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Role of catalase in the smooth muscle relaxant actions of sodium azide and cyanamide

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Abstract

The aim of this study was to determine the role of catalase in the smooth muscle relaxant actions of sodium azide and cyanamide. The effects of 3-amino-1,2,4-triazole suggested a role for this enzyme in the relaxant actions of sodium azide on rat aorta and bovine retractor penis muscle and cyanamide on rat aorta. Moreover, results obtained using a difference spectrophotometric assay based upon the oxidation of haemoglobin were consistent with the catalase-dependent oxidation of sodium azide to nitric oxide (NO) and of cyanamide to nitroxyl anion. Surprisingly, however, no free nitric oxide or nitroxyl was detected in solution using a sensitive electrode. This anomaly might be explained if the stable complexes of catalase with nitric oxide or nitroxyl do not release their respective ligand except to sites of high affinity, such as the haemoglobin employed in the difference spectrophotometric assay, or indeed, the soluble guanylate cyclase within the smooth muscle.
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1. Introduction

Sodium azide and hydroxylamine are nitrovasodilators that relax vascular and nonvascular smooth muscle through activation of soluble guanylate cyclase and elevation of cellular cyclic GMP content (Waldman and Murad, 1987). Both are prodrugs, however, and require metabolic activation before they can activate soluble guanylate cyclase. Early studies showed that metabolism by catalase and related peroxidase enzymes in the presence of the co-substrate hydrogen peroxide could explain the ability of azide and hydroxylamine to activate guanylate cyclase (Mittal et al., 1975; Miki et al., 1976). Moreover, subsequent work reporting the ability of 3-amino-1,2,4-triazole, an inhibitor of catalase and other peroxidases (Margoliash and Novogrodsky, 1957), to block azide- and hydroxylamine-induced relaxation of rat aorta (Mian and Martin, 1995) was consistent with this pathway for metabolic activation. Since nitric oxide (NO) radical can directly activate soluble guanylate cyclase (Arnold et al., 1977), it has generally been assumed that the smooth muscle relaxant actions of azide and hydroxylamine result from their metabolic conversion to this species. Biochemical studies with mammalian catalase have, however, failed to demonstrate the generation of nitric oxide from these agents, although they consistently report the formation of nitrogen dioxide and nitrogen together with two complexes: one of nitric oxide with catalase and the other of nitric oxide with compound 1, the catalase–hydrogen peroxide complex (Theorell and Ehrenberg, 1952; Nicholls, 1964; Brunelli et al., 2001). A recent report on peroxidase from the wood-rotting fungus *Phanerochaete crysosporium* suggests, however, that nitroxyl anion (NO^-) is a major product of the metabolism of azide (Tatarko and Bumpus, 1997), raising the possibility of formation of this species also by mammalian catalase. Nitroxyl anion itself cannot activate guanylate cyclase (Dierks and Burstyn, 1996) but it undergoes rapid, tissue-dependent, one-electron oxidation to nitric oxide. As a consequence, nitroxyl anion generators such as Angeli's salt are powerful smooth muscle relaxants (Fukuto et al., 1992; Nelli et al., 2001).

Cyanamide is another agent that can be metabolically activated by mammalian catalase (Nagasawa et al., 1990; Shoeman et al., 2000). The product of this reaction is believed to be nitroxyl anion which irreversibly inhibits al-

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dehyde dehydrogenase and hence, underlies the use of cyanamide as an alcohol deterrent. In spite of this presumed metabolism to nitroxyl anion, however, cyanamide is reported to lack vasodilator activity (De Master et al., 1989; Fukuto et al., 1994). It does, nevertheless, appear to possess modest relaxant activity when added to rabbit aortic rings in the presence of exogenous catalase and hydrogen peroxide (Fukuto et al., 1994).

The aim of this study was to determine whether nitric oxide radical or nitroxyl anion is the species generated following the metabolism of azide or cyanamide by mammalian catalase. Moreover, we wished to relate the production of nitric oxide or nitroxyl to the abilities of these agents to relax vascular (rat aorta) and nonvascular (bovine retractor penis) smooth muscle.

2. Materials and methods

2.1. Preparation of bovine retractor penis muscle strips

Strips of bovine retractor penis muscle were isolated and suspended for tension recording as described earlier (Liu et al., 1994). Briefly, bovine penises were obtained from a local abattoir and transported to the laboratory at ambient temperature. The retractor penis muscles were dissected out and cleared of superficial connective tissue. Some tissues were used that day and others were stored at 4 °C in oxygenated Krebs solution for use the following day. Bovine retractor penis muscle strips ~1.5-mm wide and ~1.0-cm long were cut and mounted within Ag–AgCl ring electrodes using fine silk thread (5/0). The strips were placed under 2*g* resting tension in 10 ml organ bath filled with Krebs solution at 37 °C solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, and gassed with 95% oxygen and 5% CO_2. Tissues were allowed to equilibrate for 60 min before experiments were carried out, during which time the resting tension was readjusted to 2*g*, as required.

