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Inhibition of Purified Soluble Guanylyl Cyclase by Copper Ions

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ABSTRACT. The aim of the present study was to investigate the effect of Cu(II) ions on soluble guanylyl cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2; sGC] and to test for a possible physiological role of this putative cofactor of the enzyme [Gerzer et al., *FEBS Lett.* **132:** 71–74, 1981]. $CuSO_4$ was found to inhibit NO-stimulated sGC with an IC_{50} of 2.2 ± 0.3 μM. Virtually complete inhibition of guanosine-3′,5′-cyclic monophosphate (cGMP) formation was observed at 10 μM of the copper salt. Presence of $CuSO_4$ (2 μM) did not significantly affect the potency of 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) but did markedly decrease maximal cyclase activity from 3.71 ± 0.2 μmol cGMP × mg^{-1} × min^{-1} to 1.75 ± 0.2 μmol cGMP × mg^{-1} × min^{-1}. The nonstimulated enzyme was also sensitive to $CuSO_4$ (IC_{50} of 6.2 ± 1.2 μM). Addition of glutathione, which potently complexes Cu(I) ions, induced a pronounced rightward shift of the concentration-response curves for inhibition by $CuSO_4$ of both DEA/NO-stimulated and nonstimulated guanylyl cyclase. The inhibitory effect of $CuSO_4$ was completely antagonized by the specific Cu(I) chelator neocuproine, with a half-maximal effect at 5.9 ± 0.2 μM. In contrast, the Cu(II) chelator cuprizone and several thiols, which do not form stable Cu(I) complexes, were far less protective. Our results suggest that inhibition of soluble guanylyl cyclase by $CuSO_4$ is unrelated to heme-mediated enzyme stimulation and may arise from the reversible high affinity binding of Cu(I) ions to a site of the protein that is critically involved in enzyme catalysis. BIOCHEM PHARMACOL 52;7: 1041–1045, 1996.

KEY WORDS. soluble guanylyl cyclase; $CuSO_4$-induced enzyme inhibition; glutathione; neocuproine; cuprizone; Cu(I) ions

sGC§ [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2], which catalyzes the formation of cGMP from GTP, represents the best characterized physiological target of nitric oxide. The enzyme is stimulated several hundredfold in the presence of submicromolar concentrations of NO [1, 2], resulting in a pronounced accumulation of cGMP, an important second messenger involved in a variety of physiological processes, such as smooth muscle relaxation, platelet aggregation, and modulation of neurotransmitter release [3, 4]. Impaired NO/cGMP signaling has been implicated in various cardiovascular and neurological disease states, such as ischemia-reperfusion injury of the heart, stroke, and atherosclerosis [5–7]. sGC represents a heterodimer, composed of an α-subunit with a molecular mass of 77 kDa and a 70-kDa β-subunit [8, 9]. The enzyme was reported to contain stoichiometric amounts of ferro-protoporphyrin-IX bound to histidine-105 of the β-subunit [10, 11]. NO binds with high affinity to the prosthetic heme group of sGC, resulting in formation of a ferrous-nitrosyl-heme complex and subsequent enzyme activation [12]. This hypothesis is supported by the recent observation that a heme-deficient mutant of sGC was still capable of catalyzing the conversion of GTP to cGMP but failed to respond to NO [11].

Analysis of sGC by atomic absorption spectroscopy has revealed that the enzyme contains stoichiometric amounts of copper [10], but the function of this putative cofactor has not yet been elucidated. Containing a histidine-ligated heme group with high affinity for NO, sGC resembles hemoglobin, which was reported to exhibit a high affinity copper binding site, presumably being involved in the thiol-sensitive oxidation of the ferrous heme in the β-subunits of the protein [13, 14]. To find out whether a similar mechanism accounts for the heme-mediated activation/deactivation of sGC, we studied the effects of $CuSO_4$ on the activity of the enzyme purified from bovine lung. It was found that $CuSO_4$ potently inhibited both basal and NO-

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§ *Abbreviations:* sGC, soluble guanylyl cyclase; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; cGMP, guanosine-3′,5′-cyclic monophosphate; GTP, guanosine-5′-triphosphate; GSH, glutathione.

