Published in final edited form as:

Cardiovasc Toxicol. 2008 ; 8(3): 137–144. doi:10.1007/s12012-008-9021-3.

Role of Copper and Homocysteine in Pressure Overload Heart Failure

William M. Hughes Jr, Walter E. Rodriguez, Dorothea Rosenberger, Jing Chen, Utpal Sen, Neetu Tyagi, Karni S. Moshal, Thomas Vacek, Y. James Kang, and Suresh C. Tyagi
W. M. Hughes Jr., W. E. Rodriguez, D. Rosenberger, U. Sen, N. Tyagi, K. S. Moshal, T. Vacek, S. C. Tyagi, Department of Physiology and Biophysics, School of Medicine, University of Louisville, Louisville, KY 40202, USA

J. Chen, Department of Environmental and Occupational Health Sciences, School of Medicine, University of Louisville, Louisville, KY 40202, USA

Y. J. Kang, Department of Pharmacology and Toxicology, School of Medicine, University of Louisville, Louisville, KY 40202, USA

Abstract

Elevated levels of homocysteine (Hcy) (known as hyperhomocysteinemia HHcy) are involved in dilated cardiomyopathy. Hcy chelates copper and impairs copper-dependent enzymes. Copper deficiency has been linked to cardiovascular disease. We tested the hypothesis that copper supplement regresses left ventricular hypertrophy (LVH), fibrosis and endothelial dysfunction in pressure overload DCM mice hearts. The mice were grouped as sham, sham + Cu, aortic constriction (AC), and AC + Cu. Aortic constriction was performed by transverse aortic constriction. The mice were treated with or without 20 mg/kg copper supplement in the diet for 12 weeks. The cardiac function was assessed by echocardiography and electrocardiography. The matrix remodeling was assessed by measuring matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinases (TIMPs), and lysyl oxidase (LOX) by Western blot analyses. The results suggest that in AC mice, cardiac function was improved with copper supplement. TIMP-1 levels decreased in AC and were normalized in AC + Cu. Although MMP-9, TIMP-3, and LOX activity increased in AC and returned to baseline value in AC + Cu, copper supplement showed no significant effect on TIMP-4 activity after pressure overload. In conclusion, our data suggest that copper supplement helps improve cardiac function in a pressure overload dilated cardiomyopathic heart.

Keywords

Homocysteine; Copper; Pressure overload; Heart failure; Matrix remodeling

Introduction

Copper (Cu) is a trace metal that is necessary for several enzymes to function properly. The transport of copper to cells is facilitated by proteins such as ceruloplasmin, and interestingly, other proteins such as lysyl oxidase, cytochrome c oxidase, and superoxide dismutase are dependent on copper [1,2]. Copper imbalances in humans not only impair normal function of these proteins, but can also lead to disease states. Copper metabolism has been linked to late onset neurological diseases such as Wilson's disease and Alzheimer's disease [1]. In addition

to copper metabolism, copper deficiency has been linked to diseases such as cardiomyopathy [3], myocardial infarction, severe tachycardia, and also sudden death due to rupture of the heart [4].

To reduce the effects of copper deficiency, a copper supplement has been used, specifically, against cardiomyopathy. Jiang et al. fed mice a copper supplement (20 mg/kg Cu/mouse) and found that the copper supplement reverses cardiac hypertrophy and dysfunction due to pressure overload on the heart [5].

Pressure overload occurred as a result of left ventricular wall thickening, characteristic of left ventricular hypertrophy [6]. Left ventricular hypertrophy is also associated with an increase in the electric QRS amplitude, delays in ventricular conduction, and prolonged action potentials [3,7]. Endothelial-matrix remodeling is associated with pressure overload and it occurs within the endothelium of hypertrophic hearts as a result of increased matrix metalloproteinase (MMP) activity and decreased tissue inhibitor of matrix metalloproteinase (TIMP) activity [3].

Copper deficiency can occur in the presence of homocysteine, especially elevated levels of homocysteine (hyperhomocysteinemia, HHcy). Homocysteine is a small, non-protein amino acid found in plasma. Homocysteine has been identified as one of 200 risk factors for cardiovascular disease [8]. Linnebank et al. suggest that copper deficiency occurs because homocysteine chelates copper, decreasing its availability to cells [3]. Figure 1 suggests that homocysteine is elevated in pressure overload. Homocysteine binds copper, leading to copper deficiency. Copper deficiency inactivates superoxide dismutase and activates NADPH oxidase, which may generate reactive oxygen species (ROS), fibrosis, left-ventricular hypertrophy, and endothelial dysfunction.

