FEBS Letters 345 (1994) 50–54

FEBS 14038

Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide

Implications for neurodegenerative diseases

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Received 8 April 1994

Abstract

Incubation of rat skeletal muscle mitochondria with the nitric oxide generator, *S*-nitrosoglutathione (GSNO) reversibly inhibited oxygen utilisation with all substrates tested. The visible absorption spectra of the inhibited mitochondria showed that cytochromes $c + c_1$, b and $a + a_3$ were reduced, indicating a block at the distal end of the respiratory chain. Analysis of the respiratory chain enzyme activities in the presence of GSNO localised the site of inhibition to cytochrome *c* oxidase alone. These results indicate that nitric oxide is capable of rapidly and reversibly inhibiting the mitochondrial respiratory chain and may be implicated in the cytotoxic effects of nitric oxide in the CNS and other tissues.

Key words: Mitochondrion; Parkinson's disease; Cytochrome *c* oxidase

1. Introduction

The nitric oxide (NO•) radical has been shown to be produced by cytotoxic activated macrophages (CAM) during metabolism of L-arginine to NO^-_2/NO_3 [1]. The NO• produced is capable of inhibiting a number of microbial and tumour cell targets [2,3]. CAM have been shown to induce intracellular iron loss [4–6], and inhibition of DNA synthesis [7,8], aconitase [5] and complexes I and II of the mitochondrial electron transport chain [5,6–10]. All of these enzymes have catalytically active non-haem iron, coordinated to sulphur which could be the site of interaction of NO• with these enzymes. Furthermore, the inhibitory effect of NO• is believed to be caused by its degradation of iron-sulphur centres, resulting in the release of Fe ions and Fe-nitrosyl complexes [11,12]. Such cytotoxic effects of NO• may not be restricted to activated macrophages, for example, Ca^{2+}-dependent NO• release has been described in cerebellar neurones in response to glutamate [13].

The dual observations of gliosis [14] and a deficiency of mitochondrial complex I activity in the substantia nigra in Parkinson's disease (PD) [15,16], raises the possibility that NO• released from activated microglia may be involved in neuronal loss in PD via inhibition of complex I activity. To gain a better understanding of the effects of NO• on the respiratory chain and to help determine its possible role in the pathogenesis of PD, we exposed isolated intact or permeabilised mitochondria to an NO• donor and analysed the effect of this treatment on oxygen utilisation, the visible absorption spectrum of the cytochromes and on the individual respiratory chain enzyme activities.

2. Materials and methods

2.1. Chemicals

Chemicals were obtained from Merck (Poole, UK) and Sigma Chemicals. GSNO was synthesised at Wellcome Research Laboratories as described [17].

2.2. Respiration measurements

Rat gastrocnemius muscle mitochondria were prepared as described [18]. Mitochondrial oxygen utilisation was measured polarographically at 25°C essentially as described [19] but in a final volume of 200 μl using a Clark type micro oxygen electrode (Model 5357, YSI inc. Yellow Springs, OH). Intact mitochondria (respiratory control ratio >4 with glutamate as substrate) were used for all polarographic experiments. Mitochondria that had been rendered permeable by freeze-thawing (three cycles) were used for spectrophotometric measurement of enzyme activities (see below).

2.3. Effect of GSNO

Variable amounts of GSNO (0–500 μM) were added to incubation mixtures containing: respiration medium (see [19] for details), BSA (0.5 mg/ml), DTT (50 μM) and mitochondria (0.7–1.0 mg·protein·ml^{-1}) 1 min prior to the addition of the substrate and sufficient ADP (600 nmol) to enable state III respiration to continue until anoxia, which typically occurred 3–4 min after addition of ADP. Where indicated, the

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Abbreviations: DTT, dithiothreitol; GSNO, *S*-nitroglutathione; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene-diamene.

SSDI 0014-5793(94)00424-T

incubation time before addition of substrate and ADP was varied in order to examine the effect of pre-incubation of GSNO and DTT with mitochondria. The reversibility of the NO•-dependent inhibition of mitochondria was assessed after polarographic analysis of the mitochondria with glutamate, malate and ADP. The incubation mixture was removed, centrifuged at approximately 7000 × *g* at 20°C on a bench top centrifuge for 2 min and the supernatant aspirated. Samples were carefully resuspended in respiration medium and oxygen consumption again monitored in the presence of glutamate, malate and ADP.

