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# Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta

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**1** The effects of L-cysteine were tested in rat aortic rings on responses to nitric oxide free radical ($NO^{\bullet}$), nitroxyl ($NO^{-}$) derived from Angeli's salt and endothelium-derived relaxing factor (EDRF) activated by acetylcholine, ATP and the calcium ionophore A23187. Concentrations of 300 $\mu$M or less of L-cysteine had no effect on responses.

**2** Relaxations produced by exogenous $NO^{\bullet}$ (0.25–2.5 $\mu$M) were markedly prolonged and relaxations produced by sodium nitroprusside (0.001–0.3 $\mu$M) were enhanced by 1 and 3 mM L-cysteine. The enhancements by L-cysteine of responses to $NO^{\bullet}$ and sodium nitroprusside may be attributed to the formation of S-nitrosocysteine.

**3** Relaxations mediated by the nitroxyl anion (0.3 $\mu$M) donated from Angeli's salt were more prolonged than those produced by $NO^{\bullet}$, and nitroxyl-induced relaxations were reduced by L-cysteine (1 and 3 mM).

**4** EDRF-mediated relaxations produced by acetylcholine (0.01–10 $\mu$M), ATP (3–100 $\mu$M) and the calcium ionophore A23187 (0.1 $\mu$M) were significantly reduced by 3 mM L-cysteine.

**5** The similarity between the inhibitory effects of L-cysteine on responses to EDRF and on those to nitroxyl suggests that a component of the response to EDRF may be mediated by nitroxyl anion. *British Journal of Pharmacology* (2000) **129,** 315–322

**Keywords:** Aorta (rat); EDRF; L-cysteine; nitrergic transmitter; nitric oxide; nitroxyl anion

**Abbreviations:** carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide; EDRF, endothelium derived relaxing factor; $NO^{-}$, nitroxyl anion; $NO^{\bullet}$, nitric oxide free radical; ODQ, 1H-[1,2,4]oxadiazole[4,3,-a]quinoxalin-1-one; PSS, physiological salt solution

## Introduction

The nature of the vasodilator mediator released from rabbit aorta preparations originally termed endothelium derived relaxing factor (EDRF) by Furchgott & Zawadzki (1980) was the subject of some controversy until Feelisch *et al*., (1994) produced evidence indicating that it was nitric oxide free radical ($NO^{\bullet}$). This conclusion was supported by the findings that EDRF-mediated relaxations were reduced in a concentration-dependent manner by the $NO^{\bullet}$ scavenger carboxy-PTIO in rabbit and rat aortic preparations (Akaike *et al*., 1993; Rand & Li, 1995a). However, Rand & Li (1995a) found that the concentrations of carboxy-PTIO required to reduce EDRF-mediated relaxations were about ten times greater than those required to reduce equivalent relaxations to $NO^{\bullet}$ to the same extent. This quantitative difference was attributed to the longer pathway traversed by exogenous $NO^{\bullet}$ than by EDRF, as suggested by Wood & Garthwaite (1994). Nevertheless, the possibility remained that EDRF may have contained a component other than $NO^{\bullet}$ that was less readily blocked by carboxy-PTIO. When rat aortae are near-maximally contracted, EDRF-mediated relaxations are almost completely abolished after inhibition of endothelial nitric oxide synthase (eNOS) with 10 $\mu$M $N^{G}$-nitro-L-arginine methyl ester (Martin *et al*., 1992). Furthermore, EDRF-mediated relaxations do not occur in aorta preparations from eNOS knockout mice (Chataigneau *et al*., 1999). Therefore, any component of EDRF additional to $NO^{\bullet}$ would necessarily also be a product of eNOS. One such substance is the nitroxyl anion ($NO^{-}$), which can be produced from the intermediate compound $N^{\omega}$-hydroxy-L-arginine in the pathway from L-arginine to nitrogen monoxide under the appropriate oxidative conditions (Fukuto *et al*., 1992a; Hobbs *et al*., 1994; Schmidt *et al*., 1996).

The possibility that EDRF might be the nitroxyl anion was investigated and rejected in favour of $NO^{\bullet}$ by Feelisch *et al*., (1994). One of the grounds for this was that the response of a rabbit aorta detector tissue in a superfusion cascade to EDRF released from cultured endothelial cells, like the response to $NO^{\bullet}$, was inhibited by a low concentration (5 $\mu$M) of L-cysteine. Others have also demonstrated the blockade of responses of rabbit aortic preparations to $NO^{\bullet}$ by a low concentrations of L-cysteine (Jia & Furchgott, 1993; Boeckxstaens *et al*., 1994). This effect has been attributed to inactivation of $NO^{\bullet}$ by superoxide anions that are produced by the auto-oxidation of L-cysteine (Misra, 1974; Saez *et al*., 1982).

However, at millimolar concentrations, L-cysteine enhances or prolongs responses to $NO^{\bullet}$ in vascular preparations (Arvola *et al*., 1992; Feelisch *et al*., 1994; Zamora & Feelisch, 1994) and other tissues (McLaren *et al*., 1992; Rand & Li, 1992; 1995b Liu *et al*., 1994). It has been suggested that this is because thiols such as L-cysteine react with $NO^{\bullet}$ to form S-nitrosothiols, which act as NO-carrier molecules thereby stabilizing the activity of $NO^{\bullet}$ (Stamler, 1994; Liu *et al*., 1998). S-Nitrosothiols have potent relaxant activity, have greater half-lives than free NO (Feelisch *et al*., 1994), and are more resistant than free NO to degradation by superoxide (Aleryani *et al*., 1998).

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Although millimolar concentrations of L-cysteine have been shown to enhance and prolong the actions of $NO^{\bullet}$, there are no reports of corresponding effects on responses to EDRF. Therefore, the initial aim of the present study was to investigate the effects of L-cysteine on responses to EDRF in the rat aorta, and to determine whether they corresponded to its effects on responses to exogenous $NO^{\bullet}$. When it became clear that responses to EDRF were decreased by millimolar concentrations of L-cysteine, the effect of L-cysteine on response to nitroxyl anions ($NO^{-}$) donated by Angeli's salt was also investigated since it was reported that $NO^{-}$ was inactivated by millimolar concentrations of L-cysteine (Zamora & Feelisch, 1994).