2.2. Preparation of rat aortic rings

Rat aortic rings were isolated and suspended for tension recording as described earlier (Nelli et al., 2001). Briefly, male Wistar rats of approximately 200–250 g were killed by stunning and exsanguination. The thoracic aorta was carefully removed, cleaned of fat and connective tissue, and cut into 2.5-mm-wide transverse rings with a razor blade slicing device. The endothelium was removed from all rings by gentle abrasion of the luminal surface with moist forceps. The rings were then mounted under 1*g* resting tension on stainless steel hooks within 20 ml organ baths and maintained at 37 °C in Krebs solution. 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.

2.3. Tension recording

Tension of bovine retractor penis strips and rat aortic rings was measured with Grass FTO3C isometric transducers and displayed on a Grass Polygraph model 7.

2.4. Experimental protocols with bovine retractor penis strips and rat aortic rings

Following induction of submaximal tone on bovine retractor penis strips (with guanethidine, 30–100 μM) and rat aortic rings (with phenylephrine, 10–30 nM), cumulative concentration–response curves were generated for the relaxant actions of sodium azide and cyanamide. The ability of the nitroxyl anion generator, Angeli's salt, to relax strips of bovine retractor penis was also assessed cumulatively. Relaxations are expressed as a percentage of guanethidine- or phenylephrine-induced tone.

In some experiments, the effects of the nitric oxide scavenger, haemoglobin (Martin et al., 1985), and the inhibitor of soluble guanylate cyclase, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ; Garthwaite et al., 1995), were investigated on the relaxant actions of Angeli's salt, sodium azide, and cyanamide. In these experiments, tissues were incubated with haemoglobin or ODQ at 10 μM for 20 min before addition of the relaxants.

In other experiments, the effects of the irreversible inhibitor of the catalase, 3-amino-1,2,4-triazole (Margoliash and Novogrodsky, 1957; Mian and Martin, 1995) were investigated on the relaxant actions of Angeli's salt, sodium azide, and cyanamide. In view of the irreversible nature of this blockade, tissues were incubated with 3-amino-1,2,4-triazole at 50 mM for 90 min and subsequently washed. Tone was then established in bovine retractor penis strips and rat aortic rings as described above and allowed to stabilise before addition of the relaxants.

In all experiments in which the effects of blocking drugs were examined, the concentrations of the contractile agents (guanethidine and phenylephrine) were adjusted to ensure that the levels of tone in bovine retractor penis muscle strips and aortic rings were similar to those used in control experiments.

2.4.1. Measurement of the generation of nitric oxide radical and nitroxyl anion

We wished to determine if metabolism of sodium azide (10 μM) and cyanamide (1 mM) by catalase (3000 u ml^{-1}) in the presence of a source of hydrogen peroxide (glucose 20 μM/glucose oxidase 0.3 u ml^{-1}; Murphy and Sies, 1991) led to the generation of nitric oxide radical or nitroxyl anion. It was essential to supply hydrogen peroxide continuously at low concentration in this manner since high concentrations of authentic hydrogen peroxide oxidise haemoglobin (and methaemoglobin) and thus confound the assay (Murphy and Noack, 1994). These experiments were conducted at 37 °C in phosphate buffer (50 mM, pH 7.0). The generation of nitric

oxide radical and nitroxyl was assessed using two different approaches: a spectrophotometric assay and an electrochemical assay using a sensitive electrode.

2.4.2. Spectrophotometric assay of nitric oxide radical and nitroxyl anion

This assay is based on the rapid and quantitative oxidation of haemoglobin to methaemoglobin by nitric oxide radical (Feelisch and Noack, 1987; Kelm and Schrader, 1988). The difference spectrum of haemoglobin versus methaemoglobin, recorded with a double beam spectrophotometer (Shimadzu UV 2400) displayed an absorbance maximum at 401 nm with an isosbestic point at 409 nm. The rate of formation of a nitric oxide radical-like species was taken as the rate of change of the difference spectrum and is expressed as $\Delta A_{401-409\ nm}$ min^{-1}. In some experiments, the formation of nitroxyl anion was assessed by attempting to oxidise it to nitric oxide radical by including superoxide dismutase (500 u ml^{-1}; Murphy and Sies, 1991) in the reaction mixture. $CuSO_4$ is more efficient than superoxide dismutase at oxidising nitroxyl (Nelli et al., 2000), but it could not be employed in these experiments because it reacted adversely with haemoglobin causing it to precipitate.

2.4.3. Electrochemical assay of nitric oxide radical and nitroxyl anion

Nitric oxide radical was detected using ISO-NOP200 amperometric electrodes fitted to an ISO-NO Mark II nitric oxide meter (World Precision Instruments, UK) as previously described (Nelli et al., 2000, 2001). The signals generated were captured and displayed on a MacLab (8e Series, AD Instruments, UK). Although the electrode cannot detect nitroxyl anion per se, nitroxyl can be rapidly oxidised to nitric oxide radical by $CuSO_4$ (Nelli et al., 2000). Consequently, when detection of nitroxyl anion was required, $CuSO_4$ (10 μM) was added to the reaction mixture.

