Neointima Formation in the Rat Carotid Artery Is Exacerbated by Dietary Copper Deficiency

JURANDIR J. DALLE LUCCA,* JACK T. SAARI,† JEFF C. FALCONE,* AND DALE A. SCHUSCHKE[1]*

Department of Physiology and Biophysics, University of Louisville, Louisville, Kentucky 40292, and †U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, North Dakota 58202

Dietary copper is an essential trace element with roles in both functional and structural aspects of the cardiovascular system. In particular, the vascular response to inflammatory stimuli is known to be significantly augmented in copper-deficient rats. The current study was designed to quantify the extent of injury-induced neointimal proliferation and stenosis in rats fed diets either adequate or deficient in copper. Male, weanling Sprague-Dawley rats were fed purified diets that were either adequate (CuA; 5.6 μg Cu/g) or deficient (CuD; 0.3 μg Cu/g) in copper for 4 weeks. Balloon injury was induced in the left external carotid arteries. Fourteen days after injury, histomorphometric analysis of cross-sections from carotid arteries showed increased neointimal formation in the CuD group compared with the CuA controls (neointima/media ratio: 4.55 ± 0.93 vs 1.45 ± 0.2, respectively). These results correspond with data indicating that the activity of Cu/Zn-superoxide dismutase (SOD) is depressed in rats fed this CuD diet. Because superoxide anion and redox status are known to play a key role in the extent of neointimal formation in response to injury, we propose that the exaggerated neointimal proliferation seen in the CuD group is the result of the diminished Cu/Zn-SOD activity.

[Exp Biol Med Vol. 227(7):487–491, 2002]

Key words: copper; angioplasty; restenosis; carotid artery

Copper is an essential trace element required as a co-factor in several enzymes of the cardiovascular system. In copper-deficient rats, alterations in responses to catecholamines (1), and nitric oxide (NO)-mediated agonists (2–6) as well as changes in platelet thrombus formation (7–11) and inflammation (7, 12–14) are known to occur in an environment where Cu/Zn-superoxide dismutase (Cu/Zn-SOD) activity is depressed and peroxynitrite activity is increased (4). These changes in vascular function suggest that reduced activity of the copper-dependent enzymes, dopamine β monooxygenase, soluble guanylate cyclase, and Cu/Zn-SOD can directly or indirectly affect vascular function. In addition, structurally weakened blood vessels have been attributed to reduced activity of the copper-dependent enzyme, lysyl oxidase (15)

The defects in vascular wall function and structure known to occur during dietary copper deficiency suggest that the response to balloon-induced vascular injury may also be altered in copper-deficient rats. The current study was designed to quantify the extent of injury-induced neointimal proliferation and stenosis in rats fed diets that were either adequate or deficient in copper.

Materials and Methods

Animals and Diet. This project was approved by the University of Louisville Animal Care and Use Committee. Twelve male weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12:12-hr 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 (16) was a casein-sucrose-cornstarch-based diet (no. TD 84469; Teklad Test Diets, Madison, WI) containing all known essential vitamins and minerals except for copper and iron. The copper-adequate (CuA) diet consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a copper-iron mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of $CuSO_4 \cdot 5H_2O$ per kilogram of diet. The copper-deficient (CuD) diet was the same except for replacement of copper with cornstarch in the mineral mix. Diet analysis be atomic absorption spectrophotometry (model 503; Perkin Elmer, Norwalk, CT) indicated that the CuA diet contained 5.6 mg copper/kg diet and the CuD diet contained 0.33 mg copper/kg diet. Parallel assays of National Institute of Standards and Technology (NIST; Gaithersburg, MD) reference samples (citrus leaves, no. 1572) yielded values within the specified range, which validated our copper assays.

Supported by the National Institutes of Health (grant no. DK55030-02).

[1] To whom requests for reprints should be addressed at Department of Physiology and Biophysics, Health Sciences Center A1115, University of Louisville, Louisville, KY 40292. E-mail: daschu01@louisville.edu

Received November 1, 2001.
Accepted March 14, 2002.