## Methods

### *Tissues*

Male Sprague-Dawley rats (250–400 g) were narcotised with $CO_2$ and then decapitated. The thoracic aorta was removed and freed of surrounding connective tissue and fat before cutting into 5 mm wide rings. The rings were mounted on hooks in 8 ml organ baths in physiological salt solution (PSS) at a resting tension of 2 g and allowed to equilibrate for approximately 90 min. Tension was measured isometrically using Grass FT03 force-displacement transducers and recorded on a MacLab data acquisition system.

### *Endothelium-dependent responses*

Relaxant responses to acetylcholine, ATP and the calcium ionophore A23187 were elicited in endothelium intact aortic rings that were contracted with submaximal concentrations of phenylephrine (1 $\mu$M). These relaxations were compared to those obtained in the presence of L-cysteine (100 $\mu$M–3 mM), or equal volumes of vehicle (distilled water) in time-control experiments. In most cases, L-cysteine or vehicle was added 3 min before the tissue was contracted with phenylephrine. In some experiments, the effect of 3 mM L-cysteine as the hydrochloride or as the free base was tested during a sustained relaxation induced by acetylcholine (1 $\mu$M).

### *Endothelium-independent responses*

In vessels in which endothelium-independent responses were to be observed, the endothelium was removed by gently rubbing the luminal surface of the aortic rings with forceps. Relaxant responses to $NO^{\bullet}$ (aqueous solution of nitric oxide gas) were produced after the tone was raised with phenylephrine (1 $\mu$M) and compared to responses obtained in the presence of L-cysteine. Relaxations to sodium nitroprusside and Angeli's salt (1 $\mu$M), which dissociates to release nitroxyl anions (Zamora & Feelisch, 1994), were also tested in the presence of L-cysteine. L-Cysteine and vehicle were added to the organ bath 3 min before the addition of phenylephrine. In some experiments, the effect of 3 mM L-cysteine was tested during sustained relaxations induced by sodium nitroprusside (0.1 $\mu$M).

### *Drugs and reagents*

The composition of the PSS was as follows (mM): NaCl 118.0, KCl 4.7, $MgSO_4$ 0.45, $KH_2PO_4$ 1.03, D-(+)-glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, disodium edetate 0.067, ascorbic acid 0.14. Ascorbic acid was omitted in experiments in which carboxy-PTIO was used to avoid the direct degradation of carboxy-PTIO by ascorbic acid (Akaike *et al*., 1993). The PSS was bubbled with 95% $O_2$ and 5% $CO_2$ and maintained at 37°C.

The following drugs were used: acetylcholine hydrochloride, adenosine ′5-triphosphate (ATP), L-cysteine, L-cysteine hydrochloride, isoprenaline hydrochloride, phenylephrine hydrochloride, sodium nitrite, sodium nitroprusside, and the calcium ionophore A23187 (all purchased from Sigma-Aldrich, Castle Hill, NSW, Australia), nitric oxide gas (CIG, Melbourne, Australia), carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide] (Sapphire Bioscience, Alexandria, NSW, Australia), ascorbic acid (May & Baker, Australia, Pty. Ltd.), Angeli's salt (sodium trioxodinitrate) and ODQ [1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one] (Cayman Chemical Co.). All drugs used except A23187, ODQ and Angeli's salt were dissolved in distilled water or PSS. A23187 was dissolved in 100% ethanol. ODQ was dissolved in 100% dimethyl sulphoxide. Angeli's salt was dissolved in 0.01 M NaOH and handled as described by Li *et al*. (1999). The pH values of the stock solutions of L-cysteine hydrochloride and L-cysteine free base (each 0.8 M) were 1.0 and 3.5, respectively; however, their addition to PSS in the organ bath to make a 3 mM final concentration did not produce any detectable change ($<0.05$ pH units) in the pH of the PSS. Saturated solutions of nitric oxide (2 mM) were prepared as previously described (Rajanayagam *et al*., 1993).

### *Statistical analysis of results*

Relaxations to endothelium-dependent vasodilators were expressed as percentages of the phenylephrine-induced tone. Relaxations produced by $NO^{\bullet}$ were expressed in terms of the integral of response with time, calculated as the area swept out by the relaxation (in $mm^2$), using a constant amplification (1 g = 10 mm) and time base (1 min = 6.8 mm). Values are given as means ± standard error of means. Evaluation of statistical significance was with either Student's paired *t*-test or two-way analysis of variance (ANOVA). Probability values less than 0.05 were deemed significant.

### *Ethics*

The experiments were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to guidelines laid down by the National Health & Medical Research Council of Australia.

## Results

### *EDRF-mediated relaxations*

The tension produced in aortic rings by 1 $\mu$M phenylephrine was 2.1 ± 0.1 g ($n = 18$). Acetylcholine (0.01–10 $\mu$M) produced relaxations that were maximally about 70% of the phenylephrine-induced tension (Figure 1a). L-Cysteine in concentrations up to 300 $\mu$M had no significant effect on the relaxations to acetylcholine (results not shown). Higher concentrations of L-cysteine (1 and 3 mM) significantly reduced acetylcholine-induced relaxations (Figure 1a). Equal volumes of vehicle had no significant effect on acetylcholine-induced relaxations (data not shown).

ATP (1–100 $\mu$M) induced relaxations that were maximally about 90% of the phenylephrine-induced tone. L-Cysteine at 3 mM significantly reduced ATP-induced relaxations (Figure 1b).

The calcium ionophore A23187 (0.1 $\mu$M) produced relaxations that were 75.8 $\pm$ 9.8% of the phenylephrine-induced tone. In the presence of L-cysteine (3 mM), relaxations were significantly reduced to 42.6 $\pm$ 10.6% of phenylephrine tone ($n=4$, paired $t$-test, $P<0.01$).

### *Endothelium-independent relaxants*

The tension produced by 1 $\mu$M phenylephrine in endothelium-denuded aortic rings was 2.43 $\pm$ 0.1 g ($n=11$).

*Sodium nitroprusside* Concentration-response curves to sodium nitroprusside (0.001 – 10 $\mu$M) were significantly shifted to the left by 1 and 3 mM L-cysteine (Figure 1c).

*NO•* Low concentrations of L-cysteine (1 – 300 $\mu$M) had no effect on relaxations to NO• (0.25, 0.75 and 2.5 $\mu$M; data not shown). Higher concentrations of L-cysteine (1 – 3 mM) greatly prolonged responses to NO•, as shown for one experiment in Figure 2 and for group data in Figure 3.

