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Role of copper ions and cytochrome *P*450 in the vasodilator actions of the nitroxyl anion generator, Angeli's salt, on rat aorta

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Abstract

Since copper ions catalyse the oxidation of nitroxyl anion to nitric oxide, we investigated whether this might explain the vasodilator actions of the nitroxyl generator, Angeli's salt, in rat aorta. Parallel studies were conducted with *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), since Cu ions catalyse the liberation of nitric oxide from this compound. Copper sulphate enhanced relaxation to Angeli's salt and SNAP but this resulted from reduced destruction of nitric oxide by superoxide rather than from enhanced generation of nitric oxide, since it was mimicked by superoxide dismutase and by the superoxide dismutase mimetic, $MnCl_2$. Results with the selective Cu^{2+} chelators, neocuproine and bathocuproine disulfonate, and the Cu^{2+} chelators, EDTA, cuprizone and diethyldithiocarbamate, confirmed an important role for endogenous copper in mediating relaxation to SNAP but suggested only a minor role for Angeli's salt. Relaxation to Angeli's salt was, however, powerfully blocked by proadifen, suggesting an important role for cytochrome *P*450.

Keywords: Cytochrome *P*450; Nitric oxide (NO); Nitroxyl anion; *S*-Nitrosothiol; Proadifen

1. Introduction

Nitric oxide radical (NO) is generally accepted to be the final product of the nitric oxide synthase family of enzymes. Nevertheless, interest has developed recently in the functional roles of an alternative redox form of NO, namely, nitroxyl radical (NO^-), because it is formed by a number of diverse biological reactions. Specifically, nitroxyl has been shown to be produced by nitric oxide synthase itself (Schmidt et al., 1996; Pufahl et al., 1995), although this has been challenged (Xia and Zweier, 1997). It is also produced by the oxidation of azide by peroxidase (Tatarko and Bumpus, 1997), by the decomposition of *S*-nitrosothiols in the presence of thiol (Arnelle and Stamler, 1995), by the decomposition of peroxynitrite (Khan et al., 2000) and by the reduction of nitric oxide by ferrocytochrome *c* (Sharpe and Cooper, 1998).

Nitroxyl anion is a highly unstable species but the recent introduction of a number of nitroxyl donors including Angeli's salt (sodium trioxodinitrate) (Fukuto et al., 1992a, 1993) has facilitated the elucidation of its biological actions. Of particular interest is the finding that nitroxyl is a potent relaxant of vascular and non-vascular smooth muscle (Fukuto et al., 1992a,b; Li et al., 1999). Moreover, the relaxation it produces is associated with a rise in cyclic GMP content (Fukuto et al., 1992a) and is blocked by the inhibitor of soluble guanylate cyclase, 1*H*-[1,2,4]oxadiazolo-[4,3,-a]quinoxalin-1-one (ODQ) (Li et al., 1999). These actions are surprising since nitroxyl per se is unable to stimulate soluble guanylate cyclase (Dierks and Burstyn, 1996), but strongly suggest that tissues have the ability to facilitate the one-electron oxidation of nitroxyl to nitric oxide. Indeed, it has already been shown that superoxide dismutase can catalyse this reaction (Murphy and Sies, 1991; Schmidt et al., 1996), but the levels required (~ 5000 u ml^{-1}) are vastly greater than are present in cells. Oxidation by flavin adenine dinucleotide and by methaemoglobin has also been reported (Fukuto et al., 1993), but the extent to which these contribute to tissue-dependent conversion of nitroxyl to nitric oxide has not been investigated. Using Angeli's salt as a nitroxyl donor, we have recently reported that copper ions have the

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ability to promote the rapid and efficient oxidation of nitroxyl to nitric oxide (Nelli et al., 2000). Copper does not exist in a free state in the body and consequently, these findings may suggest that a copper-containing enzyme catalyses the oxidation of nitroxyl to nitric oxide by cells.

The aim of this study was to investigate, in isolated rings of rat aorta, the effects of copper and selective chelators of copper to determine if this metal ion participates in the oxidation of nitroxyl to nitric oxide, which underlies the relaxant actions of Angeli's salt. For comparison, we also studied their effects on relaxation to the *S*-nitrosothiol, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), since the role of copper in the liberation of nitric oxide from this compound is well-established (Dicks et al., 1996; Gordge et al., 1996; Al-Sa'doni et al., 1997).

2. Materials and methods

2.1. Preparation of tissues

Male Wistar rats (200–250 g) were killed by stunning and exsanguination. The thoracic aorta was then carefully removed, cleaned of fat and connective tissue, and cut into transverse rings (2.5-mm wide). In all experiments, the endothelium was removed by gentle abrasion of the intimal surface using a moist wooden stick (successful removal of the endothelium was confirmed later by the inability of acetylcholine 1 μM to induce relaxation). Aortic rings were then mounted under 1 g resting tension on stainless steel hooks within 10 ml tissue baths and maintained at 37°C in Krebs solution (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically with Grass FTO3C transducers and displayed on a MacLab (E Series, AD Instruments) or a Grass polygraph model 7D. Tissues were allowed to equilibrate for 60 min before experiments were carried out, during which time the resting tension was readjusted to 1 g, as required.