Previous studies from our laboratory have indicated that there is a correlation between copper deficiency and cardiac diseases. The present study uses a mouse model with pressure overload heart failure via aortic constriction. We hypothesized that a copper supplement regressed left ventricular hypertrophy, fibrosis, and endothelial dysfunction in part by decreasing the MMP/TIMP axis and increasing lysyl oxidase (LOX) expression during chronic pressure overload.

Methods

Materials

Rodent diet AIN-93 was purchased from Rodent Laboratory (Rodent Laboratory Chow® 501). β -Mercaptoethanol, proteinase inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin), and tribromoethanol were purchased from Sigma (St. Louis, MO). Specific antibodies against MMP-9, TIMP3, TIMP-1, TIMP-4 and LOX were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bio-Rad protein assay reagents were purchased from Bio-Rad (Hercules, CA).

Animals

Male and female C57BL/6J mice 8–10 weeks old (Jackson Laboratory) were fed the AIN-93 diet (copper adequate) for 4 weeks [5,9]. Mice were kept in cages in a temperature- and humidity-controlled room with a 12 h light:dark cycle. Animal protocols were approved by the National Institutes of Health and the Institutional Animal Care and Use Committee at the University of Louisville.

Aortic Banding

Ascending aortic constriction (AC) was the surgery performed to create pressure overload in the left ventricle. This surgery mimics aortic stenosis and has been established as an important

mouse model [10]. To create pressure overload, an incision is made at the left “armpit,” a 6-0 suture is placed underneath the ascending portion of the aorta and loosely tied around a 26½-gauge needle. The needle is removed and the knot is tightened. Sham surgery did not require banding of the aorta. The survival rate for animals with AC was 50%.

Echocardiography

Echocardiograms were obtained using M-Mode echocardiography (SONOS 5500; Hewlett-Packard, Inc.) and a 2.5-MHz transducer on anesthetized mice. Using this machine, we could obtain a two-dimensional image of the left ventricle and measure the inner wall of the left ventricle during diastole and systole, in addition to fractional shortening. Fractional shortening was measured since it is a better indicator of the raw data. Ejection fraction squared in a mouse results in a larger error.

Electrocardiography

Mice were anesthetized and then a three-lead electrocardiogram was taken. The leads were placed on the right foreleg, left foreleg, and right hind leg. The electrocardiogram was obtained using a Heart Performance Analyzer (DigiMed).

Endothelial-myocyte Coupling Analysis

Endothelial-myocyte uncoupling was measured using a technique described previously by Rodriguez et al. [11]. Cardiac rings (2 mm in diameter) were made from each group and placed in a physiological saline solution myobath under 37°C, 95% O₂–5% CO₂, and pH 7.4. The rings were mounted between two wires (20 µM) and connected to a force transducer (World Precision Instruments). The ring was stretched and tension was measured via AcqKnowledge software. After maximal tension, acetylcholine was added over the course of 1 h. Percent relaxation was measured assuming 100% contraction occurred at maximal tension.

Tissue Collection

Four weeks post-surgery, a few animals were sacrificed and four groups were created: sham (S); sham with 20 parts per million (ppm, copper supplement) (S + Cu); aortic constriction (AC); and aortic constriction with copper supplement (AC + Cu). S + Cu and AC + Cu groups received a diet of 20 ppm Cu (20 mg/kg Cu/mouse). S and AC groups remained on the 13 ppm Cu (6 mg/kg Cu/mouse). All groups of mice remained on the diet for 12 weeks and were sacrificed to obtain tissue samples. Mice were anesthetized intraperitoneally with 0.02 ml/g body weight tribromoethanol. Heart tissue was excised, and tissues were rapidly washed twice in phosphate buffered saline. The tissue samples were stored at –80°C until further use. Plasma was collected from the renal vein and centrifuged before storage at –80°C for further use. Prior to the sacrifice, an echocardiogram and electrocardiogram was taken from each group. There were four mice in each group.

