Endothelial cell-derived nitric oxide mobilization is attenuated in copper-deficient rats

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Abstract: The attenuation of endothelium-dependent nitric oxide (NO) mediated vasodilation is a consistent finding in both conduit and resistance vessels during dietary copper (Cu) deficiency. Although the effect is well established, evidence for the mechanism remains circumstantial. This study was designed to determine the relative amount of NO produced in and released from the vascular endothelium. Using the fluorescent NO indicator, 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM), we now demonstrate the effect of a Cu-deficient diet on the production of NO from the endothelium of resistance arterioles. In one group of experiments, control and Cu-chelated lung microvascular endothelial cells (ECs) were used to assay NO production and fluorescence was observed by confocal microscopy. Weanling Sprague–Dawley rats were fed purified diets that were either Cu adequate (6.3 micrograms Cu per gram of food) or Cu deficient (0.3 micrograms Cu per gram of food) for 4 weeks. In the second series of experiments, first-order arterioles were microsurgically isolated from the rat cremaster muscle, cannulated, and pressurized with (3][N-morpholino]propanesulfonic acid) physiologic salt solution (MOPS-PSS). DAF-FM (5 μmol·L⁻¹) was added in the lumen of the vessel to measure NO release. Baseline DAF-FM fluorescence was significantly lower in Cu-chelated ECs than in controls. In response to 10⁻⁶ mol·L⁻¹ acetylcholine, fluorescent intensity was significantly less in chelated ECs and in the lumen of Cu-deficient arterioles. The results suggest that production and release of NO by the vascular endothelium is inhibited by a restriction of Cu. This inhibition may account for the attenuated vasodilation previously reported in Cu-deficient rats.

Key words: copper, nitric oxide, endothelium, microcirculation, vasoreactivity.

Introduction

The contractile state of vascular smooth muscle cells determines the caliber of the vessels and contributes to both the total peripheral resistance and the control of local tissue blood flow. In normal blood vessels, one of the major mediators of smooth muscle tone is nitric oxide (NO), which is produced in the luminal endothelial cells (ECs). The release of NO by ECs can be acutely stimulated by several mechanisms, including the activation of specific receptors on the
intracellular Ca$^{2+}$ during periods of diminished activity of vascular Cu–Zn-SOD (Schuschke et al. 2000).

We have previously presented evidence that the bioavailability of NO is diminished during copper (Cu) deficiency. This lack of available NO is seen in the attenuated arteriolar dilation response in both weaning and adult models of the Cu-deficient rat (Schuschke et al. 1992, 1995, 2000; Falcone et al. 2005). We have hypothesized that the attenuated activity of vascular copper–zinc superoxide dismutase (Cu–Zn-SOD) during inadequate Cu increases the concentration of O$_2^-$, which reacts with NO to produce ONOO$^-$ and decrease the diffusion gradient for NO (Schuschke et al. 2000).

In addition, we have evidence that suggests that NO synthesis is attenuated during Cu deficiency. This attenuation is likely caused by inhibition of the requisite mobilization of intracellular Ca$^{2+}$ during periods of diminished activity of vascular Cu–Zn-SOD (Schuschke et al. 2000).

Although the above data suggest that the production and release of NO from vascular ECs is attenuated by inadequate Cu concentrations, the amount of NO production and release has not been compared between Cu-adequate and Cu-deficient ECs. The current study was designed to document the relative amount of NO produced by ECs and the amount released by ECs in resistance arterioles. The results were determined using the fluorescent NO indicator 4-amino-5-methylamino-2',7'-difuorofluorescein (DAF-FM) (Kojima et al. 1999).

**Materials and methods**

**Animals and diet**

The use of animals was approved by the University of Louisville Animal Care and Use Committee. Male weaning Sprague–Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12 h light : 12 h dark cycle. The rats were given free access to distilled water and to one of two purified diets for 4 weeks. The basal diet was a casein–sucrose–cornstarch-based diet (no. TD84469, Teklad Test Diets, Madison, Wis.) containing all known essential vitamins and minerals except for Cu and Fe. The Cu-adequate diet consisted of the basal diet (940 g kg$^{-1}$ of total diet) with safflower oil (50 g kg$^{-1}$) and a Cu–Fe mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of CuSO$_4$·H$_2$O per kilogram of diet. The Cu-deficient diet was the same except for replacement of Cu with cornstarch in the mineral mix. Analysis of diets indicated average Cu concentrations of 6.3 and 0.3 milligrams of Cu per kilogram of diet for Cu-adequate and -deficient diets, respectively.