2.5. Measurement of catalase activity

Catalase activity was measured by a modification of the method of Suttorp et al. (1986), in which the disappearance of hydrogen peroxide was followed spectrophotometrically at 230 nm. The final reaction mixture contained 12 mM hydrogen peroxide in 50 mM sodium phosphate buffer (pH 7.0) together with either bovine liver catalase (3000 u ml^{-1}) or bovine retractor penis muscle supernatants. These supernatants were prepared by a modification of the method of Terblanche (1999). Specifically, control bovine retractor penis muscle strips set up in organ baths as indicated above (Section 2.2) and strips treated with ODQ (10 μM, 20 min) or 3-amino-1,2,4-triazole (50 mM, 90 min) were frozen in liquid N_2, pulverised in a stainless steel mortar and pestle, and extracted into five volumes of 50 mM sodium phosphate buffer (pH 7.0). The homogenates were sonicated for 60 min and then spun to 1500*g* for 10 min at 4 °C. The catalase activity of the supernatants was then assayed as above.

2.6. Drugs and reagents

Angeli's salt (sodium trioxodinitrate) and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ) were obtained from Alexis (Nottingham, UK). 3-Amino-1,2,4-triazole, catalase (bovine liver), cyanamide, glucose oxidase (*Aspergillus niger*), guanethidine sulfate, haemoglobin (bovine erythrocyte), phenylephrine hydrochloride, sodium azide, and sodium dithionite were obtained from Sigma (Poole, UK). All other chemicals were of analytical grade. All drugs were dissolved in saline (0.9%) except Angeli's salt (0.1 M) which was dissolved in 0.01 M NaOH, and ODQ (0.1 M) and cyanamide (10 M) which were dissolved in dimethylsulfoxide.

Solutions of haemoglobin were prepared as previously described (Martin et al., 1985). Briefly, a 10-fold molar ex-

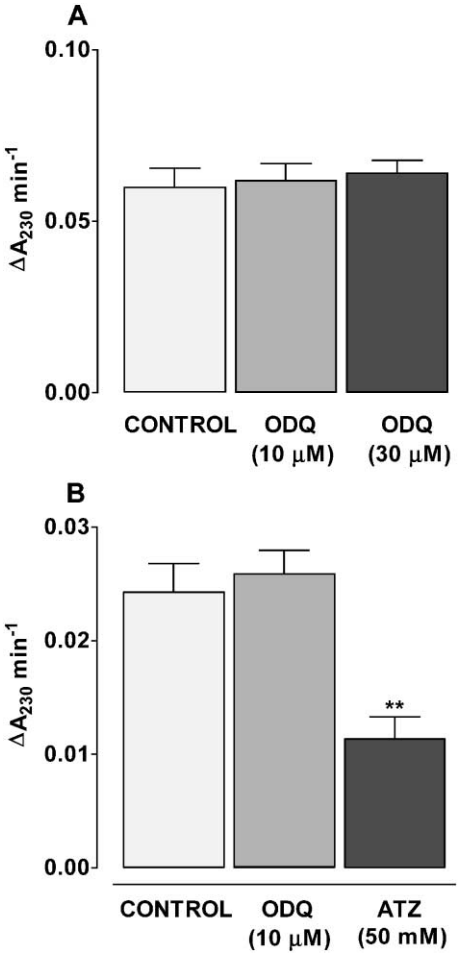

Fig. 1. Bar graphs showing: (A) the effects of an inhibitor of soluble guanylate cyclase (1*H*-[1,2,4]-oxadiazole-[4,3-*a*]-quinoxaline-1-one; ODQ; 10 or 30 μM for 20 min) on the activity of bovine liver catalase (3000 u ml^{-1}); and (B) the effects of ODQ (10 μM, 20 min) or of an inhibitor of catalase (3-amino-1,2,4-triazole; AT; 50 mM for 90 min) on the catalase activity of bovine retractor penis strips. Catalase activity was assessed as a function of the loss of absorbance of hydrogen peroxide (12 mM) at 230 nm per min (ΔA_{230} min^{-1}). Each column is the mean ± S.E.M. of eight observations. ** $P < 0.01$ indicates a significant difference from control.

