Dietary Copper Deficiency Increases Inducible Nitric Oxide Synthase-Mediated Vascular Dilation in Rat Aorta

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The attenuation of endothelium-dependent nitric oxide (NO)-mediated vasodilation is a consistent finding in both conduit and resistance vessels during copper deficiency. However, there is often no effect on systemic blood pressure in experimental animals, suggesting that peripheral vascular resistance is not altered. We hypothesized that baseline vascular smooth muscle relaxation may be maintained by a chronic increase in inducible NO synthase (iNOS) expression, as has been documented in hearts of copper-deficient rats. We used endothelium-denuded rat aortic rings to examine the role of iNOS in the regulation of vascular reactivity during dietary copper deficiency. Male weanling rats were fed a copper adequate (CuA, 5.6 mg Cu/kg diet) or copper-deficient diet (CuD, 0.33 mg Cu/kg diet) for 4 weeks. The induction of "functional" iNOS was indicated by a relaxation response to the NO precursor L-arginine or to Cu,Zn-superoxide dismutase (SOD), which preserves basal NO. Time to 50% relaxation in response to either compound was significantly shorter in the CuD than in the CuA aortas. The maximal relaxation response to L-arginine was blocked by the iNOS inhibitor L-NIL, and the relaxation response to Cu,Zn-SOD was blocked by the NO-sensitive guanylate cyclase inhibitor ODQ. Maximal activation of iNOS expression with lipopolysaccharide pretreatment did not cause a difference in vascular relaxation between dietary groups in response to L-arginine. Expression of the iNOS protein in the aortas was also not different between groups. These results suggest that although there is no apparent increase in protein expression, copper deficiency increases baseline iNOS activity in the vascular wall. J. Trace Elem. Exp. Med. 15:85–95, 2002. © 2002 Wiley-Liss, Inc.

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INTRODUCTION

The requisite role for dietary copper in nitric oxide (NO)-mediated vasodilation is well established. This copper dependency has been demonstrated in experiments showing impaired vascular smooth muscle relaxation in response to exogenous NO and NO-mediated agonists during dietary copper deficiency [1–6] and copper chelation [7–9]. Studies in copper-deficient rats implicate the inactivation of copper, zinc superoxide dismutase (Cu, Zn-SOD), and the subsequent buildup of superoxide (O$_{2}^{-}$) as the mechanism for the interruption of the NO-signaling pathway. Based on our prior studies, we proposed that NO and O$_{2}^{-}$ react to produce peroxynitrite (ONOO$^{-}$), which decreases the amount of NO available to diffuse to the vascular smooth muscle [3,5]. The increased ONOO$^{-}$ also likely reduces continued production of endothelial-derived NO by suppressing the calcium signaling in the endothelial cells [5].

Although the attenuation of NO-dependent vasodilation is a consistent finding in copper deficiency, the effect on blood pressure is less predictable. Copper deficiency may cause either hypertension [10–11] or hypotension [12–14] or have no effect on blood pressure [15–19]. The lack of an effect on blood pressure when NO-mediated vascular relaxation is decreased suggests that compensatory mechanisms are involved. One possible mechanism may be a chronic increase in inducible nitric oxide synthase (iNOS) expression such as has been documented in hearts of copper-deficient rats [20].

NO generated by iNOS in the vascular smooth muscle effector cells [21] may be less susceptible to inactivation by O$_{2}^{-}$ than endothelial-derived NO because cell-to-cell diffusion of the NO is not necessary and, unlike the endothelial isoform (eNOS), iNOS is not dependent on Ca$^{2+}$ mobilization [22]. Therefore, the current study was designed to examine the role of iNOS in the regulation of vascular reactivity during copper deficiency. The induction of "functional" iNOS was indicated by a relaxation response to the L-arginine in endothelium-denuded aortic rings that were precontracted with phenylephrine. Rings were either stimulated with lipopolysaccharide (LPS) to up-regulate iNOS expression or left untreated to test the effect of dietary copper restriction on baseline iNOS expression. Relaxation in response to Cu,Zn-SOD, which protects basally produced NO from destruction by O$_{2}^{-}$ [8], was also used as an index of iNOS activity. Blockade of the above relaxations with the iNOS-specific antagonist L-N$^{6}$-(1-Iminoethyl)-lysine (L-NIL) and ODQ, a selective inhibitor of NO-sensitive guanylate cyclase activity, were indicative of NO mediation. Western blot analysis was used to compare the presence of iNOS in the endothelium-denuded vessels from rats fed diets adequate and deficient in copper.