1535-3702/02/2277-0487$15.00

Arterial Injury Model. Animals were anesthetized by intraperitoneal injection of a ketamine (37.5 mg/kg) and xylazine (5 mg/kg) mixture. The level of anesthesia was verified before and during surgery by evaluating vibrissa movement, tail tonus, and breathing rate in response to handling. If the animal did not attain an adequate level of anesthesia in the first 7 min following administration, it was returned to the breeding colony for use in later experiments. This protocol ensured that animals remained under complete anesthesia for at least 40 min.

The left common and external carotid arteries were exposed and a balloon catheter (PE50) was passed through the external carotid artery and advanced into the aorta, inflated with saline, then pulled back to distend the common carotid artery. This procedure was repeated twice, after which the external carotid artery was ligated, and the incision was sutured.

Histomorphometric Analysis. Fourteen days after balloon injury, the rats were anesthetized and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde at a fixed perfusion pressure of 100 mmHg. The right and left common carotid arteries were removed, and the adipose and connective tissue in the adventitia were excised. The vessels were then divided into three equal segments and placed for 24 hr in 4% paraformaldehyde for further fixation.

The arterial segments were embedded in paraffin, cut on a rotary microtome (5 μm), and stained with hematoxylin and eosin (H&E). Cross-sectional rings stained with H&E were used to determine the extent of neointima formation. Analysis was performed using a Axiovert microscope (Zeiss, Jena, Germany) attached to an imaging system analyzer Image-1/Metamorph (Universal Imaging Corp., Westerchester, PA) at ×100 magnification. Microscopic images of cross-sectional rings were digitally transferred into a computerized image analyzer and calibrated for area measurement. Two independent operators using a double-blind protocol performed the area measurements by contouring the luminal, luminal plus neointimal, and total arterial threshold areas on the computer screen with a digital pointer. Data were then transferred into a Microsoft® Excel spreadsheet, and luminal, neointimal, medial, and total arterial areas were calculated. The average of these measurements were used and they did not differ by more than 0.1%.

Hepatic Copper Measurement. After removal of the carotid arteries, the median lobe of the liver was removed, weighed, and frozen at −10°C for subsequent copper analysis. Tissues were lyophilized and digested in nitric acid and hydrogen peroxide (17). Hepatic copper concentrations of individual rats were assessed by using inductively coupled argon plasma emission spectrometry (model 1140; Jarrell-Ash, Waltham, MA). Parallel assays of reference samples (no. 1477a, bovine liver from NIST yielded mineral contents within the specified range.

Statistical Analysis. Each artery was divided into three equal segments (upper, middle, and lower) whose areas did not differ when compared by one-way analysis of variance (ANOVA). Therefore, an average of the three segments was used as a single measurement for each artery. Neointima formation is reported as the neointimal area/medial area (neo/media). This is a common mathematical tool that is utilized to normalize the neointima area for individual differences in arterial sizes (18–20).

All data are expressed as mean ± SE. Comparisons between dietary groups by Student's t test. Differences were considered significant when $P < 0.05$.

Results

As shown in Table I, no significant difference in body weight was observed between CuD and CuA groups before or 14 days after arterial injury. Spectrometric analysis of hepatic copper content revealed significantly lower concentrations of copper in CuD compared to CuA rats (Table I).

No differences were observed in medial area and luminal area between uninjured carotid arteries from CuA and CuD groups. Luminal and medial area values of uninjured arteries were (mean ± SEM, in mm^2): CuA, 8.81 ± 0.77; CuD, 7.66 ± 0.49 for lumen, and CuA, 0.74 ± 0.32; CuD, 0.98 ± 0.27 for media. In contrast, the histomorphometric analysis of balloon-injured carotid arteries from CuD animals revealed a dramatic increase in neointima formation when compared with CuA animals (Table II), resulting in most cases in almost complete obstruction of the arterial lumen (Fig. 1).

Cross-sectional segments from injured carotid arteries were further analyzed under higher magnification ×400. The CuA group had concentric intimal hyperplasia consisting of totipotential mesenchymal cells and an intact intimal surface (Fig. 1). The CuD group had lumen occluding intimal proliferation with recanalization. There was also early collagen deposition with significant loss of inner medial fibers that were apparently incorporated into the neointima formation of injured CuD animals (Fig. 1). Figure 1 also demonstrates dramatic retraction of the medial wall in the CuD group.