**Nitric oxide production in cultured endothelial cells**

Human Lung Microvascular Endothelial Cells were purchased from Lonza (Walkersville, Md.) and were grown to confluence in 25 cm$^2$ flasks in endothelial basal medium-2 (EBM-2) supplemented with 5% fetal bovine serum and endothelial growth factors. The cells were then seeded into 8-well chambered coverglass plates from Nalge Nunc (Roschester, N.Y.) and grown to confluence. To make the cells Cu deficient, 4 of the wells in each chamber were treated with tetraethylenapentamine (TEPA). We have previously shown that this treatment mimics dietary Cu deficiency by significantly reducing the cellular Cu concentration and Cu–Zn-SOD activity (Lominadze et al. 1999).

Endothelial cells were treated with DAF-FM diacetate, which is the cell membrane permeant form of the NO indicator. Once inside the cells, the diacetate portion is cleaved by cellular esterases and the resultant DAF-FM is virtually “trapped” within the cells (Kojima et al. 1999). DAF-FM is an essentially non-fluorescent compound until it reacts with NO to form a fluorescent benzoazirazole. The dye was added to the media to a final concentration of 10 µmol L$^{-1}$ and allowed to incubate for 30 min. Separate wells were used to determine control levels of DAF-FM fluorescence and the fluorescent response to 10$^{-6}$ mol L$^{-1}$ acetylcholine (Ach). The ECs were visualized using a confocal laser-scanning microscope (VF10-SW; Olympus, Melville, N.Y.) with a 60× objective and equipped with a 495 nm excitation and 513 nm emission filter. The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (excitation/emission: 358/461).

All confocal images were analyzed using Matrox Inspector software (version 4.1; Dorval, Que.). The software was used to determine the relative concentration of NO by quantifying the mean pixel intensity of each cell.

**Luminal release of microvascular NO**

In situ experiments were also conducted using isolated, perfused, resistance arterioles from the rat cremaster muscle microcirculation. Five Cu-adequate and 5 Cu-deficient rats were anesthetized with sodium pentobarbital (50 mg kg$^{-1}$ i.p.). The cremaster muscle was excised and placed in a refrigerated well (0–4 °C) containing a solution of 145 mmol L$^{-1}$ NaCl, 5.0 mmol L$^{-1}$ KCl, 2.0 mmol L$^{-1}$ CaCl$_2$, 1.0 mmol L$^{-1}$ MgSO$_4$, 1.0 mmol L$^{-1}$ NaHPO$_4$, 5.0 mmol L$^{-1}$ dextrose, 2.0 mmol L$^{-1}$ pyruvate, 0.02 mmol L$^{-1}$ disodium ethylenediamine tetraacetate (Na$_2$EDTA), and 3.0 mmol L$^{-1}$ 3-(N-morpholino)propanesulfonic acid – physiological saline solution (MOPS-PSS; pH 7.4 ± 0.3), as well as 1% albumin (Falcone et al. 1993). A segment of the A1 arteriole was excised, cannulated with glass micropipettes, and secured to the pipettes with 11-0 suture. MOPS-PSS without albumin was used to bathe the vessel. The arterioles were pressurized to 90 cmH$_2$O (approximate in vivo pressure (Meininger et al. 1987)). The cannulation pipettes were resistance matched and slightly smaller in diameter than the vessels. Each pipette was connected to independent reservoirs that were set at the same hydrostatic level (i.e., no flow gradient) (Kuo et al. 1990; Falcone et al. 1993).

To measure endothelial-produced NO in intact arterioles, the membrane impermeant form of the NO-sensitive fluorescent indicator DAF-FM (24 µmol L$^{-1}$) was used to fill one of the cannulation pipettes. A 40 cmH$_2$O pressure gradient was applied across the vessel to allow the flow of DAF-FM into the vessel for 5 min. With the flow stopped, NO release was stimulated with 10$^{-6}$ mol L$^{-1}$ Ach in the bath. Adenosine (10$^{-3}$ mol L$^{-1}$), an NO-independent dilator agonist, was used as a control for the detection system. During agonist administration the intraluminal pressure was main-
tained at −3 mm Hg to prevent an increase in luminal diameter and luminal volume dilution of the dye. Fluorescent images were recorded with the Universal Imaging Software – Hardware and a Hamamatsu intensified charge-coupled device detector at time 0 and thereafter at 10 s intervals for 2 min. The images were digitized and stored for off-line processing. Emission fluorescence (535 nm) during excitation at 495 nm was measured and used as an index of NO. Images were analyzed for changes in pixel fluorescence intensity as a function of pharmacological or control applications in the bathing chamber.

