British Journal of Pharmacology (1995) 116, 3302-3308 

# The inhibitory effect of 3-amino-1,2,4-triazole on relaxation induced by hydroxylamine and sodium azide but not hydrogen peroxide or glyceryl trinitrate in rat aorta

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**1** In this study we investigated the role of catalase in relaxation induced by hydroxylamine, sodium azide, glyceryl trinitrate and hydrogen peroxide in isolated rings of rat aorta.

**2** Hydrogen peroxide (1 $\mu$M-1 mM)-induced concentration-dependent relaxation of phenylephrine (PE)-induced tone in endothelium-containing rings. In endothelium-denuded rings, however, higher concentrations (30 $\mu$M-1 mM) of hydrogen peroxide were required to produce relaxation. The endothelium-dependent component of hydrogen peroxide-induced relaxation was abolished following pretreatment with $N^G$-nitro-L-arginine methyl ester (L-NAME, 30 $\mu$M). L-NAME (30 $\mu$M) had no effect, however, on hydrogen peroxide-induced relaxation in endothelium-denuded rings.

**3** Pretreatment of endothelium-denuded rings with catalase (1000 u $ml^{-1}$) blocked relaxation induced by hydrogen peroxide (10 $\mu$M-1 mM). The ability of catalase to inhibit hydrogen peroxide-induced relaxation was partially blocked following incubation with 3-amino-1,2,4-triazole (AT, 50 mM) for 30 min and completely blocked at 90 min.

**4** Pretreatment of endothelium-denuded rings with methylene blue (MeB, 30 $\mu$M) inhibited relaxation induced by hydrogen peroxide (10 $\mu$M-1 mM), sodium azide (1-300 nM), hydroxylamine (1-300 nM) and glyceryl trinitrate (1-100 nM) suggesting that each acted by stimulation of soluble guanylate cyclase.

**5** Pretreatment of endothelium-denuded rings with AT (1-50 mM, 90 min) to inhibit endogenous catalase blocked relaxation induced by sodium azide (1-300 nM) and hydroxylamine (1-300 nM) but had no effect on relaxation induced by hydrogen peroxide (10 $\mu$M-1 mM) or glyceryl trinitrate (1-100 nM).

**6** In a cell-free system, incubation of sodium azide (10 $\mu$M-3 mM) and hydroxylamine (10 $\mu$M-30 mM) but not glyceryl trinitrate (10 $\mu$M-1 mM) with catalase (1000 u $ml^{-1}$) in the presence of hydrogen peroxide (1 mM) led to production of nitrite, a major breakdown product of nitric oxide. AT (1-100 mM) inhibited, in a concentration-dependent manner, the formation of nitrite from azide in the presence of hydrogen peroxide.

**7** These data suggest that metabolism by catalase plays an important role in the relaxation induced by hydroxylamine and sodium azide in isolated rings of rat aorta. Relaxation appears to be due to formation of nitric oxide and activation of soluble guanylate cyclase. In contrast, metabolism by catalase does not appear to be involved in the relaxant actions of hydrogen peroxide or glyceryl trinitrate.

**Keywords:** Hydrogen peroxide; catalase; 3-amino-1,2,4-triazole; glyceryl trinitrate; hydroxylamine; sodium azide; soluble guanylate cyclase; nitric oxide; nitrite

## Introduction

A wide variety of soluble cell components, capable of undergoing oxidation-reduction reactions, are important contributors to free radical production. Superoxide anion is the primary radical formed by the reduction of molecular oxygen. Hydrogen peroxide is the secondary product of spontaneous or enzymatically catalysed dismutation of superoxide anion (Freeman & Crapo, 1982), and may damage cells by its powerful oxidant properties. The breakdown of hydrogen peroxide may be catalysed by the highly specific enzyme, catalase, forming water and oxygen. Catalase is present in many cell types and acts as a natural defence against the production of hydrogen peroxide (Halliwell, 1982; Cheeseman & Slater, 1993). Additionally, many cells possess the enzyme, glutathione peroxidase, which also exerts a protective effect in biological systems by catalysing the reaction of hydrogen peroxide with reduced glutathione to form oxidised glutathione disulphide and water (Smith *et al.*, 1989).

Catalase not only promotes the decomposition of hydrogen peroxide (Chance *et al.*, 1952), but also exhibits peroxidase activity, i.e. it catalyses the oxidation of various substrates in the presence of low concentrations of hydrogen peroxide (Keilin & Hartree, 1945; Theorell & Ehrenberg, 1952; Nicholls, 1964). Two well characterized substrates for peroxidation are azide and hydroxylamine which undergo oxidation to nitrous oxide, nitrogen and nitric oxide (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964). The nitric oxide thus produced is believed to underly the ability of azide and hydroxylamine to activate soluble guanylate cyclase and promote vascular relaxation (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994).

