British Journal of Pharmacology (1995) 116, 1906–1910 © 1995 Stockton Press All rights reserved 0007–1188/95 $12.00

Discrimination by the NO-trapping agent, carboxy-PTIO, between NO and the nitrergic transmitter but not between NO and EDRF

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1 The effects of carboxy-PTIO, a scavenger of free radical nitric oxide (NO), were studied on endothelium-dependent relaxations of rat aorta and nitrergic nerve stimulation-induced relaxations of anococcygeus muscle and gastric fundus strips to test the hypothesis that endothelium-derived relaxing factor (EDRF) and the transmitter released by nitrergic nerves is free radical NO.

2 Carboxy-PTIO (10–300 μM) produced concentration-dependent reductions of relaxations elicited by exogenous NO, and relaxations mediated by EDRF released by acetylcholine and ATP in rings of rat aorta. The inhibitory effect of carboxy-PTIO was removed by washing the tissues.

3 In the rat anococcygeus muscle, carboxy-PTIO (10–300 μM) produced concentration-dependent reductions of relaxations to exogenous NO; however, in concentrations up to 2000 μM it did not reduce relaxations elicited by nitrergic nerve stimulation (1–2 Hz), in fact, concentrations of 300 μM or more slightly enhanced them.

4 In rat gastric fundus strips, carboxy-PTIO (100 and 300 μM) reduced relaxations to exogenous NO, but relaxations elicited by stimulation of the nitrergic component of non-adrenergic, non-cholinergic nerves were not affected.

5 These results suggest that EDRF is free radical NO and may be designated EDNO, but the transmitter released from nitrergic nerves does not appear to be identical to EDNO and may not be free radical NO.

Keywords: Anococcygeus muscle (rat); carboxy-PTIO; endothelium-derived relaxing factor (EDRF); gastric fundus (rat); nitrergic transmitter; nitric oxide

Introduction

Functional and morphological studies indicate that transmission at certain neuroeffector junctions is nitrergic. In summary, the nerves contain nitric oxide synthase (NOS) which catalyses the formation of NO from L-arginine and transmission depends on the functional integrity of this NOS, responses to the transmitter are mimicked by nitric oxide (NO), transmission can be inhibited by haemoglobin which inactivates NO, and the relaxation of the effector smooth muscle by both NO and the transmitter involves the activation of soluble guanylate cyclase with formation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP); however, some doubts remain about the exact nature of the transmitter (for reviews, see Rand & Li, 1995a,b). This is because a number of agents that inactivate exogenously applied NO (in aqueous solution), and thereby block responses to it, do not block responses to nitrergic nerve stimulation. Such differential blockade has been demonstrated for the following substances in various tissues: pyrogallol, a superoxide generator, in bovine retractor penis muscle (Gillespie & Sheng, 1990; Liu *et al.*, 1991; Martin *et al.*, 1994) and dog isolated ileocolonic junction (Boeckxstaens *et al.*, 1994); hydroquinone, which has been described as a free radical scavenger and is also a generator of superoxide anions, in the bovine retractor penis muscle (Gillespie & Sheng, 1990), the anococcygeus muscle (Hobbs *et al.*, 1991; Gibson *et al.*, 1992), the possum lower oesophageal sphincter (Knudsen *et al.*, 1992), and guinea-pig trachea (Hobbs *et al.*, 1991); LY83583, which inhibits guanylate cyclase but is also a superoxide anion generator, in the rat gastric fundus (Barbier & Lefebvre, 1992); 7-ethoxyresorufin, which inhibits NOS but its predominant action is due to generation of superoxiode anions, in the rat anococcygeus muscle (Li & Rand, 1993b; Rand & Li, 1993b); the enzymatic superoxide generating systems xanthine/xanthine oxidase in the mouse anococcygeus muscle (Gibson *et al.*, 1994) and hypoxanthine/xanthine oxidase in the bovine retractor penis (Martin *et al.*, 1994); and hydroxocobalamin, which is thought to trap NO by forming nitrosocobalamin, in the rat anococcygeus muscle and gastric fundus (Li & Rand, 1993a; Rajanayagam *et al.*, 1993; Rand & Li, 1993a; Jenkinson *et al.*, 1995).