2.4. Preparation of oxyhaemoglobin (HbO_2) and estimation of the rate of NO• production

Oxyhaemoglobin was prepared as described previously [20]. The rate of NO• production was determined by the spectrophotometric measurement of the production of methaemoglobin from HbO_2 at 25°C [21].

2.5. Visible absorption spectra of mitochondrial cytochromes

The spectrum of fresh or freeze-thawed mitochondria (1 mg/ml) was determined (500 nm to 630 nm) in the following buffer: 225 mM mannitol, 75 mM sucrose, 5 mM phosphate-Tris, 10 mM MOPS and 100 μM potassium EDTA, pH 7.4, using a Hitachi U-3210 spectrophotometer. A sample of mitochondria from the same preparation was used as an optical reference. The following additions were made sequentially to the sample cuvette: DTT (50 μM), GSNO (190 μM) and succinate (25 μM) or NADH (150 μM in freeze-thawed mitochondria) and the spectrum taken.

2.6. Spectrophotometric measurement of enzyme activities

NADH CoQ_1 reductase (complex I), succinate-cytochrome *c* reductase (complex II–III) and cytochrome *c* oxidase (complex IV) were measured by published methods [22–24]. Protein was estimated using fatty acid free BSA as a standard [25].

3. Results

3.1. Effects of NO• on intact mitochondria

The addition of GSNO (from 0–500 μM) with DTT (50 μM) to mitochondria caused a concentration dependent inhibition of the total oxygen utilisation by the mitochondria, although only at the highest concentrations of GSNO (190–500 μM) were the initial state III respiration rates also decreased (Fig. 1). This inhibition was seen with all substrates tested: glutamate/malate; succinate and ascorbate/TMPD (Fig. 2A). Increasing the time between exposure of mitochondria to NO• and the addition of substrate, decreased the apparent inhibition of the respiratory chain, suggesting that the inhibitory effect was either reversible or dependent upon electron transfer within the respiratory chain (Fig. 2B). For example, after 30 min of pre-incubation with 190 μM GSNO and 50 μM DTT, approximately 66% of available oxygen was consumed by mitochondria before complete inhibition compared to only 12% when the pre-incubation was for 1 min.

In the presence of DTT the rates of NO• production from GSNO ranged from 0 to 7.6 $nmol \cdot min^{-1} \cdot ml^{-1}$ for concentrations of GSNO between 0–500 μM (Fig. 2C) and were comparable to the rates of generation of NO• from activated neuronal cells and macrophages [21]. In the absence of DTT, rates were approximately fourfold less. With 190 μM GSNO (+50 μM DTT) the rate of NO• generation 15 min after the time of mixing the two

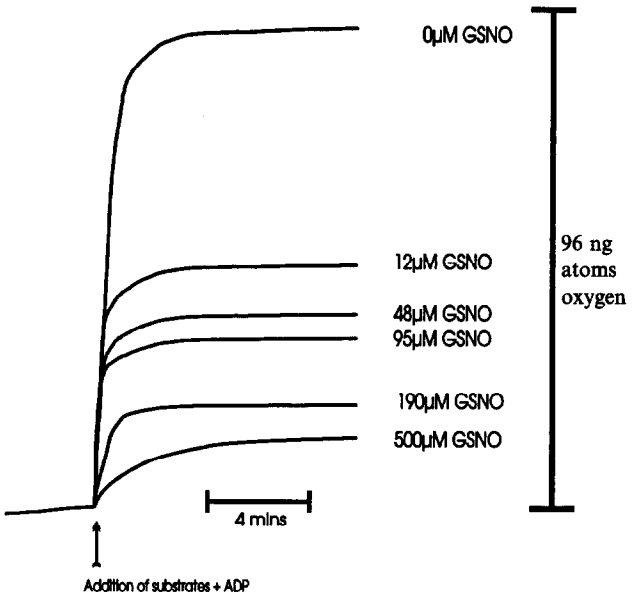

Fig. 1. Representative traces from the oxygen electrode showing the effect of increasing concentrations of GSNO on oxygen consumption by isolated rat skeletal muscle mitochondria in the presence of: GSNO and 50 μM DTT and the addition of glutamate, malate and ADP after 1 min.