In addition to its actions as an oxidant, hydrogen peroxide can also induce vascular relaxation (Burke & Wolin, 1987; Furchgott, 1991; Furchgott *et al.*, 1994). This occurs by both endothelium-dependent and endothelium-independent mechanisms. The endothelium-dependent component is thought to be mediated via enhanced endothelial synthesis of nitric oxide, whilst the endothelium-independent component is due to a direct effect on smooth muscle soluble guanylate cyclase (Burke & Wolin, 1987; Furchgott, 1991; Zembowicz *et al.*, 1993). The mechanism by which hydrogen peroxide stimulates

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soluble guanylate cyclase is not fully elucidated but it has been proposed that the presence of catalase is vital for this to occur and that the active species is compound 1, i.e. the catalase-hydrogen peroxide complex formed as an intermediate (Burke & Wolin, 1987; Wolin & Burke, 1987).

In this study, we made use of an inhibitor of catalase, 3-amino-1,2,4-triazole (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), to test the hypothesis that metabolism by catalase is necessary in order to express the relaxant activity of azide, hydroxylamine and hydrogen peroxide.

## Methods

### *Preparation of aortic rings*

Female Wistar rats of approximately 200–250 g were killed by stunning and exsanguination. The thoracic aorta was removed and cut into 2.5 mm wide transverse rings with a razor blade slicing device. In some experiments, the endothelium was removed by locating the aortic ring between two stainless steel hooks, placing a 2 g weight on the bottom hook and gently rubbing the intimal surface with a moist matchstick for 10–20 s. Endothelial denudation was deemed successful if no relaxation took place in response to acetylcholine (ACh, 1 $\mu$M).

### *Tension recording*

The aortic rings were mounted under 1 g resting tension on stainless steel hooks in 20 ml organ baths maintained at 37°C containing Krebs solution (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24, and glucose 11, and gassed with 95% $O_2$ and 5% $CO_2$. Tension was recorded isometrically by means of Grass FT03C transducers and responses were displayed on a Grass polygraph model 7. Tissues were allowed to equilibrate for 90 min before experiments were carried out, during which time the resting tension was re-adjusted to 1 g, as required.

### *Experimental protocols*

The effects of hydrogen peroxide (100 nM–1 mM) were examined on both endothelium-containing and endothelium-denuded rings, precontracted with phenylephrine (PE) 100 nM and 10 nM, respectively. In experiments in which the effects of $N^G$-nitro-L-arginine methyl ester (L-NAME, 30 $\mu$M) and methylene blue (MeB, 30 $\mu$M) were to be studied on hydrogen peroxide-induced relaxation, we ensured that the level of tone before relaxation was similar to that of untreated preparations. In order to achieve this, tissues were initially precontracted with a lower concentration of PE before addition of the inhibitors. This was necessary since both L-NAME and MeB potentiated the tone by blocking the synthesis and actions of basal nitric oxide, respectively.

In a separate series of experiments, the vasodilator actions of sodium azide (1–300 nM), hydroxylamine (1–300 nM) and glyceryl trinitrate (1–100 nM) were investigated in endothelium-denuded rings, precontracted with PE (10–30 nM). In all experiments relaxant responses are expressed as a % relaxation of PE-induced tone.

### *Use of 3-amino-1,2,4-triazole*

In certain experiments, we wished to use 3-amino-1,2,4-triazole (AT), a selective inhibitor of catalase (Heim *et al.*, 1956; Margoliash & Novogrodsky, 1958), to inhibit the endogenous catalase activity of rat aortic rings. In order to establish the conditions necessary to produce optimum inhibition, we first conducted a series of experiments using exogenous catalase (bovine liver). In these experiments, endothelium-denuded rings were incubated either with catalase (1000 u $ml^{-1}$) or with catalase together with AT (50 mM) for 30 and 90 min. As shown in the results (Figure 2), incubation of catalase with AT for 90 min was required to inhibit maximally the ability of the enzyme to block hydrogen peroxide-induced relaxation. Consequently, in all subsequent experiments involving AT, tissues were incubated with the drug for 90 min. Pretreatment with AT (50 mM, 90 min) depressed PE (10 nM)-induced tone by 31.8±2.2% ($n$=15), but we ensured that the level of tone before relaxation was similar to that of untreated preparations by increasing the concentration of PE (to 30–100 nM). AT (50 mM), when added to the tissue baths, remained in solution and had no effect on the pH of the Krebs solution. The addition of AT clearly resulted in an increase of the osmotic strength of Krebs solution. This, however, played no role in the actions of AT since addition of the inert substance, mannitol, at the same concentration (50 mM, 90 min) had no effect on relaxations induced by sodium azide (1–300 nM), hydroxylamine (1–300 nM), glyceryl trinitrate (1–100 nM) or hydrogen peroxide (10 $\mu$M–1 mM) (data not shown).

The effects of inhibiting endogenous catalase with AT (1–50 mM, 90 min) were studied on relaxations of endothelium-denuded rings induced by sodium azide (1–300 nM), hydroxylamine (1–300 nM), glyceryl trinitrate (1–100 nM) and hydrogen peroxide (10 $\mu$M) following precontraction with PE (30–100 nM).

