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Neocuproine, a selective Cu(I) chelator, and the relaxation of rat vascular smooth muscle by *S*-nitrosothiols

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1 A study has been made of the effect of neocuproine, a specific Cu(I) chelator, on vasodilator responses of rat isolated perfused tail artery to two nitrosothiols: *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and *S*-nitroso-glutathione (GSNO).

2 Bolus injections (10 μl) of SNAP or GSNO (10^{-7}–10^{-3} M) were delivered into the lumen of perfused vessels pre-contracted with sufficient phenylephrine (1–7 μM) to develop pressures of 100–120 mmHg. Two kinds of experiment were made: SNAP and GSNO were either (a) pre-mixed with neocuproine (10^{-4} M) and then injected into arteries; or (b) vessels were continuously perfused with neocuproine (10^{-5} M) and then injected with either pure SNAP or GSNO.

3 In each case, neocuproine significantly attenuated vasodilator responses to both nitrosothiols, although the nature of the inhibitory effect differed in the two types of experiment. We conclude that the ability of exogenous nitrosothiols to relax vascular smooth muscle in our *ex vivo* model is dependent upon a Cu(I) catalyzed process. Evidence is presented which suggests that a similar Cu(I)-dependent mechanism is responsible for the release of NO from endogenous nitrosothiols and that this process may assist in maintaining vasodilator tone *in vivo*.

Keywords: *S*-nitrosothiol; vasodilator responses; copper; neocuproine; vascular smooth muscle

Introduction

S-Nitrosothiols (RSNOs) are an important class of nitric oxide (NO) donor drug, capable of initiating a range of cellular processes including the prevention of platelet aggregation and adhesion (Mellion *et al*., 1983) and vasodilatation (Ignarro *et al*., 1981). It is well-established that RSNOs decompose in aqueous solution to give only NO and a disulphide:

$$2\mathrm{RSNO} \rightarrow \mathrm{RSSR} + 2\mathrm{NO}$$

There have been a number of unsuccessful attempts to relate the stability of *S*-nitrosothiols *in vitro* with their biological activity (Mathews & Kerr, 1994). The reason for this failure may be that the *in vitro* decomposition of *S*-nitrosothiols is strongly catalyzed by copper ions which are adventitiously present in the buffer used for those studies (McAninly *et al*., 1993). The rate of NO release depends, therefore, upon the source of distilled water and salts used. Copper ions at a concentration as low as 10^{-6} M are capable of effecting NO release. The decomposition of both *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (Askew *et al*., 1995; Dicks *et al*., 1996) and *S*-nitroso-L-glutathione (GSNO) (Gorren *et al*., 1996) has been examined in detail. With most S-nitrosothiols examined so far NO release is inhibited by addition of the nonspecific metal ion chelator EDTA. The kinetic study of SNAP decomposition, including the effect of added thiol and copper nitrate, has shown conclusively (Dicks *et al*., 1996) that the species responsible for catalysis is Cu(I), rather than the more commonly occurring Cu(II). Cu(I) ions are produced by the reaction of Cu(II) with a thiol: $Cu^{2+} + RS^- \rightarrow \frac{1}{2}RSSR + Cu^+$.

Confirmation of the catalytic role of Cu(I) ions comes from the observation that addition of a selective Cu(I) chelator, neocuproine (2,9-dimethyl-1,10-phenanthroline), is as effective as EDTA at inhibiting NO release from SNAP. With GSNO the overall picture is the same but with a complication that the conversion of Cu(II) into Cu(I) by glutathione is much slower than that for some other thiols, giving rise to an induction period before NO release occurs.

