Calcium Reintroduction Decreases Viability of Cardiac Myocytes from Copper-Deficient Rats

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ABSTRACT Copper deficiency leads to profound cardiac hypertrophy and failure. Myocytes were isolated from hearts of copper-deficient and copper-adequate male Holtzman rats to characterize size and function of the cells. Weanling rats were offered a semipurified diet low in copper in two separate experiments (Experiment 1, 0.45 mg Cu/kg and Experiment 2, 0.30 mg Cu/kg). Control (copper-adequate) rats drank water supplemented with cupric sulfate (20 mg Cu/L). Compared with copper-adequate rats, copper-deficient rats had lower hematocrits, liver copper concentrations and plasma ceruloplasmin activities, and higher heart weights and liver iron concentrations. When myocytes were isolated in low calcium media (1 μmol/L), cell viability was not affected by diet history. However, upon restoration to more physiologic levels of calcium (1 mmol/L), cells from copper-deficient rats were less viable, exhibiting an average loss of 34 and 40% in Experiments 1 and 2, respectively, compared with a 9.5 and 13% loss of cells, respectively, from the copper-adequate rats. Addition of the calcium channel blocker, verapamil, did not block this calcium-dependent loss of viability nor did the mitochondrial calcium channel blockers, ruthenium red and cyclosporin A. For comparison with another model of cardiac hypertrophy, the calcium sensitivity of myocytes from hypertrophied hearts of rats with arterial pressure overload was determined. It was discovered in a first experiment that viability of myocytes from copper-deficient rats was compromised by the restoration of physiologic levels of calcium (Heller et al. 1997). This preliminary observation prompted further research, which is reported here, including a second experiment assessing calcium sensitivity of copper deficiency and a third experiment assessing calcium sensitivity of cardiac myocytes from hypertrophied hearts of rats with arterial pressure overload.

MATERIALS AND METHODS

Animal care and diets. Male weanling Holtzman rats were purchased commercially (Harlan Sprague Dawley, Indianapolis, IN). Rats were fed one of two dietary treatments, copper-deficient (−Cu) or copper-adequate (+Cu), consisting of a low copper purified diet (Teklad Laboratories, Madison, WI) and either low copper drinking water or copper-supplemented drinking water, respectively. The purified diet was a modified AIN-76A diet (Prohaska 1991) and contained the following components (g/kg diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; AIN-76A vitamin mix, 10, DL-methionine, 3; choline bitartrate, 2; and ethoxyquin, 0.01. Cupric carbonate was omitted from the AIN-76 mineral mix. The purified diet contained by chemical analysis 0.45 mg Cu/kg and 47 mg Fe/kg in Experiment 1 and 0.30 mg Cu/kg and 44 mg Fe/kg in Experiment 2. Holtzman male rats fed the −Cu treatment drank deionized water, whereas the +Cu treatment groups drank water that contained 20 mg Cu/L through the addition of CuSO₄ to the drinking water. Rats were given free access to diet and drinking water. All animals were maintained at 24°C with 55% relative humidity on a 12-h light cycle (0700–1900 h). All protocols were approved formally by the University of Minnesota Animal Care Committee.

A third study was done in which young adult male Sprague-Dawley rats were offered a nonpurified laboratory rat diet (5001

KEY WORDS: • copper-deficient • cardiac myocyte • rats

Copper is essential for the development and optimal function of biological systems. Many of the required processes can be linked with known cuproenzymes. However, lack of adequate dietary copper results in a mammalian phenotype in which alterations in several biological systems have yet to be linked with specific enzyme defects. Notable in this context is a pronounced cardiac hypertrophy. Previous studies on the cardiac hypertrophy of copper deficiency have been thoroughly reviewed (Medeiros et al. 1993, Medeiros and Wildman 1997).

Several hypotheses surrounding changes in antioxidant systems via lower Cu,Zn-superoxide dismutase, connective tissue pathology via lower lysyl oxidase, and impaired energy metabolism via lower cytochrome c oxidase have been proposed. We tested the idea that overproduction of humoral growth factors such as angiotensin II might be involved, but we showed that this was unlikely (Lear et al. 1996). Typical physiologic reasons for developing hypertrophy such as hypertension and volume overload are not involved in the postnatal copper-deficient rat model. Thus, the specific trigger responsible for inducing cardiac hypertrophy in copper deficiency is not known.

