Hepatic Iron Overload May Contribute to Hypertriglyceridemia and Hypercholesterolemia in Copper-Deficient Rats

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The present study was conducted in order to determine whether hepatic iron retention in rats fed a copper-deficient diet containing fructose is associated with hypertriglyceridemia and hypercholesterolemia, and whether a reduction of iron intake will prevent elevation of blood triglycerides and cholesterol. Rats were fed from weaning either a copper-deficient (0.6 μg Cu/g) or copper-adequate (6.0 μg Cu/g) diet for 4 weeks. Half the rats consumed either an adequate level of iron (50 μg Fe/g) or a low level (17 μg Fe/g). Reduction of iron intake reduced blood levels of both triglycerides and cholesterol in rats fed a copper-deficient diet containing fructose. In addition, hepatic lipid peroxidation was also decreased. The combination of high iron, low copper, and fructose may be responsible for increased levels of risk-factor metabolites associated with heart disease.

COPPER DEFICIENCY is associated with hypercholesterolemia and hypertriglyceridemia.[1-10] Both cholesterol and triglycerides in high concentrations are considered as risk-factor metabolites associated with heart disease. Copper deficiency is also accompanied by hepatic iron overload due to the antagonistic relationship between copper and iron.[11,12] Hepatic iron overload in copper deficiency may be responsible for hypercholesterolemia and hypertriglyceridemia.

We have recently reported that rats that consumed either a copper-deficient or copper-adequate diet containing saturated fat such as beef tallow exhibited elevated plasma levels of triglycerides and cholesterol.[13] These rats also displayed elevated levels of liver iron.[13] Based on these data, we hypothesize that high levels of iron may be associated with elevated plasma cholesterol and triglycerides. If this hypothesis is correct, then a reduction of hepatic iron should prevent elevation of both cholesterol and triglycerides.

This study was designed to determine whether prevention of hepatic iron overload in copper-deficient rats will protect the rats against development of hypercholesterolemia and hypertriglyceridemia.

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), weighing approximately 40 to 45 g were fed either a copper-deficient (0.6 μg Cu/g) or copper-adequate (6.0 μg Cu/g) diet. The composition of the diets was previously described.[14] Half the rats consumed either an adequate-iron (50 μg Fe/g) or low-iron (17 μg Fe/g) diet. Deionized distilled water was freely available to all rats. Rats were housed individually in stainless steel cages in a room with a 12-hour light/dark cycle and maintained at 22°C and 50% to 60% humidity. All rats were weighed weekly.

At the end of the 4-week experimental period, rats were decapitated following an overnight fast. Livers and pancreata were removed and weighed, and liver portions were taken for copper and iron analysis.[15] Other portions of livers were used to isolate crude mitochondria.[16] Lipid peroxidation in isolated liver mitochondria was tested by measuring malondialdehyde formation by using the thiobarbituric acid–reactive substance technique as described by Paynter.[16] Isolated mitochondria were incubated in either KCl-Tris buffer or KCl-Tris buffer containing 0.12 mmol/L Fe^{2+} as an oxidation initiator.[16]

Blood was collected, and plasma was obtained upon centrifugation. Triglyceride and cholesterol levels were measured by an automated procedure with the CentrifiChem (Baker Instrument, Pleasantville, NY) using Trace reagents (Trace America, Miami, FL).

Liver protein was determined by automated procedures with the CentrifiChem using biuret reagent (Sigma Kit 541-2; Sigma Chemical, St Louis, MO). A 1-g portion of each liver was homogenized in 0.01 mol/L Tris buffer and centrifuged at 30,000 × *g* for 30 minutes. The clear supernatants were used to determine glucose 6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) and malic enzyme (ME) (EC 1.1.40) according to the method of Freedland[17] with the automated CentrifiChem procedure. Enzyme activities were expressed as international units per gram soluble protein. One unit of enzyme is defined as the amount of enzyme producing 1 μmol of measured product per minute under the conditions of the assay.

All data expressed as the mean ± SEM were analyzed by ANOVA. The independent effects of iron and copper and the interaction between iron and copper were examined by two-way ANOVA. Differences at $P < .05$ were considered significant.

