Relationship between dietary copper concentration and acetylcholine-induced vasodilation in the microcirculation of rats

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Abstract. Dietary copper deficiency has been shown to significantly reduce acetylcholine (Ach)-induced vascular smooth muscle relaxation. The current study was designed to examine the relative relationship between dietary copper and the vasodilator response to Ach in the microcirculation of the rat. Male weanling rats were fed a purified basal diet supplemented with 0.0, 3.0, 1.5 or 0.0 μg Cu/g diet for 4 weeks to provide an adequate, marginal, and deficient intakes of dietary copper. Arteriole dilation in response to increasing concentrations of acetylcholine (10^{-5} to 10^{-4} M) was measured in the in vivo cremaster muscle microcirculation for each dietary group. Liver copper and both aortic and erythrocyte Cu,Zn-SOD activity were used as indices of systemic copper status. Dilation to the increasing concentrations of Ach was only different in the 0 μg Cu supplemented group compared to the copper-adequate control values. However, the combined results showed an exponential increase in 10^{-5} M Ach-induced vasodilation as liver copper concentration increases from 0 μg Cu/g dry wt. This relationship suggests that dilation is attenuated at liver Cu concentrations below 5 μg/g dry wt. The results indicate that Ach-induced vasodilation is copper-dependent but that the pathway is not very sensitive to short-term marginal restriction of copper intake.

Keywords: Acetylcholine, copper, microcirculation, super oxide dismutase, vasodilation

1. Introduction

Dietary copper has been shown to have a role in several microvascular control mechanisms [10] including the acetylcholine (Ach)-induced relaxation pathway in vascular smooth muscle cells. Previous studies have demonstrated that copper deficiency impairs Ach-mediated dilation in both large arteries [4, 9] and in arterioles of the microcirculation [11, 13]. Similar results have been reported in isolated vascular ring preparations when copper is chelated [6]. In both the dietary and chelation models of copper deficiency, 

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deficiency the addition of the copper-dependent enzyme superoxide dismutase (Cu,Zn-SOD) restores the Ach-mediated smooth muscle relaxation to normal [6,13]. These results suggest that the diminished dilation in copper deficiency is at least in part caused by decreased cytosolic SOD activity.

While it is well documented that dietary copper is a requisite for Ach-stimulated vascular smooth muscle relaxation, the relationship between the dietary concentration of copper and the degree of relaxation has not been studied. The current study was designed to examine this relationship and determine the minimal dietary copper intake necessary to prevent attenuation of Ach-induced vasodilation. The study compares concentration-response curves to Ach in rats fed diets which contain adequate, marginal, or deficient amounts of copper. Copper status is indicated by hepatic copper concentration and both erythrocyte and aorta Cu,Zn-SOD activity. Hematocrit and cardiac hypertrophy are also reported for each diet group.

2. Materials and methods

2.1. Animals and diet

This project was approved by the University of Louisville Animal Care and Use Committee. Male weanling Sprague–Dawley rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12-h light-dark cycle. The rats were given free access to distilled water and to one of four purified diets for 4 weeks. The basal diet [1] was a casein-sucrose-cornstarch-based diet (no. TD 84469, Teklad Test Diets, Madison, WI) containing all known essential vitamins and minerals except for copper and iron. The copper-adequate (CuA6.0; 6.0 μg Cu/g) diet consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a copper-iron mineral mix which provided 0.22 g of ferric citrate (16% Fe) and 24 mg of CuSO4 · 5H2O per kilogram of diet. The marginally copper-deficient diets contained 12 and 6 mg of CuSO4 · 5H2O per kilogram of diet to provide 3.0 (CuM3.0) and 1.5 (CuM1.5) μg Cu/g diet, respectively. The copper-deficient (CuD0.0) diet was the same except for the replacement of supplemented copper with cornstarch in the mineral mix. Diet analysis was by atomic absorption spectrophotometry. Parallel assays of National Institute of Standards and Technology (NIST; Gaithersburg, MD) reference samples (apple leaves, no. 1515) yielded values within the specified range, which validated our copper assays.