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stimulated cyclase activity and that this inhibitory effect was most likely mediated by Cu(I) ions.

MATERIALS AND METHODS

Materials

Bovine lung sGC was purified from baculovirus-infected Sf9 cells as described elsewhere [11, 15]. [α-^{32}P]GTP (400 Ci/mmol) was purchased from Med Pro (Amersham, Vienna, Austria). DEA/NO [16] was obtained from Research Biochemicals International (RBI, Natick, MA, USA). Tenfold concentrated stock solutions of DEA/NO were prepared in 10 mM NaOH. Chelex® 100 chelating ion exchange resin (analytical grade) was obtained from Bio Rad Laboratories (Hercules, CA, USA). GSH, 1,10-dimethyl-2,9-phenanthroline (neocuproine), bis-cyclohexanone dihydrazone (cuprizone), and all other chemicals were purchased from Sigma (Vienna, Austria).

Determination of Guanylyl Cyclase Activity

Purified sGC (45 ng) was incubated at 37°C for 10 min in a total volume of 0.1 mL of a 50-mM triethanolamine/HCl-buffer, pH 7.4, containing 0.5 mM [α-^{32}P] GTP (200,000–300,000 cpm), 3 mM $MgCl_2$, and 1 mM cGMP. $CuSO_4$, thiols, and chelators were present as indicated. Reactions were started by adding 10-fold concentrated stock solutions of DEA/NO to the samples and stopped by $ZnCO_3$ precipitation followed by the isolation of ^{32}P-cGMP as described elsewhere [17]. For determination of basal activity, reactions were started by transferring the assay mixtures from 0 to 37°C. Basal sGC activity was not detectable when incubated on ice (not shown). Results were corrected for enzyme-deficient blanks and recovery of cGMP. For the determination of basal sGC activity, incubations were performed with 0.2 µg of enzyme for 20 min. All solutions were treated with Chelex® resin (5 g/100 mL) to remove endogenous trace metals. Data represent mean ± SE values of three experiments performed in duplicate. Parameters of the concentration-response curves were calculated according to the Hill equation.

Other Methods

NO was determined electrochemically with a Clark-type electrode (Iso-NO, World Precision Instruments, Mauer, Germany) as previously described [18]. Protein was determined by the Bradford method [19] using bovine serum albumin as standard.

RESULTS

$CuSO_4$ was found to inhibit both NO-stimulated and basal sGC activity in a concentration-dependent manner. The enzyme stimulated with 1 µM DEA/NO was inhibited by $CuSO_4$ with an IC_{50} of 2.2 ± 0.3 µM when assayed in the absence of added GSH. Enzyme inhibition was complete at