RESULTS

Table 1 summarizes data for the body weight, relative organ masses, hematocrit, and concentrations of cholesterol and triglycerides in plasma. Rats that consumed the copper-deficient diets with adequate or low iron exhibited lower body weight compared with the copper-adequate controls. Decreasing the iron intake resulted in a reduced body weight in copper-adequate rats, but increased body weight in copper-deficient rats. Adequate intake of iron caused an increase in relative liver mass in rats consuming the copper-inadequate diet. In contrast, liver mass was not increased by the combination of low iron and low copper intake. Relative heart mass was the largest in copper-deficient rats that ate the adequate-iron diet. Heart mass was higher in copper-adequate rats that ate the low-iron diet compared with copper-adequate rats that ate the adequate-iron diet. The smallest pancreas size was noted in copper-deficient rats that ate the adequate-iron diet. The combination of low copper and low iron prevented pancreatic atrophy. The hematocrit was reduced by copper deficiency. The lowest hematocrit was noted in copper-deficient rats that consumed the adequate-iron diet. However, the hematocrit was reduced by the low-iron diet in copper-adequate rats. The highest plasma cholesterol was noted in copper-deficient rats that consumed the adequate-

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Table 1. Body Weights, Relative Organ Masses, Hematocrits, and Concentrations of Cholesterol and Triglycerides in Plasma

Parameter	Copper-Deficient		Copper-Adequate	
	Adequate Fe	Low Fe	Adequate Fe	Low Fe
Body mass (g)	160 ± 3	169 ± 3	194 ± 3	185 ± 4
Relative organ mass (g/100 g)				
Liver	4.6 ± 0.1	3.4 ± 0.3	3.6 ± 0.1	3.6 ± 0.1
Heart	0.62 ± 0.03	0.51 ± 0.01	0.38 ± 0.01	0.43 ± 0.05
Pancreas	0.38 ± 0.02	0.67 ± 0.01	0.66 ± 0.01	0.67 ± 0.01
Hematocrit (%)	25 ± 0.6	32 ± 0.5	44 ± 0.5	32 ± 0.6
Cholesterol (mg/dL)	152 ± 4	117 ± 5	114 ± 2	108 ± 2
Triglycerides (mg/dL)	45 ± 4	28 ± 2	22 ± 1	28 ± 2

	ANOVA (*P*)		
	Copper	Iron	Copper × Iron
Body mass	.0001	NS	.0092
Liver	NS	.0001	.0204
Heart	.0001	NS	.0096
Pancreas	.0001	.0001	.0001
Hematocrit	.0001	.0001	.0001
Cholesterol	.0001	.0001	.0001
Triglycerides	.0001	.0001	.0001

NOTE. Results are expressed as the mean ± SEM of 10 observations per group.

Abbreviation: NS, not significant.

iron diet. Decreasing the intake of iron normalized the cholesterol concentration in copper-deficient rats. Similarly, the concentration of triglycerides was highest in copper-deficient rats that ate the adequate-iron diet. Copper-deficient rats that consumed the low-iron diet exhibited lower triglycerides. The lowest concentration of plasma triglycerides was found in copper-adequate rats that consumed the adequate-iron diet.

Hepatic copper and iron concentrations and hepatic lipid peroxidation are presented in Table 2. As expected, feeding rats a copper-deficient diet caused a reduction of hepatic copper concentration. When the low-iron diet was fed, the concentration of hepatic copper was increased. Copper deficiency resulted in elevated levels of hepatic iron. When rats consumed the low-iron diet, hepatic iron concentration was decreased. The lowest concentration of iron was found in livers of copper-adequate rats that consumed the low-iron diet. The highest lipid peroxidation occurred in the livers of copper-deficient rats that consumed the adequate-iron diet and the lowest peroxidation in copper-adequate rats that consumed the low-iron diet. Copper-deficient, low-iron rats had lipid peroxidation levels similar to those of copper-adequate controls.

The activities of G6PD and ME are also presented in Table 2. Livers of rats fed the low-iron diet contained higher activities of G6PD than those of rats fed the adequate-iron diets. Copper deficiency also decreased the activity of G6PD. ME activity was lower in copper-deficient compared with copper-adequate rats.

DISCUSSION

The data from the present study clearly show that a reduction of dietary iron intake by copper-deficient rats is capable of ameliorating the signs associated with copper deficiency. These included increased body weight, reduced liver and heart masses, and prevention of pancreatic atrophy. In addition, the hematocrit was higher and hepatic lipid peroxidation was lower. Furthermore, plasma levels of both cholesterol and triglycerides were also decreased.

The importance of copper in the metabolism of iron has been recognized since 1928, when Hart et al[18] showed that rats became anemic when fed a diet composed solely of milk and responded to administration of iron only if copper was adequately provided. Since then, other researchers reported that copper is involved in the metabolism of iron.[11,12,18-25] Due to the antagonistic relationship between copper and iron, copper deficiency results in hepatic iron overload.[12,23-25] Iron has the potential to generate reactive oxygen species that in turn cause damage to cellular components.[26,27] The prevention of hepatic iron overload has been shown to hinder free-radical generation, inhibiting the pathologies and mortality of copper-deficient rats.[23,24] The reduction of hepatic iron in the present study reduced hepatic lipid peroxidation. Since iron is a potential initiator of lipid peroxidation, it was expected that reducing the levels of hepatic iron would result in a decrease of lipid peroxidation. However, it is not simply a reduction of hepatic iron concentrations, because by decreasing hepatic iron, hepatic copper was increased. Although the reduction of hepatic iron (174 *v* 71 μg/g) was greater than the increase of hepatic copper (0.93 *v* 1.66 μg/g), one cannot ignore the fact that copper and iron work in concert.