To extend previous work, experiments designed to characterize the morphometric characteristic of cardiac myocytes were undertaken. It was discovered in a first experiment that viability of myocytes from copper-deficient rats was compromised by the restoration of physiologic levels of calcium (Heller et al. 1997). This preliminary observation prompted further research, which is reported here, including a second experiment assessing calcium sensitivity of copper deficiency and a third experiment assessing calcium sensitivity of cardiac myocytes from hypertrophied hearts of rats with arterial pressure overload.

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Table 1: Characteristics and copper status of male Holtzman rats fed copper-adequate or copper-deficient treatment for 4–5 wk after weaning.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Copper-adequate</th>
<th>Copper-deficient</th>
<th>Copper-adequate</th>
<th>Copper-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, d</td>
<td>55 ± 1.9</td>
<td>55 ± 1.9</td>
<td>48 ± 0.38</td>
<td>48 ± 0.38</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>319 ± 3.8</td>
<td>299 ± 20.5</td>
<td>254 ± 9.4</td>
<td>199 ± 8.8</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.41 ± 0.12</td>
<td>2.26 ± 0.132</td>
<td>1.73 ± 0.08</td>
<td>3.15 ± 0.202</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.46 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>0.29 ± 0.018</td>
<td>0.37 ± 0.062</td>
</tr>
<tr>
<td>Liver copper, μg/g</td>
<td>4.97 ± 0.37</td>
<td>0.51 ± 0.042</td>
<td>3.71 ± 0.14</td>
<td>0.37 ± 0.062</td>
</tr>
<tr>
<td>Liver iron, μg/g</td>
<td>56.6 ± 2.99</td>
<td>143 ± 20.52</td>
<td>61.4 ± 4.65</td>
<td>137 ± 17.32</td>
</tr>
<tr>
<td>Ceruloplasmin, units/L</td>
<td>159 ± 7.1</td>
<td>0.1 ± 0.062</td>
<td>187 ± 11</td>
<td>0.48 ± 1.42</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n = 5, Experiment 1; n = 8, Experiment 2).
2 Student’s t-test between groups, P < 0.01.
3 Hearts were not trimmed of the aortic stalk before weighing in Experiment 2.
4 A μmol of copper is 63.55 μg and a μmol of iron is 55.85 μg.

Ralsdon Purina, St. Louis, MO). This diet, which was evaluated previously, contains adequate copper (13 mg Cu/kg) (Prohaska and Hoffman 1996). These rats drank tap water.

In Experiment 1, rats began the treatments at age 21 d and were maintained for ~5 wk. Rats were killed in pairs (one +Cu and one −Cu) and averaged 55 d of age at the time of analysis (Table 1). In Experiment 2, rats began the treatments at age 20 d and were maintained for ~4 wk at which time rats were killed because growth impairment was detected (Table 1).

Rats were given an injection of heparin (1 U/g body weight); 30 min later, they were anesthetized with CO2 before decapitation. A sample of trunk blood was taken for hematocrit determination, plasma ceruloplasmin assay and determination of plasma calcium concentration. Hearts were quickly removed and a portion of the median liver lobe was excised.

Myocyte preparation. Cardiac myocytes were isolated from hearts using standard techniques (Solem et al. 1996). Briefly, hearts were perfused at 37°C with Joklik’s modified minimum essential medium containing collagenase (7g/L) (Worthington Biochemical, Lakewood, NJ) until the vascular bed deteriorated (~20 min). The tissue was then coarsely minced and digested with the collagenase-containing solution; the process was continued in a beaker placed in a gyrating water bath for an additional ~20 min in the presence of 1.0 μmol/L CaCl2. The tissue digestate was then filtered through cheesecloth and cells allowed to settle out of the filtrate. This cell preparation was resuspended and resettle two times in collagenase-free Joklik’s solution. Settling time was limited to promote recovery of live rod-shaped myocytes, which have a greater density than nonmyocytes and dead cells. A 100-μL sample was taken from the final suspension in low calcium for assessment of initial viability. Calcium was then added to the cell suspension (in several steps spread over a 30-min period) to a final concentration of 1.0 mmol/L at 37°C. Viability was reassessed at the end of this procedure.

