Dietary Copper Deficiency and Endothelium-Dependent Relaxation of Rat Aorta (43388)

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Abstract. **Endothelium-dependent relaxation of aortas was studied in dietary copper (Cu) deficiency. Male, weanling Sprague-Dawley rats were fed diets deficient (CuD, <0.5 ppm) or adequate (CuA, 5.0–5.5 ppm) in Cu for 4 weeks. Aortic rings from paired Cu-deficient and Cu-adequate rats were isolated from the descending thoracic aorta, placed in tandem tissue baths, and attached to force transducers. Aortas were contracted with phenylephrine (3×10^{-7} *M*) and the degree of force reduction was measured after successively increasing the dose of acetylcholine (10^{-8}–10^{-5} *M*), histamine 10^{-6}–10^{-3} *M*), or sodium nitroprusside (10^{-9}–10^{-6} *M*). Cu deficiency was found to significantly reduce the relaxation responses of each relaxing agent at the highest three of the four doses tested. The ability of Cu-adequate and Cu-deficient aortas to relax was not different, as indicated by their complete relaxation in response to 10^{-4} or 10^{-5} *M* papaverine. Because the relaxation responses to both acetylcholine and histamine in rat aorta are dependent on the presence of endothelium, the reduction of these responses suggests that endothelium, or its interaction with smooth muscle, was disrupted in dietary Cu deficiency. The reduction in response to sodium nitroprusside, an endothelium-independent analog of endothelium-derived relaxing factor, indicates that the interaction of endothelium-derived relaxing factor with smooth muscle was disrupted. These findings have implications regarding blood pressure regulation in Cu deficiency.**

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Dietary copper deficiency in experimental animals produces vascular defects which include structural changes in aortas (1), altered vasoactive responses to norepinephrine in portal veins and pulmonary arteries (2), enhanced microvascular protein leakage in response to endogenous histamine release (3), and reduced microcirculatory platelet thrombus formation in response to photoactivation of a light-sensitive dye (3). Structural changes in aortas may affect the endothelium (1) and some of the functional defects noted above (permeability, thrombus formation) necessarily involve endothelium. This knowledge suggested the present study, which was initiated to determine whether endothelium-dependent relaxation of aortas was affected by copper deficiency.

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Materials and Methods

Animals and Diets. Eighty male, weanling Sprague-Dawley (Harlan Sprague-Dawley, Madison, WI)[2] rats were fed *ad libitum* a purified diet (Table I) that was either copper deficient (CuD) or copper adequate (CuA). Deionized water was provided *ad libitum*. All animals were housed in quarters maintained at 22–24°C with a 12:12-hr light:dark cycle.

Diet analysis for copper by atomic absorption spectrophotometry indicated that mean copper content of the diets ranged between 0.38 and 0.55 g Cu/kg diet for five CuD diets and between 5.17 and 5.71 g Cu/kg diet for five CuA diets. Dietary iron ranged between 48 and 55 g Fe/kg diet for CuD diets and between 46 and 54 g Fe/kg diet for CuA diets.

Aortic Force Measurements. After 4 weeks on their respective diets, rats were anesthetized with intraperitoneal injections of sodium pentobarbital (65 mg/

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

kg; Vet Labs, Lenexa, KS). Blood was drawn from the inferior vena cava for the blood assays described below.

From each rat, a ring of descending thoracic aorta (3–5 mm long) was isolated from just distal to the aortic arch. Two such rings, one each from a CuA rat and a CuD rat, were attached to force transducers (Kulite Semiconductor Products, Ridgefield, NJ) and placed in tandem tissue bath chambers (Harvard Apparatus, South Natick, MA) containing Krebs-Henseleit solution (37°C, aerated with 95% O_2 and 5% CO_2). Over a 90-min equilibration period, tension on the aortas was gradually increased until a stable resting force of 2*g* was reached. Contraction of aortas was caused by addition of phenylephrine (3×10^{-7} *M*) to each chamber. When phenylephrine-induced contraction was maximal, aortas were caused to relax by successively increasing the dose of acetylcholine (10^{-8}–10^{-5} *M*), histamine (10^{-6}–10^{-3} *M*), or sodium nitroprusside (10^{-9}–10^{-6} *M*). Degree of force reduction by these relaxing agents was represented as a percentage of the force increase caused by phenylephrine. Relaxation of four pairs of aortas was also tested with 10^{-4} or 10^{-5} *M* papaverine.

