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Effect of copper on nitric oxide synthase and guanylyl cyclase activity in the rat isolated aorta

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1 The potential role of copper (Cu^{2+}) in modulating the activity of nitric oxide synthase (NOS) and guanylyl cyclase (GC) was investigated by use of diethyldithiocarbamic acid (DEDCA), a high affinity Cu^{2+} chelator.

2 DEDCA 100 μM inhibited sodium nitroprusside (SNP; 0.005–10 μM)-evoked relaxation of rat isolated aortic rings precontracted with 3 μM phenylephrine (PE). A lower concentration of DEDCA (10 μM) did not significantly attenuate SNP-evoked responses but did inhibit relaxation to the endothelium-dependent dilator, A23187 (0.01–10 μM).

3 The presence of 100 μM Cu^{2+}, but not 100 μM Fe^{2+}, alone enhanced A23187- and SNP-evoked relaxation of aortae precontracted with PE.

4 The inhibitory effect of DEDCA on SNP- and A23187-induced relaxation was reversed by equimolar concentrations of Cu^{2+} but not Fe^{2+}, indicating that DEDCA does not act via removal of haem-iron from the NOS and GC complexes.

5 Superoxide dismutase (30 μ ml^{-1}) was without effect on the inhibition of DEDCA relaxation induced by either SNP or A23187 in aortae precontracted with PE.

6 When assessed by radioimmunoassay, DEDCA inhibited SNP- and A23187-stimulated cyclic GMP formation with IC_{50} values of 0.5 μM and 50 μM, respectively.

7 These data demonstrate that Cu^{2+} plays a role in controlling NOS and GC activity in the rat aorta.

Keywords: Copper; nitric oxide synthase; guanylyl cyclase; cardiovascular disease

Introduction

The nitric oxide (NO)-guanylyl cyclase (GC) axis plays a variety of protective roles in the cardiovascular system: vasodilatation, inhibition of platelet and leukocyte activity, inhibition of vascular smooth muscle cell (VSMC) proliferation, scavenging of free radicals and modulation of lipid metabolism (Gryglewski *et al*., 1995; Darley-Usmar *et al*., 1995; Jeremy *et al*., 1996). Disruption of NO formation and actions has been widely implicated in the pathophysiology of atherosclerosis and associated cardiovascular complications, including thrombosis, myocardial infarction (MI) and reperfusion injury (McCord, 1985; Moncada & Higgs, 1993; Gryglewski *et al*., 1995; Darley-Usmar *et al*., 1995).

It has become increasingly apparent that transition elements such as Cu^{2+} may contribute to atherogenesis MI and reperfusion injury (Aalbers *et al*., 1985). For example, positive correlations between serum Cu^{2+} levels and the presence of atherosclerosis (Iskra *et al*., 1993) and acute MI have been obtained (Salonen *et al*., 1991a,b; Pucheu *et al*., 1995). Atherosclerotic lesions from man and experimental animals also contain high levels of Cu^{2+} (Smith *et al*., 1992; Swain & Gutteridge, 1995; Vlad *et al*., 1995). There is also evidence that copper mediates lipoxygenase activity and thromboxane A_2 formation in platelets (Jeremy *et al*., 1995).

The pathological impact of copper is ultimately mediated by its capacity to catalyse the generation of superoxide (O_2^-) and hydroxyl radicals from hydrogen peroxide (Denko, 1989). Among their wide range of deleterious actions, these free radicals exert damaging effects on biomembranes and oxidation of low density lipoproteins (LDL). Oxidised LDL have been widely implicated in the pathophysiology of atherosclerosis and exert deleterious effects on platelets, leukocytes, foam cells, VSMC proliferation, NO release as well as potentiating responses to vasoconstrictors (Berliner & Heinecke, 1996). Although little is known of NO-Cu^{2+} interactions, NO reacts readily with O_2^- to generate the toxic free radical, peroxynitrite ($ONOO^-$; Darley-Usmar *et al*., 1995). Thus, it is conceivable that an excess of O_2^- generated by Cu^{2+} may result in altered NO activity. The activity of both NO synthase (NOS) and guanylyl cyclase (GC) involves redox reactions catalysed by haem iron (Ignarro, 1990). Since iron and Cu^{2+} possess similar properties, it is reasonable to suggest that copper imbalance may influence the NO-GC axis which in turn may be of relevance to the pathophysiology of atherogenesis and associated cardiovascular complications.

