Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose

Ignacio LIZASOAIN*, Maria A. MORO*, Richard G. KNOWLES‡, Victor DARLEY-USMAR*† and Salvador MONCADA

The Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, U.K.

Nitric oxide (NO) and peroxynitrite both inhibit respiration by brain submitochondrial particles, the former reversibly at cytochrome c oxidase, the latter irreversibly at complexes I–III. Both GSH ($IC_{50} = 10\ \mu M$) and glucose ($IC_{50} = 8$ mM) prevented inhibition of respiration by peroxynitrite ($ONOO^-$), but neither glucose (100 mM) nor GSH (100 μM) affected that by NO. Thus, unless $ONOO^-$ is formed within mitochondria it is unlikely to inhibit respiration in cells directly, because of reactions with cellular thiols and carbohydrates. However, the reversible inhibition of respiration at cytochrome c oxidase by NO is likely to occur (e.g. in the brain during ischaemia) and could be responsible for cytotoxicity.

INTRODUCTION

Nitric oxide (NO) is generated in many mammalian tissues and is an important mediator of both physiological and pathological responses [1]. In the brain, activation of glutamate receptors causes an influx of calcium ions, leading to stimulation of the calcium-dependent NO synthase (EC 1.14.13.39), resulting in increases in cGMP (reviewed in [2]). NO synthesis in the brain has been implicated in many different physiological responses (synaptic plasticity, regulation of the cerebral circulation and neuronal and neuroendocrine function) and also in pathological conditions such as cerebral ischaemia. The brain contains high NO synthase activity compared with other tissues [3] and during cerebral ischaemia this can result in exposure of the brain to high concentrations of NO ($> 1\ \mu M$ [4]). Inhibition of NO synthase or genetic manipulation to 'knock out' expression of nNOS (the predominant NO synthase isoform in the brain) have both been shown to result in significant protection of brain tissue from ischaemic injury [5,6].

The mechanisms of cell damage by NO include inhibition of a number of cellular processes, such as DNA synthesis and mitochondrial respiration [7–9]. Some of these effects may be direct and others may arise from the reaction of NO with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) [10]. In the brain, ischaemia results in release of glutamate from compromised cells, which then activates neuronal NO synthase in susceptible oxygenated cells adjacent to the infarct [2]. Through this mechanism, damage to neuronal cells may extend from the site of the original insult over a period of several hours. The activation of the N-methyl-D-aspartate receptor by high glutamate concentrations in these oxygenated cells is also associated with formation of oxygen free-radicals such as O_2^- [11–13]. It is therefore possible that $ONOO^-$ may also mediate damage to mitochondria and other targets in the cells. It has been shown that both NO and $ONOO^-$ can disrupt mitochondrial function [8,11,14,15] and therefore either NO or $ONOO^-$ could potentially be responsible for mitochondrial damage occurring in the brain as a consequence of cerebral ischaemia. However, the sites at which NO and $ONOO^-$ interact with the respiratory chain and the mechanism of inhibition appear to be different. It has been known for some time that NO binds to cytochrome c oxidase, the terminal member of the mitochondrial respiratory chain [16,17], but only recently was it shown that it may act as an inhibitor of this enzyme at physiological concentrations of NO [8]. This reaction is reversible and competitive with oxygen [18]. In contrast, $ONOO^-$ has little or no effect on cytochrome c oxidase but inhibits respiratory complexes I–III [14].

Despite the potential significance of NO-dependent disruption of the mitochondrial electron transport chain to both neurodegenerative diseases and stroke, a comparison of the NO- and $ONOO^-$-dependent inhibition of the respiratory chain has not been reported. Furthermore, it is known that $ONOO^-$ may react rapidly with a broad range of molecules such as thiols, low molecular-mass antioxidants and sugars [10,19–21], any or all of which could conceivably scavenge this oxidant and prevent reaction with proteins such as the respiratory complexes. However, since the relative rates of reaction of $ONOO^-$ with these potential scavengers and mitochondrial electron-transfer proteins are unknown, the potential of $ONOO^-$ as an inhibitor of mitochondrial respiration remains uncertain. Because of these points we have carried out a direct comparison of the effects of NO and $ONOO^-$ on respiration in which submitochondrial particles (SMP) were exposed to NO donors and to $ONOO^-$ and the effect of these treatments on the mitochondrial respiratory chain analysed. In characterizing the actions of the two agents we found that those of $ONOO^-$, but not those of NO, were potently inhibited by the presence of GSH and glucose.