Copper Status Indices. Hearts and livers were lyophilized, wet ashed with nitric acid and hydrogen peroxide (5), and assayed for copper by inductively coupled argon plasma emission spectrometry (model 503; Perkin Elmer, Norwalk, CT). Serum copper was determined on the same instrument after dilution in nitric acid. Serum was analyzed on a Cobas Fara automated analyzer (Roche Diagnostics Systems, Nutley, NJ) for ceruloplasmin (6) and cholesterol (7).

Statistics. The Student's *t* test (8) was used to compare means of copper status indices between CuD and CuA groups of animals.

Two-way repeated-measures analysis of variance (8) was used to determine effects of copper and vasodilator on the relaxation responses of each agent. Bonferroni contrasts (8) were used for comparison of mean relaxation response at each dose of vasodilator.

Table I. Diet Composition

Ingredient	Amount (g/kg diet)
Basal diet[a]	940.0
Safflower oil[b]	50.0
Ferric citrate·n-hydrate (16% Fe)[c]	0.22
Cornstarch[d]	9.76 (CuA) 9.78 (CuD)
Cupric sulfate penta-hydrate[c]	0.02 (CuA) 0 (CuD)

[a] A casein (20%)-, sucrose (39%)-, and cornstarch (29%)-based diet containing all known essential minerals and vitamins except for iron and copper (catalog no. TD 84469; Teklad Test Diets, Madison, WI). See Johnson and Kramer (4) for exact composition.
[b] Hollywood Foods, Los Angeles, CA.
[c] J. T. Baker Chemical Co., Phillipsburg, NJ.
[d] Best Foods, Englewood Cliffs, NJ.

Results

Copper Status. Copper deficiency was verified in CuD rats by the presence of lower liver, heart, and serum copper concentrations relative to those in CuA rats (Table II). CuD rats also had lower serum ceruloplasmin concentrations, lower hematocrits, higher heart weight to body weight ratios, higher serum cholesterol concentrations, and lower body weights than CuA rats, common observations in severely copper-deficient animals (9, 10).

Contraction with Phenylephrine. The mean contractile responses to 3×10^{-7} *M* phenylephrine which preceded relaxation with each vasodilator are indicated in Table III. Copper deficiency was found to have no effect on contractile response to this dose of phenylephrine in any of the experiments.

Relaxation Responses. Relaxation as a percentage of the peak force of contraction with phenylephrine is graphically illustrated for acetylcholine (Fig. 1), histamine (Fig. 2), and sodium nitroprusside (Fig. 3). Analysis of variance indicated that copper deficiency marginally reduced the relaxation response to acetylcholine ($0.05 < P < 0.10$) and significantly reduced the relaxation responses to histamine and sodium nitroprusside ($P < 0.05$). Comparison of means (Bonferroni contrasts) at specific drug doses indicated that differences in response were significant at the three highest doses of each relaxing agent. Relaxation of aortas from both CuD and CuA rats was 100% with either 10^{-5} or 10^{-4} *M* papaverine.

Discussion

Acetylcholine was the first vasodilatory agent for which an obligatory role of intact endothelium was reported (11). Although rabbit aorta was used in that study, subsequent studies have shown acetylcholine to act similarly in blood vessels of other species, including rat aorta (12–14). Histamine has shown a similar dependence on endothelium for its ability to relax vascular smooth muscle, including that of rat aorta (12–15). Because of the clear endothelium dependence of relaxation responses to both acetylcholine and histamine in rat aorta, the reduction of these responses in copper deficiency suggests that the endothelium, or its interaction with smooth muscle, is disrupted by dietary copper deficiency.