In order to investigate the potential role of Cu^{2+} in modulating NOS and GC activity, the effect of the high affinity Cu^{2+} chelator, diethyldithiocarbamic acid (DEDCA) on these enzymes was investigated in the rat aorta by use of both biochemical and organ bath techniques. DEDCA has a high affinity for Cu^{2+}, much more than for other metals, but more importantly does not chelate calcium or magnesium (Renoux, 1982). Dithiocarbamyl chelators are a family of drugs that have been used therapeutically as antioxidants, in the detoxification of heavy metals, in the treatment of AIDS and in alcohol addiction (Renoux, 1982).

A preliminary account of this study has been presented to the British Pharmacological society (Wigmore *et al*., 1996).

Methods

Organ bath studies

Male Wistar Kyoto rats (250–350 g) were stunned and decapitated. The descending aorta was rapidly removed, cleared of adhering fat and connective tissue and cut into cylindrical segments approximately 2 mm in length. Segments were then mounted in organ baths containing oxygenated Krebs solution, maintained at 37°C and gassed with 95% O_2; 5% CO_2. Tension was measured by a Grass FTO3 force displacement transducer and data recorded on disc (MacLab). Tissues were reset to a tension of 1.5 g and left to equilibrate in the buffer

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for 30 min after which time the tension was reset. Aortic rings were pre-contracted with phenylephrine (PE; 1–3 μM) and relaxed with cumulative doses of acetylcholine (ACh; 0.01–10 μM) to assess the integrity of the endothelium. Following washout and equilibration, tissues were recontracted and relaxed with cumulative doses of the endothelium-dependent calcium ionophore A23187 (0.01–10 μM) or the endothelium-independent vasodilator, sodium nitroprusside (SNP) in the presence or absence of DEDCA and/or divalent Cu^{2+} and Fe^{2+} or superoxide dismutase (SOD).

Cyclic GMP formation

Following pre-incubation, aortic rings, in duplicate for each drug dose, were placed into polypropylene tubes containing Dulbecco's minimum essential medium (DMEM), 250 μM isobutylmethylxanthine (a non-specific phosphodiesterase inhibitor) and various concentrations of DEDCA (with and without $CuCl_2$ and Fe_2Cl_3) and incubated for 30 min at 37°C in a shaking water bath. Guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation was stimulated with 10 μM SNP and 1 μM A23187. Tubes were then incubated for a further 20 min at 37°C. Reactions were stopped by the addition of 1 M perchloric acid and the tissues sonicated (3×30 s; Soniprep, MSE). Following centrifugation at 1000 *g* for 15 min, supernatants were taken and neutralized with 1 M K_3PO_4. Aliquots were then taken and acetylated with triethylamine/acetic anhydride (1/2; v/v). Concentrations of cyclic GMP were measured by use of specific ^{125}I-radioimmunoassay kits as previously described (Jeremy *et al.*, 1996). The inter-assay and intra-assay coefficients of variation derived from 20 estimations for the cyclic GMP assay were 4% and 1%, respectively.

Drugs and materials

Diethyldithiocarbamic acid was purchased from Aldrich Chemical Co. (Poole, Dorset, U.K.). All other chemicals and drugs were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Dual range ^{125}I cyclic GMP kits were purchased from Amersham Radiochemicals (Amersham, U.K.). The Krebs solution (pH 7.4) had the following composition (mM): NaCl 120, KCl 4.7, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 5.

Analysis of data

Responses of isolated aortic rings to A23187 and SNP are expressed as % relaxation of PE-induced tone. All results are expressed as mean ± s.e.mean for six separate experiments and comparisons were made by use of Student's *t* test for unpaired samples where $P < 0.05$ was considered significant.

Results

Organ bath study

The calcium ionophore A23187 (0.01–10 μM) evoked concentration-dependent and endothelium-dependent relaxation of rat isolated aortic rings pre-contracted with PE (1–3 μM). In the presence of DEDCA (10 μM and 100 μM; 10 min) contractions to PE were not significantly altered but A23187-evoked relaxation was significantly inhibited (Figure 1a). The maximal relaxation to A23187 was reduced from 90 ± 4% ($n = 6$) to 15 ± 7% and 6 ± 3% ($n = 6$, $P < 0.001$), in the presence of 10 μM and 100 μM DEDCA, respectively (Figure 1a).