Discussion

Smooth muscle cell proliferation is a common event featured in several cardiovascular diseases. From the development of the atheromatous plague to accelerated restenosis

Table I. Body Weight and Liver Copper Concentrations in Animals Before and After Angioplasty

Group	n	Body Weight (g)			Liver Cu (mg/g dry wt)
		Arrival	Before injury	After injury	
CuA	6	35 ± 5	221 ± 31	258 ± 25	11.51 ± .28
CuD	6	35 ± 5	210 ± 17	240 ± 16	1.93 ± .28[a]

Values are mean ± SEM.
[a] $P < 0.05$ by Student's t test.

Table II. Histomorphometric Analysis of Injured Common Carotid Arteries

Group	*n*	Lumen (mm^2)	Media (mm^2)	Neointima (mm^2)	Total area (mm^2)	Neo/Media
CuA	6	1.62 ± 0.14	1.18 ± 0.06	1.62 ± 0.18	3.98 ± 0.16	1.45 ± 0.22
CuD	6	0.99 ± 0.30[a]	0.44 ± 0.07[a]	2.03 ± 0.35	2.91 ± 0.29[a]	4.55 ± 0.93[a]

Values are mean ± SEM. Lumen, luminal area; Media, medial area; Total Area, total arterial area; Neo/Media, ratio of neointima area/media area; *n*, number of animals.
[a] $P < 0.05$ by Student's *t* test.

after angioplasty, smooth muscle cell plasticity has been associated with some interesting functions. It is able to regress into its undifferentiated embryological form and from there become a highly specialized collagen producer (21). Evidence of this change in the vascular smooth muscle is clearly seen in the vascular response of the rat carotid artery to disruption of the endothelium by balloon injury (Fig. 1). In particular, the response is accentuated in the CuD group where both the intimal hyperplasia consisting of the totipotential mesenchymal cells and the luminal deposition of collagen are significantly greater in the CuD group only 2 weeks after vascular injury (Fig. 1).

The most likely mechanism for this exaggerated stenosis in the CuD group is related to the redox imbalance, which occurs during the repair reaction and which has been shown to be a major contributor to restenosis following angioplasty (22–26). Azevedo *et al.* (26) have reported that the timing of the oxidative stress corresponds with the early inflammatory and proliferative phase of vascular repair.

There are several copper-dependent antioxidant enzymes in the cardiovascular system (for review, see Ref. 27). Of these, the activity of Cu/Zn-SOD is implicated by studies showing a role for superoxide in the early vascular smooth muscle mitogenesis associated with restenosis (28, 29). We have previously shown that Cu/Zn-SOD activity is significantly reduced in the wall of the aorta from rats fed the CuD diet used in the current study (30). Further, we have reported that the concentration of plasma peroxynitrite is increased in CuD rats, indicating an increased interaction between superoxide anion and nitric oxide *in vivo* (4). Together, these results demonstrate a decrease of Cu/Zn-SOD activity and consequent increase in superoxide in CuD rats.