The endothelium dependence of vasodilators is generally related to the production and release of two possible types of mediator by the endothelial cell, endothelium-derived relaxing factor (EDRF) or arachidonic acid-derived metabolites, in particular, prostacyclin (16). EDRF, which recent evidence indicates is nitric oxide or a closely related substance (17, 18), is believed to diffuse into the smooth muscle cell and bind to and activate guanylate cyclase, thereby producing cyclic GMP (19), which in turn causes relaxation by a

Table II. Characteristics[a] of Groups of Copper-Adequate and Copper-Deficient Rats in which Aortic Relaxation Was Measured with Either Acetylcholine, Histamine, or Sodium Nitroprusside

Diet	*n*	Body wt (g)	Liver Cu (μg/g)	Serum Cu (μg/ml)	Heart Cu (μg/g)	Cerul[b] (mg/dl)	Heart wt/body wt (mg/g)	Hct[b] (%)	Chol[b] (mg/dl)
Acetylcholine experiments									
CuA	19	247 ± 5	9.9 ± 0.2	0.469 ± 0.046	15.6 ± 0.8	24.1 ± 2.4	3.68 ± 0.03	41 ± 1	98 ± 3
CuD[c]	19	207 ± 5	0.9 ± 0.1	0.032 ± 0.004	4.0 ± 0.2	3.3 ± 0.3	6.59 ± 0.47	19 ± 1	114 ± 3
Histamine experiments									
CuA	15	241 ± 7	9.3 ± 0.3	0.357 ± 0.057	14.5 ± 0.9	17.8 ± 2.4	3.67 ± 0.05	40 ± 1	107 ± 5
CuD[c]	15	194 ± 12	1.0 ± 0.1	0.032 ± 0.001	3.5 ± 0.2	4.8 ± 0.2	7.72 ± 0.85	18 ± 2	133 ± 8
Sodium nitroprusside experiments									
CuA	6	299 ± 10	11.1 ± 0.8	0.525 ± 0.080	17.3 ± 1.6	20.6 ± 3.0	3.56 ± 0.12	42 ± 1	97 ± 5
CuD[c]	6	266 ± 8	1.2 ± 0.1	0.015 ± 0.003	4.9 ± 0.2	4.3 ± 0.4	4.78 ± 0.30	30 ± 2	129 ± 10

[a] Values of variables are mean ± SE.
[b] Abbreviations used in this table: Cerul, serum ceruloplasmin; Hct, hematocrit; Chol, serum cholesteol.
[c] All CuD variables listed are significantly different from the corresponding CuS variables by the Student's *t* test.

Table III. Force of Contraction[a] in Response to 3×10^{-7} *M* Phenylephrine which Preceded Relaxation with Acetylcholine, Histamine, or Sodium Nitroprusside

Diet	*n*	Force (g)	Force (mg/mm² surface area)	Force (mg/mg wet wt)
Acetylcholine experiments				
CuA	19	2.7 ± 0.1	113 ± 5	197 ± 12
CuD	19	2.7 ± 0.1	115 ± 6	201 ± 13
Histamine experiments				
CuA	15	2.7 ± 0.1	114 ± 5	194 ± 12
CuD	15	2.9 ± 0.2	111 ± 6	202 ± 13
Sodium nitroprusside experiments				
CuA	6	5.2 ± 0.1	174 ± 7	280 ± 22
CuD	6	5.6 ± 0.6	185 ± 16	346 ± 25

[a] Values of variables are mean ± SE.

mechanism not yet delineated. Prostacyclin, on the other hand, causes vasodilation by the activation of adenylate cyclase and the resultant production of cyclic AMP (20). Because both acetylcholine and histamine cause enhanced cyclic GMP production in associated smooth muscle (13), the primary endothelium-dependent mediator of relaxation for these agents is probably EDRF. Evidence against prostacyclin being the mediator is the fact that responses to neither agent are inhibited by the cyclooxygenase inhibitor indomethacin (12, 14).