SNP evoked concentration-dependent relaxation of endothelium-denuded aortic rings pre-contracted with PE. At a concentration of 10 μM, DEDCA did not significantly inhibit SNP-evoked responses (Figure 1b). However, a higher concentration of DEDCA (100 μM) inhibited SNP-evoked relaxation to a similar extent as A23187-induced relaxation; the maximum response was reduced from 92 ± 4.4% to 7 ± 4.9% ($n = 6$; $P < 0.001$). The inhibitory effect of DEDCA on either A23187- or SNP-evoked relaxation was reversed by the addition of equimolar concentrations of Cu^{2+} (Figure 2a and b) but not Fe^{2+} (Figure 3a and b). The presence of Cu^{2+} alone significantly enhanced A23187- and SNP-evoked relaxation of PE contracted aortic rings (Figure 2a and b). In contrast, Fe^{2+} did not alter the responses to either A23187 or SNP (Figure 3a and b). Superoxide dismutase (SOD; 30 μ ml^{-1}) did not alter relaxation to either A23187 or SNP (Figure 4a and b). Furthermore, the presence of SOD did not prevent the inhibition of A23187- and SNP-evoked relaxation caused by DEDCA (Figure 4a and b).

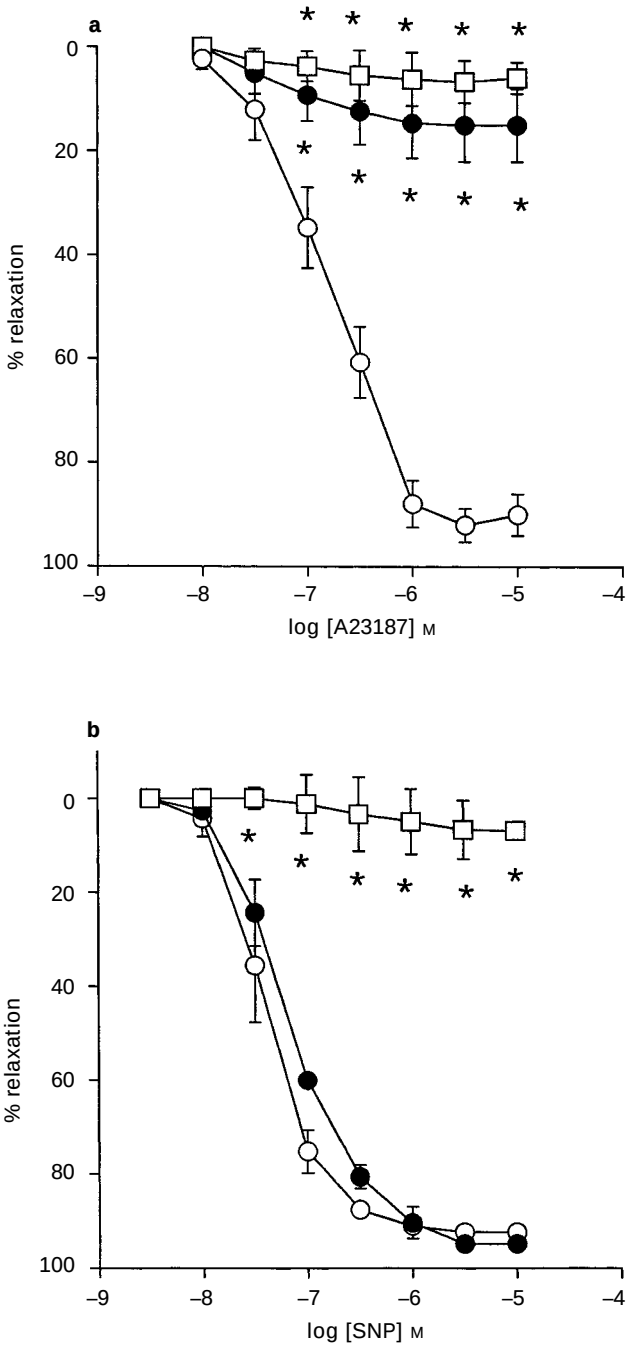

Figure 1 (a) Effect of diethyldithiocarbamic acid (DEDCA), (●) 10 and (□) 100 μM, on calcium ionophore A23187 (A23187)-evoked relaxation of rat aortic rings precontracted with phenylephrine (PE); (○) control. (b) Effect of DEDCA, (●) 10 and (□) 100 μM, on sodium nitroprusside (SNP)-evoked relaxation of rat aortic rings precontracted with PE; (○) control. Each point is the mean, $n = 6$; vertical lines show s.e.mean. *$P < 0.001$.