Copper status indices

The median lobe of the liver and the right kidney were removed, weighed, and frozen at −10 °C for subsequent Cu analysis. Liver and kidney Cu concentrations of individual rats were assessed using inductively coupled plasma emission spectrometry.

Statistical analysis

Data are presented as mean ± SEM. Effect of diet on copper indices was examined by one-way analysis of variance (ANOVA). DAF-FM diacetate fluorescence in cultured ECs was examined by two-way ANOVA and Tukey post hoc means comparison. Comparison of the change in intraluminal DAF-FM fluorescence over time between dietary groups was determined by repeated-measures ANOVA. Differences were considered significant at p < 0.05.

Results

Four weeks of dietary Cu restriction produced a significant Cu deficiency as indicated by assays of both liver and kidney Cu concentration (Table 1).

The relative amount of NO within ECs was compared between control and Cu-chelated groups, both as unstimulated control concentrations and during stimulation with 10⁻⁶ mol·L⁻¹ Ach. In both cases, the Cu-chelated ECs had significantly less intracellular NO as indicated by DAF-FM fluorescence (Figs. 1 and 2). Comparison within groups with and without ACh stimulation showed a significant Ach-induced increase in intracellular fluorescence in the Cu-chelated ECs, but not in the Cu-adequate group. Since the EC membrane is freely permeable to NO and the EC volume is relatively small, it is highly likely that the NO produced in the Cu-adequate cells readily diffused from the cell and that we would not expect to see an increase in intracellular fluorescence in this group. This diffusion of NO from the ECs was the subject of the second part of this study.

The amount of NO being released by the arteriolar wall into the lumen was determined by DAF-FM fluorescence using isolated cremasteric arterioles. In response to 10⁻⁶ mol·L⁻¹ acetylcholine, there was a rapid increase in the NO released from the Cu-adequate vessels, which peaked at about 50 s and stayed elevated through a 2 min observation period (Fig. 3). The Cu-deficient group showed a slight decrease in the NO release early in the experiment, which returned to baseline by the end of 2 min (Fig. 3). As a test of the efficacy of the detection method, NO-independent adenosine (10⁻³ mol·L⁻¹) was used as the endothelial stimulus. There was no detectable change in DAF-FM fluorescence from either dietary group (Fig. 4) in response to adenosine, confirming the specificity of the indicator for NO.

Discussion

Studies using several vascular beds and models of Cu-deficiency have shown that the NO signaling of vascular smooth muscle relaxation is Cu dependent. Studies in both large arteries (Saari 1992; Lynch et al. 1997) and in arterioles of the microcirculation (Schuschke et al. 1992, 1995, 2000; Falcone et al. 2005) of Cu-deficient rats have shown significant inhibition to agonists such as Ach, calcium ionophore A23187, and sodium nitroprusside. Similar findings have been reported for aortic and coronary artery rings that were treated with a Cu chelator to inactivate the cytosolic enzyme Cu–Zn-SOD (Omar et al. 1991; Plane et al. 1997).

Mechanistic studies of attenuated smooth muscle relaxation during Cu deficiency have suggested that NO production may be reduced and that the diffusion gradient between the inside of the endothelial cell and the surrounding tissues may be diminished. We have previously reported that the elevation of intracellular Ca²⁺ required for NO production is inhibited when Cu is inadequate (Schuschke et al. 2000). This inhibition of Ca²⁺ mobilization within the ECs likely prevents the synthesis of endothelial-derived NO because the endothelial form of NO synthase is Ca²⁺ dependent.

The current data supports the hypothesis that Cu-deficient ECs produce less NO. The results demonstrate that intracellular concentrations of NO are significantly less when Cu concentrations are reduced. These results are seen when examining both baseline and stimulated concentrations of NO within the cultured ECs (Fig. 2). Similar results have recently been reported in a Cu-deficient model of teratogenicity (Yang et al. 2007). They reported that NO levels were low in conditioned media from both Cu-deficient embryos and yolk sacs under basal conditions and with NO synthase stimulation.

In addition to a decreased NO production by ECs, we have previously hypothesized that the diffusion of NO from the ECs to the vascular smooth muscle cells may be disrupted when Cu concentration is inadequate. We have shown that dietary Cu deficiency attenuates the activity of Cu–Zn-SOD at the same time that vasorelaxation is reduced (Schuschke et al. 2000). Coincident with the loss of Cu–Zn-SOD activity and the reduction of vasorelaxation is the generation of peroxynitrite (ONOO⁻) in the Cu-deficient rats (Schuschke et al. 2000). Furthermore, the addition of exogenous Cu–Zn-SOD restores normal dilation in Cu-deficient rats (Schuschke et al. 1995). Based on these data, we have proposed that superoxide anion (O₂⁻) buildup in the absence

Table 1. Indices of copper status in rats fed diets either adequate or deficient in copper (Cu).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Liver Cu (μg·g⁻¹ dry weight)</th>
<th>Kidney Cu (μg·g⁻¹ dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu adequate</td>
<td>11.83±0.59</td>
<td>26.14±1.91</td>
</tr>
<tr>
<td>Cu deficient</td>
<td>1.28±0.16a</td>
<td>8.96±0.44a</td>
</tr>
</tbody>
</table>

Note: All values are mean ± SE.