*Nitroxyl anion* Angeli's salt (0.3 $\mu$M) produced relaxations with a mean of 80.5 $\pm$ 3.6% ($n=7$) of the phenylephrine-induced tone. In the presence of L-cysteine (1 and 3 mM) relaxations to Angeli's salt were significantly reduced as shown in Figure 4. The effect of nitrite (using sodium nitrite as the source) were also tested since dissociation of Angeli's salt yields nitrite as well as nitroxyl anions; however, no relaxation was observed until the nitrite concentration reached 300 $\mu$M (data not shown).

Relaxations produced by Angeli's salt were considerably longer lasting than were equivalent relaxations produced by NO•, as shown in Figure 5.

The free radical scavenger carboxy-PTIO (100 $\mu$M) abolished relaxations to NO• but did not affect the extent of the relaxations to Angeli's salt; although the duration of the

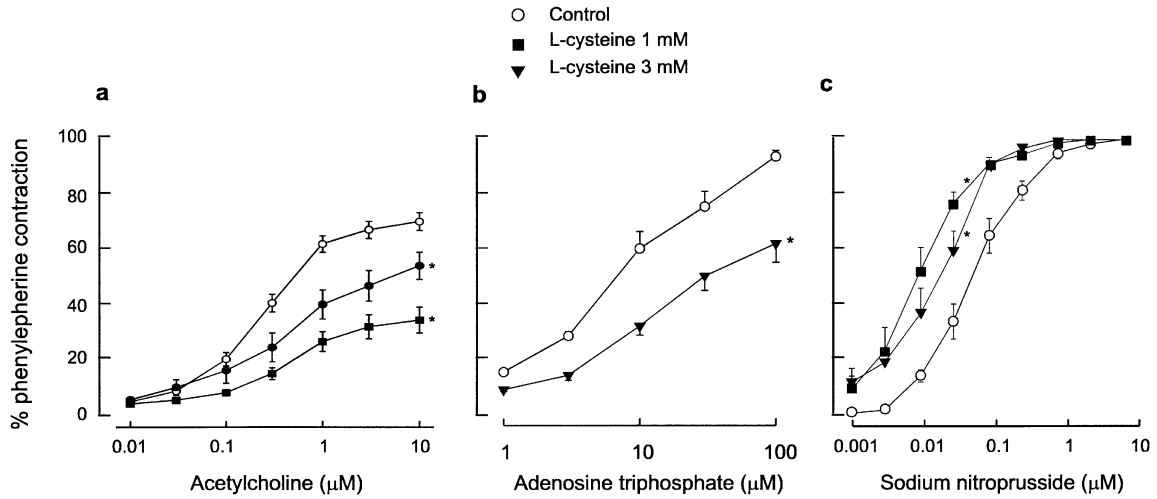


**Figure 1** The effects of L-cysteine on relaxations to (a) acetylcholine (ACh 0.01 – 10 $\mu$M) and (b) adenosine triphosphate (ATP 1 – 100 $\mu$M) in intact aortic rings, and (c) sodium nitroprusside (SNP 0.001 – 10 $\mu$M) in endothelium-denuded aortic rings contracted with phenylephrine (1 $\mu$M) and expressed as percentages of phenylephrine-induced tone. Symbols are means and T-bars indicate s.e.means ($n=4-9$). In some cases the size of the symbol was greater than the s.e.mean. *$P<0.05$ when compared to respective control curves (two-way ANOVA).

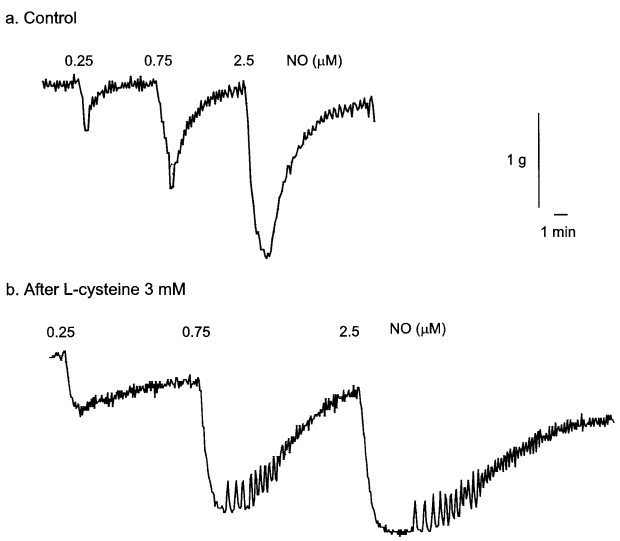


**Figure 2** Trace comparing relaxations to NO• (0.25 – 2.5 $\mu$M in the (a) absence and (b) presence of 3 mM L-cysteine in an endothelium-denuded aortic ring contracted with 1 $\mu$M phenylephrine.

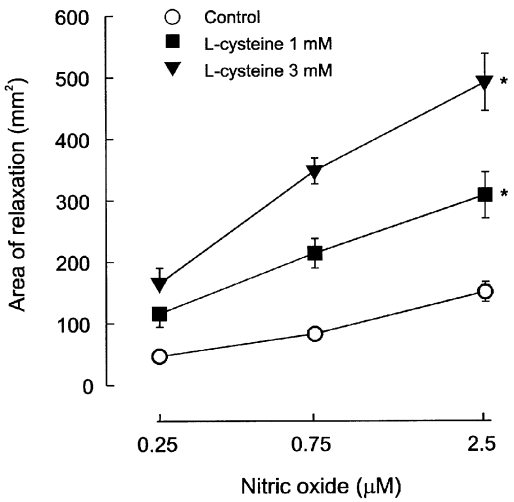


**Figure 3** Mean data of the effects of L-cysteine on the areas of relaxations to NO• (0.25, 0.75, 2.5 $\mu$M in endothelium-denuded aortic rings contracted with phenylephrine (1 $\mu$M). Responses expressed as area of relaxation (mm$^2$) with amplification and time base as in Figure 2. Symbols are means and T-bars indicate s.e.means ($n=5$). *$P<0.05$ when compared to controls area (two-way ANOVA).