High levels of cholesterol[1-7] and triglycerides[8-10] are well-established consequences of dietary copper deprivation. How-

Table 2. Hepatic Copper and Iron Concentrations, Lipid Peroxidation, and G6PD and ME Specific Activities

Parameter	Copper-Deficient		Copper-Adequate	
	Adequate Fe	Low Fe	Adequate Fe	Low Fe
Copper (μg/g wet weight)	0.93 ± 0.06	1.66 ± 0.08	5.59 ± 0.21	6.69 ± 0.26
Iron (μg/g wet weight)	174 ± 12	71 ± 4	94 ± 4	40 ± 1
Lipid peroxidation (mmol MDA/g liver)	33.8 ± 3.1	19.5 ± 2.7	21.7 ± 3.2	12.0 ± 2.1
G6PD (U/g protein)	54.0 ± 3.1	73.8 ± 3.3	69.8 ± 6.0	79.0 ± 2.9
ME (U/g protein)	11.0 ± 1.2	10.3 ± 1.1	15.4 ± 1.2	15.3 ± 1.3

	ANOVA (*P*)		
	Copper	Iron	Copper × Iron
Copper	.0001	.0001	NS
Iron	.0001	.0001	.0002
Lipid peroxidation	.0001	.0001	NS
G6PD	.0150	.0013	NS
ME	.0001	NS	NS

NOTE. Results are expressed as the mean ± SEM of 10 observations per group. One unit (U) is the amount of G6PD or ME activity that will convert 1 mmol of substrate per minute under the conditions specified in the assay.

Abbreviation: MDA, malondialdehyde.

ever, it is not simply copper deficiency that causes these abnormalities. The presence of dietary simple sugars such as sucrose, fructose, or glucose plays a major role in this process.[1-10] When the copper-deficient diets that had been used to induce copper deficiency contained complex carbohydrate such as starch, hypercholesterolemia and triglyceridemia were either ameliorated or prevented.[8-10] Exchanging the carbohydrate moiety of the diet from fructose to starch greatly reduced the levels of cholesterol and triglyceride in copper-deficient rats.[9] Dietary fructose per se is able to increase plasma lipids, but dietary starch is not.[8-10,28] In addition, intake of high levels of dietary iron in combination with copper deficiency is also responsible for elevations of plasma cholesterol[1-3] and triglycerides.[10] Investigations of the potential changes in liver cholesterol and very–low-density lipoprotein synthesis and secretion, activities of lipoprotein lipase and lecithin:cholesterol acyltransferase, and turnover of low-density lipoprotein have yielded conflicting results. Despite considerable investigation by several laboratories, the reasons for hypercholesterolemia in copper deficiency are still unclear. Mechanisms that involve the uptake and mobilization of fat from liver and adipose tissue and the rate of synthesis all play roles in governing the levels of cholesterol and triglyceride in plasma. We are not aware of any enzyme that participates in the synthesis of either cholesterol or triglycerides that is dependent on either copper or iron. Therefore, it may be that not only the synthesis but also the secretion of these metabolites is enhanced. Indeed, it has recently been reported that increased hepatic apolipoprotein A-I synthesis and secretion occurred in copper deficiency.[7]

Mobilization of fat from the periphery is governed by numerous hormones. Except for insulin, the majority of other glucoregulatory hormones act to increase the levels of blood lipids by mobilizing depot stores. However, copper deficiency is associated with elevated glucocorticoids[29] only in rats fed fructose.

Copper deficiency is associated with impaired insulin secretion[30,31] and insulin resistance, which result in "diabetes-like symptoms."[32-37] Accumulation of iron in the liver of hemochromatosis patients also results in diabetes-like symptoms.[38-40] In hemochromatosis and in high-ferritin diabetes, the removal of iron by either venesection or iron-chelating therapy resulted in improvement of glucose tolerance.[38-40] We have recently reported that a reduction of hepatic iron in copper-deficient rats restored the ability of the pancreas to secrete insulin and improved the glucose response to an oral glucose load.[41] Impaired insulin secretion could be related to increased pancreatic iron stores in copper-deficient rats.

Insulin resistance and impaired insulin secretion can be translated into relative insulin deficiency. Defects in glucose disposal and lipid abnormalities may be induced by this means. Abnormalities in serum lipids and lipoprotein composition are commonly observed in diabetic patients. When insulin resistance is prevented and when insulin becomes effective, abnormalities of glucose metabolism should be prevented, which in turn should decrease levels of triglycerides and low-density lipoprotein cholesterol.