Preparation of Samples

The tissue samples were homogenized in 250 µl of ice-cold lysis buffer (pH 7.4) containing Tris–HCl (20 mM), EDTA (1 mM), NaCl (50 mM), DTT (1 mM), and β-mercaptoethanol (10 mM). The clear supernatant of tissue samples was collected and protein concentration in the samples was determined by Bradford’s method using bovine serum albumin as a standard [12].

Western Blot Analysis

The expression of MMP-9, TIMP-1, TIMP-3, TIMP-4, and LOX was determined by Western Blot analysis. Protein samples were mixed with 1:1 v/v ratio with 2× sample loading buffer,

boiled at 90°C for 7 min, cooled, and then centrifuged at 10,000g for 5 min. Equal amounts of protein (15 µg) for each group were resolved by 12% gradient SDS-PAGE. Proteins were then electrophoretically transferred to a polyvinylidene fluoride membrane (BioRad, Hercules, CA). Transferred proteins were blocked with 5% non-fat dry milk in TBS-T (pH 7.4) containing: Tris-HCl (50 mM), NaCl (150 mM), and Tween-200 (0.1%) for 1 h at room temperature. The blots were then incubated with respective primary antibodies in blocking solution according to the supplier's recommendations. After incubation, the blots were washed with TBS-T three times for 10 mins each. The blots were incubated with appropriate secondary antibody for 1 h. After washing four times (for 10 min each wash) ECL Plus substrate (Amersham Biosciences, Pittsburgh, PA) was applied to the blots for 5 min. The protein bands were visualized by exposing the blots to X-ray film as described previously [13]. Image analysis of the protein bands was performed using UMAX PowrLock II (Taiwan). The protein expression intensity was assessed by integrated optical density [14].

Measurement of Intracellular Copper Levels

Copper concentrations in the heart were measured using Phen Green SK probe (Invitrogen). A 1 mM copper sulfate stock solution was made in phosphate buffered saline (pH 7.4). Standards from 0 to 50 µM were made, adding 50 µl of the copper probe. Fluorescence was measured at 585 nm using Spectra max spectrofluorimeter (Molecular Devices).

Measurement of Endogenous Homocysteine Levels

Homocysteine was measured by using high pressure liquid chromatography (HPLC) as described earlier [15]. HPLC analyses were performed using Class-VP 5.0 chromatograph (Shimadzu) containing a LC-10ADvp pump, a SIL-10ADvp auto-injector, a CTO-10Avp column oven, and a SPD-10Avp detector. The temperature inside the column was maintained at 37°C during analyses.

The sample preparation and mobile phase preparation were the same as described elsewhere [16]. The mobile phase consisted of 0.1 M monochloroacetic acid and 1.8 M octyl-sulfate, pH 3.2. Samples were prepared using supernatant removed after centrifugation. To 200 µl of supernatant, 100 µl of water and 300 µl of 9 M urea (pH 9.0) were added. Also, 50 µl of *n*-amyl alcohol was added to the supernatant to act as an anti-foaming agent. In order to carry out reduction of disulfides and the cleavage of protein-bound, sulfur containing amino acids, 50 µl of NaBH₄ (10% wt/vol) in 0.1N NaOH was added. This reaction was carried out in a 50°C water bath for 30 min. The reaction ended when 500 µl of 20% trichloroacetic acid was added at room temperature. Samples were centrifuged at 12,000g for 4 min and filtered using a 0.45 µM millipore filter.

Statistics

Data are presented as mean values ± SD. Significance was determined using Student's *t*-test, and a *P* value of <0.05 was considered statistically significant.

Results

Plasma Homocysteine Concentrations

Pressure overload heart failure may perturb homocysteine metabolism. Therefore, it is a major factor in elevated plasma homocysteine levels. Interestingly, our results indicate a correlation between elevated copper levels and elevated homocysteine levels within the plasma. Figure 2a shows sham + Cu and AC + Cu groups having higher levels of homocysteine than sham and AC groups. Plasma homocysteine concentration was 3.2 µM in sham; 8.2 µM in sham + Cu; 5.2 µM in AC; and 8.9 µM in AC + Cu.

Cardiac Copper Concentrations

To determine the effect of copper on cardiac function, we measured copper concentration using a fluorimetric assay. Figure 2b depicts that copper concentration in the AC heart sample, $9.5 \pm 0.87 \mu\text{M}$, was similar to copper concentration in the sham group, $8.7 \pm 0.58 \mu\text{M}$. sham + Cu and AC + Cu groups had higher copper concentration, indicating that the copper supplement (CuS) diet increased copper concentration within tissues.

