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ENDOTHELIAL CELL-DERIVED NITRIC OXIDE MOBILIZATION IS ATTENUATED IN COPPER-DEFICIENT RATS

Jeff C. Falcone¹, David Lominadze¹, W. Thomas Johnson², and Dale A. Schuschke¹

¹Department of Physiology and Biophysics, University of Louisville School of Medicine, Louisville, KY 40292

²Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202

Abstract

The attenuation of endothelium-dependent nitric oxide (NO)-mediated vasodilation is a consistent finding in both conduit and resistance vessels during dietary Cu deficiency. While the effect is well established, evidence for the mechanism is still 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, 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 which were either Cu-adequate (6.3 µg Cu/g diet) or Cu-deficient (0.3µg Cu/g diet) for 4 weeks. In the second series of experiments, first-order arterioles were microsurgically isolated from the rat cremaster muscle, cannulated and pressurized with MOPS-PSS. DAF-FM (5 µM) was added in the lumen of the vessel to measure NO release. Baseline DAF-FM fluorescence was significantly less in Cu-chelated ECs compared to controls. In response to 10⁻⁶ M Ach, 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.

Keywords

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 (EC). The release of NO by ECs can be acutely stimulated by several mechanisms including the activation of specific receptors on the EC membrane. The agonist-receptor interaction results in an increase in the intracellular concentration of free calcium, the activation of NO-synthase and the release of endothelial-derived NO.

Correspondence and reprint requests to: Dale A. Schuschke, PhD., Department of Physiology and Biophysics, Health Sciences Center A1111, University of Louisville, Louisville KY 40292, Phone: (502) 852-7553, FAX: (502) 852-7215, E-mail: daschu01@louisville.edu. E-mail for co-authors, jcfalc01@louisville.edu, dglomi01@louisville.edu, thomas.johnson@ars.usda.gov

We have previously presented evidence that the bioavailability of NO is diminished during Cu deficiency. This lack of available NO is seen in the attenuated arteriolar dilation response in both weanling and adult models of the Cu-deficient rat (Schuschke 1992; 1995; 2000; Falcone, 2005). We have hypothesized that the attenuated activity of vascular 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, 2000).

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

While the above data suggests 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 DAF-FM (4-amino-5-methylamino-2',7'-difluorofluorescein) (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 weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA). 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. The basal diet was a casein-sucrose-cornstarch-based diet (no. TD84469, Teklad Test Diets, Madison, WI, USA) containing all known essential vitamins and minerals except for Cu and Fe. The Cu-adequate diet consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a Cu-Fe mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of $CuSO_4 \cdot H_2O$ 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 mg of Cu/kg of diet for Cu-adequate and Cu-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 (Rochester, NY) 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 nonfluorescent compound until it reacts with NO to form a fluorescent benzotriazole. The dye was added to the media to a final concentration of 10 μM and allowed to incubate for 30 minutes. Separate wells were used to determine control levels of DAF-FM fluorescence and the fluorescent response to 10^{-6} M acetylcholine (Ach). The

ECs were visualized using a confocal laser-scanning microscope (FV10-SW; Olympus, Melville, NY) with a 60x objective and equipped with a 495-nm excitation and 513-nm emission filter. The nuclei were counterstained with DAPI dihydrochloride (excitation/emission: 358/461).

All confocal images were analyzed using Matrox Inspector software (Version 4.1; Dorval, Quebec, Canada). The software was used to determine the relative concentration of NO by quantitating 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 five Cu-deficient rats were anesthetized with sodium pentobarbital (50 mg/kg) via intraperitoneal injection. The cremaster muscle was excised and placed in a refrigerated well (0–4°C) containing a solution of (in mM) 145 NaCl, 5.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 1.0 NaH₂PO₄, 5.0 dextrose, 2.0 pyruvate, 0.02 disodium ethylenediamine tetraacetate (Na₂EDTA), and 3.0 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 bath the vessel. The arterioles were pressurized to 90 cmH₂O (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 µM) was used to fill one of the cannulation pipettes. A 40-cm H₂O pressure gradient was applied across the vessel to create the flow of DAF-FM into the vessel for 5 min. With the flow stopped, NO release was stimulated with 10⁻⁶ M Ach in the bath. Adenosine (10⁻³ M) is an NO-independent dilator agonist that was used as a control for the detection system. During agonist administration the intraluminal pressure was maintained at -3 mmHg 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 CCD detector at time 0 and thereafter at 10s 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 fluorescent 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 by using inductively coupled plasma emission spectrometry (ICP).