2.2. Experimental protocols

All experiments involving relaxation were conducted on endothelium-denuded aortic rings following induction of 40–60% of maximal phenylephrine-induced tone (1.92 ± 0.02 g, $n = 10$): in control tissues, this level of tone was achieved with phenylephrine at 10–30 nM. Moreover, in all experiments in which the effects of agents were examined on relaxation, we ensured that the tone in each case was also 40–60% of the maximum. Data obtained from any tissue that fell outside this range were excluded.

Agents whose effects were examined on relaxation induced by SNAP and Angeli's salt were $CuSO_4$ (1–100 μM), superoxide dismutase (250 u ml^{-1}), the superoxide dismutase mimetic $MnCl_2$ (10 μM), the selective Cu^+ chelators, neocuproine (30 μM) and bathocuproine disulfonate (30 μM), and the selective Cu^{2+} chelators, cuprizone (30 μM) and EDTA (30 μM). In each case, the agent was left in contact with the aortic rings for 20 min. The effects of the intracellular Cu^{2+} chelator, diethyldithiocarbamate (Cocco et al., 1981), were also examined on relaxation induced by SNAP and Angeli's salt. In these experiments, high concentrations (0.1–3 mM) and a long incubation time (2 h) with diethyldithiocarbamate were employed, since these have previously been shown to be necessary for effective chelation of intracellular copper (Cocco et al., 1981; MacKenzie and Martin, 1998).

In some experiments, the effects of inhibiting endogenous catalase with 3-amino-1,2,4-triazole (Margoliash and Novogrodsky, 1957) were examined on relaxation induced by azide and Angeli's salt. For these experiments, tissues were treated with a concentration of 10 mM for 90 min before relaxation was examined. In other experiments, attempts were made to determine the effects of the thiol-depleting agents, diamide and *N*-ethylmaleimide (Kosower et al., 1969), on relaxation to glyceryl trinitrate and Angeli's salt. For these experiments, tissues were treated for 20 min with either diamide or *N*-ethylmaleimide (10 μM–1 mM for both). In the final set of experiments, the effects of inhibiting cytochrome *P*450 with proadifen (Schröder, 1992; Singer et al., 1984) were examined on relaxation to Angeli's salt, glyceryl trinitrate and sodium nitroprusside. For these experiments, tissues were treated with a concentration of 100 μM proadifen for 20 min before relaxation was examined.

2.3. Drugs

Acetylcholine chloride, 3-amino-1,2,4-triazole, bathocuproine disulfonate, cuprizone (bis-cyclohexanone oxaldihydrazone), diamide, diethyldithiocarbamate, neocuproine (2,9-dimethyl-1,10-phenanthroline hydrochloride), *N*-ethylmaleimide, phenylephrine hydrochloride, proadifen (SKF 525A), *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), sodium azide, sodium nitroprusside and superoxide dismutase (Cu^{2+}–Zn^{2+}-containing form from bovine erythrocytes) were obtained from Sigma (Poole, UK). Ethylenediaminetetraacetic acid disodium salt (EDTA) was obtained from Hopkin and Wiliams (Essex, UK). Glyceryl trinitrate (10% w/w in lactose) was a generous gift from Bard Pharmaceuticals (Cambridge, UK). Angeli's salt (sodium trioxodinitrate) was obtained from Alexis (Nottingham, UK). All drugs were dissolved in saline (0.9%) except for Angeli's salt (0.01 M) which was dissolved in 0.01 M NaOH, cuprizone (0.01 M) which was dissolved in 50% ethanol and SNAP (0.01 M) which was dissolved at pH 9 in distilled water containing EDTA (0.54 mM).

2.4. Analysis of data

Results are expressed as the mean ± S.E.M. of *n* separate experiments. Relaxant responses are expressed as

percentage (%) relaxation of phenylephrine-induced tone. Statistical comparisons were made by one-way analysis of variance followed by the Bonferroni post hoc test. A probability (P) of 0.05 or less was considered significant. Graphs were drawn and logEC_{50} values calculated using a computer-based program (GraphPad, Prism).

3. Results

3.1. Effects of copper sulphate, superoxide dismutase and manganese chloride on relaxation

SNAP (1 nM–3 μM) and Angeli's salt (1 nM–3 μM) each produced concentration-dependent relaxation of phenylephrine-contracted, endothelium-denuded rings of rat aorta: the log EC_{50} value for each was -7.48 ± 0.03 and -7.51 ± 0.06, respectively (Fig. 1). Treating aortic rings for 20 min with $CuSO_4$ affected subsequent relaxation to SNAP and Angeli's salt: at a concentration of 1 μM, it produced no significant effect, at 10 μM, it enhanced the effects of the two relaxants but at 100 μM, the enhancement was lost (Fig. 1).

