A Role for Dietary Copper in Nitric Oxide-Mediated Vasodilation

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ABSTRACT

Objective: This study was designed to investigate the role of dietary copper in nitric oxide-mediated arteriolar dilation.

Methods: Male weanling Sprague–Dawley rats were fed a purified diet that was either copper-adequate (6.0 μg Cu per g diet) or copper-deficient (0.3 μg Cu per g diet) for a period of 4 weeks. Each rat was anesthetized with pentobarbital and its cremaster muscle was positioned in a Krebs’-filled bath to which graded concentrations of vasoactive agents were added. In the first series, responses to norepinephrine (NE 10⁻⁶–10⁻⁸ M) and acetylcholine (ACH 10⁻⁷–10⁻⁴ M) were compared in third-order arterioles. Second, the dilator response to 10⁻⁵ M ACH in the absence and presence of 240 U/ml Cu, Zn-superoxide dismutase (SOD) was determined. Third, arteriolar dilation was determined in response to NO-independent stimulation of soluble guanylate cyclase with hydrogen peroxide (10⁻⁷–10⁻⁵ M) and to dibutyryl cGMP (10⁻⁶–10⁻⁴ M), dibutyryl cAMP (10⁻⁶–10⁻⁴ M), and papaverine (10⁻⁴ M).

Results: The arteriole constrictor response to NE and the dilator response to hydrogen peroxide, dibutyryl cGMP and cAMP, and papaverine were not different between the dietary groups. Copper deficiency attenuated the ACH-induced dilation, but the response was restored in the presence of SOD.

Conclusions: The inactivation of cytosolic Cu, Zn-SOD by restriction of dietary copper results in the depression of NO-mediated vascular smooth-muscle relaxation probably by interaction of NO with superoxide.

KEY WORDS: rat, arterioles, superoxide dismutase, guanylate cyclase.

INTRODUCTION

Dietary copper deficiency produces several vascular effects which suggest that endothelial function is altered. For example, platelet–endothelial adhesion is inhibited, resulting in decreased thrombogenesis (22–24), and large vessel vasoconstrictor sensitivity to norepinephrine is increased in thoracic aortas and decreased in portal veins of copper-deficient rats (11). In humans, where up to two-thirds of the western population may consume less than the estimated safe and adequate daily intake of copper (10), there is an elevated blood pressure response to hand-grip stress observed in women depleted of copper (13). Recent work indicates that copper deficiency also causes decreased smooth-muscle relaxation to acetylcholine, histamine, and sodium nitroprusside in rat aortic rings (19) and depressed dilation to acetylcholine, calcium ionophore A23187, and sodium nitroprusside in 15–25-μm arterioles of the rat cremaster microcirculation (21). Because these dilator agonists all cause vascular smooth-muscle relaxation via the cGMP second messenger system, the objective of the present study was to determine if copper restriction results in specific defect(s) of the nitric oxide (NO)–cGMP dilation pathway.

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We have previously proposed that reduced activity of Cu,Zn-superoxide dismutase (SOD) or soluble guanylate cyclase might account for the attenuation of NO-mediated vascular smooth-muscle relaxation in copper deficiency. These suggestions were based on evidence that Cu,Zn-SOD activity is reduced in copper deficiency (12, 25), that superoxide anion interacts with NO and attenuates the activity of NO (6, 18), and speculation that the copper contained in guanylate cyclase (4) may play a role in its activity. Thus, we studied: (1) the effect of exogenous SOD in restoring acetylcholine-induced dilation to determine if excess superoxide anion was present which would scavenge NO, (2) the non-NO-dependent stimulation of guanylate cyclase to ensure that the enzyme was active, and (3) the ability of exogenous cGMP to elicit vasodilation to ensure that the dilator pathway distal to guanylate cyclase was functional. Dilation to cAMP which mediates prostacyclin-induced smooth-muscle relaxation was also quantified to determine whether copper deficiency has an effect on that parallel dilator pathway. In addition, since the sensitivity of large isolated arteries to norepinephrine is increased by copper deficiency (11), the vasoconstrictor response to norepinephrine was studied to determine if there is also a role for copper in microvascular smooth-muscle contraction to this agonist. The norepinephrine and cAMP experiments were also used to indicate whether vasoreactivity in general was affected or whether the inhibited dilation was pathway specific. The phenomena were studied by direct observations of the microcirculation of the rat cremaster muscle using intravital microscopy.