MATERIALS AND METHODS

Chemicals were obtained from Merck (Poole, Dorset, U.K.) and Sigma Chemicals. S-Nitroso-glutathione (SNOG) was synthesized at Wellcome Research Laboratories as described [22]. 3-Morpholinosydnonimine N-ethylcarbamide (SIN-1) was a gift from Schwarz Pharma (Monheim, Germany). The stock solution

of GSH was made in 10 μM diethylenetriaminepentaacetic acid (DTPA) in water. The final concentration of DTPA in the reaction mixtures was less than 1 μM, and we have previously shown that this has little effect on the pro-oxidant reactions of ONOO$^-$ but effectively inhibits reactions of contaminating metals, such as iron, with peroxides [20]. Human oxyhaemoglobin was prepared by the method of Paterson et al. [23].

Rat brain mitochondria were prepared by a modification of the method of Partridge et al. [24]. Adult female Wistar rats were sacrificed by decapitation and all brain tissue rostral to the cerebellum was rapidly removed and placed in ice-cold isolation buffer (0.15 M KCl/20 mM potassium phosphate, pH 7.6, at 4 °C). The brain mitochondria were prepared by homogenization and differential centrifugation followed by a Ficoll gradient [10 % (w/v) Ficoll solution in isolation buffer]. A suspension of freshly prepared brain mitochondria was exposed to three cycles of freeze–thaw followed by centrifugation (20 000 *g* for 10 min at 4 °C) to obtain a high yield of SMP. SMP from multiple rats were pooled and stored at -70 °C. The protein content was determined using bicinchoninic acid [25].

Incubation of SMP (0.5 mg of protein/ml) was carried out at 37 °C with continuous stirring in 2 ml of buffer (50 mM potassium phosphate/100 μM EGTA, pH 7.2). NADH (50 μM), succinate (5 mM) or ascorbate (Asc; 5 mM) plus N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD; 0.5 mM) were used to quantify complex I-, II-, III- or IV-dependent respiration. In experiments to test the reversibility of effects on respiration, 1 mM NADH was added to sustain oxygen consumption for more than 10 min; oxyhaemoglobin (30 μM) was added to scavenge NO.

Rat brain SMP were exposed to SIN-1 (500 μM) $\pm$ superoxide dismutase (SOD; 25–800 units/ml), SNOG (500 μM) $\pm$ dithiothreitol (DTT; 50 μM) or ONOO$^-$ (100–400 μM) in 2 ml of buffer. In another set of experiments, rat brain SMP were exposed to ONOO$^-$ (100–400 μM) or SIN-1 (500 μM) with SOD (400 units/ml) in the presence of either GSH (1–1000 μM) or glucose (0.1–100 mM). SMP respiration was measured polarographically using a Clark-type oxygen micro-electrode (Model 5357, YSI Inc., Yellow Springs, OH, U.S.A.).

NO gas in solution was measured polarographically using an NO electrode (Diamond General Development Corporation, MI, U.S.A.). The electrode was calibrated with anaerobic solutions of pure NO gas in deoxygenated water [26]. DTT (50 μM) was used to promote the release of NO from SNOG [8,27] and SOD (400 units/ml) was used to remove O_2^- which is formed from SIN-1 concomitantly with the release of NO [20,28]. ONOO$^-$ was synthesized chemically by the reaction of acidified NaNO$_2$ (1.8 M) with H$_2$O$_2$ (2.1 M) in an NaOH-quenched flow reactor, and its concentration was determined spectrophotometrically ($\epsilon_{302} = 1670$ M$^{-1} \cdot$cm^{-1}) [20,29–32]. To prepare decomposed ONOO$^-$, the addition of NaOH to the NaNO$_2$/H$_2$O$_2$ mixture was delayed for 3 min, after which time no ONOO$^-$ was present.

Results are means $\pm$ S.E.M. of at least three separate experiments unless otherwise indicated. Student's unpaired *t*-test was used to determine the significance of differences between means and $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of NO donors