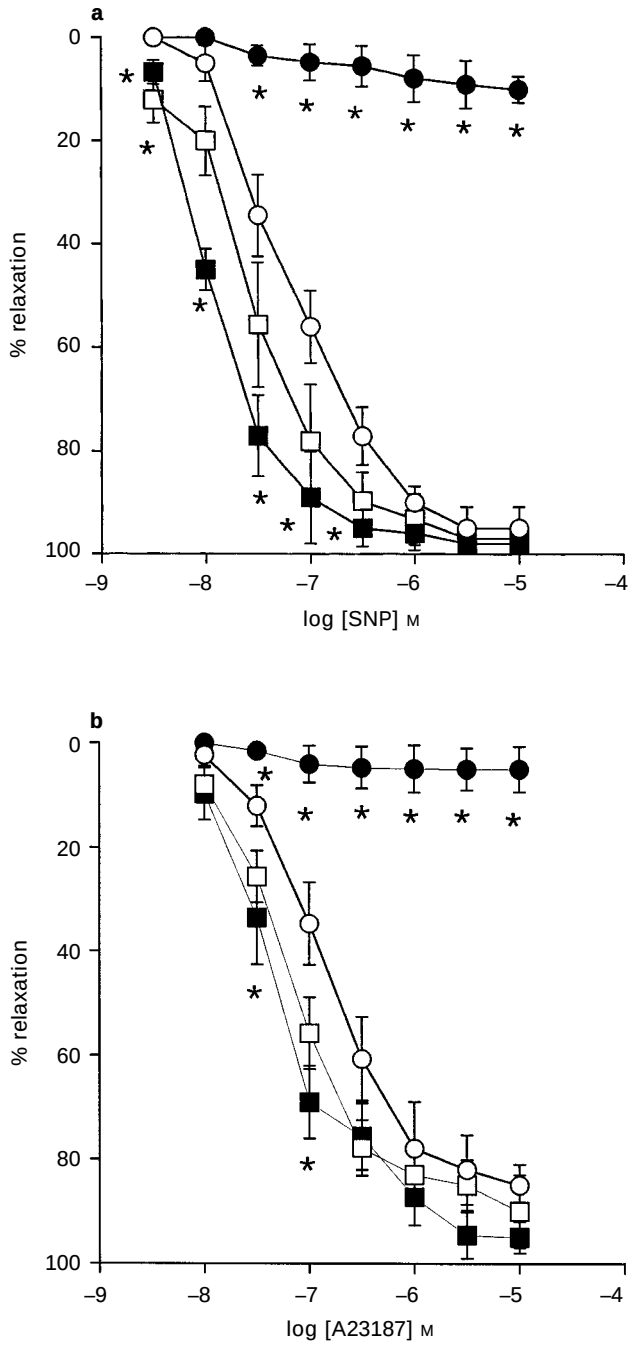

Figure 2 (a) Effect of $CuCl_2$ on the inhibition of sodium nitroprusside (SNP)-evoked relaxation by diethyldithiocarbamic acid (DEDCA) in rat aortic rings precontracted with phenylephrine (PE). (b) Effect of $CuCl_2$ on the inhibition of calcium ionophore A23187 (A23187)-evoked relaxation by DEDCA in rat aortic rings precontracted with PE. In (a) and (b): (○) control, (●) DEDCA (100 μM), (□) DEDCA (100 μM)+Cu^{2+} (100 μM), (■) Cu^{2+} (100 μM). Each point is the mean, $n=6$; vertical lines show s.e.mean $^*P<0.001$.