### *Catalase-induced formation of nitrite*

We measured nitrite, the major breakdown product of NO, formed from nitrovasodilators by the peroxidase activity of catalase. Briefly, the reaction mixtures containing phosphate buffer (pH 5.6, 0.08 M), bovine liver catalase (300 or 1000 u $ml^{-1}$), hydrogen peroxide (1 mM) and the nitrovasodilators sodium azide (10 $\mu$M–3 mM), hydroxylamine (10 $\mu$M–30 mM) and glyceryl trinitrate (10 $\mu$M–1 mM) in a final volume of 80 $\mu$l were incubated at 37°C for the times indicated in the Results. The nitrite content of the samples was measured by the formation of a diazo product using a variant of the method of Green *et al.* (1982), i.e. the samples were mixed with 80 $\mu$l of 1% sulphanilic acid (Sigma) in 2 M HCl. After 5 min, 80 $\mu$l of 1% (w/v) aqueous N-(1-napthyl) ethylenediamine dihydrochloride (Sigma) was added, and the absorbance of the pink complex was determined at 550 nm by use of a Dynatech Microplate Reader model no MR 5000/7000. A standard curve was prepared with solutions of sodium nitrite (1–30 $\mu$M) (Sigma).

### *Drugs*

Acetylcholine chloride, 3-amino-1,2,4-triazole, catalase (bovine liver), hydrogen peroxide (30%), hydroxylamine chloride, L-arginine hydrochloride, methylene blue, $N^G$-nitro-L-arginine methyl ester, phenylephrine hydrochloride and sodium azide were obtained from Sigma (Poole, Dorset), whilst glyceryl trinitrate was obtained from NAPP Laboratories (Cambridge). All drugs were dissolved and dilutions made in saline (0.9%).

### *Statistical analysis*

Results are expressed as the mean±s.e.mean for $n$ separate experiments and comparisons were made by one-way analysis of variance followed by Fisher's test. A probability of 0.05 or less was considered significant.

## Results

### *Endothelium-dependent and endothelium-independent components of hydrogen peroxide-induced relaxation*

Following induction of phenylephrine (PE, 100 nM)-induced tone (1.6±0.1g) in endothelium-containing rings of rat aorta, hydrogen peroxide (100 nM–1 mM) produced powerful concentration-dependent relaxation (maximum relaxation 96.2±1.0%, Figure 1). In endothelium-denuded rings, a lower

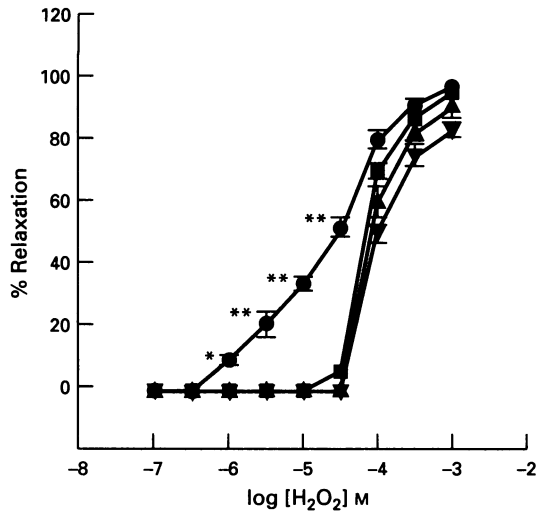


**Figure 1** Concentration-response curves showing relaxation to hydrogen peroxide ($H_2O_2$, 100 nM – 1 mM) on phenylephrine (PE)-contracted rings of rat aorta: endothelium-containing rings in the absence (●) and presence of $N^G$-nitro-L-arginine methyl ester (L-NAME, 30 $\mu$M, ▲) and endothelium-denuded rings in the absence (■) and presence of L-NAME (30 $\mu$M, ▼). Care was taken to ensure that the levels of PE-induced tone were similar in all experiments. In order to achieve this, in experiments involving endothelial removal or treatment of endothelium-containing rings with L-NAME, the concentration of PE was lowered from 100 nM to 10 nM. Levels of tone obtained in endothelium-containing rings, endothelium-denuded, endothelium-containing treated with L-NAME and endothelium-denuded rings treated with L-NAME were 1.6±0.1 g, 1.6±0.1 g, 1.7±0.1 g and 1.4±0.1 g, respectively. Note that L-NAME blocked the endothelium-dependent component of relaxation to $H_2O_2$ but had no effect on the endothelium-independent component. Each point is the mean±s.e.mean of 7 observations. $^*P<0.05$ and $^{**}P<0.005$ indicate a significant difference from relaxations obtained in endothelium-denuded rings.