In preparation for in vivo experimentation, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The trachea was cannulated to maintain airway patency and the carotid artery was cannulated to directly monitor blood pressure. The cremaster muscle was prepared for in vivo microcirculatory observation as previously described [13] and positioned over an optic port in a specially designed plexiglass bath. The bath was filled with 60 ml of a modified Kreb's solution [13] which was maintained at a temp of 35 ± 0.5°C and a pH of 7.4 ± 0.5.

The rat and tissue bath were placed on the modified stage of a Nikon MM-11 microscope so that the microcirculation could be observed by transillumination of the cremaster muscle. Closed-circuit television microscopy was used to observe and quantitate the diameters of third-order arterioles which had basal diameters of 10–12 μm. The video system was calibrated with a stage micrometer and the vessel diameters were measured with a video caliper.

Following a 1 hr equilibration period, mean arterial blood pressure was recorded and a concentration-response curve to acetylcholine (Ach) was determined for each of the four dietary groups. Five CuA6.0,
six CuM₁,₀, five CuM₁,₅, and six CuD₀,₀ rats were used for this protocol. Successive concentrations of Ach (10⁻⁷–10⁻⁴ M) were added to the tissue bath at 10 min intervals. Arteriolar diameters were measured every 2 min during the treatment and the maximal response for each concentration of Ach was used for comparison. Blood pressure was continuously monitored during the in vivo experimentation to assess the hemodynamic stability of the rat.

After the in vivo experimentation, the chest was opened and blood was withdrawn via cardiac puncture for hematocrit and erythrocyte Cu,Zn-SOD activity determinations. Liver samples for copper analysis and sections of thoracic aorta for blood vessel Cu,Zn-SOD activity were also collected. Residual blood was flushed from the aortas with heparinized saline to ensure that erythrocyte Cu,Zn-SOD activity did not confound the enzymatic activity of the vascular tissue.

2.2. Copper status indices

The median lobe of the liver was removed, weighed and frozen at −10°C for subsequent copper analysis. Tissues were lyophilized and digested in nitric acid and hydrogen peroxide [5]. Hepatic copper concentrations of individual rats were assessed by using inductively coupled argon plasma emission spectrometry (Jarrell-Ash, model 1140, Waltham, MA). Parallel assays of reference samples (no. 14772a, bovine liver) from the NIST yielded mineral contents within the specified range.

The Cu/Zn-SOD activity in red blood cells and aortas was determined using the autoxidation of pyrogallol as described by Percival et al. [7]. Tissue homogenates were treated with 0.4 vol of chloroform and ethanol (15 : 25) to precipitate the manganese SOD and then centrifuged at 14,000g for 4 min before aliquoting to microtiter plates. Fifty microliter samples were diluted in triplicate (1 : 1) with 50 mmol/l Tris buffer containing diethylenetriaminepentaacetic acid at pH 8.2. Fifty μl of 0.2 mM pyrogallol in 10 mM HCl containing 1 mM diethylenetriaminepentaacetic acid were added and absorbance at 340 nm was monitored every 10 s for 3 min at room temp.

2.3. Statistical analysis

Data are presented as mean ± SEM. Comparisons between dietary groups was by one-way ANOVA. Effects were considered significant if p < 0.05. If a significant effect was found, a Student–Newman Keuls test was used to determine which means were different. The relationship between liver copper concentration and arteriolar dilation to 10⁻⁵ M acetylcholine was determined by fitting an exponential curve to the data points (SAS/STAT, SAS Institute, Inc., Cary, NC). Because this is a nonlinear model, only an approximate R² and significance level can be calculated [3].