reagents was negligible (data not shown). The inhibition of mitochondrial oxygen utilisation by GSNO was dependent upon the presence of DTT (Table 1), consistent with the transnitrosation of NO• from GSNO to DTT and the subsequent decomposition of the less stable nitrosothiol. However, the addition of 50 μM DTT alone to respiring mitochondria had no effect on rates of oxygen utilisation (Table 1). To help to exclude the possibility that products from the decomposition of GSNO + DTT other than NO• were having an inhibitory action on mitochondria, GSNO and DTT were preincubated for 150 min at room temperature, to allow the complete decomposition of the GSNO and the oxidation of the NO• generated. Subsequent addition to mitochondria had no inhibitory effect on mitochondrial oxy-

Table 1
Effect of DTT, HbO_2, GSSG, decomposition of GSNO and washing on mitochondrial oxygen utilisation

Addition	Control	+ GSNO
	85 ± 3	85 ± 7
DTT	81 ± 4	21 ± 4
After Washing	89 ± 2	83 ± 6
HbO_2 ± DTT	74 ± 4	81 ± 6
Decomposed GSNO	90 ± 2	ND
GSSG	79 ± 10	ND

The effect of various additions upon oxygen utilisation by rat skeletal muscle mitochondria (ng atom 4 min after the addition of glutamate, malate and ADP, mean ± S.D., $n = 3$). The additions were GSNO (190 μM), DTT (50 μM), HbO_2 (24 μM), decomposed GSNO (190 μM). ND = not determined.

gen utilisation (see Table 1). The addition of oxidised glutathione (GSSG), a product of the decomposition of GSNO, to mitochondria had no significant effect on oxygen use (Table 1). The presence of oxyhaemoglobin prevented the inhibition of mitochondrial oxygen utilisation due to GSNO and DTT (Table 1), providing further evidence that NO• was the inhibitory agent.

Washing the mitochondria after inhibition with GSNO/DTT resulted in restoration of oxygen utilisation with all substrates suggesting the inhibitory effect of NO• was fully reversible (Table 1).

The visible absorption spectra of mitochondria in the region of 500–630 nm showed characteristic absorption maxima at 550 nm (cytochromes $c + c_1$), 560 nm (cytochrome b) and 601 nm (cytochromes $a + a_3$) following the addition of GSNO, DTT and succinate. These peaks were not observed, however, if any one of the three additions were omitted (data not shown). Similar results were obtained using freeze-thawed mitochondria, except that DTT was not required.

3.2. *The effects of NO• on freeze-thawed mitochondria*

The effect of NO• upon the individual respiratory chain complexes was determined spectrophotometrically, using freeze-thawed mitochondria. GSNO up to a concentration of 500 μM (+DTT 50 μM) in the assay itself failed to have any significant effect on NADH CoQ_1 reductase or succinate cytochrome *c* reductase activities (complex I and complexes II–III respectively). The activity of cytochrome *c* oxidase, however, was significantly affected with 50% inhibition occurring at 10 μM GSNO in the presence of 50 μM DTT, compared to 45 μM GSNO in the absence of DTT (Fig 3). The possibility that GSNO or NO• was reacting with the cytochrome *c* in the assay medium and rendering it resistant to oxidation was discounted as addition of ferricyanide caused virtually complete oxidation of cytochrome *c* (data not shown).

4. Discussion

GSNO has been shown to decompose principally to GSSG and NO• [27] a process accelerated in the presence of thiolate anions such as DTT. The addition of GSNO to isolated mitochondria resulted in the inhibition of oxygen utilisation when using glutamate, succinate or ascorbate/TMPD as substrate, consistent with inhibition of complex IV. Several observations suggest NO• as the cause of this inhibition. Firstly, GSNO alone has no effect on electron transport and in intact mitochondria, inhibition required the presence of DTT. Secondly, respiratory chain function was protected by the addition of HbO_2 (which has a high affinity for NO•), and thirdly, the addition of decomposed GSNO and DTT failed to inhibit oxygen utilisation by the mitochondria, presuma-