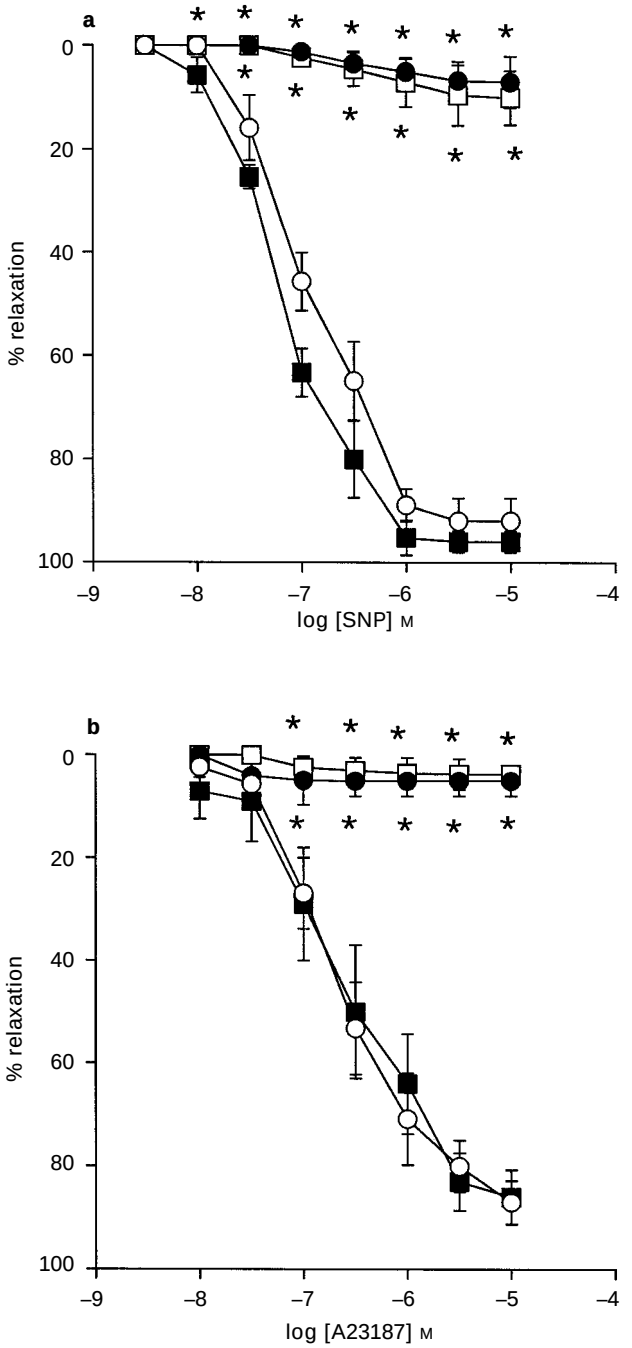

Figure 3 (a) Effect of Fe_2Cl_3 on the inhibition of sodium nitroprusside (SNP)-evoked relaxation by diethyldithiocarbamic acid (DEDCA) in rat aortic rings precontracted with phenylephrine (PE). (b) Effect of Fe_2Cl_3 on the inhibition of calcium ionophore A23187-evoked relaxation by 100 μM DEDCA in rat aortic rings precontracted with PE. In (a) and (b): (○) control, (●) DEDCA (100 μM), (□) DEDCA (100 μM)+Fe^{2+} (100 μM), (■) Fe^{2+} (100 μM). Each point is the mean, $n=6$; vertical lines show s.e.mean. $^*P<0.001$.

In parallel experiments, A23187- and SNP-stimulated increases in cyclic GMP in rat isolated aortic rings were assessed by radioimmunoassay. DEDCA inhibited increases in cyclic GMP formation stimulated by either A23187 or SNP with IC_{50} values of 0.5 and 50 μM, respectively (Figure 5).

Discussion

The present study demonstrates that the Cu^{2+} chelator, DEDCA, inhibits smooth muscle relaxation to the endothelium-dependent agonist, A23187 and to the activator of soluble GC, SNP. Addition of Cu^{2+} but not Fe^{2+} reversed the inhibitory effect of DEDCA on these responses, indicating that the present effects were not due to chelation/removal of Fe^{2+} associated with haem. The addition of Cu^{2+}, but not Fe^{2+}, caused a leftward shift of the concenetration-response curves to SNP- and A23187-induced relaxation, indicating that exogenous Cu^{2+} can exert a direct effect on both NOS and GC activity. Furthermore, in parallel experiments, DEDCA also inhibited SNP- and A23187-stimulated increases in cyclic GMP. Taken together, these data indicate that Cu^{2+} may play