Viability assessment. Viability of myocytes was assessed by determining the percentage of live rod-shaped cells out of total cells in a given sample. A 100-μL sample of suspended cells was mixed with 50 μL of solution containing trypan blue (5 g/L) and gluteraldehyde (60 g/L). An aliquot of these fixed cells was placed on a microhematocytometer, and live cells were identified by their shape and ability to exclude trypan blue.

Copper analyses and ceruloplasmin assay. Portions of liver and diet (~1 g) were weighed to the nearest milligram and wet-digested with 4 mL of concentrated HNO3 (AR select grade, Mallinckrodt, St. Louis, MO); the residue was brought to 4.0 mL with 0.1 mol/L HNO3. Samples were then analyzed for total copper and iron by flame atomic absorption spectroscopy (AAS) (Model 2380, Perkin-Elmer, Norwalk, CT). The method was checked with a certified standard, U.S. National Bureau of Standards 1577 bovine liver (Gaithersburg, MD).

Plasma copper was determined by flame AAS at 425 nm according to the manufacturer’s protocol. Samples of plasma were diluted with 49 vol of a solution containing 2.67 g/L LaCl3 × H2O. Standards were prepared in the same diluent.

Plasma ceruloplasmin activity was measured by its ability to oxidize o-dianisidine as described in detail elsewhere (Prohaska 1991).

Calcium antagonists. In Experiment 1, three +Cu and three −Cu rat myocyte preparations were used to test the hypothesis that the enhanced sensitivity to calcium observed in −Cu preparations could be attenuated or blocked by the use of calcium transport antagonists (Smogorzewski et al. 1993, Solem et al. 1996). Cells were incubated with buffer alone or buffer with the following drugs (final concentration): verapamil (40 μmol/L); ruthenium red (10 μmol/L); or cyclosporin A (5 μmol/L) for 5 min before calcium additions as described above.

Aortic constriction-induced cardiac hypertrophy. Six young adult male Sprague-Dawley rats (7 wk old), maintained on a copper-sufficient diet, were divided randomly into two groups. Three rats served as sham-operated controls and three were surgically given aortic constrictions to increase upstream aortic pressure and cardiac afterload. After sedation with xylazine (6 mg/kg, intraperitoneal) and anesthesia with ketamine (30 mg/kg, intraperitoneal), a midline abdominal incision was made and a ligature passed under the subdiaphragmatic-suprarenal segment of the abdominal aortic. A blunted 20-gauge hypodermic needle was placed alongside the aorta and the ligature tightened over both. The needle was carefully withdrawn, and the position and integrity of the ligature knot verified by inspection. The abdominal incision was closed by suturing the muscle layers and using wound clips on the skin. Sham treatment in this model consisted of all surgical steps except for the tightening of the ligature. The development of cardiac hypertrophy in the aortic-constricted group determined at the time of killing verified the efficacy of the constriction and the presence of chronic elevation of aortic pressure. After an average of 13 ± 2 d, pairs were killed and cardiac myocytes harvested, as described above. Cardiac myocyte viability was assessed.

Statistics. Dietary treatment effects were evaluated by Student’s t-test after variance equality was tested; α = 0.05 and 0.01. Data in the calcium antagonist study were analyzed by 2 × 4 factorial ANOVA. Data were analyzed using a personal computer and statistical software (Statview 4.5, Abacus Concepts, Berkeley, CA).

RESULTS

Restriction of dietary copper during the rapid growth phase of male Holtzman rats resulted in severe copper deficiency compared with rats drinking copper-supplemented water (Table 1). In Experiment 1, in which the basal diet contained more copper (0.45 mg Cu/kg) than in Experiment 2 (0.30 mg Cu/kg), there was no growth impairment during the 5-wk feeding period. In contrast, in Experiment 2, growth was impaired after 3 wk of the treatment; by 4 wk, a 22% average difference in body weight was evident (Table 1).
In both experiments, biochemical characteristics consistent with severe copper deficiency were evident in −Cu rats compared with +Cu rats, including a 90% reduction in liver copper concentration and near total loss of ceruloplasmin activity (Table 1).