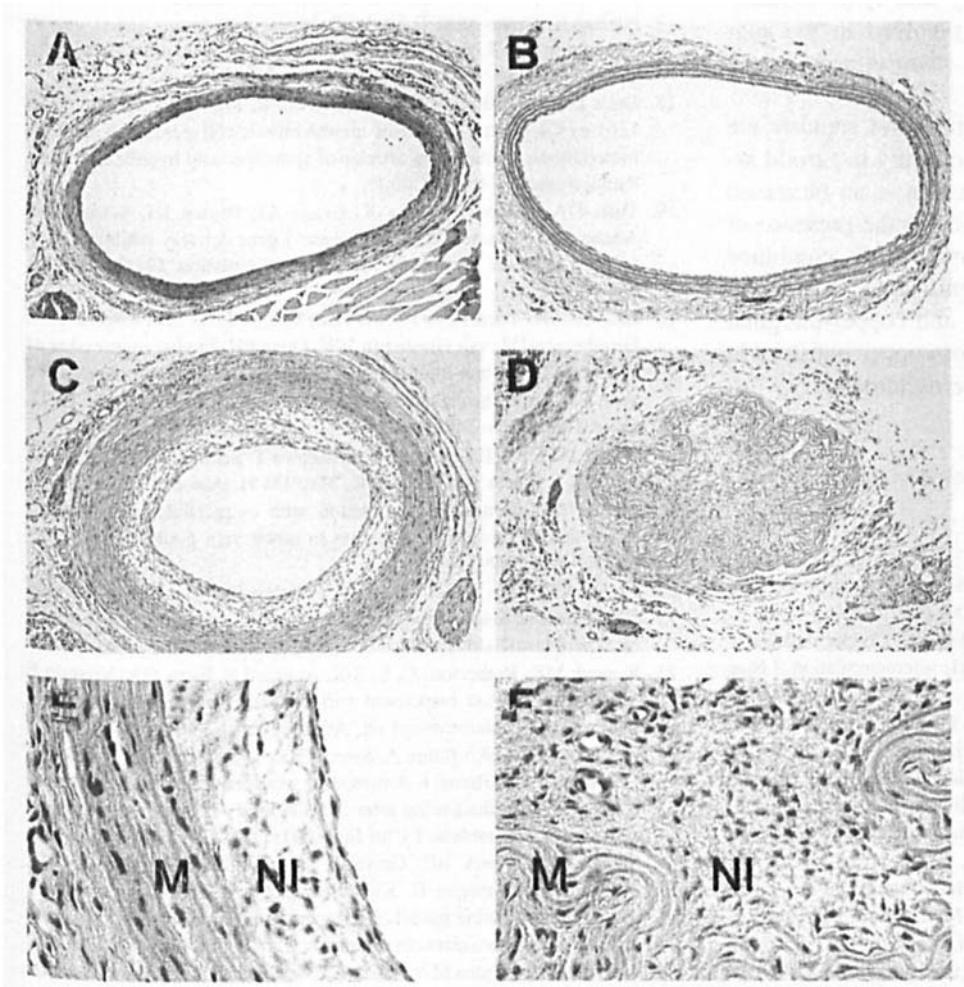

Figure 1. Photomicrographs of representative cross-sections of uninjured and balloon injured carotid arteries from rats fed CuA or CuD diets. (A) Uninjured CuA. (B) Uninjured CuD. (C and E) Injured CuA at ×25 and ×400. (D and F) Injured CuD at ×25 and ×400. M, media; NI, neointima.

Therefore, the exaggerated degree of restenosis seen in the CuD rats (Fig. 1) correlates with the inactivation of Cu/Zn-SOD by dietary copper restriction. This conclusion follows the assertion of Azevedo *et al.* (26) that oxidative stress may be a "dose-dependent" sensor of the degree of injury.

Generation of the reactive oxygen species following vascular injury occurs predominantly in the vascular smooth muscle cells (VSMC) or adventitial fibroblasts rather than in the endothelium (31, 32). Leukocyte-induced oxidative stress is also a component of the early inflammatory response to the balloon-induced injury (33). However, the respiratory burst is significantly diminished in CuD neutrophils (34). Therefore, VSMC or fibroblasts are probably the cell type involved with the balloon injury-induced oxidative stress in the CuD group.

A second possible mechanism for the augmented response seen in the CuD group involves the copper-dependent enzyme, lysyl oxidase. Lysyl oxidase is an amine oxidase in the vascular wall that initiates the covalent crosslinking of collagen and elastin. The enzyme is also potently chemotactic for VSMCs and is critical for repair of injuries in the cardiovascular system (35). However, this lysyl oxidase-dependent chemotaxis is abolished by inhibition of the enzyme's active site (35). Dietary copper deficiency is also known to inhibit lysyl oxidase activity in the rat (36). Therefore, it seems unlikely that lysyl oxidase-mediated chemotaxis of VSMCs is involved in the augmented neointimal proliferation and stenosis seen in the CuD group of the current study.

In conclusion, neointimal formation and stenosis are exaggerated following balloon-induced injury in carotid arteries of CuD rats. The likely mechanism is an increased superoxide-induced VSMC proliferation in the presence of reduced Cu/Zn-SOD activity. The current study, combined with previous data demonstrating significantly larger atherosclerotic lesions in aortas of CuD and copper-marginal rats (37), suggest an important role for copper nutriture in the vascular response to injury or atherosclerosis.