It has also been suggested that endothelium-derived relaxing factor (EDRF) may not be identical to NO (Myers *et al.*, 1990; Bates *et al.*, 1991; Vedernikov *et al.*, 1992); however, the above-mentioned agents do not discriminate between EDRF and NO. Hence, for this and other reasons (Moncada *et al.*, 1991; Feelisch *et al.*, 1994), NO as such appears to account for the activity of EDRF, but further evidence would be desirable.

The present study was designed to test the hypothesis that free radical NO accounts for the smooth muscle relaxant actions of EDRF and the transmitter released by nitrergic nerves. For this purpose, we used carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide]. It rapidly inactivates NO by reacting stoichiometrically to form the corresponding imidazolineoxyl and NO_2 free radical, which in turn reacts with water to form nitrite, nitrate and hydrogen ions (Akaike *et al.*, 1993). The effects of carboxy-PTIO were studied on EDRF-mediated relaxations of rat aorta and nitrergic nerve stimulation-induced relaxations of anococcygeus muscle and gastric fundus strips, and on NO-induced relaxations in these tissues. Preliminary communications of the findings in the present paper have been given (Rand & Li, 1994; Li & Rand, 1994).

Methods

Male Sprague-Dawley rats (250–400 g) were killed by decapitation and the proximal thoracic aorta, anococcygeus muscles and stomach were removed.

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Rings of aorta of about 6 mm in length were set up for measurement of isometric tension as described previously (Rand & Li, 1993a). Sustained contractions were produced by phenylephrine (1 μM) and endothelium-dependent relaxations were elicited by acetylcholine (0.01–10 μM) or ATP (100 μM). In some preparations the endothelium was removed and relaxations were elicited with NO (0.2–1.2 μM). After replacing the bath fluid with fresh PSS, an interval of 20 min elapsed before the next addition of phenylephrine.

Anococcygeus muscles were set up for isometric recording as described previously (Gillespie, 1972; Li & Rand, 1989). The tone was raised and nitrergic relaxations in response to field stimulation (1–2 Hz, 10 s) were revealed by guanethidine (10–30 μM). The relaxant actions of NO (0.2–1.2 μM) were determined in some preparations.

Longitudinal strips of gastric fundus were set up for isotonic recording as described by Li and Rand (1990). The tone was raised with 5-hydroxytryptamine (5-HT, 10 μM) and relaxations were elicited by NO or field stimulation in the presence of guanethidine (10 μM) and atropine (1 μM), using stimulation parameters (5 Hz, 10 s) that elicited only the nitrergic component of the NANC response.

Two preparations were made of each tissue from each donor rat. In one of each pair, the effects of carboxy-PTIO were studied after initial control responses to NO or nerve stimulation had been obtained and parallel experiments without addition of carboxy-PTIO were carried out in the other tissue from the same donor rat to serve as time controls.

The composition of the physiological salt solution (PSS) was as follows (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1 and disodium edetate 0.067. Note that we usually include ascorbic acid (0.14 mM) in the PSS, but this has been omitted in the present series of experiments because ascorbic acid reduces carboxy-PTIO to *N*-hydroxy-carboxy-PTIO, and the reduced product does not react with NO (Tsunoda *et al.*, 1994).

The following drugs were used: acetylcholine perchlorate (British Drug Houses Ltd, U.K.); atropine sulphate, guanethidine monosulphate, 5-hydroxytryptamine creatinine sulphate (serotonin), (-)-phenylephrine hydrochloride (Sigma Chemical Co., U.S.A.); carboxy-PTIO (Sapphire Bioscience); and nitric oxide (compressed gas, CIG, Melbourne). Saturated aqueous solutions of NO were prepared from NO gas as previously described (Rajanayagam *et al.*, 1993).

Data were expressed as means and standard errors. The significance of differences between means was determined by Student's *t* test or analysis of variance (ANOVA). Values of $P<0.05$ were considered as significant.