The fact that both acetylcholine and histamine utilize guanylate cyclase as a common pathway raised the possibility that the reduced relaxation response to these agents was caused by a defect in this enzyme. This possibility is made more compelling by the knowledge that guanylate cyclase in lung has been found to contain copper (21); if this copper is a functional cofactor, copper deficiency may impair enzyme activity. Furthermore, iron metabolism is known to be altered in copper deficiency (22) and the binding site by which EDRF (nitric oxide) activates guanylate cyclase is its heme group (23); this represents another possible defective site in copper deficiency.

The effect of copper deficiency on guanylate cyclase was tested indirectly by the use of the nitrovasodilator sodium nitroprusside. Nitrovasodilators bypass the endothelium and relax smooth muscle directly; they are thought to mediate their responses by interaction with smooth muscle guanylate cyclase (24), a mechanism analogous to that of EDRF (19, 25). The reduction of the sodium nitroprusside response indicates that the interaction of EDRF with smooth muscle, at or beyond guanylate cyclase activation, is disrupted in copper deficiency. Indirect indicators of copper deficiency, that is, heart weight to body weight ratio and hematocrit, indicate that the rats used for sodium nitroprusside experiments were not as deficient as the other two groups (Table II). This suggests that the responses to sodium nitroprusside were underestimated relative to those of acetylcholine and histamine.

To determine whether guanylate cyclase itself or some downstream pathway sensitive to cyclic GMP was affected by copper deficiency, relaxation response was tested with papaverine, a phosphodiesterase inhibitor. Papaverine caused complete relaxation in both CuD and CuA aortas, indicating that the responsiveness of the contractile mechanism to elevated cyclic GMP is intact. (Note that papaverine, though a nonspecific phosphodiesterase inhibitor, does not raise cyclic AMP levels in rat aorta [26].) Furthermore, the fact that the contractile response to phenylephrine was not affected by copper deficiency (Table III) indicates that the contractile mechanism does not play a role in the reduced response to either acetylcholine, histamine, or sodium nitroprusside. These conclusions regarding the contractile mechanism also indicate that any effect of copper deficiency on connective tissue, which may contribute to the series and parallel elasticity of the contractile apparatus, did not have functional significance under the conditions of these experiments.

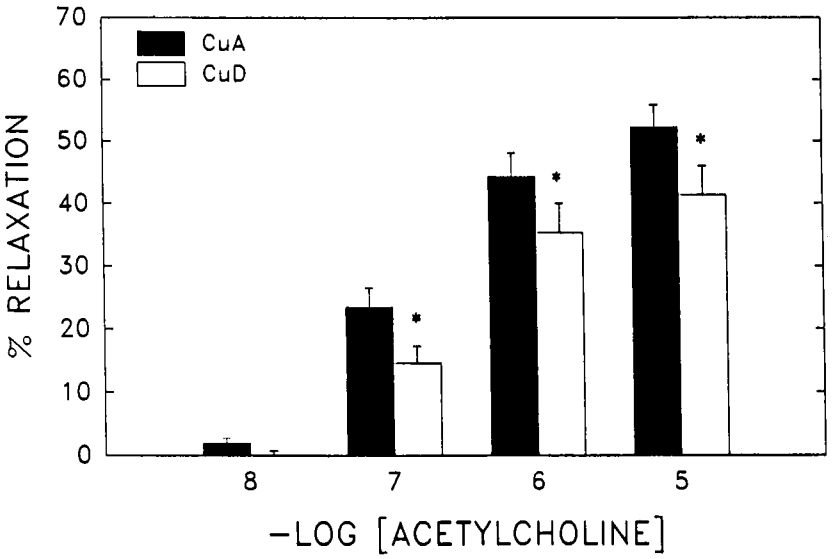

Figure 1. Effect of copper-adequate (CuA) and copper-deficient (CuD) diets on the percentage of relaxation (± SE) to acetylcholine in phenylephrine-contracted aortas. Two-way repeated-measures analysis of variance indicates that the effect of diet on the response to acetylcholine is marginal ($0.05 < P < 0.10$). However, application of Bonferroni contrasts to the means at specific concentrations indicates that copper deficiency significantly reduces the percentage of relaxation at the three highest concentrations ($^*P < 0.05$). The number of rats was 19 CuA and 19 CuD.