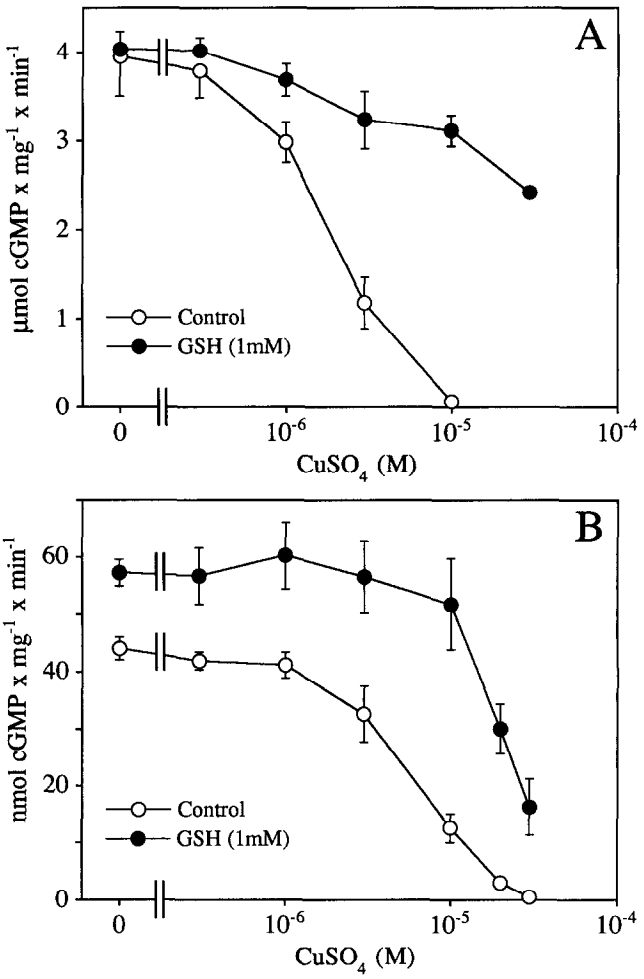

FIG. 1. Effect of $CuSO_4$ on DEA/NO-stimulated and basal sGC activity. Purified sGC (45 ng) was stimulated with 1 µM DEA/NO (A) and incubated at 37°C for 10 min with increasing concentrations of $CuSO_4$. Enzyme activity was determined in the absence (open circles) and presence (filled circles) of 1 mM GSH as described in Materials and Methods. For determination of basal activity (B), 0.2 µg of purified sGC was incubated at 37°C for 20 min with increasing concentrations of $CuSO_4$ in the absence (open circles) and presence (filled circles) of 1 mM GSH. Samples were assayed for enzyme activity as described in Materials and Methods. Data represent mean ± SE values of three experiments performed in duplicate.

10 µM $CuSO_4$ (Fig. 1A). Addition of 1 mM GSH caused a pronounced rightward shift of the concentration-response curve with only 23 ± 2% inhibition produced by 10 µM $CuSO_4$. Enzyme activity determined in the absence of DEA/NO was also sensitive to copper ions. As shown in Fig. 1B, $CuSO_4$ induced a concentration-dependent inhibition of basal guanylyl cyclase activity, with IC_{50} values of 6.2 ± 1.2 and 27.2 ± 5.7 µM in the absence and presence of added GSH, respectively. $CuSO_4$-mediated enzyme inhibition was fully reversible, as revealed by preincubation of sGC for 10 min at ambient temperature in the presence of different concentrations of $CuSO_4$, followed by determination of cGMP formation of the 10-fold diluted samples (data not shown).

To investigate whether the inhibitory effect of $CuSO_4$ was competitive with NO, sGC was incubated in the presence of increasing concentrations of DEA/NO with and without 2 μM $CuSO_4$. As shown in Fig. 2, the NO donor increased sGC activity up to 3.71 ± 0.2 μmol cGMP × mg^{-1} × min^{-1}, with an EC_{50} of 129 ± 3 nM and a maximally effective concentration of 1 – 10 μM. Presence of $CuSO_4$ did not appreciably affect the potency of DEA/NO (EC_{50} = 99 ± 7 nM) but did markedly decrease maximal cyclase activity to 1.75 ± 0.1 μmol cGMP × mg^{-1} × min^{-1}. The non-NO competitive nature of $CuSO_4$-induced enzyme inhibition was further confirmed by electrochemical experiments. It was found that 3 μM $CuSO_4$ had no significant effect on the kinetics of NO autooxidation as measured with an NO-sensitive electrode in the presence of 10 μM DEA/NO under sGC assay conditions (data not shown).

To investigate whether the observed protection of the enzyme by GSH was due to a nonspecific reduction of essential protein sulfhydryl groups, GSH, L-cysteine, D,L-penicillamine, and dithiothreitol (1 mM each) were tested for their ability to prevent $CuSO_4$-induced inhibition of sGC. When the enzyme was incubated with and without $CuSO_4$ in the absence and presence of the various thiols, GSH was the sole compound that showed a significant effect. In the absence of added thiols, 3 μM $CuSO_4$ inhibited the enzyme down to ~40% of controls (from 4.64 ± 0.85 to 2.04 ± 0.51 μmol cGMP × mg^{-1} × min^{-1}). GSH (1 mM) markedly antagonized the effect of $CuSO_4$, which produced only ~13% of inhibition (4.04 ± 0.78 μmol cGMP × mg^{-1} × min^{-1}) in the presence of this thiol. The other sulfhydryl compounds had no significant effect on the $CuSO_4$-inhibited enzyme activity (2.01 ± 0.47, 1.92 ± 0.37, and 2.46 ± 0.38 μmol cGMP × mg^{-1} × min^{-1} in the presence of 1 mM L-cysteine, D,L-penicillamine, and dithiothreitol, respectively).