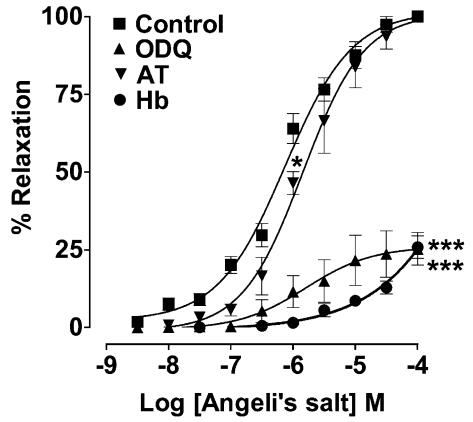

Fig. 2. Concentration–response curves showing the ability of an inhibitor of soluble guanylate cyclase (1*H*-[1,2,4]-oxadiazole-[4,3-*a*]-quinoxaline-1-one; ODQ; 10 μM for 20 min), or of catalase (3-amino-1,2,4-triazole; AT; 50 mM for 90 min), or a scavenger of nitric oxide (haemoglobin; Hb; 10 μM for 20 min) to influence the relaxation of bovine retractor penis muscle induced by Angeli's salt. Each point is the mean ± S.E.M. of six to nine observations. * $P<0.05$ and *** $P<0.001$ indicate a significant difference from control.

cess of the reducing agent, sodium dithionite, was added to aqueous solutions of Sigma haemoglobin (1 mM; supplied as mixture of haemoglobin/methaemoglobin) and this was followed by dialysis against 250 volumes of distilled water for 2 h at 4 °C. The resulting solution was stored in aliquots at −20 °C for 7 days.

2.7. Statistical analysis of data

Results are expressed as the mean ± S.E.M. of *n* separate experiments. 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 $\log EC_{50}$ values were calculated by nonlinear regression analysis using a computer-based program (GraphPad, Prism).

3. Results

3.1. Effects of ODQ, haemoglobin, and 3-amino-1,2,4-triazole on the ability of Angeli's salt to relax bovine retractor penis

Treatment with the catalase inhibitor, 3-amino-1,2,4-triazole (50 mM, 90 min), significantly reduced the catalase activity of bovine retractor penis muscle strips (Fig. 1B). In contrast, the inhibitor of soluble guanylate cyclase, ODQ (10 μM, 20 min), had no effect on the catalase activity of bovine retractor penis muscle strips. Indeed, treatment with ODQ (10 or 30 μM, 20 min) had no effect on the activity of commercially available bovine liver catalase (3000 u ml^{-1}, Fig. 1A). In the presence of guanethidine (30–100 μM)-induced tone, the nitroxyl anion generator, Angeli's salt (3 nM–100 μM), produced concentration-dependent relaxation of bovine retractor penis muscle strips (Fig. 2). Treatment of the muscle strips with ODQ or the nitric oxide scavenger (both 10 μM, 20 min) powerfully blocked this relaxation. In contrast, 3-amino-1,2,4-triazole (50 mM, 90 min) had little effect on the relaxation produced by Angeli's salt.

3.2. Effects of ODQ, haemoglobin, and 3-amino-1,2,4-triazole on the ability of sodium azide and cyanamide to relax bovine retractor penis

Sodium azide (1 nM–30 μM) produced powerful concentration-dependent relaxation of bovine retractor penis muscle strips (Fig. 3A). In contrast, cyanamide (1–100

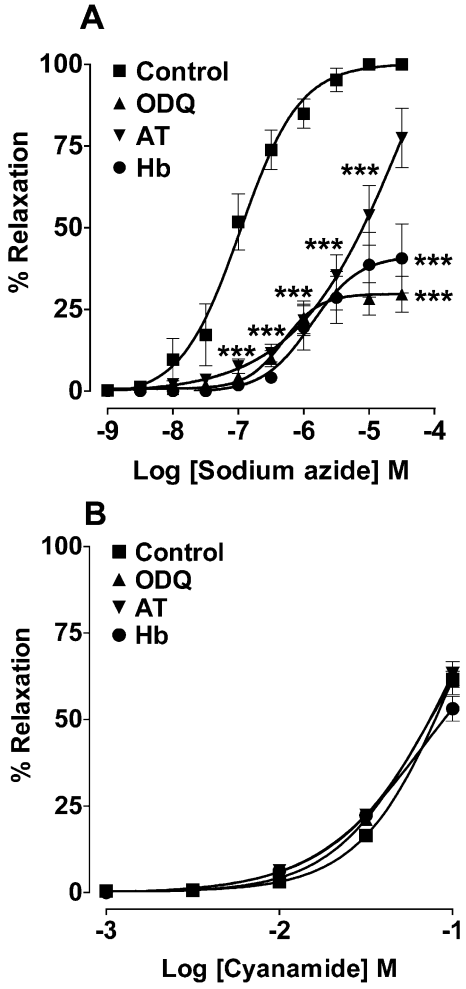

Fig. 3. Concentration–response curves showing the ability of an inhibitor of soluble guanylate cyclase (1*H*-[1,2,4]-oxadiazole-[4,3-*a*]-quinoxaline-1-one; ODQ; 10 μM for 20 min), or of catalase (3-amino-1,2,4-triazole; AT; 50 mM for 90 min), or a scavenger of nitric oxide (haemoglobin; Hb; 10 μM for 20 min) to influence the relaxation of bovine retractor penis muscle induced by (A) sodium azide and (B) cyanamide. Each point is the mean ± S.E.M. of five to nine observations. *** $P<0.001$ indicates a significant difference from control.