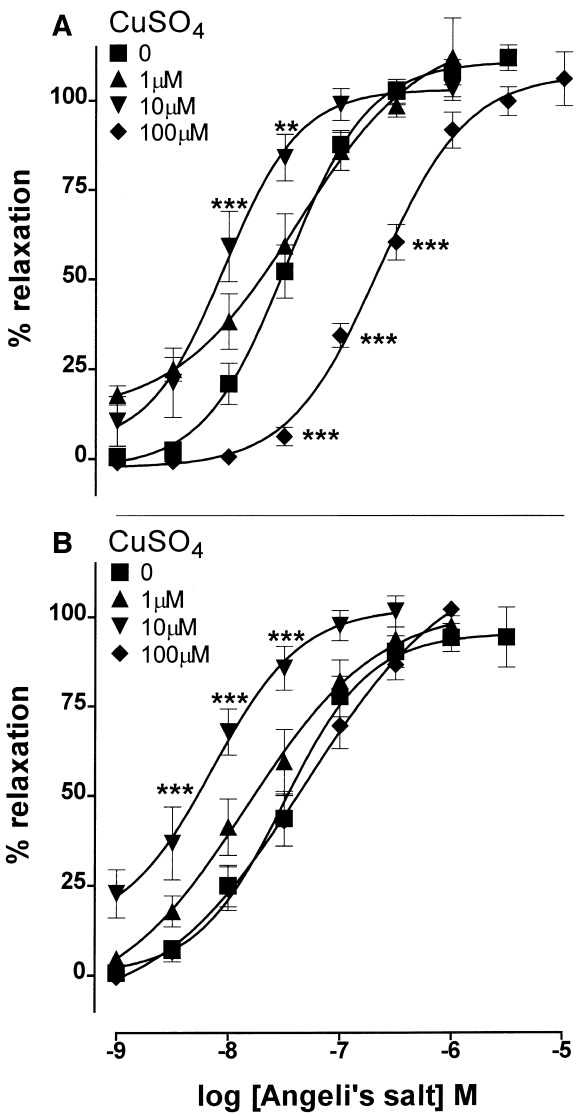

Fig. 1. Concentration–response curves showing the ability of $CuSO_4$ at concentrations of 1, 10 and 100 μM for 20 min to influence the relaxation of rat aortic rings induced by (A) SNAP and (B) Angeli's salt. Each point is the mean ± S.E.M. of 6 to 10 observations. $^{**}P < 0.005$ and $^{***}P < 0.001$ indicate a significant difference from control.

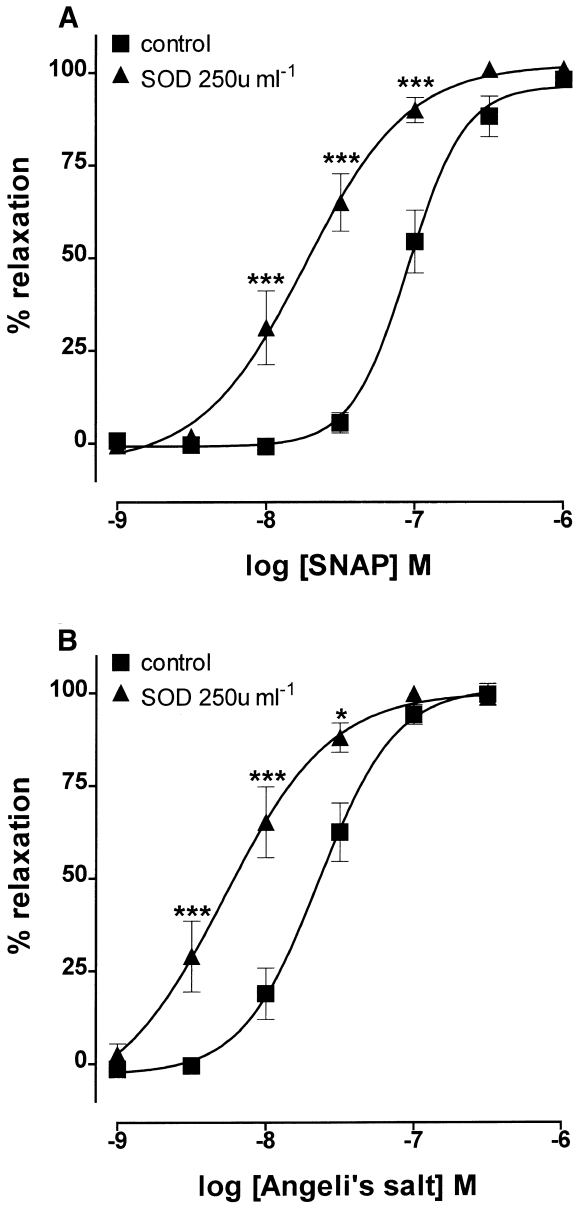

Fig. 2. Concentration–response curves showing the ability of Cu^{2+}–Zn^{2+} superoxide dismutase at 250 u ml^{-1} for 20 min to influence the relaxation of rat aortic rings induced by (A) SNAP and (B) Angeli's salt. Each point is the mean ± S.E.M. of 8 to 12 observations. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate a significant difference from control.