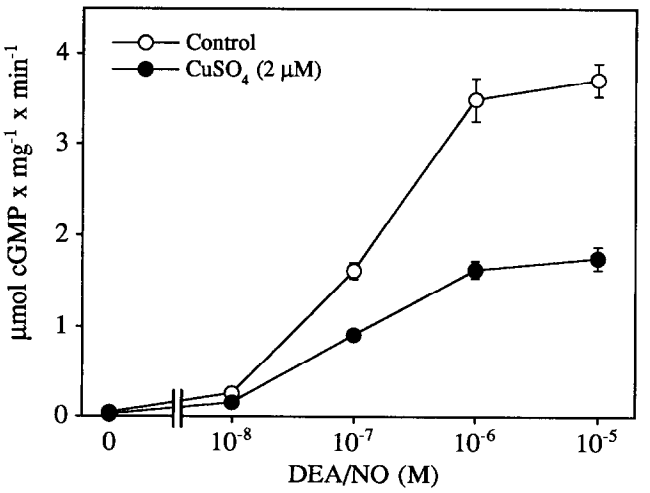

FIG. 2. Effect of $CuSO_4$ on the concentration-dependent stimulation of sGC by DEA/NO. Purified cyclase (45 ng) was incubated at 37°C for 10 min with increasing concentrations of DEA/NO in the absence (open circles) and presence (filled circles) of 2 μM $CuSO_4$. Enzyme activity was determined as described in Materials and Methods. Data represent mean ± SE values of three experiments performed in duplicate.

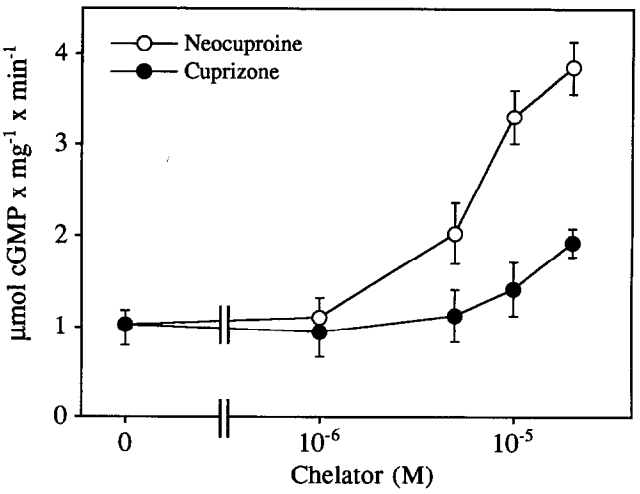

FIG. 3. Effect of copper ion chelators on $CuSO_4$-induced inhibition of sGC. Purified sGC (45 ng) was stimulated with 1 μM DEA/NO and incubated at 37°C for 10 min in the presence of 3 μM $CuSO_4$ and increasing concentrations of neocuproine (open circles) or cuprizone (filled circles). Samples were assayed for enzyme activity as described in Materials and Methods. Data represent mean ± SE values of three experiments performed in duplicate.

In earlier studies, GSH was reported to form stable complexes with Cu(I) ions [20]. In view of the fact that $CuSO_4$-induced enzyme inhibition was prevented by GSH but not by other thiols, the effect of $CuSO_4$ may have been mediated by Cu(I) ions. To address this issue, we studied the effect of neocuproine, an agent with high Cu(I) selectivity, and cuprizone, a well-established Cu(II) chelator [21] on sGC activity in the presence of 3 μM $CuSO_4$. As shown in Fig. 3, the Cu(I) chelator neocuproine almost completely restored the enzymatic activity with an EC_{50} of 5.9 ± 0.2 μM, whereas cuprizone showed much less potency (EC_{50} > 15 μM). Neither chelator had any significant effect on cGMP-accumulation in the absence of $CuSO_4$ (data not shown).

