($P < 0.05$ and $P < 0.01$ respectively) and hepatic catalase ($P < 0.05$) activities.

Cu deficiency significantly ($P < 0.01$) increased, while lactose consumption significantly ($P < 0.01$) decreased, hepatic G6PDH activity (Table 3). Hepatic LDH activity was significantly ($P < 0.05$) decreased with lactose, compared with sucrose, consumption and a significant ($P < 0.05$) carbohydrate and Cu interaction was also noted.

The hepatic and cardiac levels of Cu are reported in Table 2. AAS determinations of the hepatic and cardiac levels of Mn, Zn and iron levels are reported in Table 4. Hepatic Mn was found to be significantly increased in both Cu-deficient ($P < 0.01$) and lactose-fed animals ($P < 0.001$). Cardiac Mn was below the detection limit (i.e. < 1.0 μg/g dry weight) of the instrumentation available. Cardiac Zn was found to be significantly ($P < 0.01$) increased as a result of Cu deficiency, and there were no significant alterations of Fe in any of the experimental groups. No significant differences in hepatic or cardiac TBARS levels (Table 4) were found as a result of either Cu deficiency or dietary carbohydrate.

DISCUSSION

The results indicate that the low-Cu diets induced a mild Cu deficiency in the experimental animals. Tissue Cu and CCO activity were found to be significantly decreased as a result of the low-Cu diets in both the liver and heart, while CuZnSOD activity was significantly decreased only in hepatic tissue. However, the low-Cu diets did not significantly affect growth rate or relative heart size.

The significantly lower cardiac Cu and CCO activity in those animals fed on sucrose-, compared with lactose-containing diets, suggests that sucrose, compared with lactose, may exacerbate the effects of a dietary Cu deficiency. The observations in the present study would lend support to the results of Petering *et al.* (1986), who found that the physiological and biochemical effects of Cu deficiency are significantly exacerbated with sucrose compared with a starch–lactose (1:1, w/w) diet, and it has been suggested that the protein in milk powder is the causative factor resulting in the increased morbidity and mortality associated with milk-based Cu-deficient diets compared with those based on other protein sources (Stemmer *et al.* 1985). In the current study, although some indices of Cu status were higher in rats fed on lactose, others were unaffected and hepatic CuZnSOD activity was significantly lowered. However, there is some evidence that CuZnSOD may be induced by free radical production (Jannson *et al.* 1985) and CCO activity has been used by many investigators (e.g. Trayhurn & Jennings, 1987) as a measure of tissue mitochondrial content. Thus, it would appear that neither measurement is influenced by Cu status alone.

Increased hepatic and cardiac CCO activities (Table 2) suggest that lactose feeding may result in increased mitochondrial respiration in these tissues, with a possible concomitant rise in O_2 demand. Since it has been shown that O_2 consumption increases dramatically in arterial and cardiac tissue during experimentally induced atherosclerosis (e.g. Morrison *et al.* 1972; Stange & Papenberg, 1978; Thuesen *et al.* 1984), any increase in mitochondrial

respiration could have important implications for the development of coronary atherosclerotic lesions and cardiac dysfunction. Increased tissue O_2 consumption may increase O_2 free-radical production and thus lead to the generation of potentially harmful lipid hydroperoxides (Blake *et al.* 1987). However, when the hepatic and cardiac TBARS levels (a measure of lipid peroxidation) were determined, no significant differences among any of the dietary groups were observed. Diets deficient in Cu have previously been shown to result in increased lipid peroxidation (Paynter, 1980) and the absence of lipid peroxidation in the present study may be due either to the decreased severity of the Cu deficiency experienced by the rats, or the recognized limitations of the thiobarbituric acid assay in estimating lipid peroxidation (Slater, 1984).

Lactose feeding resulted in significantly decreased G6PDH activity, while Cu deficiency caused significantly increased G6PDH activity (Table 3). Although similar changes in G6PDH activity associated with both Cu deficiency (Lynch & Strain, 1988) and lactose feeding (Michaelis & Szepesi, 1973) have been observed previously, their importance is, as yet, unclear.

In agreement with a number of other studies using milk-based diets (e.g. Gruden, 1976; King *et al.* 1979), the present results showed that dietary lactose significantly increased hepatic Mn (Table 4). However, at least one study has shown the opposite (King *et al.* 1980), and so the exact nature of the interaction between dietary lactose and tissue Mn status is currently uncertain. In the present study hepatic Mn significantly increased with Cu deficiency (Table 4), while the activity of cardiac MnSOD significantly decreased with Cu deficiency (Table 3). The latter result is in agreement with Paynter (1980), who found that the lowered activity of tissue CuZnSOD was not compensated for by any increase in MnSOD activity in the rat, rather a decrease in MnSOD activity was observed. Murthy *et al.* (1974) found inverse relations between Zn and Cu levels only in some tissues in the rat. In the current study cardiac, but not hepatic, Zn was significantly increased by Cu deficiency. Cu deficiency has also been shown in several studies (see Fields *et al.* 1984) to decrease cellular activities of the selenoenzyme GSH-Px and a similar effect of Cu deficiency on the major biochemically active form of Se was observed in cardiac tissue (Table 3).

Observations in the current study support the work of others with respect to interactions between Cu deficiency and the apparent body status of Mn, Se and Zn. Lactose, compared with sucrose, consumption decreased the activities of cytosolic antioxidant enzymes, including the Cu-dependent enzyme CuZnSOD. Lactose consumption, however, appeared to increase other indices of Cu status, including the mitochondrial marker enzyme CCO. These results suggest that components in milk other than lactose may interact with Cu deficiency to produce the increased morbidity and mortality associated with milk-based diets.

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