Cardiac hypertrophy was evident because absolute heart weight was elevated 60 and 80%, respectively, in Experiments 1 and 2 (Table 1). Relative heart weight/body weight was also significantly elevated (P < 0.01). For example, in Experiment 1 −Cu rats averaged 7.8 ± 0.91 mg/g compared with 4.4 ± 0.32 mg/g for the +Cu rats.

After myocytes were isolated by collagenase perfusion in low calcium solutions (1 μmol/L), there was no difference in viability of the live rod-shaped cells. However, after calcium was reintroduced gradually to restore extracellular levels to 1 mmol/L, there was a pronounced loss of viability (Fig. 1). Experiment 2 was designed to verify the observations in Experiment 1, and indeed results were similar (Fig. 1). On average, there was a loss of cells from +Cu rat hearts after calcium restoration of 9.5 and 13% in Experiment 1 and 2, respectively, compared with average losses of 34 and 40% for the −Cu cardiac myocytes, P < 0.01.

To determine whether a blockade of calcium channels would prevent the cell loss in −Cu cardiac myocytes, a drug intervention experiment was designed. Myocytes from six rats in Experiment 1, three +Cu and three −Cu, were preincubated for 5 min before calcium addition with buffer alone (none) or buffer containing verapamil (an L-type calcium channel blocker), ruthenium red (mitochondrial calcium uptake uniport blocker), or cyclosporin A (an inhibitor of the calcium-dependent mitochondrial permeability transition). There were no significant effects of these calcium channel blockers on cell viability. The mean viability of the 12 +Cu preparations in low calcium was 84 ± 0.6%, this declined an average of 8.3 ± 1.2% to a viability of 77 ± 1%, when 1 mmol/L calcium was added. The myocytes from −Cu rats were as viable as their +Cu counterparts in low calcium, averaging 85 ± 0.7%. However, the response to the addition of 1 mmol/L calcium was very different (Fig. 2). The average loss of viability for the four treatment groups of −Cu myocytes was 27 ± 1.3% (P < 0.01), resulting in an average postcalcium viability of 62 ± 0.9%. Thus, there was a strong diet effect but no drug effect detected by ANOVA. These drugs did not protect the −Cu cardiac myocyte against the deleterious effects of calcium.

Plasma calcium concentration was measured by flame AAS in samples from Experiment 1. There were no significant differences between groups with +Cu rats averaging 2.44 ± 0.04 mmol/L and −Cu rats 2.39 ± 0.04 mmol/L. Therefore dietary copper deficiency had no effect on plasma calcium concentration.

A third study was done using subdiaphragmatic aortic constriction on male Sprague-Dawley rats fed a copper-adequate diet to determine whether cardiac myocytes from another hypertrophic model would also show such calcium intolerance. Surgical intervention resulted in rats with an elevated heart weight to body weight ratio due to the elevated aortic pressure above the constriction. The heart weight to body weight ratio of the constricted rats averaged 5.7 ± 0.1 mg/g compared with 4.3 ± 0.11 mg/g for the sham-operated group. The body weights of the constricted group, 228 ± 11 g (n = 3), were not different from those of the sham-operated group, 250 ± 12 g (n = 3). Cardiac myocytes harvested from the aortic-constricted group had an initial viability of 83 ± 2% compared with 88 ± 3% for cells from the sham-operated group. When calcium was restored to 1 mmol/L, viability decreased to a similar extent in both groups, aortic-constricted to 73 ± 4% and sham-operated to 79 ± 3%. These results in pressure-overload hypertrophy are clearly different from the results in the copper-deficient hypertrophy.