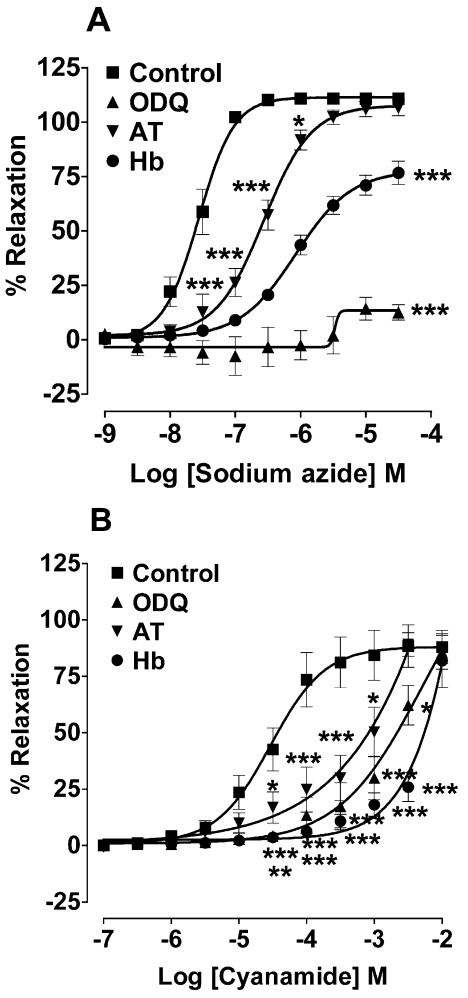

Fig. 4. Concentration–response curves showing the ability of an inhibitor of soluble guanylate cyclase (1*H*-[1,2,4]-oxadiazole-[4,3-*a*]-quinoxaline-1-one; ODQ; 10 μM for 20 min) or of catalase (3-amino-1,2,4-triazole; AT; 50 mM for 90 min), or a scavenger of nitric oxide (haemoglobin; Hb; 10 μM for 20 min), to influence the relaxation of endothelium-denuded rings of rat aorta induced by (A) sodium azide and (B) cyanamide. Each point is the mean ± S.E.M. of five to nine observations. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ indicate a significant difference from control.

mM) was a weak relaxant (Fig. 3B). Treatment with ODQ or haemoglobin (each at 10 μM for 20 min) powerfully blocked relaxation to sodium azide but had no effect on that with cyanamide. Treatment with 3-amino-1,2,4-triazole (50 mM, 90 min) inhibited relaxation to sodium azide but had no effect on that with cyanamide.

3.3. Effects of ODQ, haemoglobin, and 3-amino-1,2,4-triazole on the ability of sodium azide and cyanamide to relax rat aortic rings

Following development of phenylephrine (10–30 nM)-induced tone in endothelium-denuded rings of rat aorta, sodium azide (1 nM–30 μM) and cyanamide (0.1 μM–10 mM) each produced concentration-dependent relaxation (Fig. 4). Treatment with ODQ or haemoglobin (each at 10 μM for 20 min) powerfully inhibited relaxation to sodium azide and cyanamide. Furthermore, treatment with 3-amino-1,2,4-triazole (50 mM, 90 min) powerfully blocked relaxation to sodium azide and cyanamide.

3.4. Generation of a nitric oxide-like species following metabolism of sodium azide and cyanamide by catalase as measured spectrophotometrically

The ability of catalase (3000 u ml^{-1}) in the presence of a source of hydrogen peroxide (glucose 20 μM/glucose oxidase 0.3 u ml^{-1}) to metabolise sodium azide and cyanamide to a nitric oxide-like species was assessed by measuring the oxidation of haemoglobin to methaemoglobin using a difference spectrophotometric assay (Feelisch and Noack, 1987).

Sodium azide (10 μM) had no effect by itself, but in the presence of catalase and a source of hydrogen peroxide, promoted oxidation of haemoglobin consistent with formation of a nitric oxide-like species (Fig. 5A). No oxidation of haemoglobin took place when the source of hydrogen peroxide was omitted (data not shown). Cyanamide (1 mM) alone had no effect on haemoglobin (Fig. 5B). It also, in