Treating aortic rings for 20 min with either Cu^{2+}–Zn^{2+} superoxide dismutase (250 u ml^{-1}) or the superoxide dismutase mimetic, $MnCl_2$ (10 μM), mimicked the ability

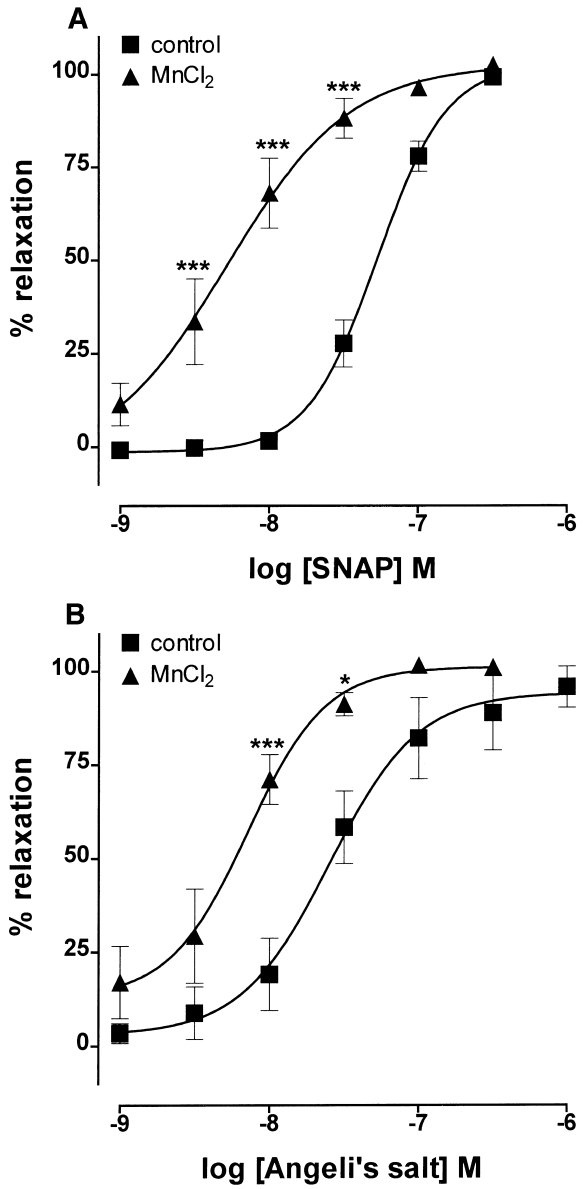

Fig. 3. Concentration–response curves showing the ability of the superoxide dismutase mimetic $MnCl_2$ at 10 μM for 20 min to influence the relaxation of rat aortic rings induced by (A) SNAP and (B) Angeli's salt. Each point is the mean ± S.E.M. of 8 observations. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate a significant difference from control.

of $CuSO_4$ to enhance relaxation to SNAP and Angeli's salt (Figs. 2 and 3).

3.2. Effects of copper chelators on relaxation

The effects of a range of copper chelators were examined on relaxation induced by SNAP and Angeli's salt. Treatment for 20 min with each of the selective Cu^{+} chelators, neocuproine (30 μM) and bathocuproine disulfonate (30 μM), inhibited subsequent relaxation to SNAP (Fig. 4(A)). In contrast, neither neocuproine nor bathocuproine disulfonate, had a significant effect on relaxation to Angeli's salt (Fig. 4(B)). Furthermore, treatment for 20 min with each of the selective Cu^{2+} chelating agents, EDTA (30 μM) and cuprizone (30 μM), failed to affect relaxation to SNAP, however, cuprizone produced a small but significant inhibition of relaxation to Angeli's salt (Fig. 4(A),(B)). Treatment for 2 h with the membrane permeant chelator of Cu^{2+}, diethyldithiocarbamate, at concentrations of 0.1 or 1 mM, had no effect on relaxation to SNAP or Angeli's salt, but at 3 mM, a small but significant inhibition was seen with both relaxants (Fig. 5).