**DISCUSSION**

Copper deficiency results in a severe, unique cardiac enlargement that is characterized by a concentric hypertrophy without hypertension (Medeiros et al. 1993). Transmission...
electronic microscopic data show enlarged myocytes with a pronounced increase in the ratio of mitochondrial to myofilibrillar volume. Mitochondria are swollen and lipid droplets are evident in the cytoplasm. Results of the present experiments clearly demonstrate that the functional integrity of isolated myocytes was also altered in some way that made them highly susceptible to toxic effects of calcium. This conclusion is supported by the robust and reproducible cell loss that occurred upon reintroduction of calcium to the cell suspensions (Fig. 1). It should be noted that many cells from Cu-deficient rats were still viable upon calcium addition. These cells remained viable for other studies lasting up to 5 h.

Another goal of this series of experiments was to characterize the morphometric properties of the isolated cardiac myocytes from −Cu hearts and to characterize their tolerance to distension and stretch. Results of these experiments, which are described elsewhere (Heller et al. 1999), clearly verify a myocyte enlargement and also identify an increase fragility to distension in hypotonic conditions. The basis for the increased calcium sensitivity of the cardiac myocytes from −Cu rats is not clear. In an attempt to obtain information about this abnormality, we tried to interfere with calcium transport processes across the cell and/or mitochondrial membrane. Part of the approach was patterned after studies on a different rodent model of cardiac failure in which myocytes showed increased calcium sensitivity; i.e., adriamycin-induced cardiac failure (Solem et al. 1996). With this model, chronic treatment of rats with adriamycin, a cancer chemotherapeutic drug with cardiotoxic effects, produces calcium intolerance in isolated myocytes that can be blocked acutely by the presence of cyclosporin A or ruthenium red in the media. Because these drugs interfere with mitochondrial calcium uptake and release, and prevent the mitochondrial permeability transition, it is likely that the calcium intolerance in this model is related to the mitochondrial abnormality. However, in the −Cu model of cardiac hypertrophy, even though mitochondria are abnormal in shape and function, the mitochondrial calcium blockers, ruthenium red and cyclosporin A, did not attenuate the cell loss of isolated myocytes. Furthermore, isolated mitochondria from −Cu rat hearts were able to accumulate calcium to the same extent as +Cu mitochondria, i.e., ~1060 nmol/mg protein (unpublished data). Thus, these data suggest that the basis for increased calcium sensitivity in the −Cu cardiac myocyte is not mitochondrial.

Another approach to identify the basis for the increased calcium sensitivity was to block calcium uptake at the cell membrane with the calcium channel antagonist, verapamil. Although the dose used was high enough to completely block movement of calcium into isolated rat cardiac myocytes via this route (Smogorzewski et al. 1993), verapamil had no effect on the calcium intolerance of the −Cu myocytes. Therefore, it is unlikely that the increased calcium sensitivity of these cells proceeds by a process involving the L-type calcium channel.

If the toxicity is a result of calcium accumulation within the myocyte, it is possible that calcium may enter the cell via an enhancement of the sodium/calcium exchanger. This intriguing possibility requires further studies.

It should be emphasized that the observed calcium sensitivity in −Cu hypertrophic cardiac myocytes is not characteristic of all models of cardiac hypertrophy because it was not seen in the hypertrophic cardiac myocytes from the aortic-constricted rats. Thus, it is possible that a unique mechanism involving abnormal myocyte calcium handling exists as a consequence of dietary copper deficiency. Studies of intracellular calcium homeostasis as affected by copper deficiency are limited. Johnson and Dufault (1993) were able to demonstrate in −Cu platelets a diminished rise in cytosolic calcium upon agonist administration. It is not clear how that observation in platelets relates to enhanced toxicity of −Cu cardiac myocytes.

The relevance of this in vitro observation is not clear. Circulating levels of total plasma calcium that we measured (~2.4 mmol/L) would result in a free calcium ion concentration similar to that used in the cell exposures (1.0 mmol/L). Thus, the enzymatic dispersal techniques and/or the transient imposition of a low calcium environment probably reveal the calcium intolerance of the isolated −Cu myocyte. However, it is interesting to point out that apoptotic and necrotic processes can be triggered by rises in cellular calcium, and apoptosis is a feature of dietary copper deficiency in certain organs such as pancreas (Rao et al. 1993).

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LITERATURE CITED