MATERIALS AND METHODS

Animals and Diet

This project was approved by the University of Louisville Animal Care and Use Committee. Forty-two 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 two purified diets for 4 weeks [1,3,4]. The basal diet [23] 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 (CuA) diet consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a copper-iron mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of CuSO\(_4\)-5H\(_2\)O per kilogram of diet. The copper-deficient (CuD) diet was the same except for replacement of copper with cornstarch in the mineral mix. Diet analysis by atomic absorption spectrophotometry indicated that the CuA diet contained 5.6 mg copper/kg diet and the CuD diet contained 0.33 mg copper/kg diet. Parallel assays of National Institute of Standards and Technology (NIST; Gaithersburg, MD) reference samples (citrus leaves, no. 1572) yielded values within the specified range, which validated our copper assays.

Preparation of Aortic Rings

Thoracic aortae were excised from rats, cleaned of connective tissues, denuded of the endothelium, and cut into 3- to 4-mm ring segments. Endothelium denudation was achieved mechanically by gently rubbing the intimal surface with a moist matchstick. The absence of an intact endothelium was determined by the absence of a relaxation response to 10 \(\mu\)M acetylcholine. Denudation was performed to remove the influence of endothelium-derived NO. Each ring was mounted between stainless-steel triangular hooks and suspended under 2-g passive tension in 20-mL organ baths containing physiological salt solution maintained at 37°C and bubbled with 95% O\(_2\)/5%CO\(_2\) gas mixture. Isometric tension was recorded with Grass FT 0.03-force displacement transducers coupled to a Grass polygraph (model 7H). The composition (in mM) of our physiological salt solution was: NaCl, 118; KCl, 4.7; CaCl\(_2\), 2.5; KH\(_2\)PO\(_4\), 1.2; MgSO\(_4\), 1.2; NaHCO\(_3\), 12.5; and glucose, 11.1. The pH of the solutions after saturation with 95% O\(_2\)/5% CO\(_2\) gas mixture was 7.4. As a routine, tissues were allowed to equilibrate for 1 h before the start of all experiments.

Drugs

The following drugs were purchased from Sigma Chemical Co (St. Louis, MO): L-phenylephrine hydrochloride (PE), acetylcholine, L-arginine hydrochloride, L-NIL, ODQ, Cu\(_2\)Zn-superoxide dismutase (SOD) and lipopolysaccharide from Escherichia coli serotype (LPS). All solutions were made in physiological salt solution.

Experimental Protocols

The first three protocols were designed as a functional test of the effect of copper deficiency on the upregulation of vascular iNOS.
Protocol 1. This series of experiments determined the relaxation effect of the NOS substrate L-arginine on PE-induced constriction in denuded aortic rings of 5 CuA and 5 CuD rats before and after iNOS blockade. Once the contraction to PE (1 μM) reached plateau levels, the aortic rings were exposed to L-arginine (1 mM). Ninety min later, tissues were rinsed at least three times with warm (37°C) physiological salt solution before the addition of 1 mM L-NIL to inhibit iNOS [24]. L-NIL has an IC₅₀ of 3.3 μM for iNOS compared to 92 μM for cNOS [25]. After a 20-min pretreatment, the aortic rings were again contracted with PE and exposed to 1 mM L-arginine. Maximal relaxation of initial PE-induced tone and time to achieve 50% relaxation of aortic rings were compared between dietary groups. Preliminary studies demonstrated that, in the absence of a blocker, there was no difference in the relaxation response between the first and second applications of L-arginine.

Protocol 2. This series of experiments determined the relaxation effect of SOD, which preserves basally produced NO from destruction by superoxide anion [8]. Relaxation to SOD was determined on phenylephrine-induced contraction in denuded aortic rings of 5 CuA and 5 CuD rats. Once the maximal contraction to phenylephrine (1 μM) was obtained, the aortic rings were exposed to SOD (240 units/mL). After the relaxation reached plateau, tissues were rinsed at least three times with warm (37°C) physiological salt solution. Then tissues were incubated with ODQ (10 μM) to inhibit the NO-sensitive guanylyl cyclase activity. After a 15-min pretreatment, the aortic rings were again contracted with phenylephrine and exposed to SOD. Maximal relaxation of initial PE-induced tone and time to achieve 50% relaxation of aortic rings were compared between dietary groups.

Protocol 3. Experiments in this series examined the effect of iNOS induction by LPS on L-arginine-induced relaxation of aortic rings from 5 CuA and 5 CuD rats. Vascular rings were incubated for 4 h with LPS (300 ng/mL). After incubation, tissues were contracted with PE (1 μM). Once the maximal contraction was obtained, the aortic rings were exposed to L-arginine (1 mM). Maximal relaxation of initial PE-induced tone and time to achieve 50% relaxation of aortic rings were compared between dietary groups.