The addition of SIN-1 (500 μM) alone to SMP had no significant effect on rates of respiration (91 $\pm$ 7, 97 $\pm$ 4 and 104 $\pm$ 5 % of control rate with NADH, succinate and TMPD/Asc as substrates respectively) and produced no detectable release of NO (Figure
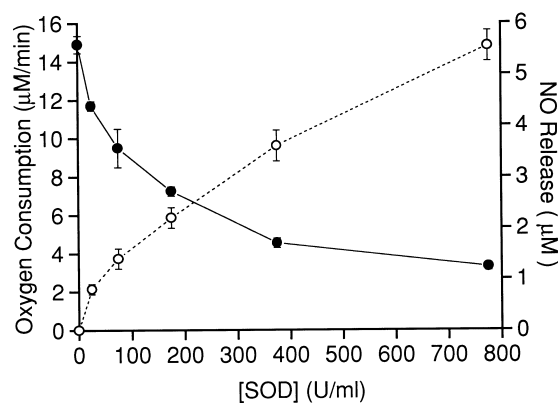
Figure 1   Production of NO and inhibition of NADH respiration by SIN-1 + SOD

SIN-1 was present at 500 μM and SOD at 0–750 units/ml as shown. ○, Nitric oxide concentrations; ●, respiration rates. The data are the means $\pm$ S.E.M. from 3–4 determinations.

Table 1   Effects of NO donors and ONOO$^-$ on SMP respiration from NADH, succinate and TMPD/Asc

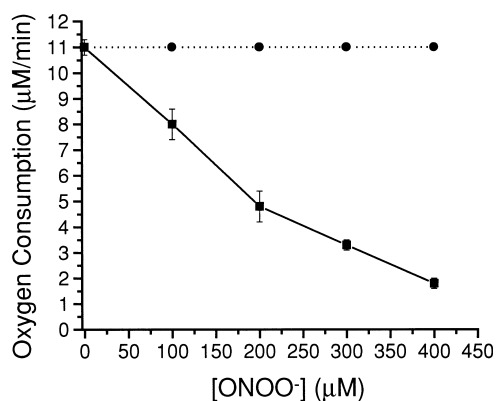The concentrations used were: SIN-1, 500 μM; SOD, 400 units/ml; SNOG, 500 μM; DTT, 50 μM; ONOO$^-$, 200 μM. The data are means $\pm$ S.E.M. from 3–6 determinations, except for that with SIN-1 alone which is mean $\pm$ range from two experiments. *Significantly different from control, $P < 0.05$; ns, no significant difference from control.

	Respiration rate (μM of O$_2$/min) from substrate		
	NADH	Succinate	TMPD/Asc
Control	11.9 $\pm$ 0.5	9.2 $\pm$ 0.9	19.5 $\pm$ 1.5
SIN-1	10.8 $\pm$ 0.8^{ns}	8.9 $\pm$ 0.4^{ns}	20.2 $\pm$ 1.0^{ns}
SIN-1 + SOD	6.0 $\pm$ 1.1*	3.5 $\pm$ 0.5*	8.5 $\pm$ 0.3*
SNOG + DTT	5.3 $\pm$ 0.8*	3.7 $\pm$ 0.2*	10.3 $\pm$ 1.4*
ONOO$^-$	5.8 $\pm$ 0.5*	3.2 $\pm$ 0.1*	16.0 $\pm$ 1.1^{ns}

1, Table 1). However, addition of SOD (25–800 units/ml) to SIN-1 caused measurable release of NO and an inhibition of NADH-dependent respiration (Figure 1) and of succinate and TMPD/Asc respiration (Table 1). The extent of inhibition of NADH respiration was dependent on the concentration of NO, with an IC$_{50}$ of 2.0 $\pm$ 0.1 μM. SIN-1 (500 μM) + SOD (400 units/ml) also inhibited succinate-dependent respiration and TMPD/Asc respiration (Table 1).

The exposure of SMP to SNOG alone did not significantly modify rates of respiration (92 $\pm$ 6, 101 $\pm$ 4 and 98 $\pm$ 3 % of control rate with NADH, succinate and TMPD/Asc as substrates respectively). Under these conditions, no release of NO was detected. However, SNOG (500 μM) + DTT (50 μM) produced similar inhibitions of NADH, succinate and TMPD/Asc respiration to those caused by SIN-1 + SOD (Table 1). DTT (50 μM) alone did not have any effect on respiration (results not shown). The steady-state concentration of NO achieved with SNOG + DTT (4.6 $\pm$ 0.5 μM) under these conditions was similar to that of SIN-1 + SOD at the concentrations tested (3.6 $\pm$ 0.3 μM).