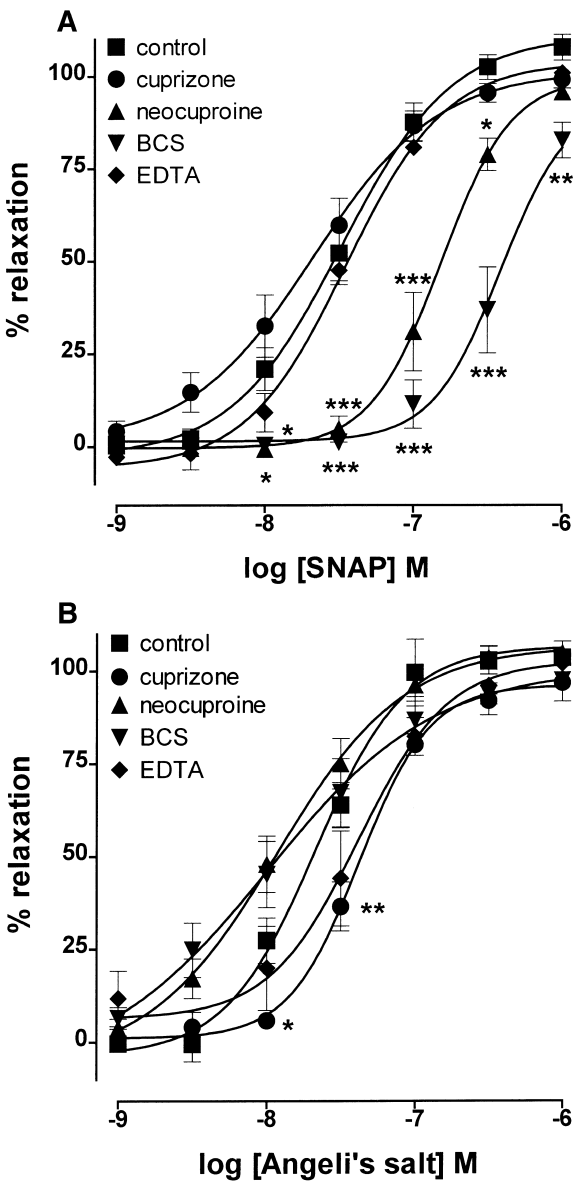

Fig. 4. Concentration–response curves showing the ability of the selective Cu^{+} chelators, neocuproine and bathocuproine disulfonate (BCS), and the selective Cu^{2+} chelators, cuprizone and EDTA, all at 30 μM for 20 min to influence the relaxation of rat aortic rings induced by (A) SNAP and (B) Angeli's salt. Each point is the mean ± S.E.M. of 8 to 15 observations. $^{*}P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$ indicate a significant difference from control.

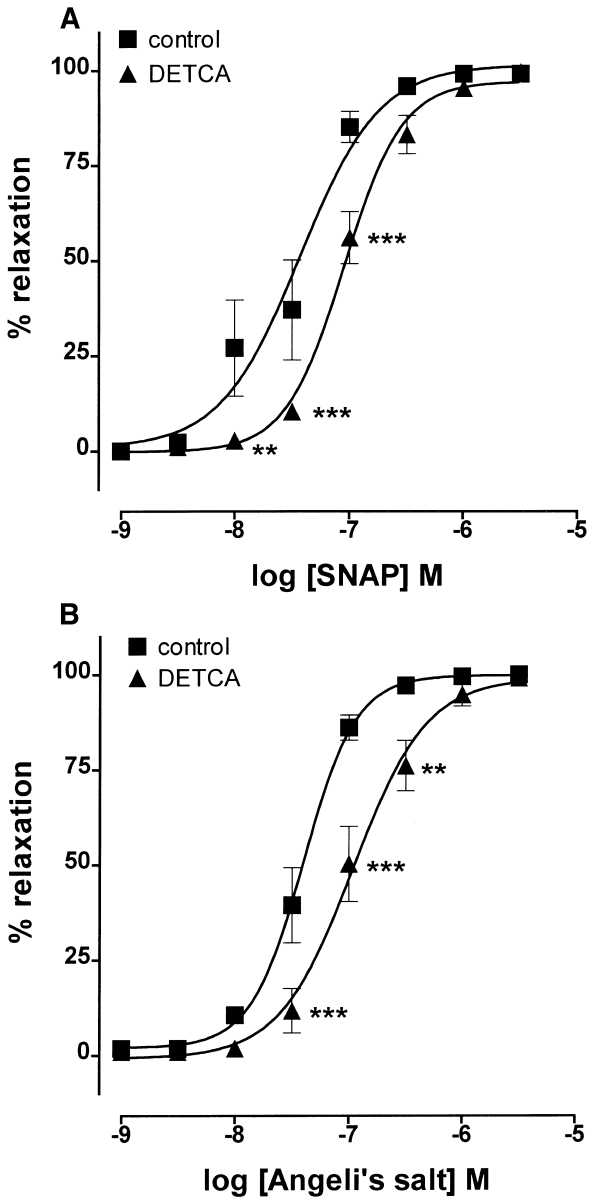

Fig. 5. Concentration–response curves showing the ability of the intracellular Cu^{2+} chelator, diethyldithiocarbamate (DETCA) at 3 mM for 2 h, to influence the relaxation of rat aortic rings induced by (A) SNAP and (B) Angeli's salt. Each point is the mean ± S.E.M. of 7 to 8 observations. $^{**}P < 0.005$ and $^{***}P < 0.001$ indicate a significant difference from control.

3.3. Effects of the catalase inhibitor 3-amino-1,2,4-triazole, thiol depletors and the cytochrome P450 inhibitor, proadifen, on relaxation

Endogenous catalase oxidises the nitrovasodilator, sodium azide, to nitric oxide (Murad et al., 1978), but its ability to oxidise nitroxyl anion has not been tested. We therefore, treated aortic rings with the catalase inhibitor, 3-amino-1,2,4-triazole (Margoliash and Novogrodsky, 1957), and examined the effects on relaxation to sodium azide and to Angeli's salt. Fig. 6 shows that treatment with 3-amino-1,2,4-triazole (10 mM, 90 min) powerfully inhibited relaxation to sodium azide but slightly enhanced relaxation to Angeli's salt.