Statistical analysis

Data are presented as mean ± SEM. Effect of diet on copper indices was by one-way 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 by repeated measures ANOVA. Differences were considered significant at *p* < 0.05.

RESULTS

Four weeks of dietary Cu produced caused 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} M acetylcholine. In both cases, the Cu-chelated ECs had significantly less intracellular NO as indicated by DAF-FM fluorescence (Fig 1 and Fig 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} M acetylcholine, there was a rapid increase in the NO released from the Cu-adequate vessels which peaked at about 50 s and stayed elevated though a 2 minute 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 minutes (Fig. 3). As a test of the efficacy of the detection method, NO-independent adenosine (10^{-3} M) was used as the endothelial stimuli. 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, 2005) of copper 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 the 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 Ca^{++} required for NO production is inhibited when Cu is inadequate (Schuschke, 2000). This inhibition of Ca^{++} mobilization within the ECs likely prevents the synthesis of endothelial-derived NO because the endothelial form of NO synthase is Ca^{++} 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 cultures ECs (Fig. 2). Similar results have recently been reported in a Cu-deficient model of teratogenicity (Yang, 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, 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, 2000). Further, the addition of exogenous Cu,Zn-SOD restores normal dilation in Cu-deficient rats (Schuschke, 1995). Based on these data, we have proposed that superoxide anion (O_2^-) build up in the absence of Cu,Zn-SOD dismutation 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, 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 region. The release of NO from the Cu-adequate arterioles (Fig. 3) parallels the Ca^{++} mobilization curve previously reported by our group (Schuschke, 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^{++} seen in arterioles from Cu-deficient rats (Schuschke, 2000). Combined these data suggest that the production of NO is decreased in Cu-deficient ECs likely caused by inadequate Ca^{++} mobilization required for activation of eNOS. This inhibition likely involves the buildup of ONOO^- (Schuschke, 2000) which inhibits the agonist-stimulated influx of external Ca^{++} (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 previously postulated (Yang, 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 Sarri 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, 2007). Further, a comparison of the relative amounts of vascular and cardiac eNOS and iNOS isoforms shows that neither is elevated in vascular tissue (Schuschke, 2000; Gobejishvili, 2002) but both are elevated in cardiomyocytes from Cu-deficient rats (Saari, 2007). While 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 quantitate 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^{++} (Schuschke, 2000) required for the synthesis of endothelial derived NO in both Cu-adequate and Cu-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