To test the reversibility of the effects of NO on respiration, sufficient substrates were added to sustain oxygen consumption for at least 10 min. SIN-1 (500 μM) + SOD (400 units/ml) were added and respiration rates determined: inhibition of respiration (to 41 $\pm$ 6, 45 $\pm$ 8 and 53 $\pm$ 5 % of control for NADH, succinate

Figure 2   Concentration-dependent inhibition of NADH respiration by ONOO⁻

■, ONOO⁻ (0–400 μM); ●, decomposed ONOO⁻ (0–400 μM). The data are means $\pm$ S.E.M. from 3–4 determinations, except for that with decomposed ONOO⁻ which is mean $\pm$ range from two experiments.

Table 2   Effects of GSH and glucose on the inhibition of NADH respiration by NO from SIN-1 + SOD and by ONOO⁻

The concentrations used were: SIN-1, 500 μM; SOD, 400 units/ml; ONOO⁻, 200 μM; GSH, 100 μM; glucose, 100 mM. The data are means $\pm$ S.E.M. from 3–4 determinations. *Significantly different from control, $P < 0.01$; ns, no significant difference from control.

	Respiration rate (% of control)
Control	100 ± 8
SIN-1 + SOD	$63 \pm 4^*$
SIN-1 + SOD + glucose	$61 \pm 4^*$
SIN-1 + SOD + GSH	$62 \pm 4^*$
Decomposed ONOO⁻	100 ± 8
ONOO⁻	$49 \pm 4^*$
ONOO⁻ + glucose	100 ± 4^{ns}
ONOO⁻ + GSH	85 ± 7^{ns}

and TMPD/Asc as substrates respectively) was maintained over the following 3 min under these conditions. Addition of oxy-haemoglobin (30 μM) reversed the inhibition caused by SIN-1 + SOD on NADH (to 109 ± 5 % of control, $n = 4$), succinate (to 107 ± 8 % of control, $n = 3$) and TMPD/Asc respiration (to 100 ± 1 % of control, $n = 3$).

Effects of ONOO⁻

The addition of ONOO⁻ (100–400 μM) to SMP produced a concentration-dependent inhibition of NADH respiration with an IC$_{50}$ of 200 ± 17 μM (Figure 2). ONOO⁻ (200 μM) also inhibited succinate-dependent respiration but not TMPD/Asc respiration (Table 1). Control experiments showed that decomposed ONOO⁻ had no effect on NADH respiration (Table 2) or on succinate respiration (control, 9.2 ± 0.9 μM/min; 200 μM decomposed ONOO⁻, 8.5 ± 0.3 μM/min). The inhibition of respiration by ONOO⁻ persisted for the duration of the experiment (up to 10 min). Addition of oxyhaemoglobin (30 μM) after ONOO⁻ did not reverse the inhibition of respiration from either NADH or succinate ($n = 3$–4).

Effects of GSH and glucose on the inhibition of respiration by ONOO⁻ and NO

Exposure of SMP to ONOO⁻ in the presence of GSH or glucose protected against the inhibitory effects of ONOO⁻ on NADH respiration (Table 2). Protection against the inhibition by 400 μM ONOO⁻ was concentration dependent with an EC$_{50}$ of 10.3 ± 1.9 μM for GSH and 7.9 ± 0.9 mM for glucose. Release of NO was not detectable under these conditions. The effects of exposure of SMP to SIN-1 (500 μM) + SOD (400 units/ml) were unaffected by the presence of GSH (100 μM) or glucose (100 mM) (Table 2). A higher concentration of GSH (1 mM) had little effect on the inhibition of respiration by SIN-1 + SOD (78 ± 4 % of the inhibition in its absence), whereas it abolished the effect of 400 μM ONOO⁻.