Since endogenous thiols play a role in the liberation of nitric oxide from the nitrovasodilator, glyceryl trinitrate (Feelisch, 1991), we made use of the thiol depletors diamide and *N*-ethylmaleimide to determine if they might also be involved in the generation of nitric oxide from nitroxyl. We found that treatment for 20 min with low concentrations (10–100 μM) of diamide or *N*-ethylmalei-

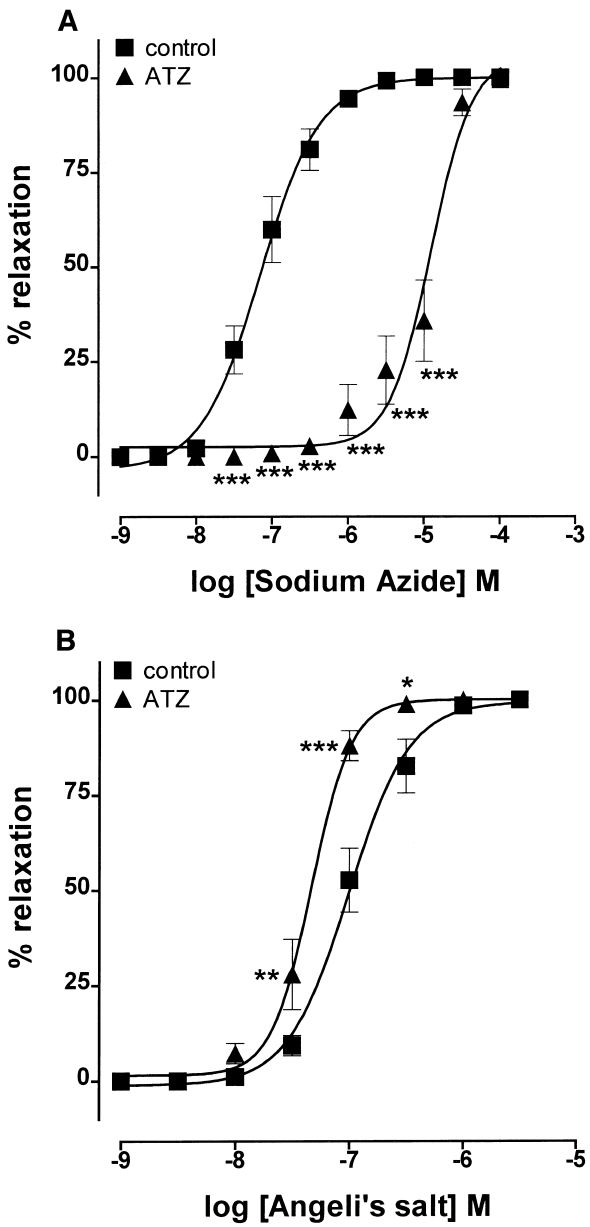

Fig. 6. Concentration–response curves showing the ability of the catalase inhibitor, 3-amino-1,2,4-triazole (ATZ) at 10 mM for 90 min, to influence the relaxation of rat aortic rings induced by (A) sodium azide and (B) Angeli's salt. Each point is the mean ± S.E.M. of 8 observations. $^{*}P < 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$ indicate a significant difference from control.

mide had no effect on relaxation to glyceryl trinitrate (1 nM–10 μM) or Angeli's salt (1 nM–30 μM), but a higher concentration (1 mM) of each depressed tone so powerfully that the effects on relaxation could not be tested (data not shown).

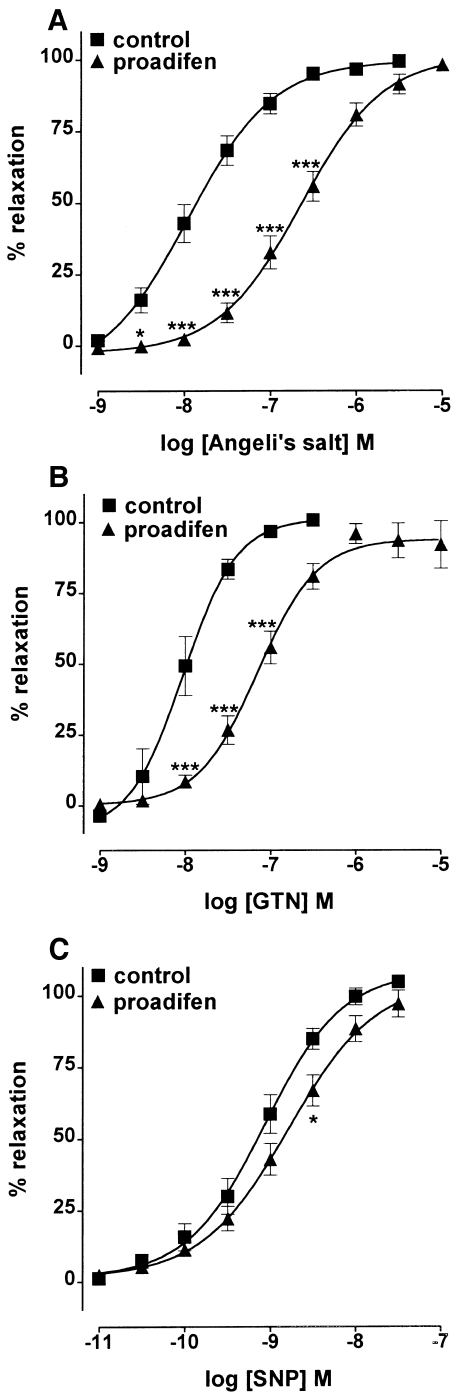

Fig. 7. Concentration–response curves showing the ability of the cytochrome *P*450 inhibitor, proadifen at 100 μM for 20 min, to influence the relaxation of rat aortic rings induced by (A) Angeli's salt, (B) glyceryl trinitrate (GTN) and (C) sodium nitroprusside (SNP). Each point is the mean ± S.E.M. of 8 to 12 observations. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate a significant difference from control.