Little is known at present of the way in which NO release from an exogenous *S*-nitrosothiol occurs *in vivo*. It could be a purely thermal process but the dominating role of the Cu(I) catalyzed process *in vitro* requires that we examine the possibility that a similar process occurs *in vivo*. All copper in living systems is complexed. However, Cu(II) complexes, in the presence of the requisite thiol, still catalyze the decomposition of *S*-nitrosothiols (Dicks & Williams, 1996). The complexes examined were those of Cu(II) with histidine, glycylglycylhistidine and caeruloplasmin. Thus, there are no *a priori* grounds for dismissing the copper-catalyzed route as a means of NO release from an *S*-nitrosothiol *in vivo*. Indeed, there is some direct evidence for this. An important property of *S*-nitrosothiols is that they prevent platelet adhesion and aggregation by releasing NO (Palmer *et al*., 1987). Significantly the antiplatelet aggregating ability of GSNO has been shown to be diminished by the presence of a selective complexing agent for Cu(I) (bathocuproine disulphonic acid) (Gordge *et al*., 1995). The authors of that study have more recently concluded that this is because of the role of a copper-containing enzyme present in platelets (Gordge *et al*., 1996). Although there has been a change of emphasis in the interpretation, the essential role of copper ions has not been questioned.

In the present study an *ex vivo* model was used, the rat isolated, perfused tail artery preparation (Flitney *et al*., 1992), to investigate whether Cu(I) catalysis plays a role in the vasodilator actions of SNAP and GSNO. The results showed that responses to both SNAP and GSNO were reversibly inihibited by neocuproine. In contrast, vasodilator responses to nitroprusside (SNP) were not affected. We conclude that relaxation of vascular smooth muscle (VSM) by SNAP and GSNO is caused in part by NO released into solution *via* a Cu(I)-dependent catalytic reaction. Our results also provide evidence that Cu(I) may contribute to the maintenance of vasodilator 'tone', either by catalyzing the decomposition of an endogenous 'store' of S-nitrosothiol (Venturini *et al*., 1993; Megson *et al*., 1995) and/or by inhibiting guanylate cyclase activity (Schrammel *et al*., 1996).

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Methods

Rat isolated tail artery preparation

Segments (1–2 cm long) of tail artery were dissected from normotensive male Wistar rats (300–400 g) following cervical dislocation. They were cannulated and perfused with pre-warmed Krebs solution pH 7.4 (composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25 NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1 and glucose 5.6, and purged with 95% O_2/5% CO_2) at a constant flow rate (2 ml min^{-1}). A Gilson Minipuls peristaltic pump was used to draw the perfusate from a reservoir. Temperature was maintained at 32 ± 2°C by superfusing preparations with pre-warmed Krebs solution, drawn from a second reservoir (flow rate: approximately 8 ml min^{-1}) by means of peristaltic pump. Perfusion pressure was monitored by a differential pressure transducer (Sensym type SCX 150NC, Farnell Electronic Components, Leeds) located upstream from the vessel. The output from the transducer was fed to a MacLab type 4e A/D converter and then to a MacIntosh LCIII computer for data storage and subsequent analysis. A short side-tube which terminated in a resealable rubber spectrum allowed bolus injections of SNAP and GSNO to be delivered into the internal perfusate. The transit time from the injection site to the lumen of the artery was approximately 3–4 s. Further details of apparatus are given elsewhere (Flitney *et al*., 1992).

Experimental protocol

The experiments were performed in a darkened laboratory since SNAP (Field *et al*., 1978), GSNO (Sexton *et al*., 1994) and sodium nitroprusside (SNP) (Wolfe & Swinehart, 1975) are all photosensitive and release NO more rapidly when exposed to light. The following protocol was used throughout. Phenylephrine (1–7 μM) was added to the Krebs solution to pre-contract the artery, generating perfusion pressures of 100–120 mmHg. Bolus microinjections (10 μl) of SNAP or GSNO (10^{-7}–10^{-3} M), prepared by serial dilution of stock solutions, were then delivered into the perfusate. These produced transient vasodilator responses which were allowed to recover fully between successive injections. The injection sequence was then repeated with SNAP and GSNO after pre-mixing with neocuproine (100 μM) for 30 min. Finally, vessels were perfused internally with Krebs solutions containing neocuproine (10 μM) and solutions of SNAP or GSNO alone were injected into the artery.

Control experiments were performed with neocuproine previously complexed with Cu(I). The neocuproine-Cu(I) complex (10 μM) was prepared by reacting glutathione, neocuproine and copper(II) acetate in molar ratios of 1 : 2: 1, respectively. Vasodilator responses to standard bolus injections of SNP (10 μM) were recorded periodically. Like SNAP and GSNO, SNP relaxes vascular smooth muscle by increasing cyclic GMP levels, but when adequately protected from light it apparently does so be reacting first with a membrane-bound protein (Kowaluk & Fung, 1990), rather than by spontaneous release of NO in solution.