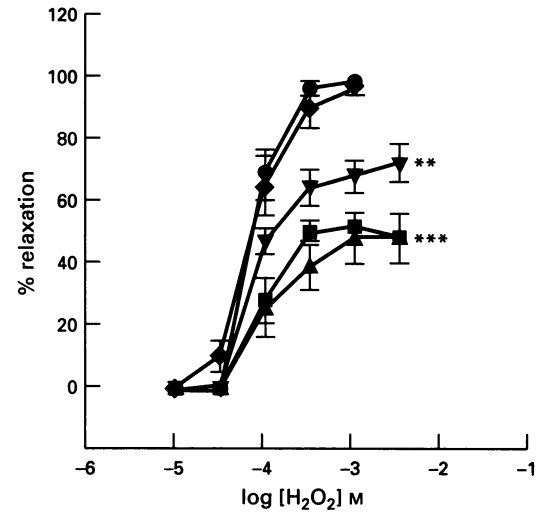


**Figure 2** Concentration-response curves showing relaxation to hydrogen peroxide ($H_2O_2$, 10 $\mu$M – 1 mM, ●) on phenylephrine-contracted endothelium-denuded rings of rat aorta and blockade of this relaxation following pretreatment with catalase (1000 u ml$^{-1}$) for 30 min (▲) or 90 min (■). Incubation with 3-amino-1,2,4-triazole (AT, 50 mM) for 30 min (▼) partially inhibited the ability of catalase to block $H_2O_2$-induced relaxation, whereas incubation for 90 min (◆) produced complete inhibition. Levels of active force for control rings, those incubated with catalase for 30 min or 90 min and those incubated with catalase and AT for 30 min or 90 min were 1.2±0.1 g, 1.3±0.1 g, 1.5±0.1 g, 1.1±0.1 g and 1.2±0.1 g, respectively. Each point is the mean±s.e.mean of 5–6 observations. $^{**}P<0.005$ and $^{***}P<0.001$ indicate a significant difference from maximal relaxation in untreated rings.

concentration of PE (10 nM) was required to induce a similar degree of tone (1.5±0.1 g) than in endothelium-containing rings, but in these, higher concentrations of hydrogen peroxide (30 $\mu$M – 1 mM) were required to produce relaxation (maximum relaxation 96.0±1.6%, Figure 1). Pretreatment of endothelium-containing rings with L-NAME (30 $\mu$M) for 15 min to inhibit NO synthesis abolished hydrogen peroxide-induced relaxation at 100 nM – 30 $\mu$M, but had no effect on relaxation induced at higher concentrations. This inhibitory action of L-NAME was reversed in the presence of L-arginine (2 mM, data not shown). L-NAME (30 $\mu$M) had no effect, however, on hydrogen peroxide-induced relaxation in endothelium-denuded rings.

### *Effects of 3-amino-1,2,4-triazole on the ability of catalase to inhibit hydrogen peroxide-induced relaxation*

Pretreatment of endothelium-denuded rings of rat aorta with catalase (1000 u ml$^{-1}$) for 30 min led to a 50.4±0.5% reduction in the maximal relaxation induced by hydrogen peroxide (10 $\mu$M – 1 mM, Figure 2). If the catalase inhibitor, 3-amino-1,2,4-triazole (AT, 50 mM), was present together with catalase during the 30 min incubation, the ability to inhibit hydrogen peroxide-induced relaxation was partially blocked. Increasing the incubation period to 90 min, however, led to complete blockade of the ability of catalase to inhibit hydrogen peroxide-induced relaxation.

### *Effects of methylene blue and 3-amino-1,2,4-triazole on relaxations induced by sodium azide, hydroxylamine, glyceryl trinitrate and hydrogen peroxide*

Sodium azide (1 – 300 nM), hydroxylamine (1 – 300 nM) and glyceryl trinitrate (1 – 100 nM) each produced powerful concentration-dependent relaxation of endothelium-denuded rings of rat aorta precontracted with PE (10 – 30 nM, Figures 3 and 4). These relaxations as well as those produced by hydrogen peroxide (10 $\mu$M – 1 mM) in endothelium-denuded rings were blocked by the inhibitor of soluble guanylate cyclase, methylene blue (30 $\mu$M, Figure 3). Pretreatment of the rings with AT (1 – 50 mM, 90 min) to inhibit endogenous catalase shifted the concentration-response curves for sodium azide and hydroxylamine (Figure 4) to the right in a concentration-dependent manner: for sodium azide the $EC_{50}$ values were shifted 11.4, 77.0, 528 and 1668 fold by AT at concentrations of 1, 5, 10 and 50 mM, respectively and for hydroxylamine shifts of 6.4, 59.9 and 190 fold were obtained, at concentrations of AT of 5, 10 and 50 mM, respectively. In contrast, AT (50 mM, 90 min) had no effect on relaxation induced by glyceryl trinitrate (1 – 100 nM, Figure 3b) or hydrogen peroxide (10 $\mu$M – 1 mM, Figure 3a). It also had no effect on the endothelium-dependent relaxation induced by acetylcholine (10 nM – 3 $\mu$M, data not shown).

### *Role of catalase and hydrogen peroxide in the formation of nitrite from nitrovasodilators*

When incubated for 1 h at 37°C in the presence of hydrogen peroxide (1 mM), catalase (1000 u ml$^{-1}$) led to the generation of nitrite from sodium azide (10 $\mu$M – 3 mM, Figure 5a) and hydroxylamine (10 $\mu$M – 3 mM, Figure 5b) but not from glyceryl trinitrate (10 $\mu$M – 1 mM, data not shown). No nitrite was generated from sodium azide or hydroxylamine by catalase in the absence of hydrogen peroxide.