The authors acknowledge the technical assistance of Sharon Young, Gwen Dahlen, and Pete Leary.

1. Kitano S. Membrane and contractile properties of rat vascular tissue in copper-deficient conditions. Circ Res **46**:681–689, 1980.
2. Schuschke DA, Reed MWR, Saari JT, Miller FN. Copper deficiency alters vasodilation in the rat cremaster muscle microcirculation. J Nutr **122**:1547–1552, 1992.
3. Schuschke DA, Saari JT, Miller FN. A role for dietary copper in nitric oxide-mediated vasodilation. Microcirculation **2**:371–376, 1995.
4. 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 **7**:83–92, 2000.
5. Saari JT. Dietary copper deficiency and the endothelium-dependent relaxation of rat aorta. Proc Soc Exp Biol Med **200**:19–24, 1992.
6. Lynch SM, Frei B, Morrow JD, Roberts LJ, Xu A, Jackson T, Reyna R, Klevay LM, Vita JA, Keaney JF. Vascular superoxide dismutase deficiency impairs endothelial vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation. Atheroscler Thromb Vasc Biol **17**:2975–2981, 1997.
7. Schuschke DA, Saari JT, Ackermann DM, Miller FN. Microvascular responses in copper deficient rats. Am J Physiol **257**:H1607–H1612, 1989.
8. Schuschke DA, Saari JT, Nuss JW, Miller FN. Platelet thrombus formation and hemostasis are delayed in the copper deficient rat microcirculation. J Nutr **124**:1258–1264, 1994.
9. Schuschke LA, Saari JT, Miller FN, Schuschke DA. Hemostatic mechanisms in marginally copper-deficient rats. J Lab Clin Med **125**:748–753, 1995.
10. Lominadze DG, Saari JT, Miller FN, Catalfamo JL, Justus DE, Schuschke DA. Platelet aggregation and adhesion during dietary copper deficiency in rats. Thromb Haemostasis **75**:630–634, 1996.
11. Lominadze DG, Saari JT, Miller FN, Catalfamo JL, Schuschke DA. Von Willebrand factor restores impaired platelet thrombogenesis in copper-deficient rats. J Nutr **127**:1320–1327, 1997.
12. Kishore V, Wokocha B, Fourcade L. Effect of nutritional copper deficiency on carrageenin edema in the rat. Biol Trace Elem Res **23**:97–107, 1990.
13. Schuschke DA, Saari JT, Miller FN. The role of the mast cell in acute inflammatory responses of copper deficient rats. Agents Actions **42**:19–24, 1994.
14. Lentsch AB, Kato A, Saari JT, Schuschke DA. Augmented metalloproteinase activity and acute lung injury in copper deficient rats. Am J Physiol **281**:L387–L393, 2001.
15. Owen CA. Biochemical Aspects of Copper, Copper Proteins, Ceruloplasmin and Copper Protein Binding. Park Ridge, NJ: Noyes, 1982.
16. Johnson W, Kramer T. Effect of copper deficiency on erythrocyte membrane proteins in rats. J Nutr **117**:1085–1090, 1987.
17. Nielsen F, Zimmerman T, Shuler T. Interactions among nickel, copper and iron in rats: Liver and plasma content of lipids and trace elements. Biol Trace Elem Res **4**:125–143, 1982.
18. Dalle Lucca JJ, Borges ACR, Ponchirolli R, Melo SACS, Ihara SSM, Lindsey CJ, Paiva TB. Role of smooth muscle cell membrane potential in neointima formation in arteries of spontaneously hypertensive rats. Pathophysiol **7**:245–250, 2001.
19. Tulis DA, Durante W, Liu X, Evans AJ, Peyton KJ, Schafer AI. Adenovirus-mediated heme oxygenase-1 gene delivery inhibits injury-induced vascular neointima formation. Circulation **104**:2710–2715, 2001.
20. Lamfers ML, Lardenoye JH, de Vries MR, Aalders MC, Engelse MA, Grimbergen JM, van Hinsbergh VW, Quax PH. *In vivo* suppression of restenosis in balloon-injured rat carotid artery by adenovirus-mediated gene transfer of the cell surface-directed plasmin inhibitor ATF. BPTI. Gene Ther **8**:534–541, 2001.
21. Zhang WD, Bai HZ, Sawa Y, Yamakawa T, Kadoba K, Taniguchi K, Masuda J, Ogata J, Shirakura R, Matsuda H. Association of smooth muscle cell phenotypic modulation with extracellular matrix alterations during neointima formation in rabbit vein grafts. J Vasc Surg **30**:169–183, 1999.
22. Lafont AM, Chai Y-C, Cornhill JF, Whitlow PL, Howe PH, Chisholm GM. Effect of α-tocopherol on restenosis after angioplasty in a model of experimental altherosclerosis. J Clin Invest **95**:1018–1025, 1995.
23. Konneh MK, Rutherford C, Li S-R, Änggard E, Ferns GA. Vitamin E inhibits the intimal responseto balloon catheterinjury in the carotid artery of the cholesterol-fed rat. Atherosclerosis **113**:29–39, 1995.
24. Freyschuss A, Stiko-Rahm A, Swedenborg J, Henriksson P, Björkhem I, Berglund L, Nilsson J. Antioxidant treatment inhibits the development of intimal thickening after balloon injury of the aorta in hypercholesterolemic rabbits. J Clin Invest **91**:1282–1288, 1993.
25. Schneider JE, Berk BC, Gravanis MB, Santoian EC, Cipolla GD, Tarazona N, Lassegue B, King SB. Probucol decreases neointimal formation in a swine model of coronary artery balloon injury: A possible role for antioxidants in restenosis. Circulation **88**:627–628, 1993.
26. Azevedo LCP, Pedro MA, Souza LC, de Souza HP, Janiszewski M, da