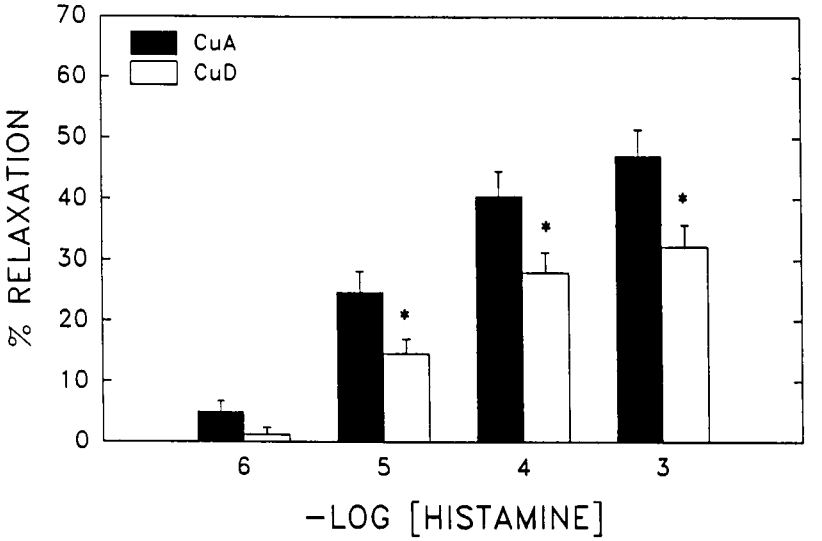

Figure 2. Effect of copper-adequate and copper-deficient diets on the percentage of relaxation (± SE) to histamine in phenylephrine-contracted aortas. Two-way repeated-measures analysis of variance indicates a significant effect of diet on the response to histamine ($P < 0.05$). Bonferroni contrasts indicate that copper deficiency significantly reduces the percentage of relaxation at each of the three highest concentrations ($^*P < 0.05$). The number of rats was 15 CuA and 15 CuD.

In addition to the possible impairment of guanylate cyclase noted above, impairment of endothelium-dependent responses in copper-deficient rats may be caused by altered activity of other copper-dependent enzymes (see Prohaska [9] for review of enzymes). For example, reduced activity of copper-dependent lysyl oxidase could result in a disruption of underlying connective tissue, thus reducing endothelial integrity. Although damage to arterial collagen and elastin in copper deficiency is well established, structural damage to endothelium appears minimal (27), having been reported in only one instance (1). However, alteration of connective tissue may place a mechanical stress on the endothelium that could alter endothelial function. Such a possibility is suggested by recent evidence that mechanical factors can affect EDRF release (28).

Another mechanism by which endothelial function might be altered in copper deficiency is by reduction of activity of copper-dependent antioxidant enzymes (i.e., serum ceruloplasmin and superoxide dismutase [29]), thus making endothelium or smooth muscle vulnerable to oxidative damage. A related possibility, not involving