### *Inhibition by 3-amino-1,2,4-triazole of catalase-dependent formation of nitrite from sodium azide*

If AT (1 – 100 mM) was present during the 1 h incubation, the catalase (1000 u ml$^{-1}$)-dependent formation of nitrite from azide (0.3 mM) in the presence of hydrogen peroxide (1 mM)

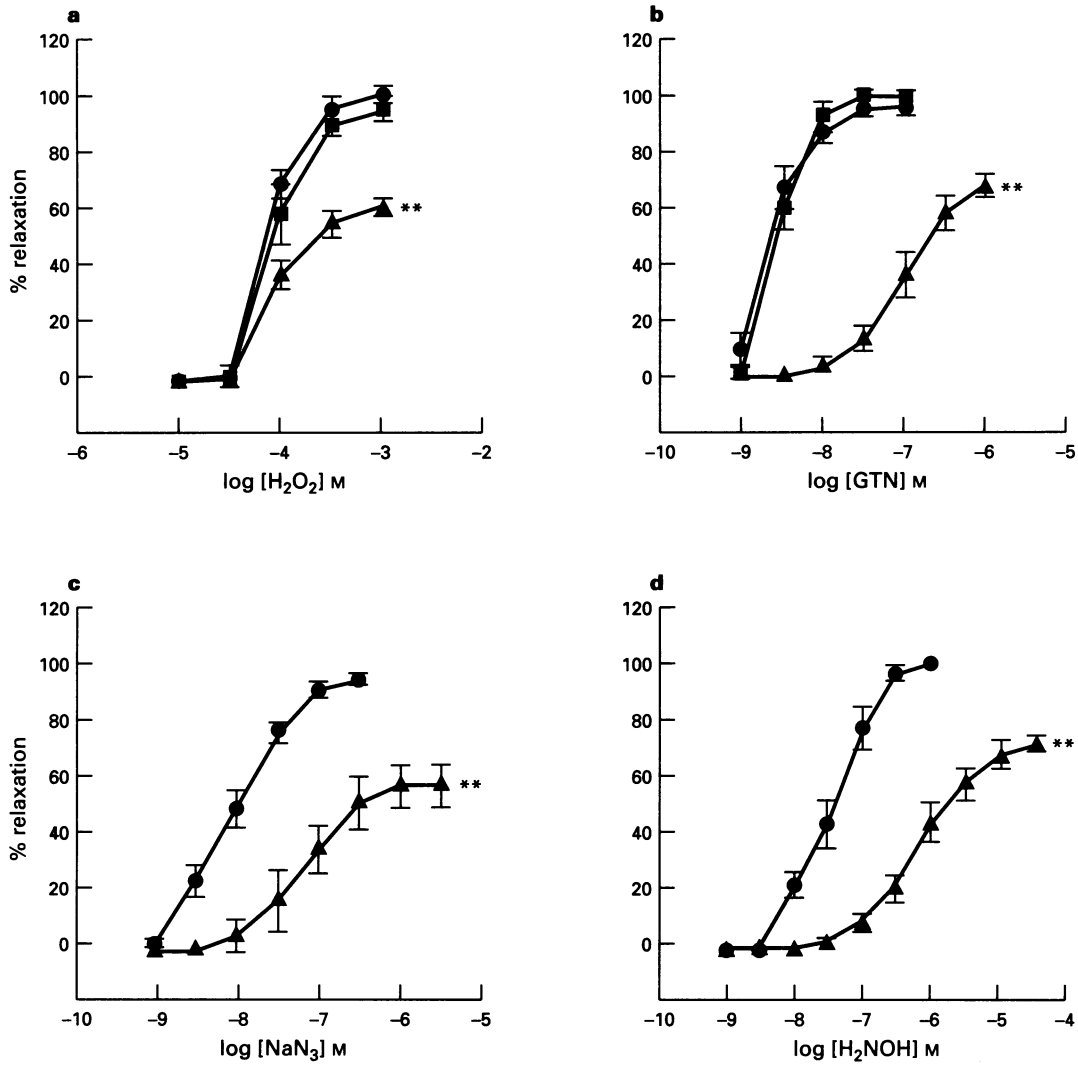


**Figure 3** (a and b) Concentration-response curves showing relaxation to (a) hydrogen peroxide ($H_2O_2$, 10 $\mu$M – 1 mM, ●) and (b) glyceryl trinitrate (GTN, 1 – 100 nM, ●) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta, blockade by methylene blue (MeB, 30 $\mu$M, ▲) and the lack of effect of inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 50 mM, 90 min, ■) on this relaxation. (c and d) Concentration-response curves showing relaxation to (c) sodium azide ($NaN_3$, 1 nM – 3 $\mu$M, ●) and (d) hydroxylamine ($NH_2OH$, 1 nM – 30 $\mu$M, ●) on phenylephrine-contracted endothelium-denuded rings and the blockade by MeB (30 $\mu$M, ▲) of this relaxation. Levels of active force for control rings and rings treated with MeB or AT were 1.1 ± 0.1 g, 1.2 ± 0.1 g and 1.2 ± 0.1 g, respectively. Each point is the mean ± s.e.mean of 5 – 12 observations. $^{**}P < 0.005$ indicates a significant difference from maximal relaxation in untreated rings.