Luz PL, Laurindo FRM. Oxidative stress as a signaling mechanism of the vascular response to injury: the redox hypothesis of restenosis. Cardiovasc Res **47**:436–445, 2000.
27. Saari JT, Schuschke DA. Cardiovascular effects of dietary copper deficiency. BioFactors **10**:359–375, 1999.
28. Li PF, Dietz R, von Harsdorf R. Differential effect of hydrogen peroxide and superoxide anion on apoptosis and proliferation of vascular smooth muscle cells. Circulation **96**:3602–3609, 1997.
29. Lauridino FRM, da Luz PL, Uint L, Rocha TF, Jaeger RG, Lopes EA. Evidence for superoxide radical-dependent coronary vasospasm after angioplasty in intact dogs. Circulation **83**:1705–1715, 1991.
30. Schuschke DA, Percival SS, Saari JT, Miller FN. Relationship between dietary copper and acetylcholine-induced vasodilation in the microcirculation of rats. BioFactors **10**:321–327, 1999.
31. Nunes GL, Robinson K, Kalnych A, King SB, Sgoutas DS, Berk BC. Vitamines C and E inhibit O_2^- production in pig coronary arteries. Circulation **96**:3593–3601, 1997.
32. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci U S A **94**:14483–14488, 1997.
33. Serrano CV, Ramires JA, Venturinelli M, Arie S, D'Amico E, Zweier JL, Pileggi F, da Luz PL. Coronary angioplasty results in leukocyte and platelet activation with adhesion molecule expression: evidence of inflammatory responses in coronary angioplasty. J Am Coll Cardiol **29**:1276–1283, 1997.
34. Babu U, Failla ML. Copper status and function of neutrophils are reversibly depressed in marginally and severely copper-deficient rats. J Nutr **120**:1700–1709, 1990.
35. Li W, Liu G, Chou I-N, Kagan HM. Hydrogen peroxide-mediated, lysyl oxidase-dependent chemotaxis of vascular smooth muscle cells. J Cell Biochem **78**:550–557, 2000.
36. Ruckker RB, Romero-Chapman N, Wong T, Lee J, Steinberg M, Mcgee C, Clegg S, Reiser K, Kosonen T, Uriu-Hare JY, Murphy J, Keen CL. Modulation of lysyl oxidase by dietary copper in rats. J Nutr **126**:51–60, 1996.
37. Iona M, Hamilton J, Gilmore WS, Strain JJ. Marginal copper deficiency and atherosclerosis. Biol Trace Elem Res **78**:179–189, 2000.