Carotid nitric oxide synthases are elevated in dietary copper deficiency

Jack T. Saari, Loren E. Wold, Jinhong Duan, Jun Ren, Hanqian L. Carlson, Ann M. Bode, Alex B. Lentsch, Huawei Zeng, Dale A. Schuschke

U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202, USA
Department of Pharmacology, Physiology and Therapeutics, School of Medicine, University of North Dakota, Grand Forks, ND 58202, USA
Hormel Institute, University of Minnesota, Austin, MN 55912, USA
Department of Surgery, University of Cincinnati Medical Center, Cincinnati, OH 45267, USA
Department of Physiology and Biophysics, School of Medicine, University of Louisville, Louisville, KY 40292, USA

Abstract

Dietary copper (Cu) deficiency leads to cardiac morphological and functional defects suggestive of heart failure. However, simultaneous cytoprotective events also appear to occur. The molecular mechanisms responsible for this complex alteration of cardiac function by Cu deficiency have not been elucidated. Because prior work has implicated altered nitric oxide (NO) metabolism in this altered function, we have examined this pathway in further detail. Male Sprague–Dawley rats were fed diets that were either Cu adequate (6 mg Cu/kg diet) or Cu deficient (<0.5 mg Cu/kg diet) for 5 weeks. Endothelial NO synthase (NOS) and inducible NOS (iNOS) protein expressions, as measured by Western blot analysis, were 58% and 40% higher, respectively, in Cu-deficient than in Cu-adequate rat hearts. Cardiac NOS activity, as measured by conversion of 3H-arginine to 3H-citrulline, was 130% higher in Cu-deficient than in Cu-adequate rats. NFκB is a known transcription factor for iNOS. Activation of NFκB, determined by an ELISA for the p65 subunit, was found to be 33% higher in Cu-deficient than in Cu-adequate rats. Coupled with prior evidence of elevated cardiac nitrate/nitrite production in Cu-deficient rats, these data suggest multiple pathways for enhanced NO production that may contribute to altered cardiac function under dietary Cu deficiency.

Keywords: Nitric oxide synthase; Heart; Copper deficiency; NFκB; eNOS; iNOS

1. Introduction

Dietary copper (Cu) deficiency displays a cardiomyopathy that includes cardiac hypertrophy, fibrosis, derangement of myofibrils and impaired cardiac contractile and electrophysiological function [1–6]. Cu-deficient hearts have also been shown to undergo events specifically associated with cardiac failure, including reexpression of fetal genes, inability to respond to an adrenergic stimulus and apoptosis [7–11].

Molecular/cellular pathways leading to cardiomyopathy and potential failure are being gradually developed for cardiac disease of other origins. Two general sets of pathways are portrayed, one leading to overt failure, the other leading to a series of cytoprotective or survival events [12,13]. Both failure and survival pathways may utilize nitric oxide (NO) [14]. The former is generally initiated by severe inflammation or oxidative stress, which causes induction of inducible NO synthase (iNOS) and production of large amounts of NO, which, when combined with high levels of reactive oxygen species (ROS), can be highly damaging. Survival pathways, on the other hand, can be initiated by milder oxidative stress. They utilize ROS as signaling molecules that trigger cascades of events that may...
include production of NO by either endothelial NOS (eNOS) or iNOS, which then leads to cytoprotection [15]. The latter pathways are in some contexts characterized by the term preconditioning in that the events set forth serve to protect hearts against subsequent, more severe oxidative stress [16,17]. Prior studies have shown that oxidative stress is enhanced in Cu deficiency [18] and in hearts in particular [19]. Further, we have shown that NO production is elevated in hearts of Cu-deficient rats [20] and, in preliminary work, that the cardiac iNOS protein is elevated [21]. Thus, the signaling pathways that apply to other cardiomyopathies may also apply to that of dietary Cu deficiency. The present study was designed to further examine this possibility by (a) determining whether elevation of NO production in Cu deficiency could be confirmed by another method, total NOS activity; (b) confirming the elevation of iNOS; (c) determining whether eNOS was affected; and (d) assaying for activation of a transcription factor, NFkB, which is known to be activated by oxidative stress and has been implicated in the up-regulation of iNOS.