was inhibited only slightly: maximum inhibition of 36.4 ± 1.2% was obtained with AT at 100 mM (data not shown). If, however, the duration of the incubation was extended to 3 h together with a reduction of the catalase concentration to 300 u $ml^{-1}$, the ability of AT to block formation of nitrite was enhanced: maximum inhibition of 77.7 ± 6.3% was obtained with AT at 100 mM (Figure 6).

## Discussion

The major new insight provided by this study came from the use of 3-amino-1,2,4-triazole (AT), an inhibitor of catalase (Heim *et al.*, 1956; Margoliash & Novogrodski, 1958), to examine the role of this enzyme in the vascular relaxation induced by certain nitrovasodilators and hydrogen peroxide.

The three nitrovasodilators employed, sodium azide, hydroxylamine and glyceryl trinitrate all produced powerful, concentration-dependent relaxation in endothelium-denuded rings of rat aorta. It is well established that the relaxant actions of these agents proceed through activation of soluble guanylate cyclase and elevation of smooth muscle guanosine 3′ 5′-cyclic monophosphate (cyclic GMP) content (Arnold *et al.*, 1977; Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994). Consistent with this view is our ability to inhibit relaxation by all three agents with methylene blue, an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1979; Holzmann, 1982). In order for the nitrovasodilators to stimulate soluble guanylate cyclase, they must first be chemically modified to yield the active principle, nitric oxide (Katsuki *et al.*, 1977; Waldman & Murad, 1987; Murad, 1994). For glyceryl trinitrate, this has been proposed to occur via metabolism by glutathione-S-transferase (Armstrong *et al.*, 1980) or cytochrome $P_{450}$ (Schröder, 1992), or by a direct reduction by tissue thiols (Feelisch, 1991). In contrast, the seminal work of Murad and his colleagues (Mittal *et al.*, 1975) showed that activation of soluble guanylate cyclase in tissue homogenates by azide and hydroxylamine required the presence of high molecular weight factors, later identified as catalase and other peroxidase enzymes (Miki *et al.*, 1976). The conversion to nitric oxide requires the presence of low concentrations of hydrogen peroxide and thus occurs as a consequence of the peroxidase activity of catalase (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954; Nicholls, 1964; Klebanoff & Nathan, 1993).

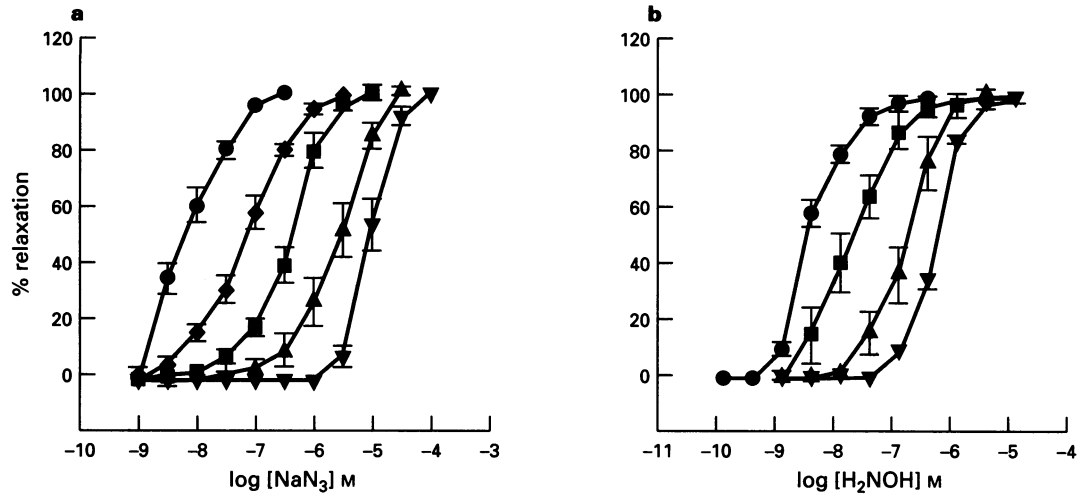


**Figure 4** (a) Concentration-response curves showing relaxation to sodium azide ($NaN_3$, ●) on phenylephrine (PE)-contracted endothelium-denuded rings of rat aorta and concentration-dependent shifts to the right following inhibition of endogenous catalase with 3-amino-1,2,4-triazole (AT, 90 min) at 1 mM (◆), 5 mM (■), 10 mM (▲), and 50 mM (▼). (b) Concentration-response curves showing relaxation to hydroxylamine ($H_2NOH$, ●) and concentration-dependent shifts to the right following inhibition of endogenous catalase with AT (90 min) at 5 mM (■), 10 mM (▲), and 50 mM (▼). Levels of active force in control and in AT (50 mM)-treated rings were 1.3±0.1 g and 1.2±0.1 g, respectively. Each point is the mean±s.e.mean of 5–8 observations.