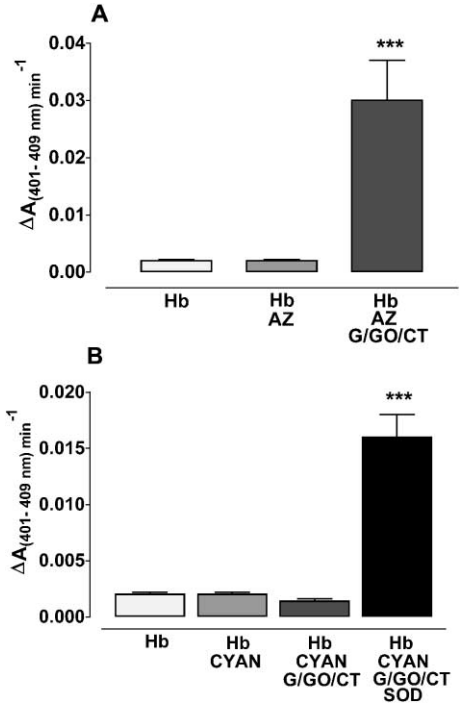

Fig. 5. Bar graph in which the oxidation of haemoglobin (Hb), assessed by a difference spectrophotometric assay ($\Delta A_{401-409\ nm}$ min^{-1}), was used as an index of formation of a nitric oxide-like species. Columns show oxidation of haemoglobin produced by: (A) sodium azide (AZ, 10 μM) and (B) cyanamide (CYAN, 1 mM), each alone, and following incubation with glucose (20 μM)/glucose oxidase (0.3 u ml^{-1}) and catalase (3000 u ml^{-1}) (G/GO/CT). Note that in the case of cyanamide, oxidation occurred only upon the addition of superoxide dismutase (SOD, 500 u ml^{-1}). Each column is the mean ± S.E.M. of five to eight observations. *** $P<0.001$ indicates a significant difference from control.

contrast to sodium azide, failed to promote the oxidation of haemoglobin in the presence of catalase and a source of hydrogen peroxide. Cyanamide did, however, promote the oxidation of haemoglobin when superoxide dismutase (500 u ml^{-1}) was present together with catalase and a source of hydrogen peroxide.

3.5. Generation of nitric oxide radical following metabolism of sodium azide and cyanamide by catalase as measured electrochemically

The ability of catalase (3000 u ml^{-1}) in the presence of a source of hydrogen peroxide to metabolise sodium azide and cyanamide to nitric oxide radical was assessed electrochemically using a sensitive electrode. In addition, the formation of nitroxyl anion was assessed by the addition of $CuSO_4$, which rapidly oxidises nitroxyl to nitric oxide radical (Nelli et al., 2000).

Sodium azide (10 μM) and cyanamide (1 mM) each generated small signals in the nitric oxide detector (Fig. 6), but these were artifacts since they were not blocked by haemoglobin (10 μM; data not shown). Surprisingly, neither of these agents led to the generation of detectable nitric oxide radical when they were metabolised by catalase (3000 u ml^{-1}) in the presence of a source of hydrogen peroxide. Moreover, nitroxyl anion was not generated either, since the subsequent addition of $CuSO_4$ (10 μM) failed to elicit a nitric oxide signal. However, subsequent addition of the nitroxyl anion releaser, Angeli's salt (10 μM), in both cases, leads to the generation of a powerful nitric oxide signal which was blocked by haemoglobin (10 μM; 97.7 ± 0.7%, $n = 5$, $P < 0.001$) and by carboxy-PTIO (1 mM; 85.6 ± 3.2, $n = 5$, $P < 0.001$) showing that the assay conditions permitted detection of nitroxyl anion and nitric oxide radical.

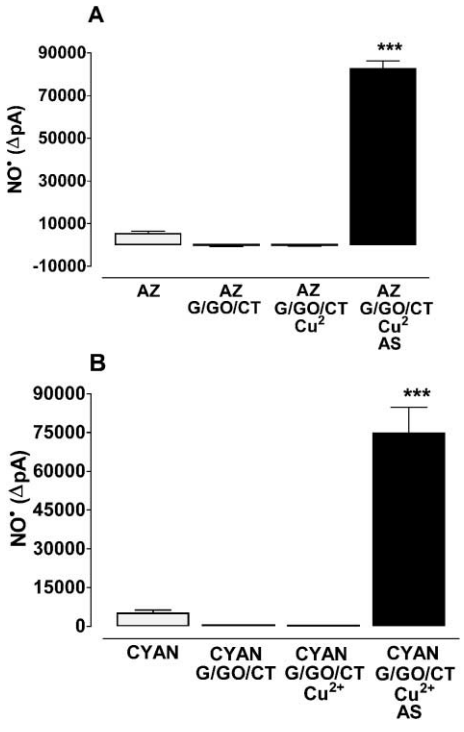

Fig. 6. Bar graph in which the current (ΔpA) generated by a sensitive electrode was used to assess the formation of nitric oxide radical. Columns show the generation of the signal from: (A) sodium azide (AZ, 10 μM) and (B) cyanamide (CYAN, 1 mM), each alone, and following incubation with glucose (20 μM)/glucose oxidase (0.3 u ml^{-1}) and catalase (3000 u ml^{-1}) (G/GO/CT). In each case, the third column shows the effect of adding $CuSO_4$ (Cu^{2+}, 10 μM) which oxidises nitroxyl anion to nitric oxide radical. The fourth column shows the effect of adding the nitroxyl anion generator, Angeli's salt (10 μM). Each column is the mean ± S.E.M. of five to six observations *** $P < 0.001$ indicates a significant difference from control.