Another explanation for the hypercholesterolemia and hypertriglyceridemia in rats fed the adequate-iron diet could be the direct effect of iron on cellular components. Iron may be damaging to intracellular organelles like many other chemicals such as phosphorus, chloroform, and carbon tetrachloride. All of these toxic chemicals damage liver cells and induce hepatic fatty changes. Indeed, copper-deficient rats that consumed the adequate-iron diet exhibited the largest liver size. Increased liver size could result from lipid accumulation, which in turn causes hepatic fibrosis.[42-44] In the present study, when dietary iron was reduced, the liver size of copper-deficient rats was reduced to normal.

Both G6PD and ME are considered lipogenic because they provide a source of reduced nucleotides such as nicotinamide adenine dinucleotide phosphate (NADPH) that are imperative for lipogenesis to function. NADPH is required for several other reductive processes such as maintaining glutathione (GSH) in a reduced form. However, these enzymes participate in different enzymatic pathways located at different subcellular fractions. Consumption of the adequate-iron, low-copper diet resulted in decreased activity of G6PD. A decreased activity could indicate a reduced ability to use glucose intracellularly. In contrast, it has been reported that iron-deficient rats exhibited a greater metabolic clearance rate of blood glucose and increased glucose utilization.[45] However, rats in the present study that had been fed the low-iron diet were not as iron-deficient as those described by Farrell et al.[45] In the present study, when dietary iron was reduced, the activity of G6PD was increased. The increased activity of G6PD in rats fed the low-iron diet could indicate normalization of the phosphogluconate oxidative pathway for the synthesis and disposal of pentoses as a source of reducing equivalents for the reduction of NADP to NADPH, to maintain GSH in a reduced form and to use NADPH for fatty acid synthesis.

ME plays a role in a process that provides a source of NADPH for fatty acid synthesis. However, ME also fits into another pathway that supplies acetyl groups for the fatty acid chain. The activity of ME was reduced by copper deficiency, but was unaffected by levels of dietary iron. We have no explanation for this dietary effect.

The hematocrit was elevated in rats fed the low-iron diet, but only in copper-deficient rats. In contrast, copper-adequate rats fed the low-iron diet exhibited a reduced hematocrit. It is usually accepted that consumption of a low-iron diet results in anemia. One would expect to find a further reduction of the hematocrit in copper-deficient rats fed the low-iron diet. However, consumption of a copper-deficient diet that was low in iron prevented hepatic iron retention and was responsible for ameliorating the anemia. Amelioration of anemia could also be responsible for improvement of the well-being of copper-deficient rats fed the low-iron diet, as assessed by prevention of histopathological changes in the heart and pancreas and mortality.[24,46]

Reduction of both cholesterol and triglycerides, which are considered risk-factor metabolites associated with heart disease, should be beneficial. In addition, animals in the present study should benefit from a reduction of triglycerides, because hypertriglyceridemia is associated with increased superoxide production.[47] Copper is considered an antioxidant due to its function in copper-dependent superoxide dismutase (SOD).[48] Copper-deficient rats are more susceptible to oxygen radicals due to the reduced activity of SOD.[49] The reduction of copper,

which causes a simultaneous increase of liver iron, requires a greater degree of antioxidant protection. Dietary copper deficiency causes increased hepatic and circulating concentrations of GSH[50] and increased synthesis of GSH in isolated hepatocytes in vitro.[51] GSH is an antioxidant, and the demand for it is increased by agents that stimulate peroxide and hydroperoxide formation. Iron could be such an agent. It has been reported that GSH is a regulator of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) reductase.[52-56] Hypercholesterolemia in copper deficiency is accompanied by an increase in HMG CoA reductase activity.[57,58] Inhibition of GSH synthesis resulted in a reduction of cholesterol synthesis in copper-deficient rats.[59] These results indicate that increases in GSH brought about by copper deficiency are responsible for the hypercholesterolemia of copper deficiency.[60] However, the possibility that the combination of copper deficiency with hepatic iron overload induces reactive oxygen species, which in turn increase the demand for GSH, cannot be ruled out. Iron has been implicated in lipid peroxidation and ischemic myocardial damage.[61-63] High stored-iron levels or high iron intake may affect the incidence of coronary disease.[61-63]

Diets consumed in industrialized societies, including the United States, are relatively high in simple sugars,[64] in fat, and in fat saturation.[65] In addition, these diets are low in copper.[66] When consumption of these diets results in lipemia, it may be due to elevated levels of hepatic iron. The use of dietary or nutritional supplements in the United States is extensive.[67] Sales of dietary supplements increased sixfold from 1972 to 1987.[68] If the same type of interaction that has been reported here also occurs in humans, then major considerations should be taken into account when formulating dietary recommendations and advice.

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