The concentration of contaminating copper in Krebs solution (3.2 μM) was determined by atomic absorption spectroscopy by use of a Perkin Elmer X60 instrument.

Reagents

S-Nitroso-*N*-acetyl-D,L-penicillamine (Field *et al*., 1978) and *S*-nitrosoglutathione (Hart, 1985) were prepared in the laboratory by standard procedures. The reagents used in the syntheses were obtained from Aldrich Ltd or Sigma Ltd. Stock solutions of SNAP and GSNO (10 mM) were prepared in 10% dimethyl sulphoxide (DMSO) and stock solutions of neocuproine (10 mM) in 25% ethanol. Control experiments showed that the highest working concentrations of DMSO (1%) and ethanol (2.5%) alone did not cause vasodilatation of the artery. Neocuproine, sodium nitroprusside, phenylephrine (PE) and met-haemoglobin (met-Hb) were obtained from Sigma Ltd. Met-Hb was reduced to the ferro- form (Hb) by sodium dithionite (Sigma Ltd.) by the method previously described (Martin *et al*., 1985). Krebs solution components were obtained from BDH and were Aristar grade. N^G-monomethyl-L-arginine (L-NMMA) was a gift from Dr Harold Hodson of the Wellcome Research Laboratories, Beckenham, Kent.

Statistical analyses

The amplitude of the vasodilator response to SNAP or GSNO is given as the maximum fall in pressure expressed as a percentage of that existing immediately before injection. Results presented are means $\pm$ s.e.mean for 5–7 experiments. Repeated dose ANOVAs and *post-hoc* Dunnet's tests have been applied where appropriate to establish statistical significance of results ($P < 0.05$ was accepted as significant).

Results

Figure 1a shows log dose-response curves obtained by injecting increasing concentrations of SNAP alone and of SNAP premixed with neocuproine (100 μM) into precontracted arteries. Similar recordings were obtained with GSNO (Figure 1b). The vasodilator effect of both compounds was severely attenuated in the presence of neocuproine ($P = 0.0001$ for SNAP and GSNO), which caused a marked rightward shift in the dose-response curves, increasing the ED_{50} value for each compound approximately 100 fold. Addition of neocuproine (10 μM) alone to the reservoir containing the perfusate either produced a sustained vasodilator response or else it elicited a vasoconstriction, both of which were fully reversible on washout. Occasionally there was no effect of neocuproine on phenylephrine-induced tone. The magnitude of any constrictor effect (mean $\pm$ s.e.mean $= 17.9 \pm 3.1\%$, $n = 7$ vessels) was considerably less (by a factor of approximately 3) than that produced by adding either haemoglobin (5 μM: $56.5 \pm 4.9\%$; $n = 8$) or the nitric oxide synthase inhibitor N^G-L-monomethyl-L-arginine (N^G-L-NMMA 100 μM: $57.1 \pm 6.1\%$; $n = 10$) to the perfusate. At higher concentrations (100 μM), neocuproine invariably produced a sustained vasodilator response. Control experiments showed that the pre-prepared stoichiometric complex of neocuproine and Cu(I) (10 μM; see Methods) had no effect on agonist-induced tone.

Vasodilator responses to bolus injections of SNAP or GSNO (Figure 1) administered while vessels were being continuously perfused with Krebs and neocuproine (10 μM) were also significantly impaired ($P = 0.0006$ and 0.0175 respectively), and under these conditions the maximum vasodilator response was depressed too. The effect was greater for SNAP than GSNO. Again, the pre-prepared neocuproine-Cu(I) complex had no effect on responses to SNAP (Figure 1a; GSNO was not tested). Finally, vasodilator responses to SNP (10 μM) were unaffected by the presence of neocuproine in the internal perfusate: the amplitudes were $44.3 \pm 3.7\%$ ($n = 7$) in normal Krebs solution and $43.2 \pm 5.3\%$ ($n = 7$) in Krebs solution containing neocuproine.