2. Materials and methods

2.1. Animals and diets

Male weanling Sprague–Dawley rats (n=50) were fed a Cu-adequate or Cu-deficient diet. Diets were composed of 940.0 g of Cu-free, iron (Fe)-free basal diet (Catalog #TD 84469, Teklad Test Diets, Madison, WI); 50.0 g of safflower oil; and 10.0 g of a Cu–Fe mineral mix per kilogram of diet. The basal diet was a casein-based (200 g/kg), sucrose-based (386 g/kg), cornstarch-based (295 g/kg) diet containing all known essential vitamins and minerals except for Cu and Fe. Cu and Fe were added in a cornstarch-based mineral mix that provided 0.22 g of ferric citrate (16% Fe) and either 0 or 24 mg of added CuSO4·5H2O per kilogram of diet. Analysis of diets indicated average Cu concentrations of 6 and 0.3 mg of Cu per kilogram of diet for Cu-adequate and Cu-deficient diets, respectively.

After consuming their respective diets for 5 weeks, each rat was anesthetized with an intraperitoneal injection of Na pentobarbital (65 mg/kg). Blood was withdrawn from the inferior vena cava and hematocrit was measured. The median lobe of the liver was removed for trace element analysis by inductively coupled plasma emission spectroscopy (ICP). The heart was removed for trace element analysis by ICP and the following assays.

2.2. NOS activity

For NOS activity measurements and Western blotting (described below), isolated cardiomyocytes were used; the isolation procedure has been described previously [22]. NOS activity was evaluated by the 3H-arginine to 3H-citrulline conversion assay that was first described by Bredt and Snyder [23] and modified by Samson et al. [24,25]. Plated ventricular myocytes (~300,000/well) were placed in Hanks' Balanced Salt Solution (HBSS) medium (20 mM HEPES, 1% penicillin–streptomycin, 0.1% BSA) for 20 min at 37°C before replacement with HBSS containing 1 μCi/ml 3H-arginine with Traysylol (0.2 KIU/ml). The cells were then incubated for 60 min before the reaction was terminated by aspiration of the incubation medium and replacement with iced HBSS containing 5 mmol/L L-arginine and 4 mmol/L EDTA. Five minutes later, the termination medium was removed and cells were lysed with 20 mmol/L Tris (with 5 mmol/L L-arginine and 4 mmol/L EDTA). Following sonication, the total lysate was centrifuged (600×g, 4°C, 10 min). An aliquot of the supernatant fraction was diluted with 1:1 (v/v) H2O/Dowex-50W (20–50, 8% cross-linked), mixed vigorously and loaded on a polypropylene Econo-Column (Bio-Rad Laboratories, Hercules, CA). The gel bed was washed three times with 2 ml distilled water, and all

All variables were significantly different in Cu-deficient (CuD) compared with Cu-adequate (CuA) rats, P<.05.

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>NOS activity</th>
<th>eNOS/iNOS protein</th>
<th>NFkB activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CuA (n=11)</td>
<td>CuD (n=11)</td>
<td>CuA (n=6)</td>
</tr>
<tr>
<td>Liver Cu (μg/g)</td>
<td>12.0±0.3</td>
<td>1.8±0.4</td>
<td>9.8±0.3</td>
</tr>
<tr>
<td>Liver Fe (μg/g)</td>
<td>217±22</td>
<td>361±53</td>
<td>326±7</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>345±14</td>
<td>294±11</td>
<td>326±14</td>
</tr>
<tr>
<td>Heart weight (mg/g)</td>
<td>4.9±0.3</td>
<td>7.1±0.3</td>
<td>5.2±0.1</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>40±1</td>
<td>18±1</td>
<td>40±1</td>
</tr>
</tbody>
</table>

Fig. 1. NOS activity as determined by 3H-arginine to 3H-citrulline conversion; n=11 per dietary treatment. *Diet effect is significant (P<.05, t test).
effluent was collected. \(^3\)H-citrulline was counted by scintillation and was regarded as proportional to NOS activity.