Cytochrome *P*450 is known to contribute to the generation of nitric oxide from glyceryl trinitrate (Schröder, 1992), and consequently, we investigated the effects of inhibiting this pathway with proadifen. We found that treating aortic rings for 20 min with proadifen (100 μM) produced powerful blockade of relaxation induced by Angeli's salt and glyceryl trinitrate but had a minor effect to that of sodium nitroprusside (Fig. 7).

4. Discussion

Nitroxyl anion, the one-electron reduced species of nitric oxide cannot activate soluble guanylate cyclase (Dierks and Burstyn, 1996). Nevertheless, nitroxyl anion generators, such as Angeli's salt, promote vascular relaxation, almost certainly through rapid, tissue-dependent oxidation of nitroxyl to nitric oxide, since relaxation is associated with an increase in cyclic GMP levels and is blocked by ODQ (Fukuto et al., 1992a; Li et al., 1999). Moreover, there has been speculation that endogenous superoxide dismutase might be responsible for this oxidation step (Murphy and Sies, 1991; Schmidt et al., 1996). Our recent findings show, however, that although superoxide dismutase can indeed catalyse this reaction, the amount of enzyme required is vastly greater than is present in cells (Nelli et al., 2000). This study also revealed that copper ions efficiently catalyse the oxidation of nitroxyl anion to nitric oxide and prompted us to determine if this process could explain the ability of the nitroxyl anion generator, Angeli's salt, to promote vascular relaxation. This seemed possible because endogenous Cu^{+} ions were already known to contribute to the generation of nitric oxide from *S*-nitrosothiols, and so underlie the vasorelaxant and platelet inhibitory actions of this class of compound (Gordge et al., 1995, 1996; Al-Sa'doni et al., 1997).

Our first experiments involved determining if adding copper sulphate augmented the relaxation of endothelium-denuded rings of rat aorta induced by Angeli's salt and by the *S*-nitrosothiol, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). We found that copper sulphate did indeed augment relaxation to both agents: this effect was optimal at 10 μM (6.2-fold and 3.5-fold leftward shifts in EC_{50} values for Angeli's salt and SNAP, respectively) but was lost at 100 μM, perhaps due to copper-induced generation of hydroxyl radical by the Fenton reaction. Previous experiments using a nitric oxide detector (Nelli et al., 2000), demonstrated that a concentration of 10 μM copper sulphate was sufficient to stimulate, albeit sub-optimally, the generation of nitric oxide from Angeli's salt but much higher levels (optimum 10 mM) were required for generation from SNAP. It was therefore likely that the augmentation of relaxation was explained by a property of copper sulphate distinct from its ability to enhance the generation of nitric oxide from these agents. Copper ions are known to scavenge superoxide anion in an analogous manner to

superoxide dismutase (Huber et al., 1987; Sorenson, 1995), and so we investigated whether protection of nitric oxide from destruction by superoxide might underlie the augmented relaxation to Angeli's salt and SNAP. There was no reason to suspect that superoxide anion would react directly with the negatively charged nitroxyl anion. We found, in fact, that the Cu^{2+}–Zn^{2+} isoform of superoxide dismutase did indeed augment relaxation to both Angeli's salt and SNAP and the magnitude of this effect was similar to that produced by copper sulphate (10 μM). Moreover, manganese chloride, which also acts as a superoxide scavenger (Kasten et al., 1994; MacKenzie et al., 1999), but does not catalyse the oxidation of nitroxyl to nitric oxide (Nelli et al., 2000), augmented relaxation to both Angeli's salt and SNAP in an analogous manner to copper sulphate or Cu^{2+}–Zn^{2+} superoxide dismutase. Thus, it seemed certain that augmentation of the relaxant actions of Angeli's salt and SNAP by copper sulphate resulted from protection of the nitric oxide they produce rather than from enhanced production. This conclusion is supported by the finding that copper sulphate, superoxide dismutase and manganese chloride themselves produce relaxation of endothelium-containing but not endothelium-denuded vessels by protecting basal nitric oxide from destruction by superoxide (Kasten et al., 1994; MacKenzie et al., 1999).