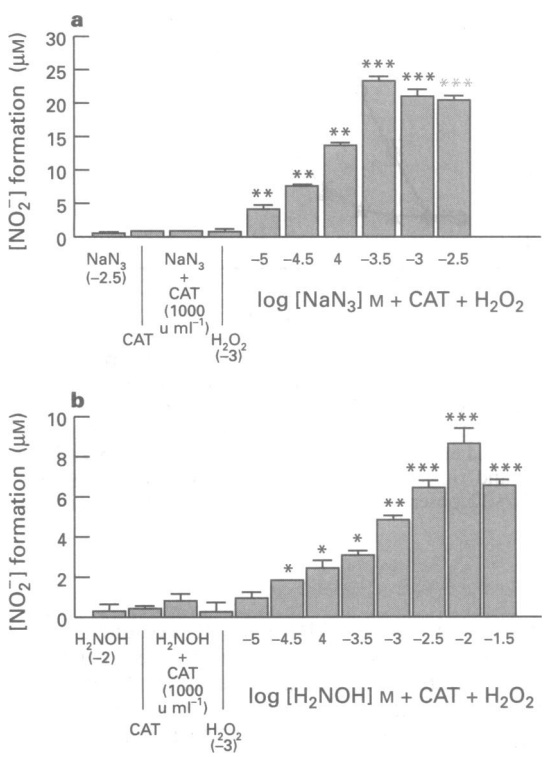


**Figure 5** Catalase (CAT)-dependent formation of nitrite from (a) sodium azide ($NaN_3$) and (b) hydroxylamine ($NH_2OH$) in the presence of hydrogen peroxide ($H_2O_2$) in a cell-free system. The reaction mixture contained phosphate buffer (pH 5.6, 0.08 M), CAT (1000 u $ml^{-1}$), $H_2O_2$ (1 mM) and $NaN_3$ (10 μM–3 mM) or $NH_2OH$ (10 μM–30 mM) in a final volume of 80 μl and was incubated for 1 h at 37°C. Each column is the mean±s.e.mean of 6 observations $^*P<0.05$, $^{**}P<0.005$ and $^{***}P<0.001$ indicate a significant difference from reaction mixtures containing catalase and the respective nitrovasodilator but not $H_2O_2$.

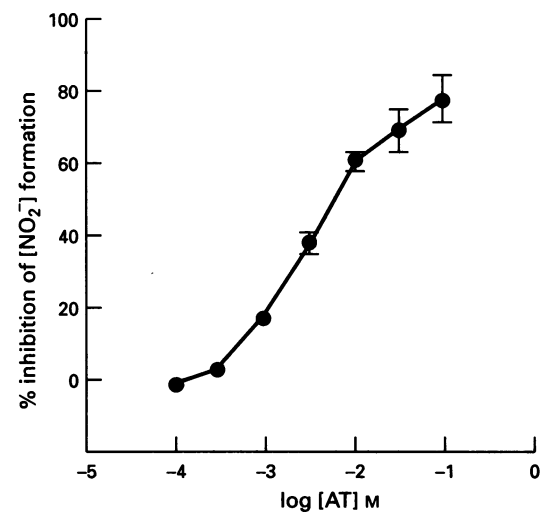


**Figure 6** The inhibition by 3-amino-1,2,4-triazole of catalase-dependent formation of nitrite from sodium azide in the presence of hydrogen peroxide. The reaction mixture contained phosphate buffer (pH 5.6, 0.08 M), catalase (300 u $ml^{-1}$), hydrogen peroxide (1 mM), 3-amino-1,2,4-triazole (AT, 1–100 mM) and sodium azide (0.3 mM) and was incubated for 3 h at 37°C. Each point is the mean±s.e.mean of 6–20 observations.

Our own experiments using bovine liver catalase are consistent with this concept: we found that nitrite, a major stable product of nitric oxide, could be generated from azide, and that conversion took place only in the presence of hydrogen peroxide. A recent study (Markert *et al.*, 1994) failed to detect nitrite formation from hydroxylamine, but under the conditions of our experiments we found that it could indeed generate significant levels of nitrite, although it was a less effective substrate than azide. Despite this difference, azide and hydroxylamine were almost equipotent as relaxants of rat aorta, perhaps suggesting an additional catalase-independent route of metabolism of the latter to nitric oxide. In contrast to azide and hydroxylamine, no nitrite was generated when glyceryl trinitrate was incubated with catalase in the presence of hydrogen peroxide, consistent with its distinct pathway for activation.