2.3. Western blot analysis for eNOS and iNOS

Isolated ventricular myocytes were collected and sonicated, and the supernatant fractions were centrifuged at 7000 \(\times G\) for 30 min at 4°C. Total cell homogenates from the pellets were used for immunoblotting of eNOS and iNOS. We confirmed that these membrane fractions did not contain any detectable collagens. Membrane proteins (50 \(\mu g/lane\)) were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes. The membranes were blocked (4% Block Ace, Dainippon Pharmaceutical, Osaka, Japan) and then incubated for 12 h at 4°C with anti-eNOS or anti-iNOS mouse IgG monoclonal antibodies (1:1000, BD Transduction Laboratories, Lexington, KY). Membranes were then washed and incubated with a horseradish-peroxidase-conjugated antihouse IgG (1:5000) for 1.5 h. After immunoblotting, the film was scanned and the intensity of immunoblot bands was determined with a calibrated densitometer (Model GS-800, Bio-Rad Laboratories).

2.4. NF\(\kappa\)B activation

Whole isolated hearts were used for this assay. Heart tissue was homogenized in 10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.6% NP-40, 0.5 mM PMSF, 1 \(\mu g/ml\) leupeptin, 1 \(\mu g/ml\) aprotonin, 10 \(\mu g/ml\) soybean trypsin inhibitor, 1 \(\mu g/ml\) pepstatin on ice. Protein concentration was determined by the bicinchoninic acid assay with trichloroacetic acid precipitation using BSA as a reference standard. Ten micrograms of extract was analyzed for NF\(\kappa\)B activation using the TransAM NF\(\kappa\)B p65 assay (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The ELISA-based kit detects the p65 subunit of the NF\(\kappa\)B complex bound to oligonucleotide containing a consensus binding site.

2.5. Statistics

Data were expressed as means±S.E.M. and analyzed by use of the Student’s \(t\) test or the Mann–Whitney Rank Sum Test. Differences were regarded as significant at \(P<.05\).

3. Results

Dietary Cu deficiency in rats fed low-Cu diets was confirmed for each of the three groups of animals for which designated assays — NOS activity, eNOS and iNOS protein and NF\(\kappa\)B activation — were performed (Table 1). The direct index of Cu status, liver Cu concentration, was depressed in all three groups. At least one indirect index of reduced Cu status was confirmed in each of the three groups. These included elevated heart weight, reduced body
4. Discussion

This study has corroborated our previous finding [20] that cardiac NO production is elevated in hearts of Cu-deficient rats. The potential cause for this elevation was examined by measurement of two isoforms of NOS. Finding of an elevation of cardiac eNOS and iNOS proteins is consistent with the elevation in NO production and, thus, extends our findings of altered cardiac NO metabolism in dietary Cu deficiency.

Because of the close relationship described in the literature between NFκB and iNOS activity, particularly in hearts [27], activation of NFκB was examined and found to be elevated. This is consistent with increased NFκB-mediated transcription of iNOS, although definitive proof of this relationship in Cu-deficient hearts requires additional study. It was important to examine the effect of Cu deficiency on NFκB activation directly in the heart because findings on the relation between Cu status and NFκB in the literature are fairly diverse and sometimes inconsistent. For instance, immune cells show either no change with Cu deficiency [28] or depressed activation with Cu toxicity [29]. Tumor cells show depression of both NFκB activation and protein with copper chelation, a presumed mimic of Cu deficiency [30]. Vascular cells, when exposed to Cu-induced oxidative stress, undergo increased NFκB activation [31]. In hearts, gene expression of a subunit of IκB kinase, which is necessary for activation of NFκB, was depressed by dietary Cu deficiency [11], but until now, the effect of dietary Cu deficiency on cardiac NFκB activation per se had not been examined. In answer to this, the present study demonstrated by measurement of its p65 subunit that NFκB activation is enhanced. In support of this finding, a recent study, published in abstract [32], has shown that the genetic message for the p50 (NFκB1) subunit of NFκB is also elevated with dietary Cu deficiency. The latter two findings, in view of the apparent depression of the upstream IκB kinase, clearly call for additional examination of the activation mechanism of cardiac NFκB in Cu deficiency.