Our next approach to explore the potential role of copper ions in the vasodilator actions of Angeli's salt was to make use of selective chelators of this metal ion. Previous reports indicate that the selective chelators of the Cu^{+} ion, neocuproine and bathocuproine disulfonate, but not the chelator of Cu^{2+}, cuprizone, block the ability of SNAP to inhibit human blood platelets (Gordge et al., 1995, 1996). Moreover, neocuproine blocks the ability of SNAP and *S*-nitroso-glutathione to relax the rat isolated perfused tail artery (Al-Sa'doni et al., 1997). In keeping with the conclusion from these studies that endogenous Cu^{+} plays a role in the generation of nitric oxide from *S*-nitrosothiols, we found that the Cu^{+} chelators, neocuproine and bathocuproine disulfonate, but not the Cu^{2+} chelators, cuprizone and EDTA, powerfully inhibited the ability of SNAP to relax rat aortic rings. The results with Angeli's salt were less straightforward. We showed previously that Cu^{+} and Cu^{2+} are equi-effective in oxidising nitroxyl released from Angeli's salt to nitric oxide (Nelli et al., 2000). Nevertheless, the Cu^{+} chelators, neocuproine and bathocuproine disulfonate, had no significant effect on relaxation to Angeli's salt, and of the Cu^{2+} chelators tested, cuprizone produced a small but significant inhibition and EDTA produced an apparent block, which failed to reach statistical significance. A previous report demonstrated that the membrane-permeant chelator of Cu^{2+}, diethyldithiocarbamate, inhibited relaxation of rat aorta to Angeli's salt (Pino and Feelisch, 1994) and we confirmed this, although the magnitude of the inhibition was small (2.5-fold rightward shift at EC_{50}). Caution is required in the interpretation of this effect, however, since the copper-chelating action of diethyldithiocarbamate leads to inhibition of Cu^{2+}–Zn^{2+} superoxide dismutase (Cocco et al., 1981) and soluble guanylate cyclase (Plane et al., 1997), leading respectively, to enhanced destruction and reduced effectiveness of nitric oxide (MacKenzie and Martin, 1998). A further complicating factor is that Cu^{2+}–Zn^{2+} superoxide dismutase has been proposed as an endogenous converter of nitroxyl to nitric oxide (Murphy and Sies, 1991; Schmidt et al., 1996). Taken together, however, our findings with these chelators suggest that if endogenous copper does contribute to the tissue-dependent oxidation of nitroxyl to nitric oxide, then the magnitude of this contribution is small.

In view of the limited ability of copper chelators to inhibit Angeli's salt-induced relaxation, we investigated the potential involvement of pathways known to contribute to nitric oxide formation by established nitrovasodilators. Specifically, sodium azide is converted to nitric oxide by catalase and other peroxidases (Murad et al., 1978; Katsuki et al., 1977; Mian and Martin, 1995), but although inhibition of these enzymes with 3-amino-1,2,4-triazole (Margoliash and Novogrodsky, 1957) powerfully blocked azide-induced relaxation of rat aorta, we observed no blockade of that induced by Angeli's salt. Indeed, 3-amino-1,2,4-triazole enhanced Angeli's salt-induced relaxation slightly, perhaps as a consequence of the accumulation of hydrogen peroxide, which itself promotes vasodilatation (Mian and Martin, 1995). Thus, catalase and peroxidase enzymes are unlikely to contribute to the oxidation of nitroxyl to nitric oxide. An alternative pathway for conversion of nitrovasodilators, including glyceryl trinitrate and sodium nitroprusside to nitric oxide, is through reduction by tissue thiols (Feelisch, 1991). Consequently, we attempted to investigate the potential role of this pathway in converting nitroxyl to nitric oxide. We found, however, that the thiol-depleting agents, diamide and *N*-ethylmaleimide (Kosower et al., 1969; Siegle et al., 1993), depressed tone so powerfully that their effects on relaxation by Angeli's salt and other nitrovasodilators could not be tested. In any event, L-cysteine is a powerful scavenger of nitroxyl anion (Pino and Feelisch, 1994) and thiols in general are powerful reducing agents. It is therefore, unlikely that endogenous thiols have a role in mediating the oxidation of nitroxyl to nitric oxide. Cytochrome *P*450 is believed to contribute to the generation of nitric oxide from glyceryl trinitrate (Schröder, 1992) and this enzyme system is reported to lead to the generation of nitric oxide from Angeli's salt under anaerobic conditions (Shibata et al., 1977). We therefore examined the effects of proadifen (SKF 525A), an inhibitor of multiple isoforms of cytochrome *P*450 (Schröder, 1992; Singer et al., 1984), on relaxation to glyceryl trinitrate and Angeli's salt. As expected, we found that proadifen strongly blocked the relaxation to glyceryl trinitrate (7.5-fold rightward shift at EC_{50}), but relaxation to Angeli's salt was blocked even more effectively (20.5-fold rightward shift at EC_{50}). In order to

test the selectivity of proadifen, we examined its effect on relaxation by sodium nitroprusside, which had previously been reported to be unaffected by this agent (Singer et al., 1984). Although we found a small block at one of the eight concentrations of sodium nitroprusside tested, it was clear that proadifen was not an effective inhibitor of this relaxant. Thus, our findings with proadifen, together with those of Shibata et al. (1977), strongly suggest a role for cytochrome *P*450 in the production of nitric oxide from the nitroxyl generator, Angeli's salt. Interestingly, proadifen also inhibits acetylcholine-induced relaxation in rabbit aorta (Singer et al., 1984), thus supporting the previous suggestion that nitroxyl anion might be the primary product of nitric oxide synthase (Schmidt et al., 1996) or indeed the endothelium-derived relaxing factor (EDRF) (Ellis et al., 2000).

In conclusion, although copper ions efficiently catalyse the one-electron oxidation of nitroxyl anion to nitric oxide (Nelli et al., 2000), our experiments with selective chelating agents suggest a minor role for this metal ion in the relaxant actions of the nitroxyl anion generator, Angeli's salt, in rat aorta. In contrast, our experiments with proadifen suggest a more important role for oxidation by cytochrome *P*450.

Acknowledgements

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