DISCUSSION

We have shown that the addition of SIN-1 + SOD or SNOG + DTT to rat brain submitochondrial particles results in the inhibition of oxygen consumption when using NADH, succinate or TMPD/Asc as substrate, consistent with inhibition of complex IV (Scheme 1). Several observations implicate NO in these effects: (1) SIN-1 alone and SNOG alone do not inhibit respiration and do not produce significant concentrations of NO, although they do both when in the presence of SOD and DTT respectively; (2) SIN-1 + SOD and SNOG + DTT inhibit respiration at similar released concentrations of NO; and (3) haemoglobin reverses the inhibition of respiration by NO-donating mixtures. These effects of NO on respiration are consistent with those previously reported for NO from donors, aqueous solutions of NO gas or NO synthase [7,8,11,15]. The inhibition of NADH respiration by NO occurred with an IC$_{50}$ of 2 μM. NO concentrations higher than this have been reported in the brain during cerebral ischaemia [4], suggesting that inhibition of respiration by NO is likely to occur during such episodes and may play a role in the neurodegeneration which results, either because of progression to irreversible inhibition of respiration (see below) or because of the irreversible consequences of prolonged ATP depletion. Moreover, the inhibition of cytochrome c oxidase is competitive with oxygen and occurs through the concerted binding of NO to the oxygen-binding site in a redox state which is only populated during turnover [18]. One consequence of this mechanism of inhibition is that the IC$_{50}$ of NO is approx. 0.1 μM under anaerobic conditions and increases to approx. 0.6 μM at a concentration of 50 μM O_2, which, *in vivo*, may result in great inhibitory effects in regions of tissue with compromised blood flow [18].
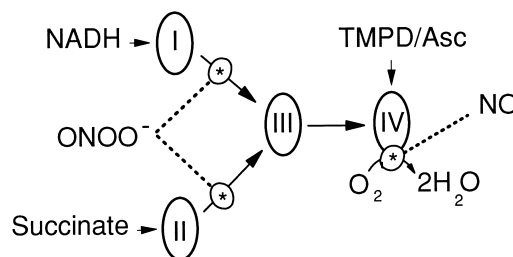
Scheme 1   Schematic representation of mitochondrial respiration through complexes I–IV with the substrates NADH, succinate and TMPD/Asc

The sites of inhibition by NO and ONOO⁻ are indicated by the dotted lines.

On the other hand, we have found that ONOO⁻ inhibits respiration at complexes I–III and II–III without affecting TMPD/Asc respiration (complex IV, Scheme 1), confirming previous reports [7,14]. Although SIN-1 in the absence of SOD generates ONOO⁻ [20], the amounts formed from 500 μM SIN-1 are not sufficient to inhibit respiration. In contrast to the effect of NO, the effects of ONOO⁻ are persistent and are not reversed by haemoglobin.

Interestingly, the effects of ONOO⁻ on respiration could be prevented by either GSH or glucose; this is consistent with our previous observations that ONOO⁻ reacts with these compounds to form NO donors [31,32]. NO release from the NO donors formed did not occur under the conditions used in these experiments (in the presence of EGTA), but may well occur in intact cells. The potency of GSH (EC_{50} 10 μM) and of glucose (EC_{50} 8 mM), compared with their normal cytosol concentrations (1–5 mM and 5–10 mM respectively), suggests that these cellular constituents will strongly inhibit the effects of ONOO⁻ on mitochondrial respiration in intact cells and *in vivo*. Moreover, it is likely that other cellular thiols and carbohydrates will also contribute to preventing the direct effects of ONOO⁻ [10,19-21,31–33]. In contrast, the effect of NO on respiration was not inhibited by either glucose (100 mM) or GSH (100 μM). Higher concentrations of GSH (> 1 mM) may modulate the effect of NO on respiration to some extent, and this suggests that GSH depletion might make cells more susceptible to the cytotoxicity of NO.

It has been proposed [7] that ONOO⁻ causes toxicity to neuronal cells via mitochondrial dysfunction, since its addition to neurons in primary culture resulted in the inhibition of succinate-cytochrome *c* reductase and release of lactate dehydrogenase into the culture medium. However, our observation that direct effects of ONOO⁻ are suppressed by cellular constituents such as GSH suggests that this effect of ONOO⁻ on cellular respiration must be indirect, either by generation of NO or by other, non-specific, effects.

In summary, our data indicate that the direct effect of NO itself on respiration is at complex IV, with complexes I–III functioning normally. Thus, the initial effect of NO on the respiratory chain would be the reversible inhibition of mitochondrial respiration at cytochrome *c* oxidase. However, O_2^- is a by-product of mitochondrial respiratory electron transport and its production increases in the presence of mitochondrial inhibitors such rotenone, antimycin A, cyanide [33] and presumably, by analogy, NO itself. The site of this additional O_2^- formation is believed to be within complexes I and III. Thus a sustained production of NO, together with this resulting formation of O_2^- (and perhaps also the depletion of antioxidant defenses) may, because of formation of ONOO⁻ within the mitochondria, cause the irreversible inhibition of complexes I–III leading to cytotoxicity. This mechanism could contribute significantly to tissue damage during ischaemia in the brain. However, if ONOO⁻ is not formed in the immediate vicinity of a redox site within the respiratory chain, it is unlikely to inhibit respiration directly. This mechanism could also reconcile the apparent contradiction between the present and previous data [8,11] showing that NO does not directly affect complexes I and II, and the observations showing inhibition of these complexes in cells exposed to NO, e.g. from cytotoxic activated macrophages [9].