MATERIALS AND METHODS

Approval for this project was obtained from the University of Louisville Animal Care and Use Committee. Male weanling Spague-Dawley rats (Charles River Laboratories, Wilmington, MA1) were housed in stainless-steel cages in a temperature- and humidity-controlled room with a 12-hr light–dark cycle. They were given free access to distilled water and a purified diet (0.4 μg Cu per g diet) that was made copper-adequate by the addition of 6 μg of copper per gram diet (CuA) or was left deficient by not adding copper (CuD diet). The basal diet (8) contained sucrose (39%), casein (20%), cornstarch (20%), safflower oil (5%), and vitamins and minerals as formulated by Teklad Test Diets (Madison, WI, #TD 84469). The animals were fed their respective diets for 4 weeks. Samples of each diet were dry-ashed (5), dissolved in aqua regia, and assayed for copper content by atomic absorption spectroscopy (Perkin Elmer, Model 503. Norwalk, CT).

In preparation for in vivo experimentation, the rats were anesthetized with sodium pentobarbital (50 mg/kg I.P) and tracheal cannula were inserted to maintain a patent airway. A carotid artery cannula connected to a transducer was used to monitor blood pressure and heart rate. The skin of the right scrotum was opened and the cremaster muscle was incised longitudinally, keeping the principal nerves and blood vessels to the muscle intact. The cremaster was spread with sutures over a coverslip in the bottom of a specially designed plexiglass bath containing modified Kreb's solution [NaCl (113 mM), NaHCO3 (25 mM), dextrose (11.6 mM), CaCl2 · 2H2O (2.6 mM), MgSO4 · 7H2O (1.2 mM), and KH2PO4 (1.2 mM)]. The Kreb's solution was replaced every 15 min and was maintained at pH 7.4 ± 0.05 by bubbling nitrogen and carbon dioxide into the bath. An indwelling heater coil with a negative feedback system was used to maintain the bath at 35 ± 0.5°C. Animals were placed on a heating pad to maintain rectal temperature at 37°C.

The animal and tissue bath were positioned on a modified stage of a Nikon MM-11 microscope so that the microcirculation could be observed by transillumination of the cremaster muscle. A closed-circuit television system was used to view the microcirculation. The magnification of the system was determined with a stage micrometer and the vessel diameters were measured directly on the television monitor.

Following the surgical preparation and preceding each experiment, there was a 1-hr equilibration period. The experimental protocols involved the topical administration of several vasoactive agonists and determination of the dilator and constrictor responses in third-order (10–25 μm) arterioles. In the first protocol, concentration–response curves were compared for the vasodilator acetylcholine and the vasoconstrictor norepinephrine. Previous work has shown that dilation to acetylcholine is attenuated in both the microvessels (21) and in the aorta (19) of copper-deficient rats. The present protocol was to determine whether the attenuated dilator response was specific for the acetylcholine pathway or if there is a generalized alteration of vasoreactivity in copper-deficient rats. In six CuA and six CuD rats, successive concentrations of acetylcholine (10−7–10−4M) were given at 10-min intervals and the diameters were recorded for each concentration. The bath was then replaced twice with fresh Kreb's solution and allowed to reequilibrate. Successive con-

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centrations of norepinephrine (10^{-9}–10^{-6} M) were then added to the bath and the diameters were again recorded.

The second protocol was designed to determine if supplementation of cytosolic Cu.Zn-SOD restores acetylcholine-stimulated dilation to normal in copper-deficient rats. In five CuA and six CuD rats, the maximal dilator response to 10^{-5} M acetylcholine was determined and then the bath was changed twice with fresh Krebs' solution. After reequilibration 240 U/ml of Cu.Zn-SOD was added to the bath for a 20-min pretreatment. Acetylcholine (10^{-5} M) was then applied in the presence of the SOD and the maximal dilation was recorded.