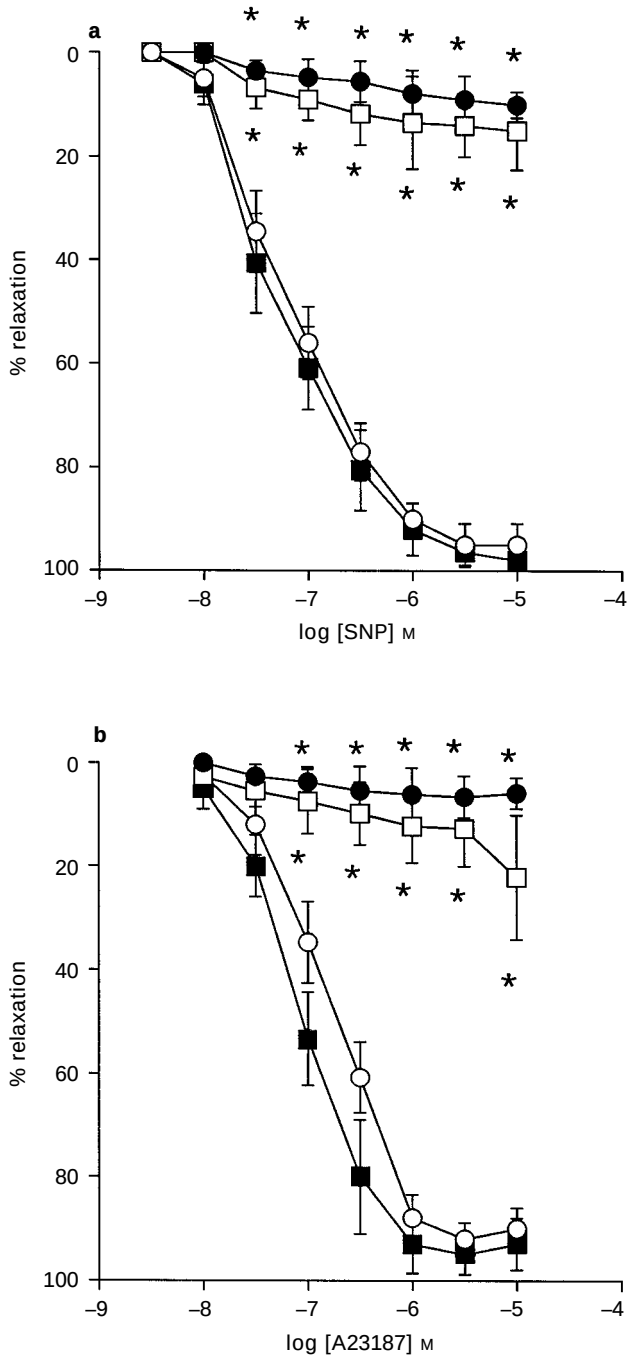

Figure 4 (a) Effect of superoxide dismutase (SOD) on the inhibition of sodium nitroprusside (SNP)-evoked relaxation by diethyldithiocarbamic acid (DEDCA) in rat aortae precontracted with phenylephrine. (b) Effect of SOD on the inhibition of A23187-evoked relaxation by DEDCA in rat aortic rings precontracted with PE. In (a) and (b): (○) control, (●) DEDCA (100 μM), (■) SOD (30 u ml^{-1}) (□) DEDCA (100 μM)+SOD (30 u ml^{-1}). Each point is the mean, $n=6$; vertical lines show s.e.mean. $^*P<0.001$.

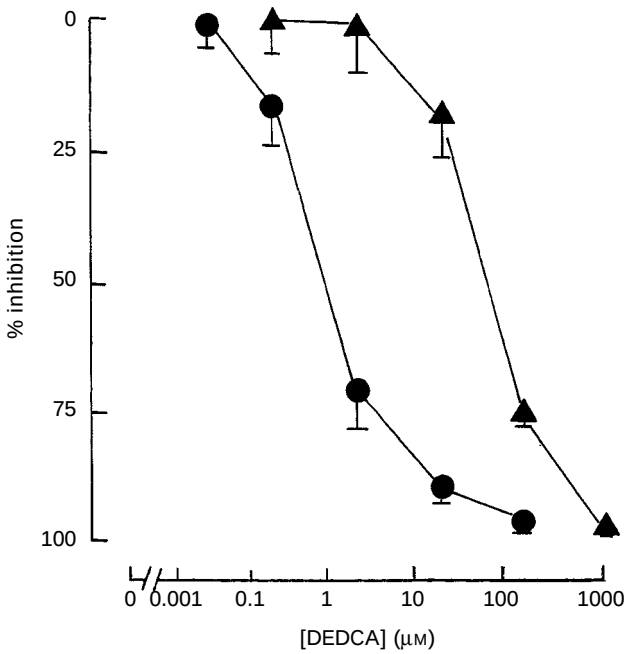

Figure 5 Effect of diethyldithiocarbamic acid (DEDCA) on (●) calcium ionophore A23187- and (▲) sodium nitroprusside-stimulated cyclic GMP formation in rat isolated aortae. Each point is the mean, $n=6$; vertical lines show s.e.mean.

a role in controlling the activities of both enzyme complexes. It is by virtue of ferrohaem groups, with the spectral properties of a cytochrome P_{450} possessed by both these enzymes, that NOS and GC function as catalytic entities (Ignarro, 1990; Moncada & Higgs, 1993; Bredt & Snyder, 1995). Thus, since the formation of NO and cyclic GMP are driven by Fe^{2+} it is perhaps not surprising that these enzymes are susceptible to modification by exogenous Cu^{2+}.