DISCUSSION

In the present study, we have demonstrated that recombinant bovine lung sGC was inhibited in a reversible manner by micromolar concentrations of $CuSO_4$. Previous reports have shown that Cu(II) ions induced rapid oxidation of hemoglobin to methemoglobin, a reaction that was prevented by addition of certain thiols [14]. Because oxidation of the prosthetic heme group by drugs like methylene blue [22] or the quinoxaline derivative ODQ [23, 24] leads to inhibition of NO-stimulated sGC, we speculated that $CuSO_4$-mediated enzyme inhibition may also result from oxidation of the heme-iron, leading to a reduced NO sensitivity of sGC. However, our data indicate that the inhibi-

tory effect of Cu(II) ions was not due to interference with NO stimulation of sGC because (i) $CuSO_4$ inhibited both basal and NO-stimulated enzyme activity with similar potency, (ii) enzyme inhibition was not overcome at higher concentrations of the NO donor DEA/NO, and (iii) $CuSO_4$ did not induce superoxide-mediated inactivation of NO as revealed from electrochemical determination of NO released from DEA/NO in the absence and presence of the copper salt. Furthermore, our results suggest that the inhibitory effect of $CuSO_4$ on sGC is mediated by Cu(I) rather than by Cu(II). Inhibition of the enzyme was almost completely prevented by the Cu(I) chelating compounds neocuproine and GSH, whereas several other thiols and cuprizone, which do not form stable Cu(I) chelates, were much less effective. Based on these results, we suggest that the inhibition of sGC by $CuSO_4$ arises from high affinity binding of Cu(I) ions to a site of the protein critically involved in its catalytic function. The mechanism accounting for reduction of Cu(II) to Cu(I) remains to be clarified. The reaction may be driven by GSH, which was endogenously present in the sGC preparations we used, resulting in final assay concentrations of ~4 μM. This finding is supported by recent data from our laboratory showing that low concentrations of GSH are effective in reducing Cu(II) to Cu(I), whereas appreciable formation of Cu(I)-GSH chelates occurs only at high concentrations of the thiol [25].

Cu(I)-mediated inhibition of sGC may not play a major role under normal physiological conditions because intracellular GSH levels are in the millimolar range [26] and copper occurs primarily in chelated redox-inactive forms [27]. However, the observed effect may become significant under different pathophysiological conditions. The Cu(I)-chelating capacity of cells could be reduced upon depletion of intracellular GSH pools as occurs in situations of oxidative stress [28]. On the other hand, peroxynitrite, which is extensively produced in atherosclerosis [29], was reported to release Cu(I) from the storage protein caeruloplasmin, thereby enhancing lipid peroxidative processes and formation of atherosclerotic lesions [30]. Another study has demonstrated that redox-active copper is mobilized in the course of myocardial ischaemia, causing heart injury through production of hydroxyl radicals [27]. Thus, impaired accumulation of cGMP induced by free Cu(I) ions may become relevant in pathological situations and contribute to the progress of certain disease states.