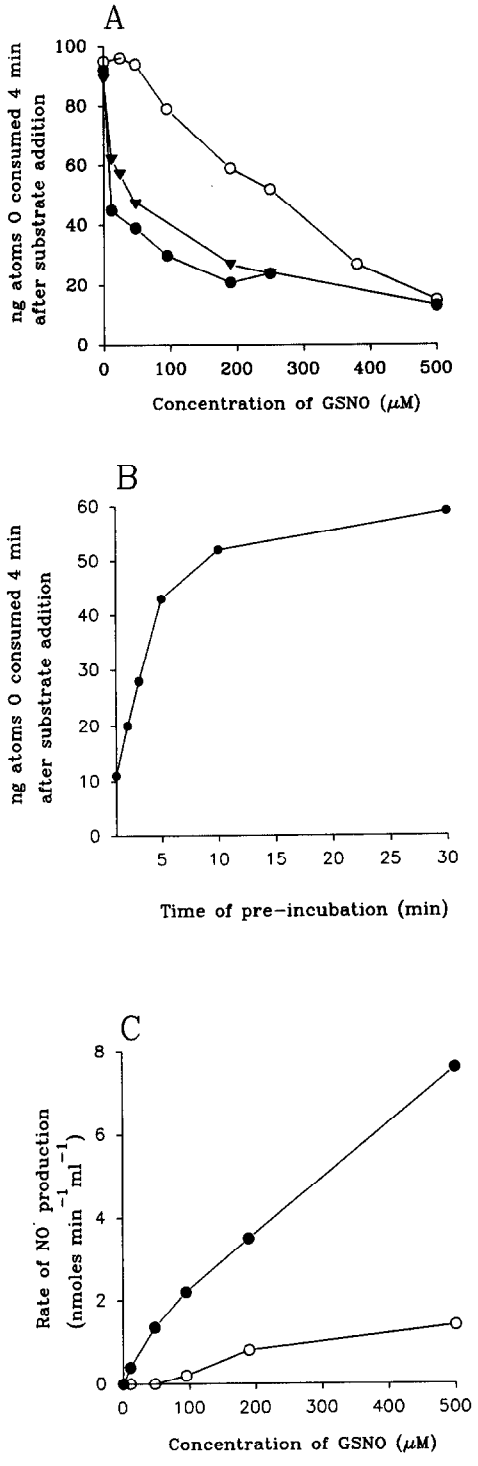

Fig. 2. (A) The effect of GSNO and DTT on skeletal muscle mitochondrial respiration using: glutamate/malate (●), succinate (▼) and ascorbate/TMPD (○) as substrate. Results are expressed as the amount of oxygen consumed 4 min after the addition of substrate and ADP (the time taken for the zero GSNO incubation to use >95% of the available oxygen in the incubation mixture). (B) The effect of incubation of GSNO (190 μM) and DTT (50 μM) with mitochondria prior to the addition of substrates and ADP. Substrates (glutamate/malate) and ADP were added after various times and the oxygen consumption calculated. (C) The rate of NO• generation by GSNO. Incubations were set up in respiration medium containing various concentrations of GSNO (○) or GSNO with DTT (●).

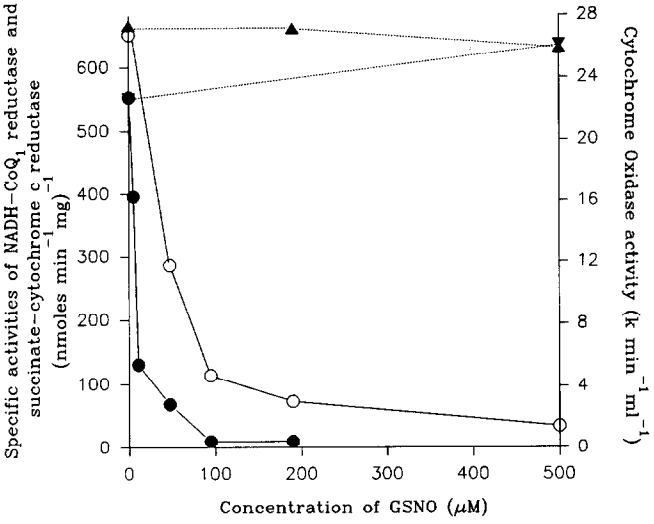

Fig. 3. The effect of varying GSNO concentration on the specific activities of respiratory chain complexes in freeze-thawed mitochondria; ○, cytochrome *c* oxidase; ●, cytochrome *c* oxidase in the presence of 50 μM DTT; ▲, NADH-CoQ_1 reductase in the presence of 50 μM DTT; ▼, succinate cytochrome *c* reductase in the presence of 50 μM DTT.

bly because most of the NO• release from GSNO took place within the first 15 min and was subsequently oxidised to nitrate and nitrite. Consequently, it can be concluded that neither GSNO per se nor its stable oxidation products (GSSG, nitrate and nitrite) had any effect upon mitochondrial respiratory chain function at the concentrations used.