We have previously reported that dilation to agents which evoke smooth-muscle relaxation through nitric oxide (NO) generation is significantly depressed in copper-deficient rats (21). Therefore, the third protocol involved the use of hydrogen peroxide to stimulate soluble guanylate cyclase by a non-NO-dependent mechanism (4, 26). A concentration-response curve to hydrogen peroxide (10^{-7}–10^{-5} M) was determined for both CuA (n = 6) and CuD (n = 5) rats.

The fourth protocol was designed to study vasodilation mediated by specific second messengers. We have previously reported that the nonselective phosphodiesterase inhibitor papaverine elicits normal dilation in both copper-adequate and copper-deficient rats (21). In the current study, we examined the concentration-response curves to dibutyryl cGMP (10^{-6}–10^{-4} M) and to dibutyryl cAMP (10^{-6}–10^{-4} M). Successive concentrations of each were added to the bath every 10 min and the maximal responses were recorded. To determine the maximal dilator capacity of the arterioles, 10^{-4} M papaverine was added after the last concentrations of the cyclic nucleotides.

The median lobe of the liver was removed from each animal. The livers were lyophilized and digested in nitric acid and hydrogen peroxide (16). Copper concentrations of the livers were determined by inductively coupled plasma emission spectroscopy (Jarrell-Ash, Model 1140). Parallel assays of reference samples (#1477a, bovine liver) from the National Institute of Standards and Technology (Gaithersburg, MD) yielded mineral contents within the specified ranges.

Statistical analysis compared groups by using Student's t-test for unpaired data. Differences were considered significant at \( P < 0.05 \). Values are means ± SEM.

RESULTS

Three samples of each diet were analyzed for copper content. The mean copper value for the CuA diet was 5.67 µg per g diet and for the CuD diet 0.32 µg per g diet. The average liver copper concentrations for these animals were 12.2 µg Cu per g dry weight in the CuA group and 1.8 µg Cu per g dry weight in the CuD group.

The results of the comparisons between the responses to norepinephrine and acetylcholine in the same vessels are shown in Fig. 1. These experiments demonstrate that after 4 weeks of dietary copper deficiency the vasodilator response to acetylcholine was depressed (96% dilution in the CuA group versus 33% in the CuD group to 10^{-5} M ACH), whereas the vasoconstrictor response to norepinephrine was not altered (Fig. 1).

The second set of experiments showed that supplementation of Cu.Zn-SOD restores the dilator response of third-order arterioles (A3) to normal in CuD rats (Fig. 2). In these experiments, the CuD group response to 10^{-5} M acetylcholine (28%) was significantly depressed compared to the CuA group (100%). Pretreatment with Cu.Zn-SOD had no effect of its own on arteriole diameters but significantly increased dilation to acetylcholine in the CuD group (Fig. 2). The Cu.Zn-SOD also had no effect on the response to acetylcholine in the CuA group.

In the third experimental protocol, non-NO-dependent vasodilation to hydrogen peroxide was the same be-
**Figure 2.** Arteriolar dilation to $10^{-5}$ M acetylcholine alone and in the presence of superoxide dismutase (SOD). $^*P < 0.05$ for comparison between copper-adequate and copper-deficient groups. $^P < 0.05$ for comparison of the response to acetylcholine (ACH) with and without SOD in the copper-deficient group.

**Figure 4.** The effect of increasing concentrations of dibutyryl cGMP and maximal dilation to $10^{-4}$ M papaverine in copper-adequate and copper-deficient rats.

Figure 3. The effect of increasing concentrations of hydrogen peroxide on arteriolar diameter in copper-adequate and copper-deficient rats.

**Figure 5.** The effect of increasing concentrations of dibutyryl cAMP and maximal dilation to $10^{-4}$ M papaverine in copper-adequate and copper-deficient rats.

**DISCUSSION**

The NO signaling system is one pathway by which vascular smooth-muscle relaxation occurs. This pathway involves either the generation of NO by agonist activation of NO-synthase or the donation of NO from a nitrovasodilator. The NO diffuses to the vascular smooth-muscle cells where it is a potent and efficient activator of soluble guanylate cyclase (GC-S). GC-S then forms cGMP, which, in turn, causes protein phosphorylation and inhibition of smooth-muscle contraction (20).