Discussion

Previous chemical studies have shown that the decomposition of *S*-nitrosothiols to release NO is strongly catalyzed by Cu(I) ions. The strongest evidence to support Cu(I) as the catalytic species is the inhibition caused by the selective Cu(I) inhibitor neocuproine. The results described here show that neocuproine also strongly inhibits vasodilator responses of rat isolated tail artery to bolus injection of either SNAP or GSNO, demonstrating that Cu(I)-catalyzed release of NO from S-nitrosothiols is important in a functionally intact, *ex vivo* physiological system.

The nature of the inhibitory effect was found to depend upon the experimental protocol, specifically on the manner in which GSNO and SNAP were administered: whether (a) pre-mixed with neocuproine before injection or (b) injected without pre-mixing into vessels that were continually perfused with Krebs solution containing neocuproine. The dose-response curves to bolus injections of SNAP or GSNO pre-mixed with neocuproine demonstrated a marked rightward shift of the curve but with similar maximum responses at the higher doses. On the other hand, dose-response curves to SNAP or GSNO alone when injected into arteries continuously perfused with Krebs solution containing neocuproine were shifted to the right and maximum responses to both compounds were significantly diminished too. It is important to emphasize here that responses to bolus injections of SNP were not impaired in this type of experiment, since this clearly demonstrates that prolonged exposure of vessels to neocuproine *per se* does not impair vascular smooth muscle relaxation directly.