The reduction of all the cytochromes in the presence of GSNO indicated that the respiratory chain was inhibited at cytochrome a_3 which, together with $Cu^{2+}{}_B$, forms part of the oxygen binding site of cytochrome *c* oxidase. The addition of both succinate and NADH (in freeze-thawed mitochondria) caused reduction of the cytochromes in the presence of GSNO and oxygen suggesting that complexes I, II and III were not significantly inhibited by GSNO or NO•. The localisation of the defect to complex IV and normal function of complexes I to III is further supported by measurement of the activity of segments of the respiratory chain (Fig. 3). Although inhibition of cytochrome *c* oxidase by GSNO in freeze-thawed mitochondria was not totally dependent upon the presence of DTT, the free thiols released from within the mitochondria by freeze-thawing may have been sufficient to catalyse the release of NO• from GSNO. These results are consistent with those of the cytochrome spectra, in which freeze-thawed mitochondria only required substrate and GSNO for the appearance of the reduced cytochrome peaks, whereas intact mitochondria also required DTT.

The kinetics of the inhibition of cytochrome *c* oxidase by NO• described here are such that complete inhibition can be seen within 4 min of adding substrates and ADP to mitochondria. The effect of incubation of the resting enzyme with NO• on its ESR (electron spin resonance) spectrum has been reported previously [28] and suggest that NO• reacts with the Cu^{2+}_B centre in a reversible fashion. Binding of NO• to the reduced form of cytochrome *c* oxidase is believed to occur at the haem iron of cytochrome a_3, NO• being the sixth ligand. Reduced cytochrome *c* oxidase has been shown to react rapidly with NO• with a *k* value of $4 \times 10^7\ M^{-1} \cdot s^{-1}$ but would probably not be able to compete for oxygen at this site [29]. From the present data, it is not clear which redox form of cytochrome *c* oxidase is binding NO•, however electron transfer appears to be necessary for inhibition.

There have been other reports in the literature on the effect of NO• or activated macrophages on mitochondrial function. Polarographic analysis of digitonin permeabilised L1210 cells after incubation with authentic NO• solutions for 5 min [30] found that complexes I and II were inhibited, oxygen utilisation being reduced to 16.4 and 44.1% of control levels respectively. An earlier study [10] using cytotoxic macrophages to injure L1210 cells found that complexes I, II and to a lesser extent complex IV, were inhibited (92%, 81% and 15%, respectively) as determined polarographically with digitonin treated cells. The difference in enzyme inhibition seen in these two studies and the present work may, in part, be explained by the different methodologies, in particular the use of whole cells rather than isolated mitochondrial fractions. In the two studies mentioned above, the use of digitonin prior to the cells being assayed may allow NO• to dissociate from cytochrome *c* oxidase, and therefore inhibition of complex IV would not be expected on the basis of the present report. The inhibition of complexes I and II may be more permanent, possibly as a result of more prolonged exposure to NO•. We suggest that the initial (acute) effect of NO• exposure on the respiratory chain is the inhibition of mitochondrial respiration at cytochrome *c* oxidase. Longer term effects may indeed involve the inhibition of complexes I and II reported previously, perhaps leading to cytotoxicity. This inhibition could be due to reaction with NO• itself or with peroxynitrite, a product of the reaction between NO• and superoxide [31]. Superoxide production is a by-product of mitochondrial respiratory electron transfer and has been shown to increase in the presence of mitochondrial inhibitors such as rotenone, antimycin A and cyanide [32]. The site of this additional superoxide formation is believed to be within complexes I and III. NO•, as an inhibitor of cytochrome oxidase, may contribute to the inhibition of complexes I and II by further promoting free radical or peroxynititrite formation, or both. Whether such mechanisms are relevant to Parkinson's disease and other neurodegenerative disorders obviously requires further investigation, but it is of interest to note that inhibition of cytochrome oxidase is required for the irreversible loss of complex I activity in a chemical model of Parkinson's disease [33].

Acknowledgements: The work described herein was financed by the Sir Jules Thorn Trust. Ubiquinone-1 (CoQ_1) was a generous gift from the Eisai Chemical Corporation (Japan).

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