Electric Properties of Pressure Overload Hearts

Left ventricular hypertrophy is associated with a prolonged QRS complex and an increase in RR intervals. Copper deficient hearts have also shown similar results. Our results indicate that the AC group had a prolonged QRS complex compared to the sham group and the AC + Cu group. The QRS complex for the AC group was $14.0 \pm 2.0 \text{ ms}$; $13.0 \pm 1.0 \text{ ms}$ for the sham group; and $13.0 \pm 1.0 \text{ ms}$ for AC + Cu group. The RR interval for the AC group was increased, reflected in the heart rate of 423 beats per min (BPM). The sham group had a heart rate of 475 BPM, and the AC + Cu group had a heart rate of 445 BPM.

Echocardiogram Data Suggest Hypertrophy

Echocardiography data suggest that mice hearts, aged 24–26 weeks, subject to chronic pressure overload had poor diastolic and systolic functions relative to mice hearts in the sham group (Fig. 3). The inner diameter during diastole of the left ventricle (LVIDd) was $0.314 \pm 0.03 \text{ cm}$ in mice with aortic constriction, compared to an LVIDd of $0.320 \pm 0.02 \text{ cm}$ in those with aortic constriction and copper supplementation. A wider LVIDd signifies a thicker left ventricular wall, an indicator of hypertrophy. The LVIDd in sham mice was $0.271 \pm 0.04 \text{ cm}$; in sham mice with copper supplementation, it was $0.311 \pm 0.04 \text{ cm}$. Fractional shortening serves as an index of cardiac contractile function and these data are in accord with LVIDd data. Sham mice had a fractional shortening of $61.9 \pm 4.3\%$, compared to a fractional shortening of $57.7 \pm 5.2\%$ in sham + Cu. The AC mice hearts had a fractional shortening value of $56.4 \pm 4.9\%$, compared to the fractional shortening value of $62.8 \pm 3.1\%$ for AC + Cu. Physiologically, a lower fractional shortening value signifies a decrease in contractility and in relaxation of the cardiac muscle.

Endothelial-myocyte Uncoupling Due to Pressure Overload

Cardiac ring samples from AC mice were less responsive to acetylcholine than those samples from AC + Cu mice and sham mice. After cardiac ring studies, the data were normalized as percentages and the Effective Dose, 50% (ED_{50}) was taken from the percent relaxation. The ED_{50} for AC mice was near $10^{-5.6}$, whereas, the ED_{50} for AC + Cu mice was $10^{-8.5}$, which is a 1,000-fold difference (Fig. 4).

Western Blot Analyses of MMPs, TIMPs, and LOX

Western Blot analyses suggest that copper has little effect on the MMP/TIMP levels as well as the LOX level. TIMP-1 expression was increased in both sham + Cu and AC + Cu groups (Fig. 5b). There is no significance with MMP-9 (Fig. 5a), LOX (Fig. 7), TIMP-3 (Fig. 6a), and TIMP-4 (Fig. 6b), but there are some trends that these data might suggest. MMP-9 expression may be increased due to pressure overload, and the copper supplement may decrease expression of MMP-9. LOX expression may also be increased due to pressure overload and the copper supplement reduces this expression.

Discussion

This study suggests that copper supplementation reduces the pathological effects of chronic pressure overload-induced cardiac dysfunction. In vivo and in vitro data suggest this may be

true; however, the molecular data from this study suggest the copper supplement has little effect after chronic pressure overload. Our data indicate a correlation between a copper supplement and plasma homocysteine levels (Fig. 2). This correlation is in agreement with previous studies which state that intracellular homocysteine release was increased by copper ions [17]. However, Tamura et al. state that a long-term intake of copper (7 mg Cu/day) might reduce the total homocysteine concentration in plasma [18]. Based on this data, we concluded that copper has deleterious effects in that it increases homocysteine levels.

Other studies show that one sign of left ventricular hypertrophy is an increased left ventricular wall thickness during diastole [19]. This was true in our study as indicated by the echocardiogram data. LVIDd in AC mice was 18% wider than LVIDd in sham mice. Another sign of LVH is a prolonged QRS complex. Chronic copper deficiency has also resulted in a prolonged QRS complex [16]. Our study revealed that both phenomena occurred: a heart subject to chronic copper deficiency and LVH via aortic constriction displayed a prolonged QRS complex when compared to the sham heart and AC + Cu hearts. Copper supplement ameliorated the prolonged QRS complex, suggesting that copper has a role in improving the electrical conduction in cardiac tissue.