Our conclusions contrast with those of Omar *et al.* (1990, 1991) who ascribed the inhibitory effect of DEDCA on NO-mediated response in bovine isolated coronary artery to inhibition of SOD activity, an enzyme which is Cu^{2+}-dependent and inhibited by DEDCA (Heikkila *et al.*, 1976). SOD inactivates O_2^- by catalyzing its dismutation to give hydrogen peroxide (Darley-Usmar *et al.*, 1995). Thus, the inhibition of SOD activity should, theoretically, enhance deactivation of NO through reaction with O_2^- to form $ONOO^-$. However, in the present study, exogenous SOD failed to alter effects on DEDCA-inhibited SNP- or A23187-induced relaxation. The present effects of DEDCA are unlikely to be mediated by removal of SOD-associated copper since exposure to concentrations of DEDCA as high as 10 mM for at least 30 min is required to inhibit SOD activity (Omar *et al.*, 1991). In the present study, the activities of NOS and GC were completely inhibited following 10 min pretreatment with 10 μM and 100 μM DEDCA, respectively. It is also possible that the addition of exogenous Cu^{2+} potentiates endogenous SOD activity which in turn would be commensurate with the conclusions of Omar *et al.* (1991). However, Darley-Usmar *et al.* (1995) have argued that SOD is unlikely to influence the reaction between NO and O_2^- in the vessel wall since this reaction is so fast that it is only effectively limited by the rate of diffusion of the two radicals. For SOD to be effective it must be close to the site of NO and O_2^- generation and therefore control of these reactions may be mediated by 'extracellular' SOD which is bound to the endothelial surface (Abrahamsson *et al.*, 1992). However, if this was the case and DEDCA inhibits extracellular SOD then in the present study exogenous SOD would have been expected to attenuate the effect of DEDCA, which it did not.

Vedernikov *et al.* (1992) showed that DEDCA inhibits NO release in rat isolated aortic rings but that it had no effect on soluble GC activity. These authors postulated that the effect of DEDCA was due to reactions of Fe^{2+}-DEDCA complexes which may react with NO, thereby negating its action. However, no empirical evidence was presented for this proposal by these authors. Furthermore, in the present study the presence of both DEDCA and Fe^{2+} elicited no effect on GC or NOS activity.

Notwithstanding the potential role for Cu^{2+} in modulating NOS and GC, in certain pathological circumstances increased free Cu^{2+} may interact deleteriously with NO. High levels of