Oxidative stress in the heart has the potential to trigger two generalized sets of molecular pathways, one oriented to failure, the other to survival [12,13]. Triggering of the failure pathway appears to require a massive oxidative insult that stimulates production of inflammatory cytokines via either p38 MAPK- or NFκB-mediated routes [33,34]. This is followed by apoptosis mediated by either NO-dependent or -independent means and subsequent contractile dysfunction and cardiac failure. The contribution of NO to cardiac failure in this scenario is likely dependent on its combination with superoxide to form the highly reactive and relatively long-lived peroxynitrite radical [14,35]. Prior studies have produced a large body of evidence that both oxidative stress and signs of cardiac failure, including apoptosis and contractile dysfunction, occur in Cu-deficient hearts (see Section 1). Largely missing has been evidence of the molecular mechanisms by which this is occurring, including NO-mediated events. Although not definitive of mechanism, findings of the present study of an elevation of NFκB and iNOS suggest that Cu-deficient hearts could be using an NO-mediated pathway to failure. Although peroxynitrite has not been measured in Cu-deficient hearts, evidence for its generalized production is supported by its elevation in plasma of Cu-deficient rats [36] and by the elevation of 3-nitrotyrosine in the neural tube of Cu-deficient mouse embryos [37].

Induction of survival or cytoprotective pathways can occur at lower levels of oxidative stress than those that trigger failure. This is evidenced by the phenomenon of preconditioning, the process whereby low levels or short bouts of oxidative stress (e.g., ischemia–reperfusion) can protect the heart against the damaging effects of subsequent infarcts. Potential survival pathways, among which preconditioning events are imbedded, include signaling cascades involving phosphoinositol-3 (PI-3) kinase, phospholipase C (PLC), ras-mediated activation of MAPKs [38] and hypoxia-inducible factor-1 [39]. At least two of these pathways (PI-3 kinase and PLC) have been shown to rely on NO [40,41]. PI-3 kinase, known to be activated by growth hormone receptor stimulation (presumably by ROS) [38,42], can cause activation of eNOS, NO from which can go on to reduce apoptosis and prevent functional defects [40,43]. PLC can also be activated by oxidative stress and initiate activation of protein kinase C (PKC) [38], which, in turn, stimulates NFκB-mediated induction of iNOS, the NO produced, thus affording protection [43]. Preconditioning is a complex phenomenon that utilizes parts of both of these pathways; activation of eNOS is thought to contribute to an acute early phase of preconditioning [44], while a prolonged late phase involves activation by eNOS and induction of iNOS [45].

The present study supports elevated production of NO by pathways utilizing both eNOS and iNOS and, thus, suggests the possibility that chronic dietary Cu deficiency acts as a preconditioner. Prior evidence that Cu deficiency acts like preconditioning is the finding that Cu-deficient hearts are relatively protected against ischemia–reperfusion injury, showing better contractile recovery and less damage, as indicated by reduced cardiac enzyme release, when compared with Cu-adequate hearts [3]. Further, cyclic GMP mediated by NO excitation is associated with classic or early preconditioning [44] and cardiac cyclic GMP is elevated in
Cu deficiency [20]. In light of the known activation of PI-3 kinase by growth factor receptors [38,42], indirect evidence that the PI-3-kinase/eNOS pathway may contribute to cytoprotection is the finding that cardiac insulin-like growth factor-1 receptors are up-regulated and that blockade of those receptors inhibits the enhanced contractility observed in cardiomyocytes from Cu-deficient hearts [46]. The elevation of NFκB and iNOS suggests possible participation of the PLC pathway, but activation of PLC and/or downstream activation of PKC will need to be shown to confirm that possibility.

In summary, findings of elevation of NOS activity and increases in eNOS and iNOS proteins confirm earlier evidence of increased NO production in hearts of Cu-deficient rats. Although the specific role of NO in Cu-deficient hearts has not been established, findings that both eNOS and iNOS proteins were elevated are consistent with the possibility that both cardiac failure and survival pathways are up-regulated, thus agreeing with functional evidence of the concurrent occurrence of failure and survival events. Molecular pathways need to be further delineated in dietary Cu deficiency not only to determine more specifically the role of NO in events influencing heart pathology but also to assess the role of other signaling molecules affected by Cu deficiency. This will then allow determination of the relative contributions of failure and survival pathways at any given level of Cu status and, ultimately, the level of dietary Cu required to maintain heart health.

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References