Endothelial-myocyte uncoupling is a phenomenon we have observed in heart failure models. This occurs when MMPs are activated due to an increase in the production of reactive oxygen species (ROS). Our results confirm that in pressure overload, there is a decrease in responsiveness to acetylcholine, which serves as a neurotransmitter to decrease heart rate. This study suggests that endothelial-myocyte uncoupling due to pressure overload is improved with the copper supplement. Recently, though, studies suggest that the interaction between copper and homocysteine inhibits the formation of nitric oxide via generation of ROS, promoting endothelial dysfunction [20-22].

As mentioned, our molecular data suggest the copper supplement has little effect after chronic pressure overload. Figures 5-7 depict representative MMP-9, TIMP-1, TIMP-3, TIMP-4, and LOX analyses. It has been well established that MMP activity is elevated during pressure overload and consequently TIMP activity is diminished [3]. The data show a tendency for these events to occur, but it is not significant. The LOX data suggest that during pressure overload there is an increased LOX expression and that the copper supplement has little effect on expression compared to sham. These data are contradictory to a previous study which states that copper promotes the activation of lysyl oxidase [23]. This finding would signify that a copper supplement should increase the expression of LOX, unless there is an alternative response to copper within rodents. Xia et al. state that rodents are resistant to high copper intakes, especially mice [24]. It might be possible, then, that when mice are given a high-copper diet, they reduce the intake of copper and consequently copper-dependent enzymes are constitutively expressed.

In conclusion, this study suggests that copper supplementation may help to improve cardiac function after pressure overload heart failure. However, it also presents a possible deleterious correlation between elevated copper levels and elevated homocysteine levels.

Acknowledgements

This study was supported in part by the NIH grants HL-74185 and HL-88012 to SCT.

References

1. Macreadie IG. Copper transport and Alzheimer's disease. *European Biophysics Journal* 2007;37(3): 295–300.10.1007/s00249-007-0235-2 [PubMed: 18004558]