4. Discussion

Consistent with previous reports (Mittal et al., 1975; Waldman and Murad, 1987), we found that the powerful relaxant actions of sodium azide in vascular (rat aorta) and nonvascular (bovine retractor penis) smooth muscle were due to its metabolic transformation to an activator of soluble guanylate cyclase since they were blocked by ODQ (Garthwaite et al., 1995). This activator is almost certainly nitric oxide since these relaxations were powerfully inhibited by haemoglobin (Martin et al., 1985). Cyanamide, although somewhat less potent, was also a powerful relaxant of rat aorta and this action too was blocked by ODQ and haemoglobin, suggesting a similar role for metabolic transformation and activation of soluble guanylate cyclase. This action on rat aorta is, however, in sharp contrast to its effects on rabbit aorta, where cyanamide is devoid of relaxant activity except in the presence of additional added catalase and hydrogen peroxide (Fukuto et al., 1994). Rabbit aorta therefore, appears to lack the ability of rat aorta to metabolically transform cyanamide to a species that activates soluble guanylate cyclase. Bovine retractor penis muscle also lacked the ability to metabolically transform cyanamide to an activator of soluble guanylate cyclase since relaxation in this tissue was poor, taking place only at exceptionally high concentrations (>10 mM), and this was unaffected by ODQ or haemoglobin. This weak relaxant activity was therefore, probably the result of a nonselective action arising from the high concentrations of the drug required.

It is well accepted that catalase and other peroxidase enzymes, when present together with a source of hydrogen peroxide, can metabolically transform sodium azide to a species that activates soluble guanylate cyclase (Mittal et al., 1975; Miki et al., 1976). Consistent with this concept, we found that the sodium azide-induced relaxation of rat aorta and bovine retractor penis muscle was powerfully blocked by the inhibitor of catalase, 3-amino-1,2,4-triazole (Margoliash and Novogrodsky, 1957; Mian and Martin, 1995). Furthermore, we confirmed that 3-amino-1,2,4-triazole had indeed blocked the catalase activity of bovine retractor penis muscle strips, although the small size of rat aortic rings prevented us from making similar measurements in this tissue.

In addition, despite inhibiting a number of haem-containing enzymes such as soluble guanylate cyclase, nitric oxide synthase, and cytochrome P_{450} (Feelisch et al., 1999), ODQ did not affect the catalase activity of bovine retractor penis muscle strips or indeed, the activity of bovine liver catalase. It was likely therefore, that the inhibitory actions of ODQ on relaxant responses to azide or cyanamide did not involve inhibition of catalase but arose from inhibition of soluble guanylate cyclase.

Cyanamide is another agent that can be metabolically transformed by catalase, but the product of the reaction is believed to be nitroxyl anion (Fukuto et al., 1994; Shoeman et al., 2000), as opposed to the nitric oxide presumed to be formed from sodium azide (Arnold et al., 1977). Our finding that 3-amino-1,2,4-triazole powerfully inhibits cyanamide-induced relaxation of rat aorta is consistent with this pathway for metabolic transformation by catalase. In contrast, the weak relaxation induced by cyanamide seen only at concentrations exceeding 10 mM on bovine retractor penis muscle was unaffected by 3-amino-1,2,4-triazole, again suggesting that it occurred by a nonselective action. We did, however, find that Angeli's salt which releases nitroxyl anion spontaneously, powerfully relaxed bovine retractor penis muscle. Moreover, this relaxation was blocked by ODQ and haemoglobin indicating that bovine retractor penis muscle, like vascular (Fukuto et al., 1992; Nelli et al., 2001) and other nonvascular (rat anococcygeus; Li et al., 1999) smooth muscle, has the ability to metabolically transform nitroxyl anion to nitric oxide. Nitroxyl anion itself, however, lacks the ability to activate soluble guanylate cyclase (Dierks and Burstyn, 1996).

The above findings with 3-amino-1,2,4-triazole are entirely consistent with a role for catalase in the relaxant actions of sodium azide in rat aorta and bovine retractor penis, and cyanamide in rat aorta but not bovine retractor penis. Accordingly, we conducted a series of experiments to determine which oxide of nitrogen (nitroxyl anion versus nitric oxide radical) was produced upon metabolism of sodium azide and cyanamide by mammalian catalase in the presence of a source of hydrogen peroxide. In these experiments, we employed two different but highly sensitive detection systems, namely a difference spectrometric method based upon the rapid oxidation of haemoglobin by nitric oxide to methaemoglobin (Feelisch and Noack, 1987; Kelm and Schrader, 1988) and a nitric oxide-sensitive electrode (Nelli et al., 2000). Using the difference spectrophotometric assay, we found that catalase together with a source of hydrogen peroxide (glucose/glucose oxidase system; Murphy and Sies, 1991) metabolised sodium azide to a species that promoted the oxidation of haemoglobin. No oxidising species was, however, generated from cyanamide, but in keeping with a previous report (Murphy and Sies, 1991), the inclusion of superoxide dismutase in the reaction mixture did lead to the oxidation of haemoglobin. $CuSO_4$ is a more efficient oxidant of nitroxyl to nitric oxide than is superoxide dismutase (Nelli et al., 2000), but it could not be employed in these experiments because it denatured the haemoglobin used in the assay. Our findings are therefore consistent with metabolism of sodium azide to nitric oxide radical by catalase. They are also consistent with metabolism of cyanamide to nitroxyl anion (Nagasawa et al., 1990; Shoeman et al., 2000), which is subsequently oxidised by superoxide dismutase to nitric oxide radical (Murphy and Sies, 1991).