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References

1. Waldman S and Murad F, Cyclic GMP synthesis and function. *Pharmacol Rev* **39:** 163–195, 1987.
2. Ignarro LJ, Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. *Blood Vess* **28:** 67–73, 1991.
3. Moncada S, Palmer RMJ and Higgs EA, Nitric oxide—physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:** 109–142, 1991.
4. Garthwaite J and Boulton CL, Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* **57:** 683–706, 1995.
5. Bruckdorfer KR, Jacobs M and Rice-Evans C, Endothelium-derived relaxing factor (nitric oxide), lipoprotein oxidation and atherosclerosis. *Biochem Soc Transactions* **18:** 1061–1063, 1990.
6. Coyle JT and Puttfarcken P, Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262:** 689–695, 1993.
7. Lefer AM and Lefer DJ, Pharmacology of the endothelium in ischemia-reperfusion and circulatory shock. *Annu Rev Pharmacol Toxicol* **33:** 71–90, 1993.
8. Koesling D, Böhme E and Schultz G, Guanylyl cyclases, a growing family of signal transducing enzymes. *FASEB J* **5:** 2785–2791, 1991.
9. Schulz S, Yuen PST and Garbers DL, The expanding family of guanylyl cyclases. *Trends Pharmacol Sci* **12:** 116–120, 1991.
10. Gerzer R, Böhme E, Hofmann F and Schultz G, Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS Lett* **132:** 71–74, 1981.
11. Wedel B, Humbert P, Harteneck C, Foerster J, Malkewitz J, Böhme E, Schultz G and Koesling D, Mutation of His-105 of the β1-subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc Natl Acad Sci USA* **91:** 2592–2596, 1994.
12. Traylor TG and Sharma VS, Why NO. *Biochemistry* **31:** 2847–2849, 1992.
13. Rifkind JM, Copper and the oxidation of hemoglobins. In: *Metal Ions in Biological Systems*, Vol. 12 (Ed. Sigel H), pp. 191–232. Marcel Dekker, New York, 1981.
14. Smith RC, Reed VD and Webb TR. Reversal of copper(II)-induced methemoglobin formation by thiols. *J Inorg Biochem* **52:** 173–182, 1993.
15. Humbert P, Niroomand F, Fischer G, Mayer B, Koesling D, Hinsch K-D, Gausepohl H, Frank R, Schultz G and Böhme E, Purification of soluble guanylyl cyclase from bovine lung by a new immunoaffinity chromatographic method. *Eur J Biochem* **190:** 273–278, 1990.
16. Maragos CM, Morley D, Wink DA, Dunams TM, Saavedra JE, Hoffman A, Bove AA, Isaac L, Hrabie JA and Keefer LK, Complexes of NO with nucleophiles as agents for the controlled biological release of nitric oxide–vasorelaxant effects. *J Med Chem* **34:** 3242–3247, 1991.
17. Schultz G and Böhme E, Guanylate cyclase. GTP pyrophosphate-lyase (cyclizing), E.C.4.6.1.2. In: *Methods of Enzymatic Analysis*, Vol. 4 (Eds. Bergmeyer HU, Bergmeyer J and Graßl M), pp. 379–389. Verlag Chemie, Weinheim, Germany, 1984.
18. Mayer B, Klatt P, Werner ER and Schmidt K, Kinetics and mechanism of tetrahydrobiopterin-induced oxidation of nitric oxide. *J Biol Chem* **270:** 655–659, 1995.
19. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
20. Spear N and Aust SD, Hydroxylation of deoxyguanosine in DNA by copper and thiols. *Arch Biochem Biophys* **317:** 142–148, 1995.
21. Gordge MP, Meyer DJ, Hothersall J, Neild GH, Payne NN and Noron-Hadutra A, Copper chelation-induced reduction of the biological activity of S-nitrosothiols. *Br J Pharmacol* **114:** 1083–1089, 1995.
22. Mayer B, Brunner F and Schmidt K, Inhibition of nitric oxide synthesis by methylene blue. *Biochem Pharmacol* **45:** 367–374, 1993.
23. Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt

K and Mayer B, Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one. *Mol Pharmacol* **48:** 184–188, 1995.
24. Schrammel A, Behrends S, Schmidt K, Koesling D and Mayer B, Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol Pharmacol* **50:** 1–5, 1996.
25. Gorren ACF, Schrammel A, Schmidt K and Mayer B, Decomposition of S-nitrosoglutathione in the presence of copper ions and glutathione. *Arch Biochem Biophys* **330:** 219–228, 1996.
26. Tietze F, Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal Biochem* **27:** 502–522, 1969.
27. Chevion M, Jiang YD, Harel R, Berenshtein E, Uretzky G and Kitrossky N, Copper and iron are mobilized following myocardial ischemia—possible predictive criteria for tissue injury. *Proc Natl Acad Sci USA* **90:** 1102–1106, 1993.
28. Bray TM and Taylor CG, Tissue glutathione, nutrition, and oxidative stress. *Can J Physiol Pharmacol* **71:** 746–751, 1993.
29. Beckmann JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM and White CR, Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe Seyler* **375:** 81–88, 1994.
30. Swain JA, Darley-Usmar V and Gutteridge JMC, Peroxynitrite releases copper from caeruloplasmin—implications for atherosclerosis. *FEBS Lett* **342:** 49–52, 1994.