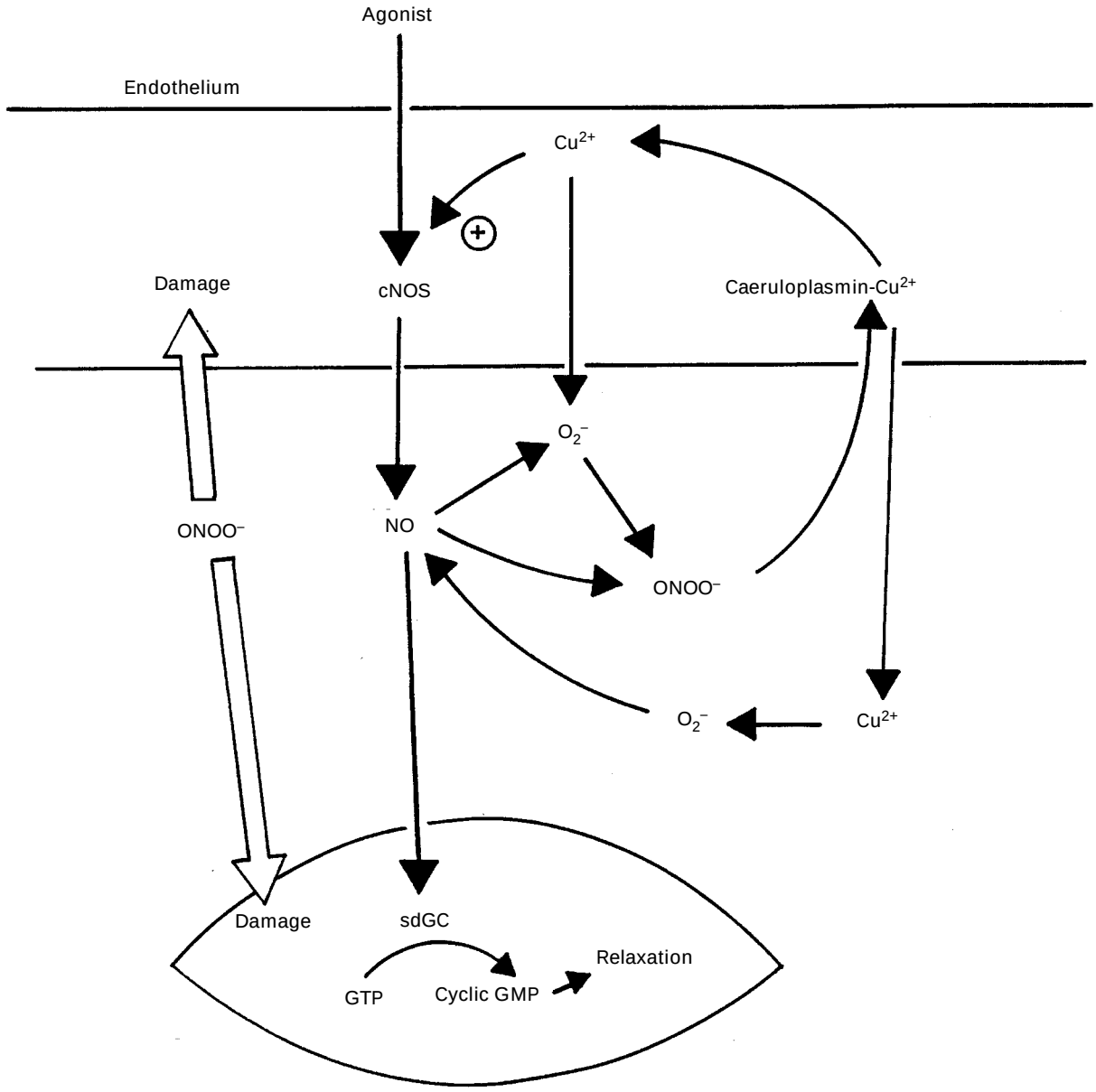

Figure 6 Hypothetical model of deleterious interactions between Cu^{2+}, NO and free radicals. Apart from the direct activation of NO synthase (NOS) and soluble guanylyl cyclase (sdGC), Cu^{2+} possesses the potential to instigate an amplification cascade that may reduce NO production and enhance cytotoxic free radical generation. Thus, locally high levels of superoxide (O_2^-), liberated for example from neutrophils, may react with NO to generate peroxynitrite ($ONOO^-$) which in turn has been shown to dissociate Cu^{2+} from its binding sites on caeruloplasmin. Furthermore, dismutation of O_2^- (catalysed by superoxide dismutase) results in the generation of H_2O_2. In the presence of Cu^{2+}, H_2O_2 can undergo Fenton reactions to generate the highly destructive hydroxyl free radical, OH• as well as more O_2^- which in turn would further augment the above cascade.

Cu^{2+} have been found in the serum of atherosclerotic men and of patients with a recent episode of myocardial infarction as well as in atherosclerotic lesions (Smith *et al*., 1992; Iskra *et al*., 1993; Vlad *et al*., 1995; Swain & Gutteridge, 1995; Tan *et al*., 1992). Since a decrease in NO-mediated responses is associated with these conditions, this would seem to mitigate against Cu^{2+} as playing a role in modulating NOS and GC activity. However, studies on aortic tissue from cholesterol-fed rabbits have shown that although endothelium-dependent responses are attenuated in atherosclerotic vessels, NO production is increased (Minor *et al*., 1990), possibly by the induction of NOS in VSMCs. Cu^{2+} also catalyses the generation of O_2^- (Denko, 1989) which reacts with NO to produce the cytotoxic radical, $ONOO^-$ (Darley-Usmar *et al*., 1995) which releases Cu^{2+} from caeruloplasmin (Swain *et al*., 1994). Thus, in atheromatous lesions or areas of ischaemia, increased NO levels could lead to an amplification cascade involving Cu^{2+}-induced O_2^- generation and the formation of $ONOO^-$ which would elicit further local release of copper and perpetuation of the cascade (Figure 6).

In conclusion, endogenous copper appears to play a role in controlling the activities of both NOS and GC in the rat aorta. This property may be of relevance not only to the pathophysiology of cardiovascular disease but also to inflammatory cascades. The present data may also provide insights into the modes of action of copper chelators in the treatment of alcohol addiction and AIDS and into their possible use as antioxidants.

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