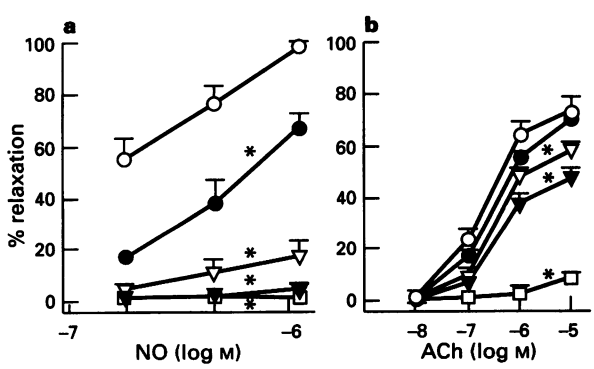

Figure 1 Effects of carboxy-PTIO (10–300 μM) on relaxations of rat aortic rings elicited by nitric oxide (0.2–1.2 μM) in endothelium-denuded rings (a) and by acetylcholine (ACh, 0.01–10 μM) in endothelium-intact rings (b). Concentrations of carboxy-PTIO are: (○) 0 (control); (●) 10 μM; (▽) 30 μM; (▼) 100 μM; (□) 300 μM. Symbols are mean with s.e.mean of 5–6 experiments. *Indicates that concentration-response curve differs significantly ($P<0.05$, two-way ANOVA) from the control curve.

Results

Rings of aorta

In endothelium-denuded preparations, carboxy-PTIO (10–300 μM) concentration-dependently inhibited relaxations elicited by exogenous NO (0.2–1.2 μM) as shown in Figure 1a. Carboxy-PTIO (10–300 μM) also produced concentration-dependent inhibition of relaxations elicited by 0.01–10 μM acetylcholine in endothelium-intact preparations (Figure 1b). The concentrations of carboxy-PTIO required to reduce responses to acetylcholine were about 10 times greater than those

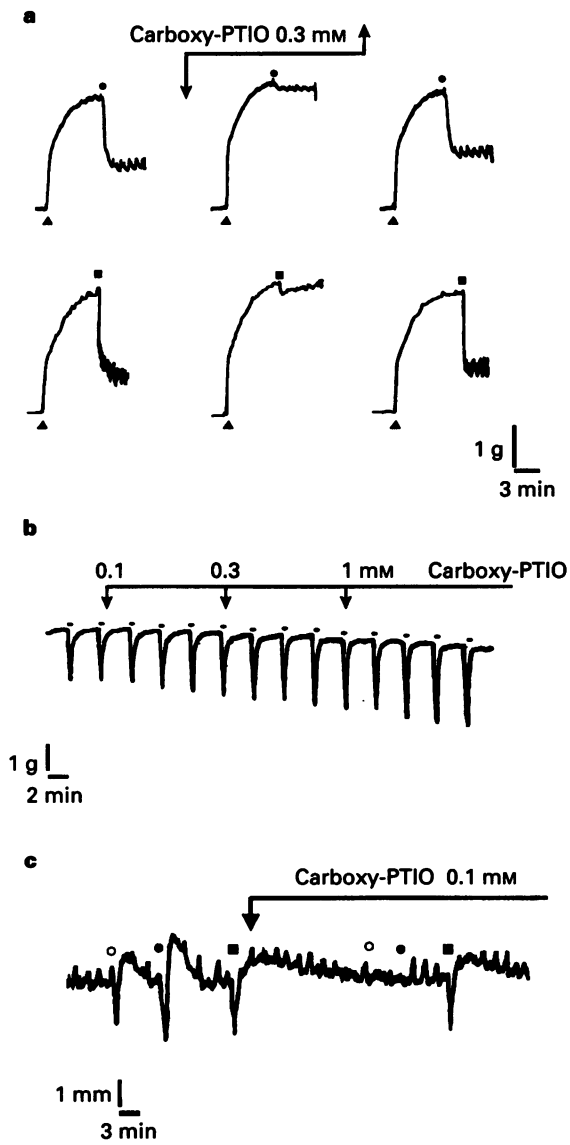

Figure 2 (a) In endothelium-intact aortic rings in which tone was raised with phenylephrine (1 μM, applied at ▲), 300 μM carboxy-PTIO blocked relaxations induced by acetylcholine (1 μM, indicated by ●, upper panel) and ATP (100 μM, indicated by ■, lower panel), and after washing out carboxy-PTIO the relaxations were restored. W indicates replacement of fresh PSS. (b) In an anococcygeus muscle, 100–1000 μM carboxy-PTIO failed to reduce relaxations induced by nitrergic nerve stimulation (2 Hz for 10 s at 3 min intervals), and slightly enhanced them. (c) In a gastric fundus strip, relaxations elicited by exogenous nitric oxide (0.5 and 1.2 μM, indicated by (○) and (●) respectively) were blocked by 100 μM carboxy-PTIO but relaxations elicited by NANC nerve stimulation (5 Hz for 10 s indicated by (■)) were not affected. The tone of each preparation was raised as described in Methods. Similar results to those illustrated were obtained in at least 4 separate experiments.