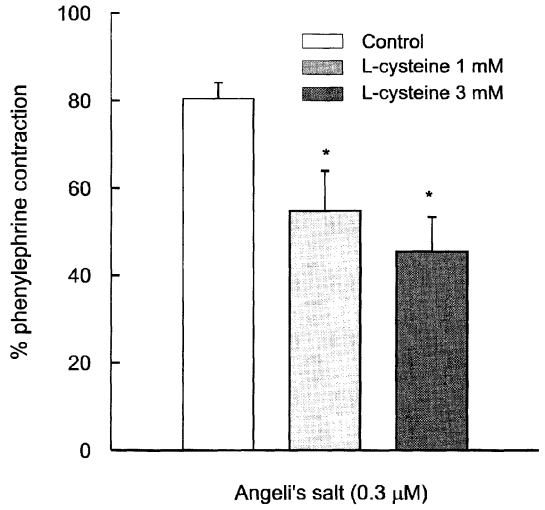


**Figure 4** Mean data for the effects of L-cysteine (1 and 3 mM) on relaxations to Angeli's salt (0.3 $\mu$M) in endothelium-denuded aortic rings. Responses are expressed as a percentage of phenylephrine-induced tone. Symbols are means and T-bars indicate s.e.means ($n=4$). *$P<0.05$ when compared to control responses (paired $t$-test).

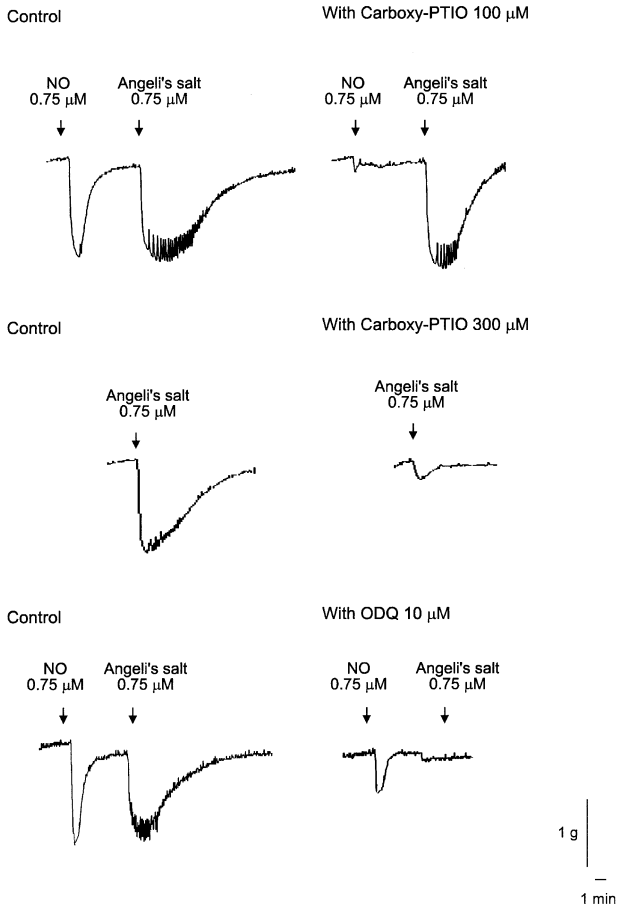


**Figure 5** Traces illustrating the effects carboxy-PTIO (100 and 300 $\mu$M) and ODQ (10 $\mu$M) on relaxations to NO$^\bullet$ (0.75 $\mu$M) and Angeli's salt (0.75 $\mu$M) in endothelium-denuded rat aorta.

relaxation was decreased (Figure 5, top tracings). However, a higher concentration of carboxy-PTIO (300 $\mu$M) almost abolished the response to Angeli's salt (Figure 5, middle tracings).

The soluble guanylate cyclase inhibitor ODQ (10 $\mu$M) abolished relaxations to Angeli's salt although those to NO$^\bullet$ were not completely abolished even at a higher than usual concentration of ODQ (Figure 5 bottom tracings).

*Isoprenaline* Relaxations to isoprenaline (0.01 – 10 $\mu$M) in endothelium-denuded preparations were not affected by the addition of L-cysteine (1 and 3 mM; data not shown).

### *Effect of L-cysteine during sustained relaxations*

These experiments were carried out to determine whether L-cysteine had direct actions that affected relaxant responses of aortic rings. Neither L-cysteine nor L-cysteine hydrochloride (3 mM) had any effect on resting tension in aortic rings. However, during relaxations to acetylcholine (1 $\mu$M) in endothelium-intact preparations or sodium nitroprusside (0.1 $\mu$M) in endothelium-denuded preparations the hydrochloride salt of L-cysteine (3 mM) had a biphasic effect on tension consisting of an initial short-lasting relaxation and then a slowly developing contraction (Figure 6a). The free base of L-cysteine produced only a slowly developing sustained contraction (Figure 6b,c). Despite this difference in action between the two forms of L-cysteine, their effects on responses mediated by EDRF, NO$^\bullet$ and Angeli's salt as described above did not differ.

## Discussion

The major finding of this study was that the effects of L-cysteine ($\geq$1 mM) on responses to NO$^\bullet$ did not correspond with its effect on responses to EDRF in rat aortic rings: responses to NO$^\bullet$ were prolonged whereas responses to EDRF were reduced. Previously, Arvola *et al.* (1992) found that 1 mM L-cysteine augmented responses to sodium nitrite and SIN-1, but did not augment responses to EDRF activated by acetylcholine in the rat mesenteric artery.

### *Low ($\leq$300 $\mu$M) concentrations of L-cysteine*

Low concentrations of L-cysteine (1 – 300 $\mu$M) had no effect on response to EDRF or NO$^\bullet$ in rat aortic rings. However, Jia & Furchgott (1993) and Feelisch *et al.* (1994), using rabbit aortic preparations, reported that low concentrations of L-cysteine blocked responses to NO$^\bullet$ and EDRF, and attributed its effect to formation of superoxide during auto-oxidation of L-cysteine (Misra, 1974; Saez *et al.*, 1982). Aortic preparations from the rabbit are considerably more sensitive to NO$^\bullet$ than those from the rat, responding in the nM range [75 nM produced >2 g relaxations in the study by Jia & Furchgott (1993)], whereas the threshold for relaxations of the rat aorta is more than 100 nM. It is possible that an inhibitory effect of L-cysteine may not be manifested with the higher concentrations of NO$^\bullet$ required to produce relaxations in rat aortic rings if the amount of superoxide that was generated was not sufficient to inactivate a substantial portion of it.