2. Trumbo P, Yates AA, Schlikcer S, Poos M. Dietary reference intakes: Vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the American Dietetic Association* 2001;101:294–301.10.1016/S0002-8223(01)00078-5 [PubMed: 11269606]
3. Linnebank M, Lutz H, Jarre E, Vielhaber S, Noelker C, Struys E, et al. Binding of copper is a mechanism of homocysteine toxicity leading to COX deficiency and apoptosis in primary neurons, PC12 and SHSY-5Y cells. *Neurobiology of Disease* 2006;23(3):725–730.10.1016/j.nbd.2006.06.010 [PubMed: 16876425]
4. Reiser S, Smith JC Jr, Mertz W, Holbrook JT, Scholfield DJ, Powell AS, et al. Indices of copper status in humans consuming a typical American diet containing either fructose or starch. *The American Journal of Clinical Nutrition* 1985;42:242–251. [PubMed: 4025196]
5. Jiang Y, Reynolds C, Xiao C, Feng W, Zhou Z, Rodriguez W, et al. Dietary copper supplementation reverses hypertrophic cardiomyopathy induced by chronic pressure overload in mice. *The Journal of Experimental Medicine* 2007;204:657–666.10.1084/jem.20061943 [PubMed: 17339407]
6. Kopp SJ, Klevay LM, Feliksik JM. Physiological and metabolic characterization of a cardiomyopathy by chronic copper deficiency. *Heart and Circulatory Physiology* 1983;14:H855–H866.
7. Oikarinen L, Nieminen MS, Viitasalo M, Toivonen L, Jern S, Dahlöf B, et al. QRS duration and QT interval predict mortality in hypertensive patients with left ventricular hypertrophy. The Losartan Intervention for Endpoint Reduction in Hypertension Study. *Hypertension* 2004;43(5):1029–1034.10.1161/01.HYP.0000125230.46080.c6 [PubMed: 15037560]
8. Castelli WP. Lipids, risk factors and ischemic heart disease. *Atherosclerosis* 1996;124:S1–S9.10.1016/0021-9150(96)05851-0 [PubMed: 8831910]
9. Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents—final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. *The Journal of Nutrition* 1993;123:1939–1951. [PubMed: 8229312]
10. Tarnavski O, McMullen JR, Schinke M, Nie Q, Kong S, Izumo S. Mouse cardiac surgery: Comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. *Physiological Genomics* 2004;16(3):349–360.10.1152/physiolgenomics.00041.2003 [PubMed: 14679301]
11. Rodriguez W, Tyagi N, Joshua I, Passmore J, Fleming J, Falcone J, et al. Pioglitazone mitigates renal glomerular vascular changes in high-fat, high-calorie-induced type 2 diabetes mellitus. *American Journal of Physiology Renal Physiology* 2006;291:F694–F701.10.1152/ajprenal.00398.2005 [PubMed: 16609149]
12. Tyagi N, Moshal KS, Tyagi SC, Lominadze D. gamma-Aminobutyric acid A receptor mitigates homocysteine-induced endothelial cell permeability. *Endothelium* 2007;14(6):315–323.10.1080/10623320701746164 [PubMed: 18080868]
13. Tyagi N, Moshal KS, Sen U, Lominadze D, Ovechkin AV, Tyagi SC. Ciglitazone ameliorates homocysteine-mediated mitochondrial translocation and matrix metalloproteinase-9 activation in endothelial cells by inducing peroxisome proliferators activated receptor-gamma activity. *Cellular and Molecular Biology* 2006;52:21–27. [PubMed: 17543202]
14. Moshal KS, Zeldin DC, Sithu SD, Sen U, Tyagi N, Kumar M, et al. Cytochrome P450 (CYP) 2J2 gene transfection attenuates MMP-9 via inhibition of NF-kappaB in hyperhomocysteinemia. *Journal of Cellular Physiology* 2008;215(3):771–781.10.1002/jcp.21356 [PubMed: 18181170]
15. Sen U, Tyagi N, Kumar M, Rodriguez W, Tyagi S. Cystathionine-beta synthase gene transfer and 3-deazaadenosine ameliorate inflammatory response in endothelial cells. *American Journal of Physiology Cell Physiology* 2007;293(6):C1779–C1787.10.1152/ajpcell.00207.2007 [PubMed: 17855772]
16. Malinow MR, Kang SS, Taylor LM, Wong PW, Coull B, Inahara T, et al. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. *Circulation* 1989;79:1180–1188. [PubMed: 2785871]
17. Hultberg B, Andersson A, Isaksson A. Copper ions differ from other thiol reactive metal ions in their effects on the concentration and redox status of thiols in HeLa cell cultures. *Toxicology* 1997;117:89–97.10.1016/S0300-483X(96)03554-8 [PubMed: 9057888]

18. Tamura T, Turnlund JR. Effect of long-term, high-copper intake on the concentrations of plasma homocysteine and B vitamins in young men. *Nutrition* (Burbank, Los Angeles County, Calif.) 2004;20(9):757–759.10.1016/j.nut.2004.05.011
19. Elsherif L, Ortines RV, Saari JT, Kang YJ. Congestive heart failure in copper-deficient mice. *Experimental Biology and Medicine* 2003;228(7):811–817. [PubMed: 12876300]
20. Emsley A, Jeremy JY, Gomes G, Angelini GD, Plane F. Copper interacts with homocysteine to inhibit nitric oxide formation in the rat isolated aorta. *British Journal of Pharmacology* 1999;126:1034–1040.10.1038/sj.bjp. 0702374 [PubMed: 10193785]
21. Shukla N, Koupparis A, Jones RA, Angelini GD, Persad R, Jeremy JY. Penicillamine administration reverses the inhibitory effect of hyperhomocysteinemia on endothelium-dependent relaxation and superoxide formation in the aorta of the rabbit. *European Journal of Pharmacology* 2006;531:201–208.10.1016/j.ejphar.2005.12.003 [PubMed: 16451799]
22. Mansoor MA, Bergmark C, Haswell SJ, Savage IF, Evans PH, Berge RK, et al. Correlation between plasma total homocysteine and copper in patients with peripheral vascular disease. *Clinical Chemistry* 2000;46:385–391. [PubMed: 10702526]
23. Rucker RB, Kosonen T, Clegg MS, Mitchell AE, Rucker BR, Uriu-Hare JY, et al. Copper, lysyl oxidase and extracellular matrix protein cross-linking. *The American Journal of Clinical Nutrition* 1998;67:996S–1002S. [PubMed: 9587142]
24. Xia B, Llanos RM, Mercer JFB. ATP7A transgenic and nontransgenic mice are resistant to high copper exposure. *The Journal of Nutrition* 2007;693–697.