Western Blot Analysis. Western immunoblotting analysis was performed to determine the relative amounts of iNOS expressed in the aortas from rats fed the CuA (n = 6) and CuD (n = 6) diets. As with the functional ring experiments, the endothelium was denuded by rubbing with a matchstick. A small sample of each vessel was taken for histological examination (H & E stained and 400X magnification) to confirm that the endothelium was removed. Frozen aortic specimens were powdered in a prechilled pulverizer and homogenized on ice with a T25 Basic homogenizer (IKA Works) in sample buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 50 μg/mL
phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/mL pepstatin A, and 10% glycerol (vol/vol). The homogenate was then incubated on a rocking platform with 20 mM 3-[(3-cholamidopropyl) dimethylammonio]1-propanesulfonate (CHAPS) for 2 h at 4°C. After centrifugation at 14,000 × g for 30 min, the supernatant was collected as total cellular protein. Protein concentration was determined using the modified Bradford method (Protein Assay Kit II; Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Protein samples (50 μg/lane) were separated by electrophoresis under reducing, denaturing conditions in 8% polyacrylamide/SDS gels and transferred by electroblotting onto nitrocellulose blots. Equal loading and transfer efficiency were carefully recorded by making photocopies of blots dyed with reversible Ponceau staining. After being blocked overnight in 5% nonfat milk (Bio-Rad), blots were incubated with an anti-mouse iNOS polyclonal antibody (Transduction Laboratories) and subsequently with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Transduction Laboratories). Signal detection was facilitated with enhanced chemiluminescence (ECL, Amersham).

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 [26]. 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. 1477a, bovine liver) from the NIST yielded mineral contents within the specified range.

Statistical Analysis

Results are presented as mean ± SEM. Statistical evaluation of the data was performed by Student's t test for independent observations. Values were considered to be statistically different at P < 0.05.

RESULTS

Rats that were fed a CuD diet for 4 weeks developed anemia and had significantly lower liver copper concentrations than the rats that were fed a CuA diet (Table I). The diet did not, however, affect the growth rate of the rats, as indicated by the body weight at the time of experimentation (Table I).

After induction of tone with phenylephrine, L-arginine (1 mM) produced relaxation of endothelium-denuded aortic rings from both CuA and CuD rats (Fig. 1). The L-arginine-induced relaxation was significantly attenuated by a 20-min pretreatment with the iNOS inhibitor L-NIL (Fig. 1). There was no significant difference in the maximal relaxation between dietary groups. However, the time to 50% relaxation induced by L-arginine was significantly shorter in the CuD group than in the CuA values (P = 0.017; Fig. 2).
TABLE I. Body Weight and Copper Status of CuA and CuD Animals

<table>
<thead>
<tr>
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<th>CuA</th>
<th>CuD</th>
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<tr>
<td>Body weight (g)</td>
<td>231.8 ± 8.58</td>
<td>215.1 ± 9.01</td>
</tr>
<tr>
<td>Hepatic copper (µg/g dry wt)</td>
<td>11.28 ± 0.55</td>
<td>3.21 ± 0.49*</td>
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<tr>
<td>Hemotocrit (%)</td>
<td>41.3 ± 0.54</td>
<td>28.3 ± 1.18*</td>
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Values are mean ± SEM.
*P < 0.05 compared to the CuA control group.

SOD produced relaxation of endothelium-denuded aortic rings of both CuA and CuD rats (Fig. 3). This relaxation response was almost abolished in both dietary groups by a 15-min pretreatment with ODQ, a potent inhibitor of the NO-cGMP pathway (Fig. 3). The maximal relaxation in response to the exogenous SOD was not different between dietary groups. However, as with the L-arginine-induced relaxation, the time to 50% relaxation in response to SOD was significantly (P = 0.035) shorter in the CuD group (Fig. 4).

In a separate experiment, aortic rings were pretreated with LPS for 4 h to fully stimulate the expression of iNOS in the vasculature. L-arginine produced nearly immediate relaxation of aortic rings after incubation with LPS, but there were no significant differences in maximal relaxation (Fig. 5) or time to 50% relaxation (Fig. 6) between CuA and CuD animals.

Although there was an apparent difference in the response to iNOS-generated NO between the dietary groups, there was no difference in iNOS protein expression as determined by Western blotting (Fig. 7).