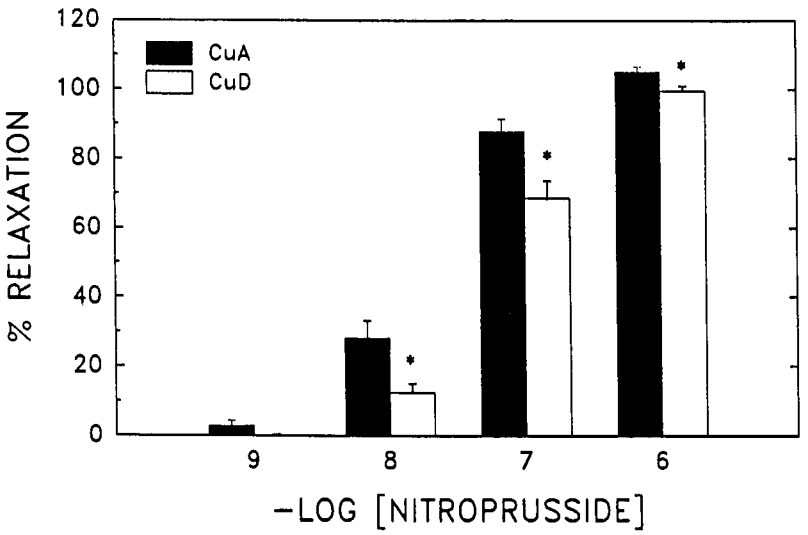

Figure 3. Effect of copper-adequate and copper-deficient diets on the percentage of relaxation (± SE) to sodium nitroprusside in phenylephrine-contracted aortas. Two-way repeated-measures analysis of variance indicates a significant effect of diet on the response to sodium nitroprusside ($P < 0.05$). Bonferroni contrasts indicate that copper deficiency significantly reduces the percentage of relaxation at each of the three highest concentrations ($^*P < 0.05$). The number of rats was six CuA and six CuD.

structural damage, is that excessive superoxide may increase EDRF (nitric oxide) destruction, thus limiting its ability to activate guanylate cyclase. This suggestion is supported by the observation that superoxide dismutase can enhance endothelium-dependent relaxation (30, 31).

Consequences of an impaired endothelium-dependent relaxation are that it may play a role in hypertension or atherosclerosis (32). Blood pressure was not measured in the present study, but weanling rats made copper deficient in general are not hypertensive and may even be hypotensive (33, 34). However, if adult rats are made copper deficient, they become hypertensive (35, 36). This suggests the hypothesis that the findings of the present study represent an early, subtle manifestation of the hypertension that develops in adult copper-deficient rats. The reduction of endothelium-dependent relaxation in resistance vessels of copper-deficient animals has recently been observed in skeletal muscle microcirculation (37). Because resistance vessels are the primary determinant of blood pressure, this observation adds additional weight to the argument that endothelium dysfunction can contribute to the hypertension of copper deficiency.

A phenomenon that may be related to the depressed endothelium-dependent relaxation observed in this study is the hypercholesterolemia often seen in copper deficiency (see Table II and Ref. 10). Hypercholesterolemia induced by cholesterol feeding is known to reduce endothelium-dependent relaxation in both large and small blood vessels (38–40). The lack of effect of this type of hypercholesterolemia on nitroprusside-induced relaxation (38, 39), however, indicates that cholesterol effects are focused on the endothelium, whereas those of copper deficiency also affect smooth muscle. The vessels of this study were not microscopically examined, but copper-deficient animals, while showing some structural abnormalities, have not shown overt atherosclerotic lesions (27). However, the fact that reduced endothelium-dependent responses have been observed in hypercholesterolemic animals without atherosclerosis (41) leaves open the possibility of a relation between copper-deficient hypercholesterolemia and an altered endothelium.

CuD rats had significantly lower body weights than CuA rats, which suggests the possibility that growth depression might have played a role in the depressed responses to acetylcholine, histamine, and sodium nitroprusside. This does not appear likely because previous work has shown that severe food restriction enhances vasodilation to the endothelium-dependent vasodilator bradykinin and has no effect on the response to the endothelium-independent vasodilator prostacyclin (42).

The reduced response of rat aorta to acetylcholine and histamine in copper deficiency indicates a disruption of endothelium-dependent relaxation. The reduced response to sodium nitroprusside, coupled with a lack of effect of copper deficiency on the papaverine response, indicates that the defect may be an alteration of guanylate cyclase activity. The latter suggestion, however, does not rule out the possibility of a reduced production of EDRF by the endothelial cell. Further study of the effect of dietary copper deficiency and its role in blood pressure regulation, hypercholesterolemia, and atherosclerosis appears warranted in light of these findings.

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