Although biochemical evidence supporting the role of catalase in the activation of soluble guanylate cyclase by azide and hydroxylamine is strong (Miki *et al.*, 1976; Katuski *et al.*, 1977; Craven *et al.*, 1979), direct proof of the involvement of this enzyme in vascular relaxation is lacking. Consequently, we attempted to test the role of catalase directly by employing AT,

an established inhibitor of this enzyme which acts irreversibly by binding to compound 1, i.e. the catalase-hydrogen peroxide complex formed as an intermediate (Heim *et al.*, 1956; Margoliash & Novogrodski, 1958). In our biochemical experiments we found that AT did indeed block the catalase-dependent conversion of azide to nitrite in the presence of hydrogen peroxide: blockade was only slight during a 1 h incubation (maximum inhibition 36.4±1.2%) but was increased (to maximum of 77.7±6.3%) if incubation was extended to 3 h, consistent with its known slow onset of action (Heim *et al.*, 1956; Margoliash & Novogrodski, 1958). Following a 90 min incubation of endothelium-denuded rings of rat aorta with AT, relaxation induced by azide and hydroxylamine was inhibited in a concentration-dependent manner, thus providing direct evidence of metabolism of these agents to nitric oxide by catalase. The action was highly selective since relaxation induced by another nitrovasodilator, glyceryl trinitrate, was completely unaffected, consistent with its metabolism by a separate pathway. Despite inhibiting the relaxant actions of both azide and hydroxylamine, it was evident that AT at all concentrations tested more powerfully inhibited the actions of the former (maximum shifts in $EC_{50}$ values for azide and hydroxylamine of 1668 fold and 190 fold, respectively with 50 mM AT), again suggesting a possible additional activation pathway for the latter. Furthermore, we found that AT had no effect on the endothelium-dependent relaxation induced by acetylcholine in rat aortic rings, which contradicts the proposed generation of hydroxylamine as an intermediate in the endothelial production of nitric oxide from L-arginine (DeMaster *et al.*, 1989; Schmidt *et al.*, 1990).

In a separate group of experiments we investigated the mechanisms underlying the relaxant actions of hydrogen peroxide in rat aortic rings. We found that low concentrations (100 nM–1 mM) selectively produced relaxation in endothelium-containing rings and this was abolished by L-NAME and methylene blue, inhibitors of nitric oxide synthase and soluble guanylate cyclase, respectively. This component of relaxation thus appeared to result from enhanced production of nitric oxide, as had previously been suggested (Furchgott, 1991; Zembowicz *et al.*, 1993; Furchgott *et al.*, 1994). Higher concentrations of hydrogen peroxide (30 μM–1 mM), however, produced relaxation of endothelium-denuded rings and this too was inhibited by methylene blue suggesting involvement of soluble guanylate cyclase. Biochemical studies have indeed shown that low concentrations of hydrogen peroxide activate soluble guanylate cyclase in tissue homogenates, but higher concentrations lead to inhibition of the enzyme (White *et al.*, 1976), presumably as a result of its oxidant actions, thus mimicking the well-characterized inhibitory actions of the oxidants, methylene blue and potassium ferricyanide (Gruetter *et al.*, 1979; Holzmann, 1982). The mechanism by which hydrogen peroxide stimulates soluble guanylate cyclase remains obscure, but the ability of catalase to potentiate this action has led to the suggestion that an intermediate in its metabolism, perhaps compound-1 is the active species (Burke & Wolin, 1987; Wolin & Burke, 1987). Our experimental findings with the catalase inhibitor, AT, however, lend no support to this hypothesis. We are confident that AT is indeed an effective inhibitor of this enzyme since it inhibited the ability of exogenous bovine liver catalase to block hydrogen peroxide-induced relaxation in rat aortic rings and stimulate nitrite formation from azide and hydroxylamine. Furthermore, it appeared to block endogenous smooth muscle catalase, as indicated by the selective blockade of azide- and hydroxylamine-induced relaxation of rat aortic rings. Despite these actions, AT at even the highest concentration tested (50 mM) failed to affect hydrogen peroxide-induced relaxation of rat aortic rings, suggesting that metabolism by catalase is not required for expression of relaxant activity. An alternative explanation is therefore required to explain the requirement for catalase in the activation of soluble guanylate cyclase by hydrogen peroxide (Burke & Wolin, 1987; Wolin & Burke, 1987). Although speculative at this stage, in view of the dual actions of hydrogen peroxide, i.e. stimulant at low concentrations and inhibitory at high concentrations (White *et al.*, 1976), it is possible that catalase, by destroying hydrogen peroxide, will lower its concentration to such an extent that only the stimulant and not the inhibitory actions are seen.

In conclusion, our study using AT, an inhibitor of catalase, provides direct proof that metabolism of azide and hydroxylamine to nitric oxide by catalase underlies the relaxant actions of these nitrovasodilators. In contrast, our study lends no support to the proposed concept that metabolism of hydrogen peroxide by catalase is necessary for expression of its vasodilator activity.

K.B.M. is supported by a University of Glasgow Postgraduate Scholarship. We are grateful to the B.H.F. for financial support.

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*(Received June 12, 1995*
*Revised August 9, 1995*
*Accepted August 23, 1995)*