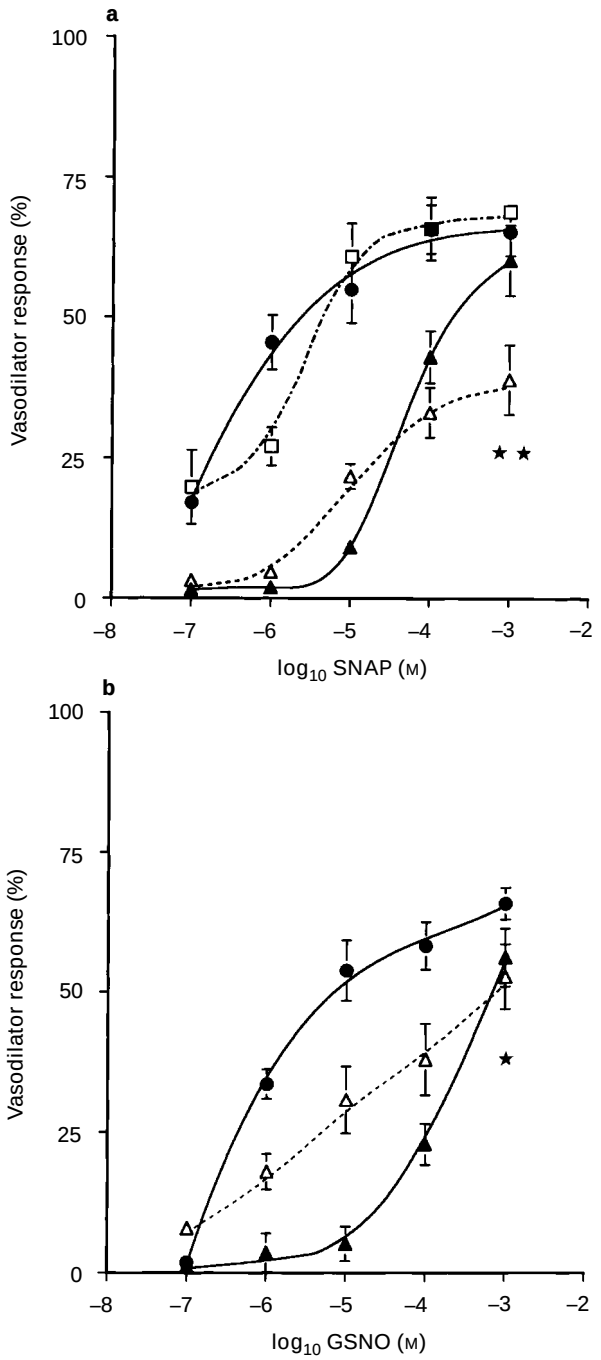

Figure 1 (a) Log dose-response curve for effect of SNAP alone (●) and of SNAP pre-mixed with neocuproine (▲), SNAP pre-mixed with Cu(I)-neocuproine complex (□) and SNAP injected into a vessel continuously perfused with Krebs buffer containing neocuproine (△). Responses to SNAP were significantly attenuated when pre-mixed with neocuproine ($P=0.0001$, 2-factor, repeated dose ANOVA) and when injected into perfusate containing neocuproine ($P=0.0006$), but not when pre-mixed with Cu(I)-neocuproine complex ($P>0.05$). (b) Log dose-response curve for effect of GSNO alone (●), GSNO pre-mixed with neocuproine (▲) and GSNO injected into a vessel continuously perfused with Krebs buffer containing neocuproine (△). Responses to GSNO were significantly attenuated when pre-mixed with neocuproine ($P=0.0001$, 2-factor, repeated dose ANOVA) and when injected into perfusate containing neocuproine ($P=0.0175$). *Post hoc* significance testing of 10^{-3} M doses (compared to control) were assessed by Dunnet's test. (*$P<0.05$; **$P<0.01$). Data shown are means and vertical lines indicate s.e.mean.

The period of time that arteries were exposed to neocuproine appears to have been critical in determining the nature of the inhibitory effect. This was negligible (approximately 3 ms) in the 'pre-mix' experiments, but considerable (0.5 – 1 h) in those experiments which involved continuous infusions of Krebs containing neocuproine. The results from the two kinds of experiment can be understood if we postulate a dual mechanism of action for neocuproine.

First, a 'direct' effect on Cu(I)-catalyzed release of NO from exogenous *S*-nitrosothiols (SNAP and GSNO) in solution; and second, an 'indirect' effect, due to inhibition of a Cu(I)-dependent process, or processes, occurring within the tissue. Interestingly, the continuous presence of neocuproine in the perfusate produced significantly less inhibition than pre-mixing the chelator with GSNO ($P=0.034$, 2-factor, repeated dose ANOVA) but not with SNAP ($P=0.145$). These results suggest that endogenous Cu(I), which may not be chelated in pre-mix experiments, is more effective at accelerating the decomposition of GSNO than SNAP.

There can be little doubt that the concentration of neocuproine (100 μM) was sufficient to chelate the trace amounts of Cu(I) likely to be present in the 'pre-mix' solutions. Atomic absorption spectroscopy showed that total copper in Krebs solution was typically in the region of 3 μM. The ability to give near maximal responses at the highest doses of SNAP or GSNO under these conditions can be attributed to the formation of Cu(I) after injection (i.e. while *en route* to, and within, the artery), by reaction of Cu(II) in the internal perfusate with trace amounts of contaminating ('parent') thiols used in the synthesis of SNAP and GSNO. The 'indirect' process(es) would be significantly affected if sufficient time is allowed for neocuproine to chelate all contaminating Cu(I) in the Krebs solution and also any cellular Cu(I), either by entering vascular smooth muscle cells directly and/or by acting as an external 'sink'. Both are more likely to occur when vessels are being continuously perfused with Krebs solution containing neocuproine.

The indications from the results of the latter type of experiment are that at least two copper-dependent mechanisms might be at work within the tissue. First, the variable vasoactive responses observed when vessels were continuously perfused with neocuproine (10 μM) alone raises the possibility that vascular smooth muscle tone may be regulated, at least in part, by Cu(I)-catalyzed release of NO from endogenous *S*-nitrosothiols. It will be recalled that the magnitude of the sustained vasoconstrictor effect was substantially less than that produced by continuous infusions of either haemoglobin (5 μM) or L-NMMA (100 μM), so this source of NO is likely to be less important than endothelium-derived NO in regulating vessel tone. Second, it was shown recently (Schrammel *et al*., 1996) that copper ions inhibit both basal and NO-stimulated soluble guanylate cyclase activity and that Cu(I) is more effective than Cu(II) in this regard. Neocuproine may therefore exert opposing effects on vascular tone: inhibition of NO release from endogenous *S*-nitrosothiols, causing vasoconstriction, and a simultaneous vasodilator action, due to removal of the inhibitory effect of

Cu(I) on guanylate cyclase activity. Clearly, the net effect will depend upon the relative contributions from each process and this may well account for the variable nature of the responses seen under these conditions.

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