### *Prolongation of responses to NO$^\bullet$ by high ($\geq$1 mM) concentrations of L-cysteine*

Relaxations elicited by NO$^\bullet$ in aortic rings were markedly prolonged by millimolar concentrations of L-cysteine, in conformity to previous findings (Arvola *et al.*, 1992; Feelisch *et al.*, 1994; Zamora & Feelisch, 1994). A possible explanation

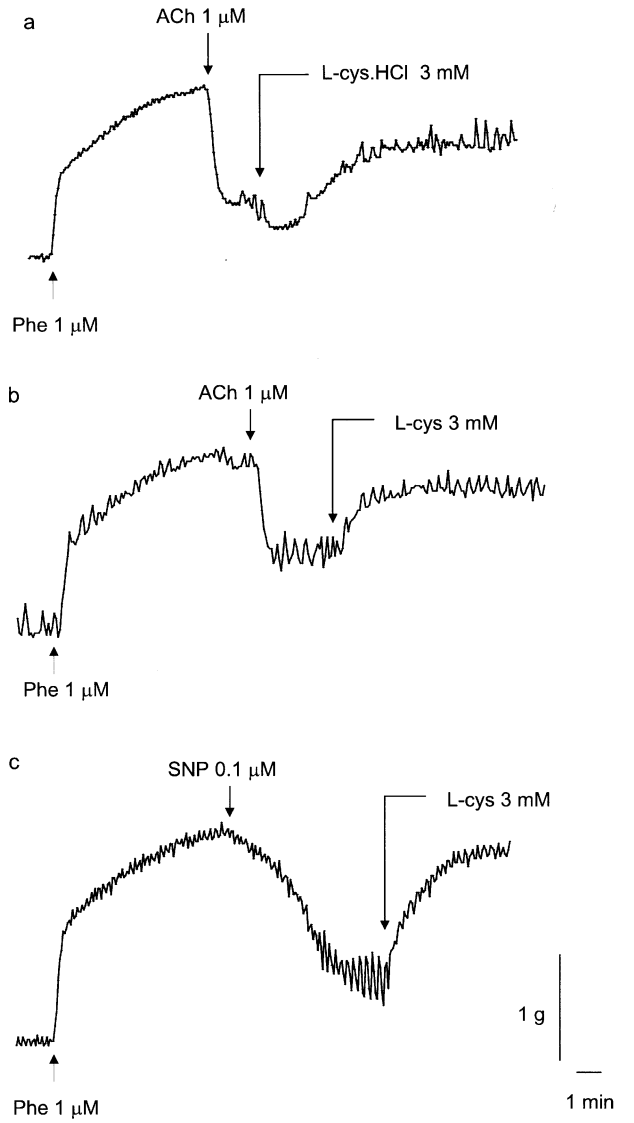


**Figure 6** Traces illustrating the effects of (a) L-cysteine hydrochloride (L-cys.HCl) and (b) the free base of L-cysteine (3 mM) during sustained relaxations induced by acetylcholine (ACh, 1 $\mu$M) in endothelium-intact aortic rings, and by (c) sodium nitroprusside (SNP, 0.1 $\mu$M) in endothelium-denuded aortic rings contracted with 1 $\mu$M phenylephrine (Phe).

for this effect is the formation of S-nitrosocysteine (Liu *et al*., 1998, Kharitonov *et al*., 1995). An alternative explanation arises from the observation that responses to nitroxyl ($NO^-$) were much more prolonged than those to $NO^\bullet$. This suggests the possibility that $NO^\bullet$ may be reduced to nitroxyl in a reaction that may be coupled to oxidation of L-cysteine, and it is the nitroxyl that results in prolongation of the response. The difficulty in accepting the latter explanation is that it would be expected that any L-cysteine still in a reduced form would react with the nitroxyl to block responses to it. Both S-nitrosocysteine and nitroxyl might be involved since Wong *et al*., (1998) reported that S-nitrosothiols react with thiols to generate nitroxyl and dithiol.

### *Relaxations produced by nitroxyl and their impairment by L-cysteine (3 mM)*

Angeli's salt, which dissociates to form nitroxyl ($NO^-$) and nitrite anions (Zamora & Feelisch, 1994), produced relaxations of rat aortic rings that were considerably longer lasting than were equivalent relaxations produced by $NO^\bullet$. The possibility that nitrite anions might account for the observed relaxations was discounted because concentrations of sodium nitrite at least 100 fold greater were required to produce relaxations of a similar magnitude to those produced by Angeli's salt.

Angeli's salt is slightly more potent than $NO^\bullet$ as a relaxant; according to Zamora & Feelisch (1994), the $ED_{50}$ values of Angeli's salt and $NO^\bullet$ for relaxation of the rat aorta were $3.16 \pm 0.8$ and $4.03 \pm 0.49$ $\mu$M, respectively. Since responses to both $NO^\bullet$ and Angeli's salt were reduced or blocked by the soluble guanylate cyclase ODQ (as also observed in the rat anococcygeus muscle by Li *et al*., 1999), it is unlikely that Angeli's salt acts on a different site from that of $NO^\bullet$. It has been reported that $NO^\bullet$ is the only redox form of nitrogen monoxide that activates soluble guanylate cyclase (Dierks & Burstyn, 1996). These findings could only be reconciled if nitroxyl was more stable than $NO^\bullet$ in the PSS in the organ bath, but that after it entered smooth muscle cells it was oxidised to $NO^\bullet$. However, in the present study, relaxations to Angeli's salt were completely abolished when guanylate cyclase was inhibited by ODQ, whereas those to free radical NO were only reduced, indicating that there may be a difference between these redox forms of nitrogen monoxide in activating soluble guanylate cyclase.

The persistence of the response to nitroxyl anion was surprising since it was reported to react with oxygen yielding peroxynitrite (Hughes & Nicklin, 1971) and the protonated form (HNO) dimerizes to hyponitrous acid ($H_2N_2O_2$), which decomposes to yield $N_2O$ and $H_2O$ (Zamora & Feelisch, 1994; King & Nagasawa, 1999). According to Hughes & Cammack (1999), who quote a rate constant for the dimerization of HNO of $1.9 \times 10^9$ $dm^3$ $mol^{-1}$ $s^{-1}$, the lifetime of nitroxyl at around neutral pH would be milliseconds. However, this supposition is not in accord with our observations since the relaxations were of a longer duration than were relaxations to $NO^\bullet$.

The relaxant action of Angeli's salt was abolished in the presence of L-cysteine, as has previously been reported by others using various nitroxyl donors and other vascular tissues (Fukuto *et al*., 1993; Zamora & Feelisch, 1994; Zamora *et al*., 1995). Zamora & Feelisch (1994) suggested a number of enzymatic and non-enzymatic mechanisms that might explain this effect. A two-stage reaction resulting in the formation of hydroxylamine with oxidation of cysteine to cystine (Doyle *et al*., 1988) is, in our view, the most convincing.