REFERENCES

1 Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) Pharmacol. Rev. **43**, 109–142
2 Garthwaite, J. and Boulton, C. L. (1995) Annu. Rev. Physiol. **57**, 683–706
3 Salter, M., Knowles, R. G. and Moncada, S. (1991) FEBS Lett. **291**, 145–149
4 Malinski, T., Bailey, F., Zhang, Z. G. and Chopp, M. (1993) J. Cereb. Blood Flow Metab. **13**, 355–358
5 Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C. and, Moskowitz M. A. (1994) Science **265**, 1883–1885
6 Nowicki, J. P., Duval, D., Poignet, H. and Scatton, B. (1991) Eur. J. Pharmacol. **204**, 339–340
7 Bolaños, J. P., Heales, S. J. R., Land, J. M. and Clark, J. B. (1995) J. Neurochem. **64**, 1965–1972
8 Cleeter, M. W. J., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. and Schapira, A. H. V. (1994) FEBS Lett. **345**, 50–54
9 Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J. C., Amber, I. J. and Lancaster, J. R., Jr. (1990) in Nitric Oxide from L-Arginine: A Bioregulatory System (Moncada, S. and Higgs, E. A., eds.), pp. 189–223, Elsevier, Amsterdam
10 Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. and Freeman, B. A. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1620–1624
11 Brown, G. C. and Cooper, C. E. (1994) FEBS Lett. **356**, 295–298
12 Lafon-Cazal, M., Pietri, S., Culcasi, M. and Bockaert, J. (1993) Nature (London) **364**, 535–537
13 McCord, J. M. (1985) N. Engl. J. Med. **312**, 159–163
14 Radi, R., Rodriguez, M., Castro, L. and Telleri, R. (1994) Arch. Biochem. Biophys. **308**, 89–95
15 Schweizer, M. and Richter, C. (1994) Biochem. Biophys. Res. Commun. **204**, 169–175
16 Tiesjema, R. H. and Van Gelder, B. F. (1974) Biochim. Biophys. Acta. **347**, 202–214
17 Brudvig, G. V., Stevens, T. H. and Chan, S. I. (1980) Biochemistry **18**, 5275–5285
18 Torres, J., Darley-Usmar, V. and Wilson, M. T. (1995) Biochem. J. **312**, 169–173
19 Radi, R., Beckman, J. S. and Freeman, B. F. (1991) J. Biol. Chem. **266**, 4244–4250
20 Hogg, N., Darley-Usmar, V. M., Wilson, M. T. and Moncada, S. (1992) Biochem. J. **281**, 419–424
21 Van der Vliet, A., Smith, D., O'Neil, C. A., Kaur, H., Darley-Usmar, V., Cross, C. E. and Halliwell, B. (1994) Biochem. J. **303**, 295–301
22 Hart, T. W. (1985) Tetrahedron Lett. **26**, 2013–2016
23 Paterson, R. A., Eagles, P. A. M., Young, D. A. B. and Beddell, C. R. (1976) Int. J. Biochem. **7**, 117–118
24 Partridge, R. S., Monroe, S. M., Parks, J. K., Johnson, K., Parker, W. D., Eaton, G. R. and Eaton, S. S. (1994) Arch. Biochem. Biophys. **310**, 210–217
25 Hill, H. D. and Straka, J. G. (1988) Anal. Biochem. **170**, 203–208
26 Palmer, R. M. J., Ferrige, A. G. and Moncada, S. (1987) Nature (London) **327**, 524–526
27 Park, J. W. (1988) Biochem. Biophys. Res. Commun. **152**, 916–920
28 Feelisch, M., Ostrowski, J. and Noack, E. (1989) J. Cardiovasc. Pharmacol. **14**, 513–522
29 Blough, N. V. and Zafiriou, O. C. (1985) Inorg. Chem. **24**, 3502–3504
30 Hughes, M. N. and Nicklin, H. G. (1968) J. Am. Chem. Soc. 450–452
31 Moro, M. A., Darley-Usmar, V. M., Goodwin, D. A., Read, N. G., Zamora-Pino, R., Feelisch, M., Radomski, M. W. and Moncada S. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 6702–6706.
32 Moro, M. A., Darley-Usmar, V. M., Lizasoain, I., Su, Y., Knowles, R. G., Radomski, M. W. and Moncada, S. (1995) Br. J. Pharmacol. **116**, 1999–2004
33 Turrens, J. F. and Boveris, A. (1980) Biochem. J. **191**, 421–427