![Graph](image)

**Fig. 1.** Maximal relaxation of denuded rat aortas to L-arginine without and with iNOS blockade by L-NIL. Results are presented as mean ± SEM from five rats in each dietary group. *P < 0.05 compared to the untreated control group.
DISCUSSION

NO is a major mediator of vascular smooth muscle relaxation. This regulation is of particular significance in the large arterioles that determine peripheral vascular resistance and arterial conductance. Under physiological conditions, the primary producer of NO in the vasculature is the eNOS isoform. A diet deficient in copper significantly attenuates NO-mediated vascular relaxation induced by
Fig. 4. Time to 50% relaxation induced by SOD in denuded rat aortas of CuA and CuD animals. Results are presented as mean ± SEM from five rats in each dietary group. *P < 0.05 compared to the CuA group.

endothelial cell stimulation [1–6] but does not diminish the relaxation capacity of the smooth muscle [1–3]. Interestingly, the anticipated increase in blood pressure associated with the depression of NO-mediated dilation during copper deficiency usually does not occur [15–19].

The current study suggests that during the reduction of eNOS-generated NO caused by copper deficiency, iNOS-generated NO may have a role in maintaining baseline vascular smooth muscle tone. Inducible NOS protein is not a constitutively expressed enzyme but requires transcriptional activation mediated by cytokines. This activation is demonstrated in the current study by pretreatment of the endothelium-denuded aortic rings with LPS for 4 h. Using a functional index for iNOS, relaxation in response to the NOS substrate L-arginine occurred

Fig. 5. Maximal relaxation of denuded rat aortas to L-arginine after a 4-h pretreatment with LPS. Results are presented as mean ± SEM from five rats in each dietary group. No significant difference was observed.
in the aortic rings from both dietary groups. There was no difference in the maximal relaxation or the time to 50% relaxation between dietary groups (Figs. 5 and 6) when pretreated with LPS. These results demonstrate that during maximal inflammatory stimulation, the CuD diet does not alter iNOS-induced generation of NO.

However, in the absence of any inflammatory stimuli, relaxation of PE-contracted aortic rings occurred significantly sooner in the CuD group when stimulated with L-arginine (Fig. 2) even though maximal relaxation was not different between dietary groups (Fig. 1). Surprisingly, the total relaxation response (percentage of PE-induced constriction) was not different between vascular rings with and without LPS pretreatment (Figs. 1 and 5), although time to 50% relaxation was much quicker after LPS pretreatment (Figs 2 and 6). Because these results occurred in aortic rings that had been denuded of the endothelium, it is likely that the relaxation response is dependent on iNOS-generated NO. This was confirmed in both dietary groups by blockade of the relaxation with the iNOS selective inhibitor L-NIL (Fig. 1). Previous data also support a role for the iNOS isoform during copper deficiency. Because dietary copper restriction decreases calcium mobilization [5,27], the calcium-independent iNOS may be less impacted than the calcium-dependent isoforms eNOS and nNOS in the CuD rat model.

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**Fig. 7.** Western blot determination of expression of iNOS in endothelium-denuded aortas of rats fed either a CuD or CuA diet. Each lane represents two pooled aortas. The positive control (left lane) is liver from a rat pretreated with LPS (6 mg/kg) for 8 h. No significant difference between CuA and CuD aortas was observed.
The addition of exogenous SOD to preserve basally produced NO caused a relaxation response similar to that seen with L-arginine. Although total relaxation of PE-constricted aortic rings was less in response to SOD than to L-arginine (Figs. 1 and 3), the time to 50% relaxation with SOD was significantly shorter in the CuD group than in the CuA controls (Fig. 4). Inhibition of this relaxation by ODQ, a blocker of the NO-sensitive guanylate cyclase (Fig. 3), confirms the role of NO in the relaxation response. These results suggest that there is a basal level of NO in the vascular wall that is independent of the endothelium and is elevated by the CuD diet. This is coincident with the increase in NO in hearts and urine of CuD rats [28]. Further, based on the L-arginine results (Fig. 2), there appears to be a greater capacity for iNOS-mediated synthesis of NO in the CuD vasculature.

The results of the Western blotting for the iNOS protein in segments of endothelium-denuded aorta did not demonstrate a significant difference between the CuA and CuD groups. These results suggest that although there is a functional increase in baseline activity, there may be only a minimally detectable upregulation of iNOS expression caused by a diet deficient in copper. This would explain why relaxation occurs so much faster after 4 h of LPS exposure (Figs. 2 and 6) but does not explain the functional difference in iNOS-dependent NO-mediated relaxation without inflammatory stimuli (Figs. 2 and 4). Because there was no significant increase in the expression of iNOS protein by Western blotting, the iNOS activity may be increased by post-translational modification in the CuD group.

In summary, the current study demonstrates that dietary copper deficiency increases the role of iNOS in the generation of NO in the vascular wall in the absence of any inflammatory stimuli. These results may explain why baseline blood pressure is not consistently altered by the reduction of eNOS generated NO [15–19]. However, the compensation may not be adequate to prevent the exacerbation of stress-induced increases in blood pressure reported during copper deficiency in humans [29].

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REFERENCES