3. Results

Two samples of each diet were analyzed for copper content. The mean copper concentrations of the CuA₆,₀, CuM₃,₀, CuM₁,₅, and CuD₀,₀ diets were, respectively, 5.90, 2.77, 1.48, and 0.32 μg Cu/g diet. Mean dietary iron concentrations for the diets were 33.62, 32.56, 35.07, and 36.64 μg Fe/g diet. The marginal and severely deficient concentrations of dietary copper produced a graded reduction in liver copper concentrations (Fig. 1) and also decreased both erythrocyte and aortic Cu,Zn-SOD activity compared to the copper-adequate controls (Fig. 1). Maximal decrease in Cu,Zn-SOD activity in the erythrocyte occurred at 1.5 μg Cu/g diet (Fig.1) and in the aorta at 3.0 μg Cu/g diet (Fig. 1).
Fig. 1. Comparison of the hepatic copper concentration (top-left), erythrocyte Cu,Zn-SOD activity (top-right) and aortic Cu,Zn-SOD activity (bottom) in rats fed a basal diet supplemented with 6.0 (n = 7), 3.0 (n = 5), 1.5 (n = 5), or 0.0 (n = 7) μg Cu/g diet. Bars with different letters are statistically different (p > 0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CuA6.0</th>
<th>CuM3.0</th>
<th>CuM1.5</th>
<th>CuD0.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Body wt. (g)</td>
<td>218 ± 13^A</td>
<td>204 ± 10^A</td>
<td>194 ± 7^A</td>
<td>173 ± 9^B</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>128 ± 4^A</td>
<td>112 ± 5^B</td>
<td>110 ± 3^B</td>
<td>100 ± 6^B</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>46.2 ± 1.6^A</td>
<td>44.4 ± 1.6^A</td>
<td>45.0 ± 1.3^A</td>
<td>19.7 ± 2.1^B</td>
</tr>
<tr>
<td>Heart wt/body wt. ratio</td>
<td>0.43 ± 0.02^A</td>
<td>0.51 ± 0.04^A</td>
<td>0.49 ± 0.01^A</td>
<td>0.94 ± 0.09^B</td>
</tr>
</tbody>
</table>

All values are mean ± SEM.

Whole animal indices of copper deficiency are given in Table 1. Body weight, as an index of growth rate, was only different in the CuD0.0 group compared to the CuA6.0 controls. Mean arterial blood pressure was statistically lower in the copper marginal and deficient groups compared to the CuA6.0 group but not to an extent which would be considered hypotensive. Other indices of copper deficiency including hematocrit and cardiac hypertrophy (as determined by heart weight-to-body weight ratio) were also significantly different in the CuD0.0 fed group compared to the CuA6.0 group.

Concentration-response curves to increasing concentrations of Ach showed a significant depression of the dilation response in only the CuD0.0 group compared to the adequate and marginal copper groups.
Fig. 2. Comparison of the dilation response to increasing concentrations of acetylcholine in rats fed a basal diet supplemented with 6.0 (n = 5), 3.0 (n = 6), 1.5 (n = 5), or 0.0 (n = 6) \( \mu \)g Cu/g diet. *p > 0.05 for comparisons between dietary groups for each concentration of agonist.

Fig. 3. Dilation of arterioles (% of baseline diameter) in response to treatment with \( 10^{-5} \) M acetylcholine in rats with varying liver copper concentrations. The best fit of an exponential function to the data was found to be as follows; dilation = 207 - 74 \times \exp(-0.31 \times \text{liver Cu}). The correlation coefficient was estimated to be \( r = 0.71 \) and the significance of the fit of the curve to the data points was \( p < 0.008 \).