Abbreviations

AC	Ascending aortic constriction
Cu	Copper
DTT	Dithiothreitol
ED₅₀	Effective dose, 50 percent
EDTA	Ethylenediaminetetraacetic acid
Hcy	Homocysteine
HHcy	Hyperhomocysteinemia
HPLC	High pressure liquid chromatography
LVH	Left ventricular hypertrophy
LVIDd	Left ventricular inner diameter during diastole
LOX	Lysyl oxidase
MMP	

	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide
oxidase	phosphate oxidase
ppm	Parts per million
QRS	Electric QRS complex
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TIMP	Tissue inhibitor of matrix metalloproteinase

Fig. 1. Copper-deficient model of endothelial dysfunction. **(a)** Copper deficiency results in fibrosis, left ventricular hypertrophy (LVH), and endothelial dysfunction due to homocysteine chelating copper. Endothelial dysfunction arises from an increased reactive oxygen species (ROS) production and as a consequence, increased levels of MMPs. **(b)** Comparison of copper-adequacy and copper-deficiency. When copper is adequate, copper binds to ceruloplasmin for cellular intake and activates LOX, CytC, SOD, and COX. When copper is deficient, it binds to homocysteine before entering the cell, thereby decreasing methyltetrahydrofolate reductase (MTHFR) levels; increasing homocysteine levels; and inactivating LOX, CytC, SOD, and LOX

Fig. 2.

(a) Plasma homocysteine levels and (b) cardiac copper concentrations taken from four groups: sham (S); sham with copper supplement (S + Cu); aortic constriction (AC); and aortic constriction with copper supplement (AC + Cu). Plasma homocysteine levels were measured using HPLC. Cardiac copper concentrations were measured using a spectrofluorimeter. Levels and concentrations represent mean $\pm$ SD values taken from $n = 4$. * Denotes sham versus S + Cu, $P < 0.05$. ** Denotes AC versus AC + Cu, $P < 0.05$. *** Denotes sham versus AC, $P < 0.05$.

Fig. 3. Representative M-mode echocardiograms for four groups: (a) sham [S]; (b) sham with copper supplement [S + Cu]; (c) aortic constriction [AC]; and (d) aortic constriction with copper supplement [AC + Cu]. White arrow suggests dilatation in (c)

Fig. 4. Endothelial-myocyte coupling as represented by percent relaxation due to response to acetylcholine (ACh). Four groups are presented: sham (S); sham with copper supplement (S + Cu); aortic constriction (AC); and aortic constriction with copper supplement (AC + Cu). ED_{50} are indicated by the dotted lines

Fig. 5. Representative Western Blot analyses for the expression of (a) MMP-9 and (b) TIMP-1 proteins. Representative scans are displayed above bar graphs indicating the scan values from four groups: sham (S); sham with copper supplement (S + Cu); aortic constriction (AC); and aortic constriction with copper supplement (AC + Cu). Scan values were given arbitrary scan units (AU) and are expressed as mean $\pm$ SD values taken from $n = 4$. * Denotes sham versus S + Cu, $P < 0.05$. ** Denotes AC versus AC + Cu, $P < 0.05$

Fig. 6. Representative Western Blot analyses for the expression of (a) TIMP-3 and (b) TIMP-4 proteins. Representative scans are displayed above bar graphs indicating the scan values from four groups: sham (S); sham with copper supplement (S + Cu); aortic constriction (AC); and aortic constriction with copper supplement (AC + Cu). Scan values were given arbitrary scan units (AU) and are expressed as mean $\pm$ SD values taken from $n = 4$

Fig. 7.

Representative Western Blot analyses for the expression of LOX protein. Representative scans are displayed above bar graphs indicating the scan values from four groups: sham (S); sham with copper supplement (S + Cu); aortic constriction (AC); and aortic constriction with copper supplement (AC + Cu). Scan values were given arbitrary scan units (AU) and are expressed as mean $\pm$ SD values taken from $n = 4$