- Elliott SJ. Peroxynitrite modulates receptor-activated Ca^{2+} signaling in vascular endothelial cells. *Am. J. Physiol* 1996;270:L954–L961. [PubMed: 8764220]
- Falcone JC, Kuo L, Meininger GA. Endothelial cell calcium increases during flow-induced dilation in isolated arterioles. *Am. J. Physiol* 1993;264:H653–H659. [PubMed: 8447477]
- Falcone JC, Saari JT, Kang YJ, Schuschke DA. Vasoreactivity in an adult model of marginal copper deficiency. *Nutr Res* 2005;25:177–186.
- Gobejishvili L, Saari JT, Adeagbo ASO, Schuschke DA. Dietary copper deficiency increases inducible nitric oxide synthase-mediated vascular dilation in rat aorta. *J. Trace Elem. Exp. Med* 2002;15:85–95.
- Kojima H, Urano Y, Kikuchi K, Higuchi T, Hirata Y, Nagano T. Fluorescent indicators for imaging nitric oxide production. *Angew Chem Int Ed Engl* 1999;38:3209–3212. [PubMed: 10556905]
- Kuo L, Davis MJ, Chilian WM. Endothelium-dependent, flow-induced dilation of isolated coronary arterioles. *Am J Physiol* 1990;259:H1063–H1070. [PubMed: 2221113]
- Lynch SM, Frei B, Morrow JD, Roberts LJ, Xu A, Jackson T, Reyna R, Klevay LM, Vita J, Keaney JF Jr. Vascular superoxide dismutase deficiency impairs endothelial vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation. *Arterioscler. Thromb. Vasc. Biol* 1997;17:29–38.
- Meininger GA, Fehr KL, Yates MB. Anatomic and hemodynamic characteristics of the blood vessels feeding the cremaster skeletal muscle in the rat. *Microvasc. Res* 1987;33:81–97. [PubMed: 3561270]
- Omar HA, Cherry PD, Mortelliti MP, Burke-Wolin T, Wolin MS. Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and -independent nitrovasodilator relaxation. *Circ. Res* 1991;69:601–608. [PubMed: 1651814]
- Plane F, Wigmore S, Angelini GD, Jeremy JY. Effect of copper on nitric oxide synthase and guanylyl cyclase activity in the rat isolated aorta. *Br. J. Pharmacol* 1997;121:345–350. [PubMed: 9154347]
- Saari JT. Dietary copper deficiency and endothelium-dependent relaxation of rat aorta. *Proc. Soc. Exp. Biol. Med* 1992;200:19–24. [PubMed: 1315059]
- Saari JT, Dahlen GM. Nitric oxide and cyclic GMP are elevated in the hearts of copper-deficient rats. *Med Sci Res* 1998;26:495–497.
- Schuschke DA, Reed MWR, Saari JT, Miller FN. Copper deficiency alters vasodilation in the rat cremaster muscle microcirculation. *J. Nutr* 1992;122:1547–1552. [PubMed: 1619479]
- Schuschke DA, Saari JT, Miller FN. A role for dietary copper in nitric oxide-mediated vasodilation. *Microcirculation* 1995;2:371–376. [PubMed: 8714818]
- Schuschke DA, Falcone JC, Saari JT, Fleming JT, Percival SS, Young SA, Pass JM, Miller FN. Endothelial cell calcium mobilization to acetylcholine is attenuated in copper-deficient rats. *Endothelium* 2000;7:83–92. [PubMed: 10865936]
- Yang SL, Keen CL, Lanoue L, Rucker RB, Uriu-Adams JY. Low nitric oxide: a key factor underlying copper deficiency teratogenicity. *Free Rad. Biol. Med* 2007;43:1639–1648. [PubMed: 18037129]

Figure 1. Representative confocal microscopy images of Cu-adequate and Cu-deficient endothelial cells stained with DAF-FM diacetate to demonstrate the relative amount and location of intracellular NO. The nuclei have been counterstained with DAPI.

Figure 2.

Comparison of the fluorescent intensity of DAF-FM in cultured lung microvascular endothelial cells between Cu-adequate and Cu-deficient groups. The letter “A” represents a significant difference ($p < 0.05$) between groups during control and Ach stimulation. The letter “B” represents a significant difference within groups during control and Ach stimulation.

Figure 3.

Changes in endothelial cell NO from intact isolated arterioles in response to stimulation with 10^{-6} M acetylcholine. The curves are average responses in arterioles isolated from rats fed either a Cu-adequate (n=5) or a Cu-deficient (n=5) diet for four weeks. Repeated measures ANOVA indicated a significant main effects of diet ($p < 0.01$) and time ($p < 0.001$) as well as an interaction between diet and time ($p < 0.001$).

Figure 4.

Changes in endothelial cell NO from intact isolated arterioles in response to stimulation with NO-independent adenosine (10^{-3} M). The curves are average responses in arterioles isolated from rats fed either a Cu-adequate (n=5) or a Cu-deficient (n=5) diet for four weeks. Repeated measures ANOVA indicated that there was no significant effect of diet or time.

Table 1

Indices of copper status in rats fed diets either adequate or deficient in copper.

	Cu-adequate	Cu-deficient
Liver Cu ($\mu\text{g/g}$ dry wt.)	11.83 ± 0.59	$1.28 \pm 0.16^*$
Kidney Cu ($\mu\text{g/g}$ dry wt.)	26.14 ± 1.91	$8.96 \pm 0.44^*$

All values are mean $\pm$ SE.*
p<0.05 compared to control.