The relaxant action of nitroxyl was not decreased by carboxy-PTIO at a concentration of 100 $\mu$M, which abolished the response to $NO^\bullet$, although its duration did decrease. However, at 300 $\mu$M of carboxy-PTIO, relaxations to nitroxyl ions were almost abolished. Since it is presumed that carboxy-PTIO selectively scavenges only the free radical form of nitrogen monoxide, an explanation for this effect is that nitroxyl is oxidized to $NO^\bullet$, possibly coupled to reduction of carboxy-PTIO. Since there is a considerably lower concentration of nitroxyl ions than of carboxy-PTIO, the larger remaining portion of non-reduced carboxy-PTIO would be sufficient to scavenge all of the free radical NO produced by oxidation of the nitroxyl ions.

### *Impairment of responses to EDRF by high ($\geqslant$1 mM) concentrations of L-cysteine*

EDRF-mediated relaxations produced by acetylcholine were reduced by 1–3 mM L-cysteine in aortic rings. EDRF-mediated relaxations produced by ATP or by the calcium ionophore A23187 were also inhibited by 3 mM L-cysteine, indicating that its effect was not likely to be due to an action on

receptors that could conceivably alter their activation by their respective agonists. Other authors have shown that the thiol homocysteine can reduce the activity of endothelium-dependent relaxations in rabbit and rat aorta and the pancreatic vascular bed of rats (Lang *et al*., 1997; Emsley *et al*., 1999; Quere *et al*., 1997) while having little or no effect on relaxations induced by NO donors.

We considered a number of possibilities that could account for the inhibitory effect of L-cysteine. It is unlikely that the inhibition of responses to EDRF by L-cysteine can be attributed to a reduction in EDRF synthesis since it has previously been shown that thiols such as glutathione, N-acetylcysteine and dithiothreitol do not inhibit the activity of eNOS, obtained from bovine aortic cells (Zembowicz *et al*., 1993). Furthermore, millimolar concentrations of the thiol dithiothreitol are often included in studies on NOS activity to ensure the stability of the enzyme (Di Iulio *et al*., 1997). The inhibitory effect was not due to inactivation of endothelium-derived $NO^{\bullet}$ by superoxide formation since relaxations to exogenous $NO^{\bullet}$ were substantially enhanced. It was not due to a counteracting contractile action of L-cysteine since this would also be expected to reduce relaxant responses to $NO^{\bullet}$ and sodium nitroprusside, but these were enhanced.

A possible effect of L-cysteine may be that the concentration within the endothelial cells may have been raised to an extent that an appreciable portion of the $NO^{\bullet}$ formed was converted intracellularly to the polar, hydrophilic nitroso-L-cysteine, which, unlike $NO^{\bullet}$, does not as readily pass through cell membranes (Kowaluk & Fung, 1990), and would be trapped within the endothelial cells.

A further possibility arises from our observation that L-cysteine blocks the response to the nitroxyl anion. Thus it is reasonable to infer that at least a portion of EDRF may exist in the nitroxyl form. This is consistent with observations that EDRF released from cultured endothelial cells has a negative charge and did not pass through an anion exchange column whereas $NO^{\bullet}$ did (Cocks *et al*., 1985; Long *et al*., 1987). Fukuto *et al*. (1992b) reported that nitroxyl could be formed from the intermediate compound N-hydroxy-L-arginine during the oxidation of L-arginine by NOS. The possibility that nitroxyl might be present in EDRF could explain the finding by Mian & Martin (1995) that superoxide anions generated by xanthine oxidase and hypoxanthine did not completely block endothelium-dependent relaxations in rat aortic rings even after treatment with DETCA to eliminate the activity of Cu/Zn SOD. Li *et al*. (1999) found that superoxide generated by pyrogallol had a negligible effect on relaxations mediated by nitroxyl derived from Angeli's salt in the rat anococcygeus muscle.

As mentioned in the Introduction, carboxy-PTIO was less effective at blocking relaxations to EDRF than to $NO^{\bullet}$ in the rat aorta (Rand & Li, 1995a). In studies on the effect of carboxy-PTIO on EDRF-mediated relaxations, it has been found that a relatively high concentration of 300 $\mu$M is required to greatly reduce or abolish them (Rand & Li, 1995a; Yoshida *et al*., 1998), whereas $NO^{\bullet}$-induced relaxations are abolished by 100 $\mu$M or less (Rand & Li, 1995a). In the present study, we found that nitroxyl-mediated relaxations produced by Angeli's salt were greatly reduced by 300 $\mu$M carboxy-PTIO, which is consistent with the possibility that there is a nitroxyl component in EDRF.

In the study by Feelisch *et al*. (1994), EDRF was released from cultured endothelial cells and detected in a bioassay cascade, using the sensitive rabbit aorta preparation, and was identified as $NO^{\bullet}$. This finding can be reconciled with the view that freshly generated EDRF in the myoendothelial junction contains nitroxyl anions, but the time lapse from the generation of the EDRF to its detection in a cascade allowed the oxidation of any nitroxyl anions originally present to $NO^{\bullet}$.

### *Effects of L-cysteine on tone*

L-Cysteine (3 mM, as the base or as the hydrochloride) had no effect on the resting tone of aortic rings. However, when added during relaxations produced by acetylcholine in rings with intact endothelium or sodium nitroprusside in endothelium-denuded rings, L-cysteine hydrochloride produced a transient relaxation followed by a slowly developing contraction whereas L-cysteine base produced only a slowly developing contraction. Others (Jia & Furchgott, 1993; Zamora & Feelisch, 1994; Furchgott & Jothianandan, 1998) have reported that L-cysteine hydrochloride produced a transient relaxation, which Furchgott & Jothianandan (1998) attributed to its marked acidity that generated ideal conditions for nitrosylation of cysteine with residual nitrite. In contrast, in reports that presumably refer to the base, Fujioka *et al*., (1993) found that L-cysteine caused contractions in dog coronary arterial strips, and Asano & Hidaka (1983) found that cysteine enhanced contractions of vascular tissue to several contractile agonists. The possibility that a contractile action of L-cysteine counteracted EDRF-induced relaxations is unlikely because it did not decrease relaxations induced by isoprenaline or sodium nitroprusside, probably because the relaxations were rapid in onset whereas the contractile action of L-cysteine developed slowly. The difference in the timing of relaxant and contractile drives may also explain the findings that sodium nitroprusside-induced relaxations were enhanced by L-cysteine (presumably because of nitrosocysteine formation), but L-cysteine produced a slowly developing contraction when added during a sustained sodium nitroprusside-induced relaxation.