(Fig. 2). The percentage dilation of arterioles to \( 10^{-5} \) M acetylcholine was plotted against liver copper concentration for each rat (Fig. 3). A reasonable model of this relationship was provided by fitting an exponential curve to the data points. The estimated correlation for this nonlinear model is \( r = 0.71 \) and the significance of the fit is \( p < 0.008 \). The model indicates that, as liver copper concentration decreased below about 5 \( \mu \)g/g dry weight, percentage dilation also decreased.
4. Discussion

Several investigators have published evidence that nitric oxide (NO)-mediated vascular smooth muscle relaxation is copper-dependent. Studies in both large arteries [4,9] and in arterioles [11,13] of copper-deficient rats have shown significant inhibition of dilation responses to agonists which stimulate NO production. These include Ach, calcium ionophore A23187, and sodium nitroprusside as well as authentic NO. Further studies have shown that the defect is specific to this dilator pathway since neither vascular smooth muscle relaxation capacity [11] nor NO-independent vasodilation [13] is influenced by copper deficiency. The addition of exogenous SOD restores the Ach-mediated dilation to normal [6,13] demonstrating that control of cellular superoxide anion concentrations by Cu,Zn-SOD is necessary for NO-mediated vasodilation.

In the current study, Ach-induced dilation of the microvascular arterioles was significantly depressed in the CuD0.0 dietary group (Fig. 2). These results are similar to previously published results with the same animal model [11,13]. The attenuated dilation was not seen in either of the marginal copper-fed groups (Fig. 2) suggesting that in an otherwise nutritionally uncompromised rat, copper-dependent vasodilation is not very sensitive to short-term moderate restriction of dietary copper.

Previous work has suggested that the depressed Ach-induced vasodilation is the result of decreased activity of Cu,Zn-SOD during dietary copper-deprivation or chelation [4,6,13]. The effects of inactivation of this copper enzyme likely include greater scavenging of NO by superoxide anion [4,13], increased endothelial membrane peroxidation [4] and decreased endothelial cell Ca2+ mobilization [12] which is a requisite for synthesis of endothelial NO. However, the current study demonstrates a difference in the vasodilator response to Ach between the CuD0.0 and CuM1.5 groups (Fig. 2) when there is no difference in SOD activity (Figs 2 and 3). These results suggest that, in addition to an effect of reduced Cu,Zn-SOD activity, there may be an effect of copper deficiency on the Ach-induced endothelial-smooth muscle relaxation pathway. This concept is supported by Plane et al. [8] who suggested that, independently of decreased SOD activity, copper may have a role in controlling nitric oxide synthase and guanylyl cyclase. These enzymes are components of this relaxation pathway.

Although Cu,Zn-SOD activity is significantly lower in the CuD0.0 rats compared to control values (Fig. 1), liver copper concentration appears to be a better indicator of changes in vasodilator physiology. Figure 3 illustrates the relationship between the arteriolar dilation response to 10−5 M Ach and the copper concentration in the liver. Examination of this exponential curve suggests that the percentage dilation decreases at liver copper concentrations below about 5 μg/g dry weight. Previous results comparing liver copper and venular bleeding time showed that hemorrhage was prolonged at about 4 μg Cu/g dry liver weight [14]. These previous data combined with the current data provide evidence of dramatic changes in endothelial cell function when liver copper concentrations fall below about 5 μg/g dry weight.

Also coincident with the attenuated Ach concentration-response curve in the CuD0.0 group (Fig. 2) is the presence of both anemia and cardiac hypertrophy (Table 1) similar to previously reported results in severely copper-deficient rats [2,14]. The effects of these cardiovascular changes on Ach-induced vasodilation in the copper-deficient rat are not known. However, since mean blood pressure is not significantly different in the CuD0.0 group compared to either CuM group (Table 1), the depressed vasodilation in the CuD0.0 group (Fig. 2) does not appear to be associated with a hemodynamically compromised animal.

In summary, the current research demonstrates a significant relationship between liver copper and Ach-induced vascular smooth muscle relaxation in arterioles. The sensitivity of this relaxation pathway to dietary copper-restriction is similar to results previously reported in a study of the role of copper in hemostatic mechanisms. We conclude that diets which result in liver copper concentrations below 5 μg/g
dry weight cause significant depression of copper-dependent microvascular control mechanisms. The results also endorse the hypothesis of Klevay and Saari [2] that liver copper probably is the best index of copper nutrition.

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