When identical experiments with catalase were repeated using a sensitive nitric oxide electrode (Nelli et al., 2000), the outcomes were quite different to those obtained using the difference spectrophotometric assay. Specifically, no nitric oxide radical was detected when sodium azide or cyanamide was reacted with catalase in the presence of a source of hydrogen peroxide. Furthermore, upon addition of $CuSO_4$, which rapidly oxidises nitroxyl to nitric oxide (Nelli et al., 2000), no signal was detected with either substrate, suggesting that no nitroxyl anion was being generated either. Subsequent addition of the nitroxyl anion generator Angeli's salt, did, however, produce a powerful nitric oxide signal, showing that the assay for nitroxyl and nitric oxide was functioning effectively.

An explanation is therefore required to account for the failure of the nitric oxide electrode to detect any nitric oxide or nitroxyl anion when the spectrophotometric assay so readily demonstrated the ability of catalase to produce nitric oxide radical from sodium azide and nitroxyl anion from cyanamide. The failure cannot be accounted for by a difference in sensitivity of the two assays; the nitric oxide electrode has a threshold sensitivity of ~3 nM (Nelli et al., 2000) and the difference spectrophotometric assay is roughly similar (~10 nM; Feelisch and Noack, 1987; Kelm and Schrader, 1988). An explanation may, however, be found in the existing literature. Specifically, although the catalase-dependent formation of nitric oxide from sodium azide and of nitroxyl from cyanamide has been inferred, no direct measurement of these products has ever been made. With sodium azide, the products are nitrogen dioxide and nitrogen, together with two complexes: one of nitric oxide with catalase and the other, of nitric oxide with compound 1, the catalase–hydrogen peroxide complex (Theorell and Ehrenberg, 1952; Nicholls, 1964; Brunelli et al., 2001). The latter complex is particularly stable and results in prolonged inhibition of enzyme activity, although this can be reversed upon dialysis (Brown, 1995; Brunelli et al., 2001). Thus, our failure to detect free nitric oxide in solution using the nitric oxide detector could be readily explained by the formation of this stable complex. This complex may, however, be able to transfer its bound nitric oxide to sites of higher affinity such as haemoglobin, thus explaining the facile detection of nitric oxide when the difference spectrophotometric assay was employed. Indeed, such a direct transfer of nitric oxide from the complex to the high affinity haem site of soluble guanylate cyclase rather than the release of free nitric oxide in solution, might even explain the process of activation of this effector system following metabolism of azide by catalase

(Mittal et al., 1975; Arnold et al., 1977). An analogous mechanism might occur with cyanamide since formation of nitroxyl has never been measured directly but simply inferred from the formation of its breakdown product, nitrogen dioxide (Nagasawa et al., 1990). Thus, our inability to detect free nitroxyl in solution using the nitric oxide detector might be explained by the formation of a stable nitroxyl-catalase complex, which does not liberate nitroxyl readily except to sites of high affinity. In this case, the high affinity of nitroxyl for superoxide dismutase (Murphy and Sies, 1991), which additionally promotes its rapid oxidation to nitric oxide, may explain its facile detection using the difference spectrophotometric assay.

In conclusion, experiments with the catalase inhibitor, 3-amino-1,2,4-triazole, provide strong evidence for the role of this enzyme in the relaxation produced by azide on rat aorta and bovine retractor penis muscle and by cyanamide on rat aorta. Moreover, our experiments using a haemoglobin-based difference spectrophotometric assay (Feelisch and Noack, 1987; Kelm and Schrader, 1988) are consistent with the concept of catalase-dependent metabolism of azide to nitric oxide and cyanamide to nitroxyl anion. Surprisingly, however, no free nitric oxide or nitroxyl was detected in solution using a sensitive nitric oxide electrode. This apparent anomaly might be explained if the stable complexes of catalase with nitric oxide or nitroxyl do not release their respective oxide of nitrogen except to sites of high affinity, such as the haemoglobin employed in the difference spectrophotometric assay. The possibility that an analogous transfer mechanism, rather than release of the free oxide of nitrogen in solution, could be involved in the activation of soluble guanylate cyclase by sodium azide and cyanamide warrants further investigation.

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