### *Conclusions*

L-Cysteine ($\geqslant$1 mM) substantially enhanced relaxant responses of rat aortic rings to $NO^{\bullet}$, abolished response to nitroxyl anions ($NO^{-}$) generated by Angeli's salt, and significantly reduced responses to EDRF. These findings suggest that EDRF in the biophase of the myoendothelial junction is not identical to $NO^{\bullet}$. The findings could be explained by assuming that EDRF consists of an admixture of $NO^{\bullet}$ and nitroxyl anions in the period between its generation in endothelial cells and its action on the adjacent vascular smooth muscle.

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## References


AKAIKE, T., YOSHIDA, M., MIYAMOTO, Y., SATO, K., KOHNO, M., SASAMOTO, K., MIYAZAKI, K., UEDA, S. & MAEDA, H. (1993). Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factors/nitric oxide through a radical reaction. *Biochemistry,* **32,** 827–832.

ALERYANI, S., MILO, E., ROSE, Y. & KOSTKA, P. (1998). Superoxide-mediated decomposition of biological S-nitrosothiols. *J. Biol. Chem.,* **273,** 6041–6045.

ARVOLA, P., PORSTI, I., VUORINEN, P., HUHTAJA, H., METSA-KETELA, T. & VAPAATALO, H. (1992). L-Cysteine augments the vasorelaxation induced by sodium nitrite and SIN-1 but not that due to acetylcholine. *Eur. J. Pharmacol.,* **214,** 289–292.

ASANO, M. & HIDAKA, H. (1983). Alterations in pharmacological receptor activities of rabbit arteries by sulfhydryl reagents. *Jpn. J. Pharmacol.,* **33,** 227–240.

BOECKXSTAENS, G.E., DE MAN, J.G., DE WINTER, B.Y., HERMAN, A.G. & PELCKMANS, P.A. (1994). The pharmacological similarity between nitric oxide and the nitrergic neurotransmitter in the canine ileocolonic junction. *Eur. J. Pharmacol.,* **264,** 85–89.

CHATAIGNEAU, T., FÉLÉTOU, M., HUANG, P.L., FISHMAN, C., DUHAULT, J. & VANHOUTTE, P.M. (1999). Acetylcholine-induced relaxation in blood vessels from endothelial nitric oxide synthase knockout mice. *Br. J. Pharmacol.,* **126,** 219–226.

COCKS, T.M., ANGUS, J.A., CAMPBELL, J.H. & CAMPBELL, G.R. (1985). Release and properties of endothelium derived relaxing factor (EDRF) from endothelial cells in culture. *J. Cell Physiol.,* **123,** 310–320.

DIERKS, E.A. & BURSTYN, J.N. (1996). Nitric oxide ($NO^{\bullet}$), the only nitrogen monoxide redox form capable of activating soluble guanylate cyclase. *Biochem. Pharmacol.,* **51,** 1593–1600.

DI IULIO, J.L., LI, C.G. & RAND, M.J. (1997). Determination of nitric oxide synthase activity in rat, pig and rabbit prostate glands. *Eur. J. Pharmacol.,* **337,** 245–249.

DOYLE, M.P., MAHAPATRO, S.N., BROENE, R.D. & GUY, J.K. (1988). Oxidation and reduction of hemoproteins by trioxodinitrate(II) The role of nitrosyl hydride and nitrite. *J. Am. Chem. Soc.,* **110,** 593–599.

EMSLEY, A.M., JEREMY, J.Y., GOMES, G.N., ANGELINI, G.D. & PLANE, F. (1999). Investigation of the inhibitory effect of homocysteine and copper on nitric oxide-mediated relaxation of rat isolated aorta. *Br. J. Pharmacol.,* **126,** 1034–1040.

FEELISCH, M., TE POEL, M., ZAMORA, R., DEUSSEN, A. & MONCADA, S. (1994). Understanding the controversy over the identity of EDRF. *Nature,* **368,** 62–65.

FUJIOKA, H., HORIIKE, K., TAKAHASHI, M., ISHIDA, T., KINOSHITA, M. & NOZAKI, M. (1993). Triphasic vascular effects of thiol compounds and their oxidized forms on dog coronary arteries. *Experientia,* **49,** 47–50.

FUKUTO, J.M., CHIANG, K., HSZIEH, R., WONG, P. & CHAUDHURI, G. (1992a). The pharmacological activity of nitroxyl: a potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.,* **263,** 546–551.

FUKUTO, J.M., HOBBS, A.J. & IGNARRO, L.J. (1993). Conversion of nitroxyl (HNO) to nitric oxide (NO) in biological systems: the role of physiological oxidants and relevance to the biological activity of HNO. *Biochem. Biophys. Res. Comm.,* **196,** 703–713.

FUKUTO, J.M., WALLACE, G.C., HSZIEH, R. & CHAUDHURI, G. (1992b). Chemical oxidation of N-hydroxyguanidine compounds. Release of nitric oxide, nitroxyl and possible relationship to the mechanism of biological nitric oxide generation. *Biochem. Pharmacol.,* **43,** 607–613.

FURCHGOTT, R.F. & JOTHIANANDAN, D. (1998). Transnitrosation reactions involved in the sensitisation of rabbit aorta for relaxations by thiols. *Pharmacol. Toxicol.,* **83,** (Supp. 1), 44–46.

FURCHGOTT, R.J. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288,** 373–376.

HOBBS, A.J., FUKUTO, J.M. & IGNARRO, L.J. (1994). Formation of free nitric oxide from L-arginine by nitric oxide synthase: direct enhancement of generation by superoxide dismutase. *Proc. Nat. Acad. Sc, U.S.A.,* **91,** 10992–10996.

HUGHES, M.N. & CAMMACK, R. (1999). Synthesis, chemistry, and applications of nitroxyl ion releasers sodium trioxodinitrate or Angeli's salt and Piloty's acid. *Meth. Enzymol.,* **301,** 279–287.

HUGHES, M.N. & NICKLIN, H.G. (1971). Autoxidation of hydroxylamine in alkaline solutions. *J. Chem. Soc. (A),* 164.

JIA, L. & FURCHGOTT, R.F. (1993). Inhibition by sulfhydryl compounds of vascular relaxation induced by nitric oxide and endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.,* **267,** 371–378.