We have previously proposed that inhibition of this NO signaling pathway by restriction of copper could involve two different enzymes in which copper is a cofactor (19, 21). The first enzyme is GC-S. Copper as well as iron are transition metals which serve as components of this enzyme (4). The iron is the metal component of the heme moiety of the GC-S. NO binding to the heme site is responsible for a confor-
tional change in the GC-S and activation of the catalytic site of the enzyme (20). If the copper is a functional cofactor with the iron in the NO--heme binding site, then NO may no longer be able to activate the GC-S when copper is inadequate. Alternatively, iron metabolism is known to be altered in dietary copper deficiency (7) and may be a mechanism by which the NO--heme binding is prevented and the NO activation of GC-S is depressed in the CuD animals.

Hydrogen peroxide activates GC-S by a NO-independent mechanism (1, 2, 26). Also, basal GC-S activity is known to be independent of heme content even though heme-free GC-S does not respond to NO (4). In our studies, the microvessel dilatation to hydrogen peroxide was not different between groups (Fig. 3) although dilation to acetylcholine was significantly decreased in the CuD group compared to the CuA group (Figs. 1 and 2 and Ref. 21). Thus, our results suggest that the general activity of the GC-S is not affected by dietary copper deficiency. Further, the normal dilator response to the phosphodiesterase inhibitor papaverine in the CuD rats (Figs. 4 and 5 and Refs. 19 and 21) suggests that cGMP generation by GC-S is not inhibited by copper deficiency. Therefore, our results indicate that if copper is a functional component of GC-S, its role is at the NO-binding site, but it is not a requisite for the basal activity of the enzyme.

The second possible mechanism for a defect in the NO signaling pathway during copper deficiency involves the antioxidant enzyme Cu,Zn-SOD. Nitric oxide is known to be inactivated by superoxide anions (6, 18) and this inactivation can be reversed by Cu,Zn-DOS (1). Omar et al (17) have proposed that a direct interaction with the GC-S enzyme may be the mechanism of inhibition by the superoxide anion. The activity of cytosolic Cu,Zn-SOD is dependent on the presence of copper at the catalytic site (3) and is known to be depressed in our rat model of copper deficiency (9), which suggests that there would be a buildup of superoxide anions in the CuD group. In the present study, the addition of Cu,Zn-SOD significantly improved arteriolar dilation to acetylcholine in the CuD group where the dilator response to the agonist alone was depressed (Fig. 2). These results coincide with previous work showing that dilation to acetylcholine and to sodium nitroprusside inhibited by diethylthiocarbamate (DETCa) could be restored with exogenous SOD (17). We propose that there is an increase in superoxide anions in copper deficiency caused by a decrease in the activity of Cu,Zn-SOD. The resultant additional superoxide anion concentration attenuates the dilator response to NO-mediated agents in the microvasculature of these animals by scavenging NO.

In addition to the NO signaling pathway, another vascular dilator pathway involves the endothelial release of prostacyclin, activation of adenylate cyclase, and the resultant production of cAMP. Dietary copper deficiency is known to inhibit prostacyclin synthesis in the rat aorta (14, 15). However, it is not clear whether there are physiological consequences of this inhibition in the microvasculature. In the present study, both dibutyryl cAMP and dibutyryl cAMP elicited normal dilator responses in CuD rats compared to the CuA controls (Figs. 4 and 5). These results demonstrate that the smooth-muscle relaxation mechanisms that respond to these second messengers are not inhibited by copper deficiency.

The present results suggest that the inactivation of cytosolic Cu,Zn-SOD by restriction of dietary copper results in the depression of NO-mediated vascular smooth-muscle relaxation. Even though copper is a component of GC-S and copper deficiency may alter the heme-binding site for NO on the enzyme, the restoration of dilation to acetylcholine by exogenous SOD suggests the Cu,Zn-SOD is the enzyme that is the most sensitive to copper restriction in the NO signaling system. Further, normal dilator responses to cGMP and cAMP suggest that the specific dilator mechanisms to these two second messengers are intact and the normal response to norepinephrine (Fig. 1) demonstrates that there is not a generalized vasodepressive effect of copper deficiency. Rather, our data suggest that depressed endothelial function in copper deficiency occurs via defects in specific signal transduction mechanisms.

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REFERENCES