*a p < 0.05 compared with control.
of Cu–Zn-SOD leads to a reaction between the NO and O$_2^-$ to produce ONOO$^-$ and reduce the concentration gradient between the ECs and the vascular smooth muscle (Schuschke et al. 2000).

By examining the amount of NO released into the lumen of resistance arterioles, the current data supports the hypothesis that Cu deficiency results in a decrease in the diffusion gradient of NO between the ECs and the surrounding...
Fig. 4. Changes in endothelial cell nitric oxide (NO) from intact isolated arterioles to stimulation with NO-independent adenosine (10^{-3} \text{ mol} \cdot \text{L}^{-1}). The curves are average responses in arterioles isolated from rats fed either a copper (Cu)-adequate (n = 5) or a Cu-deficient (n = 5) diet for 4 weeks. Repeated-measures analysis of variance indicated that there was no significant effect of diet or time.

region. The release of NO from the Cu-adequate arterioles (Fig. 3) parallels the Ca^{2+} mobilization curve previously reported by our group (Schuschke et al. 2000). The NO release from the Cu-deficient group (Fig. 3) is significantly less than that from the Cu-adequate group, which is similar to the attenuated mobilization of Ca^{2+} seen in arterioles from Cu-deficient rats (Schuschke et al. 2000). Combined, these data suggest that the production of NO is decreased in Cu-deficient ECs, and this is likely caused by inadequate Ca^{2+} mobilization, which is required for activation of eNOS. This inhibition likely involves the buildup of ONOO− (Schuschke et al. 2000), which inhibits the agonist-stimulated influx of external Ca^{2+} (Elliott 1996). Further, the NO that is generated may be quenched by O_{2}^{-} reducing the diffusion gradient of NO.

The concept of a compromised pool of NO during Cu deficiency has been postulated previously (Yang et al. 2007) and is supported by the current data. The current results suggest that the available pool of NO is very near the carrying capacity of the Cu-adequate ECs. Any stimulation of eNOS activity above baseline produces a rapid release of NO from the vasculature (Fig. 3) without any increase in the amount of NO maintained within the ECs (Fig. 2). In the Cu-deficient group, the baseline pool is significantly lower than controls and although stimulation of eNOS increases the intracellular pool of NO, the intracellular concentration is not beyond the carrying capacity of the ECs (Fig. 2). Since the intracellular pool of NO is smaller, the diffusion of NO from the Cu-deficient ECs is limited (Fig. 3).

Even though there is convincing evidence that Cu deficiency diminishes the NO bioavailability in ECs, this effect appears to be cell specific. Previous work by Saari and Dahlen (1998) reported an elevation of cardiac nitrate–nitrite production in Cu-deficient rats. This result is likely associated with the increased activity of cardiac NOS (Saari et al. 2007). Furthermore, a comparison of the relative amounts of vascular and cardiac eNOS and iNOS isoforms shows that neither is elevated in vascular tissue (Schuschke et al. 2000; Gobejishvili et al. 2002), but that both are elevated in cardiomyocytes from Cu-deficient rats (Saari et al. 2007). Although it is not known whether the bioavailability of NO or NO signal transduction is altered in the heart when Cu is inadequate, the upregulation of the synthetic machinery is clearly different in the heart and vasculature. The role of Cu in these disparate responses remains to be clarified.

In summary, circumstantial evidence of the role of Cu in NO-mediated signal transduction has been deduced from attenuated vascular reactivity studies in Cu-deficient rats and from vascular anomalies in Cu-deficient rat embryos. This is the first study to quantify the EC production of NO and the stimulated release of the mediator from resistance arterioles. The stimulated release of NO from intact vascular endothelium (Fig. 3) follows very closely the mobilization of intracellular Ca^{2+} (Schuschke et al. 2000) required for the synthesis of endothelial-derived NO in both Cu-adequate and -deficient groups. In conclusion, the results demonstrate that the bioavailability of NO produced and released by the vascular endothelium is significantly attenuated by Cu deficiency.

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References


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