KHARITONOV, V.G., SUNDQUIST, A.R. & SHARMA, V.S. (1995). Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J. Biol. Chem.,* **270,** 28148–28164.

KING, S.B. & NAGASAWA, H.T. (1999). Chemical approaches towards generation of nitroxyl. *Meth. Enzymol.,* **301,** 211–221.

KOWALUK, E.A. & FUNG, H.L. (1990). Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols. *J. Pharmacol. Exp. Ther.,* **255,** 1256–1264.

LANG, D., HUSSAIN, S.A. & LEWIS, M.J. (1997). Homocysteine inhibits endothelium-dependent relaxation in isolated rabbit aortic rings. *Br. J. Pharmacol.,* **120,** 145P.

LI, C.G., KARAGIANNIS, J. & RAND, M.J. (1999). Comparison of the redox forms of nitrogen monoxide with the nitrergic transmitter in the rat anococcygeus muscle. *Br. J. Pharmacol.,* **127,** 826–834.

LIU, X., GILLESPIE, J.S. & MARTIN, W. (1994). Non-adrenergic, non-cholinergic relaxation of the bovine retractor penis muscle: role of S-nitrosothiols. *Br. J. Pharmacol.,* **111,** 1287–1295.

LIU, Z., RUDD, A., FREEDMAN, J.E. & LOSCALZO, J. (1998). S-Transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. *J. Pharmacol. Exp. Ther.,* **284,** 526–534.

LONG, C.J., SHIKANO, K. & BERKOWITZ, B.A. (1987). Anion exchange resins discriminate between nitric oxide and EDRF. *Eur. J. Pharmacol.,* **142,** 317–319.

MARTIN, G.R., BOLOFO, M.L. & GILES, H. (1992). Inhibition of endothelium-dependent vasorelaxation by arginine analogues: a pharmacological analysis of agonist and tissue dependence. *Br. J. Pharmacol.,* **105,** 643–652.

MCLAREN, A.M., LI, C.G. & RAND, M.J. (1992). Cysteine differentially affects nicotine- and nitric oxide-induced relaxations in rat gastric fundus. *Proc. Austral. Physiol. Pharmacol. Soc.,* **23,** 176P.

MIAN, K.B. & MARTIN, W. (1995). Differential sensitivity of basal and acetylcholine-stimulated activity of nitric oxide to destruction by superoxide anion in rat aorta. *Br. J. Pharmacol.,* **115,** 993–1000.

MISRA, H.P. (1974). Generation of superoxide free radical during the autoxidation of thiols. *J. Biol. Chem.,* **219,** 2151–2155.

QUERE, I., HILLSIRE-BUYS, D., BRUNSCHWIG, C., CHAPAL, J., JANBON, C., BLAYAC, J.P., PETIT, P. & LOUBATIERES-MARIANI, M.M. (1997). Effects of homocysteine on acetylcholine- and adenosine-induced vasodilatation of pancreatic vascular bed in rats. *Br. J. Pharmacol.,* **122,** 351–357.

RAJANAYAGAM, M.A., LI, C.G. & RAND, M.J. (1993). Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle. *Br. J. Pharmacol.,* **108,** 3–5.

RAND, M.J. & LI, C.G. (1992). Differential effects of cysteine and glutathione on NO and stimulation-induced relaxations of the rat anococcygeus muscle. *Proc. Austral. Physiol. Pharmacol. Soc.,* **23,** 175P.

RAND, M.J. & LI, C.G. (1995a). Discrimination by the NO-trapping agent, carboxy-PTIO, between NO and the nitrergic transmitter but not between NO and EDRF. *Br. J. Pharmacol.,* **116,** 1906–1910.

RAND, M.J. & LI, C.G. (1995b). Nitric oxide in the autonomic and enteric nervous systems. In: *Nitric Oxide in the Nervous System.* Vincent, S.R. (ed). Academic Press, London. pp 228–279.

SAEZ, G., THORNALLEY, P.J., HILL, H.A.O., HEMS, R. & BANNISTER, J.V. (1982). The production of free radicals during the autoxidation of cysteine and their effect on isolated rat hepatocytes. *Biochim. Biophys. Acta,* **719,** 24–31.

SCHMIDT, H.H.H., HOFMANN, H., SCHINDLER, U., SHUTENKO, Z., CUNNINGHAM, D. & FEELISCH, M. (1996). No. NO from NO synthase. *Proc. Natl. Acad. Sci., USA,* **93,** 14492–14497.

STAMLER, J.S. (1994). Redox signalling: nitrosylation and related target interactions of nitric oxide. *Cell,* **78,** 931–936.

WONG, P.S., HYUN, J., FUKUTO, J.M., SHIROTA, F.N., DEMASTER, E.G., SHOEMAN, D.W. & NAGASAWA, H.T. (1998). Reaction between S-nitrosothiols and thiols: generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry,* **37,** 5362–5371.

WOOD, J. & GARTHWAITE, J. (1994). Models for the diffusional spread of nitric oxide: implications for neural nitric oxide signalling and its pharmacological properties. *Neuropharmacology,* **33,** 1235 – 1244.

YOSHIDA, M., AKAIKE, T., GOTO, S., TAKAHASHI, W., INADOME, A., YONO, M., SESHITA, H., MAEDA, H. & UEDA, S. (1998). Effect of the NO scavenger carboxy-PTIO on endothelium-dependent vasorelaxation of various blood vessels from rabbits. *Life Sci.,* **62,** 203 – 211.

ZAMORA, R. & FEELISCH, M. (1994). Bioassay discrimination between nitric oxide and nitroxyl using L-cysteine. *Biochem. Biophys. Res. Comm.,* **201,** 54 – 62.

ZAMORA, R., GRZESIOK, A., WEBER, H. & FEELISCH, M. (1995). Oxidative release of nitric oxide accounts for guanylyl cyclase stimulating, vasodilator and anti-platelet activity of Piloty's acid: a comparison with Angeli's salt. *Biochem. J.,* **312,** 333 – 339.

ZEMBOWICZ, A., HATCHETT, R.J., RADZISZEWSKI, W. & GRYGLEWSKI, R.J. (1993). Inhibition of endothelial nitric oxide synthases by ebselen. Prevention by thiols suggests the inactivation by ebselen of a critical thiol essential for the catalytic activity of nitric oxide synthase. *J. Pharmacol. Exp. Ther.,* **267,** 1112 – 1118.

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