reducing comparable responses to NO. The inhibition of acetylcholine-induced relaxations was not due to an anti-muscarinic action of carboxy-PTIO since relaxations elicited by ATP were also greatly inhibited: in the presence of 300 μM carboxy-PTIO, relaxations induced by 100 μM ATP were 12.2 ± 4.7% of the control responses ($n=4$, $P<0.01$, Student's t test). The effect of 300 μM carboxy-PTIO in inhibiting acetylcholine- and ATP-induced relaxations in aortic rings is illustrated in Figure 2a, which also shows that these EDRF-mediated relaxations were restored when carboxy-PTIO was washed out.

Anococcygeus muscles

In rat anococcygeus muscles, carboxy-PTIO (10–300 μM) concentration-dependently inhibited relaxations to exogenous NO (0.2–1.3 μM) (Figure 3). The inhibition closely resembled that observed in aortic rings (cf. Figure 1a).

In contrast, relaxations induced by nitrergic nerve stimulation (1–2 Hz, 10 s) were not reduced by carboxy-PTIO (10–2000 μM), and the higher concentrations of carboxy-PTIO (>300 μM) slightly enhanced the relaxations and also slightly reduced the muscle tone, as shown in one experiment in Figure 2b. These effects of carboxy-PTIO (10–200 μM) were consistenly observed in 6 experiments. In time-control experiments, there was no appreciable change in stimulation-induced relaxations or tone.

Gastric fundus

Relaxations of rat gastric fundus strips induced by exogenous NO (0.5 and 1.2 μM) were totally blocked by carboxy-PTIO (100 μM) but relaxations elicited by stimulation of non-adrenergic and non-cholinergic nerves (5 Hz for 10 s) were not affected, as shown with 100 μM carboxy-PTIO in Figure 2c. This differential effect occurred consistently with preparations from 4 rats. A higher concentration of carboxy-PTIO (300 μM) also failed to affect stimulation-induced relaxations. With the parameters of stimulation used, vasoactive intestinal polypeptide (VIP) does not contribute significantly to the stimulation-induced relaxations (Li & Rand, 1990).

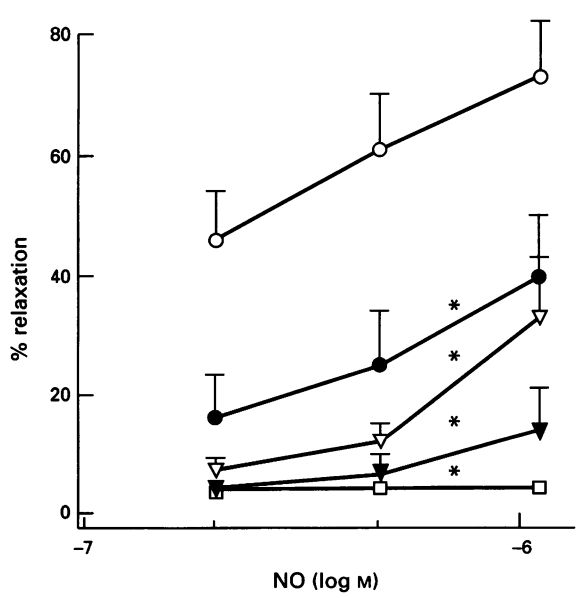

Figure 3 Effect of carboxy-PTIO (10–100 μM) on relaxations elicited by nitric oxide (0.2–1.2 μM) in rat anococcygeus muscles. Concentrations of carboxy-PTIO are: (○) 0 (control); (●) 10 μM; (▽) 30 μM; (▼) 100 μM; (□) 300 μM. Symbols are means with s.e.mean of 5 experiments. *Indicates that concentration-response curve differs significantly ($P<0.05$, two-way ANOVA) from the control curve.

Discussion

There have been a number of suggestions that EDRF is an adduct of NO rather than the free radical (Myers *et al.*, 1990; Bates *et al.*, 1991; Vedernikov *et al.*, 1992; Mülsch, 1994). However, cogent evidence has been adduced for the identity of EDRF and NO (Feelisch *et al.*, 1994). This evidence is strengthened by our finding that selective inactivation of free radical NO by carboxy-PTIO inhibited EDRF-mediated relaxations induced by acetylcholine and ATP in rat aortic rings. This confirms and extends a previous finding that carboxy-PTIO and some related PT-imidazolineoxyl N-oxides blocked acetylcholine-induced relaxations in rabbit aortic rings (Akaike *et al.*, 1993), and PTIO prevented the hypotension of endotoxin shock, indicating that the vasodilatation produced by the product of inducible NOS is also mediated by an EDRF-like factor (Yoshida *et al.*, 1994).

The concentration of carboxy-PTIO required to reduce EDRF-mediated relaxations in rat aortic rings was about 10 fold higher than that required to reduce responses to exogenous NO to a similar extent. This quantitative difference presumably reflects the supposition by Wood & Garthwaite (1994) that the longer diffusion pathway of exogenous NO to the smooth muscle enables it to be inactivated more easily, and there is no reason to suppose that EDRF differs from free radical NO, at least in the rat aorta.

Many substances that impair endothelium-dependent relaxations by blocking NOS or by inactivating NO, increase phenylephrine-induced tone. This effect was not regularly produced by carboxy-PTIO in rat aortic rings, perhaps because of a weak counteracting relaxant activity, as was observed in anococcygeus muscles.

In striking contrast to the effectiveness of carboxy-PTIO in blocking EDRF-mediated relaxations, it failed completely to reduce nitrergic nerve stimulation-induced relaxations of the anococcygeus muscle and gastric fundus, even though the potency of carboxy-PTIO in blocking responses to NO did not differ between anococcygeus muscles and aortic rings. A possible explanation of the difference is that carboxy-PTIO failed to gain access to the nitrergic neuroeffector junction, but did not reach the space between endothelial cells and medial smooth muscle. This is unlikely for at least two reasons. Firstly, as pointed out by Gillespie & Sheng (1989), the gap between the NANC nerve varicosities and smooth muscle cells in the rat anococcygeus muscle is about 260 nm whereas that between the abluminal surface of endothelium cells and the adjacent smooth muscle cells is 50–100 nm. Since carboxy-PTIO readily entered the latter, smaller space it would be expected to gain access to the wider neuroeffector junction. Secondly, it is well known that haemoglobin, which has a molecular weight more than 2,000 times that of carboxy-PTIO, does block nitrergic nerve stimulation-induced relaxations in the anococcygeus muscle (Gillespie & Sheng, 1989) and hence does penetrate to the neuroeffector junction. In this connection it is worth noting that haemoglobin is equally effective in blocking responses to aqueous NO in the rat aorta and anococcygeus muscle, but it is about 40 times more effective in blocking responses to EDRF in the rat aorta than to the nitrergic transmitter in the anococcygeus muscle (Rand, Li & La, unpublished observations). The possible reason for the effectiveness of haemoglobin but the lack of effectiveness of carboxy-PTIO in inactivating the nitrergic transmitter is that the latter is selective for radical NO whereas haemoglobin sequesters not only radical NO but also NO from a number of NO-donating compounds, and the nitrergic transmitter may be a NO-donating substance.

The concentration of NO released from nitrergic nerve terminals is not known; however, the maximal concentration of NO released by bradykinin measured at the surface of the endothelial cells is about 1 μM (Malinsky *et al.*, 1993). If the concentration of NO released from nitrergic nerve terminals is about the same as from endothelial cells, it would be expected that carboxy-PTIO would have blocked its effect. Relaxations

of the anococcygeus muscle and gastric fundus elicited by nitrergic nerve stimulation as in the present experiments are reasonable well matched by about 1 μM exogenous NO (see Figure 1c and also, for example: Rajanayagam *et al.*, 1993; Li & Rand, 1993a). Nevertheless, it is possible that the concentration of NO in the neuroeffector junction may be considerably greater than 1 μM; however responses to NO were virtually abolished by 100 μM carboxy-PTIO whereas a 20 fold greater concentration (2 mM) was completely devoid of an inhibitory effect on responses to the nitrergic transmitter. It should be noted that the stimulation frequencies used to elicit transmitter release, although sufficient to elicit distinct relaxation responses, were far short of those producing maximal relaxations, and the duration of the period of stimulation was only 10 s. In our experience, reductions of either the frequency or duration of stimulation, and hence of the quantity of transmitter released, clearly attenuates the relaxations; therefore, we conclude that the concentration of transmitter in the neuroeffector junction was not diminished by carboxy-PTIO.

Wood & Garthwaite (1994) recently suggested that the failure of various NO-inactivating agents to block responses to nitrergic nerve stimulation is due to the extremely rapid diffusion of NO released from nerve terminals across the neuroeffector junction, such that unless the inactivating agents reduce the half-life of NO to the sub-millisecond range, the responses to nitrergic nerve stimulation would not be affected. The rate constant for the reaction with NO at pH 7.4 is 1.01×10^4 $M^{-1}s^{-1}$ (Akaike *et al.*, 1993). If carboxy-PTIO is in a much higher concentration than NO, the rate constant can be considered as first-order, and the half-life of NO can be calculated as the natural logarithm of 2 divided by the rate constant: thus, the half-life of NO in the presence of large excess of carboxy-PTIO would be less than 70 μs. Therefore, since 70 μs is clearly in the sub-millisecond range, the objection to drawing a conclusion about the lack of its effect on responses to the nitrergic transmitter raised by Wood & Garthwaite (1994) does not appear to apply. Consequently, it is reasonable to conclude that the nitrergic transmitter, at least in the rat anococcygeus muscle and gastric fundus, does not behave like free radical NO.

An unexpected finding with carboxy-PTIO was that it enhanced nitrergic nerve stimulation-induced relaxations in the anococcygeus muscle. It is not clear whether this enhancement was related to the slight reduction of resting tension caused by high concentrations of carboxy-PTIO. On the other hand, this effect may be due to blockade of an autocrine action of NO generated in the immediate vicinity of the enzyme in mediated feedback inhibition of NOS (Mayer *et al.*, 1992; Rogers & Ignarro, 1992; Assreuy *et al.*, 1993; Rengasamy & Johns, 1993; Dinerman *et al.*, 1994). As mentioned in the Introduction, there is no dispute about the essential role of NOS in nitrergic transmission; however, this is not evidence that the NO produced by it is in fact the ultimate mediator of transmission. An enhancement of the microbical action of NO was produced by PTIO (Yoshida *et al.*, 1993), but it is not clear that this is related to the enhancement of the nitrergic relaxations by carboxy-PTIO.

Most of the agents that discriminate between responses to NO and those to the nitrergic transmitter (see Introduction) are superoxide generators. It has been suggested recently by Martin and his colleagues (1994) that the nitrergic transmitter is selectively protected against inactivation by superoxide dismutase (SOD). They found that after inhibition of the Cu/Zn-containing SOD with diethyldithiocarbamate (dithiocarb), transmitter-induced relaxations of the bovine retractor penis muscle were almost completely blocked by pyrogallol or hypoxanthine/xanthine oxidase and were then restored by SOD. Furthermore, the weak blocking activity of LY 83583 was increased about ten fold by pretreatment with dithiocarb (Martin *et al.*, 1994). It follows from the postulate of Wood & Garthwaite (1994) that if the rate of inactivation of the nitrergic transmitter by superoxide is too slow to be of any consequence for the transmission process because of the presence of endogenous SOD, a differential effect of superoxide generators would not be evidence that the transmitter was not NO. It will be of interest to determine whether inhibition of SOD removes the differential effect of superoxide generators between NO and the nitrergic transmitter in tissues other than the retractor penis. It is worth noting that the lack of effect of carboxy-PTIO on nitrergic nerve stimulation-induced relaxations was not affected by dithiocarb pretreatment (Li & Rand, unpublished observations), confirming that it has a different mode of action from that of superoxide generators.

In conclusion, our findings support the view that EDRF is in fact NO radical, but suggest that the nitrergic transmitter differs from EDRF, is not free radical NO, and may be a NO-adduct.

This work was supported by a Programme Grant awarded by the National Health & Medical Research Council and a grant from the Smoking & Health Research Foundation of Australia.

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(Received February 16, 1995
Revised May 12, 1995
Accepted May 26, 1995)