

Transcellular regulation of cell respiration by nitric oxide generated by activated macrophages

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Abstract A macrophage cell line (J774), activated with interferon- γ and endotoxin to express the inducible form of NO synthase (iNOS), immediately inhibited the cellular respiration of co-incubated L-929 fibroblasts or non-activated J774 macrophages. The inhibition was potent, rapid and reversible when the NO was removed by adding oxyhaemoglobin or by inhibiting iNOS. Exogenously added NO also rapidly and reversibly inhibited cellular respiration over the same range of NO concentrations. This inhibition was competitive with oxygen and due to direct inhibition of cytochrome oxidase. Thus, NO generated by one cell can regulate the respiration of adjacent cells, supporting the hypothesis that NO may be a physiological and/or pathological regulator of cellular respiration, via its inhibition of cytochrome oxidase.

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Key words: Nitric oxide; Macrophage; Mitochondrion; Mitochondrial respiration; Inflammation; Cytotoxicity

1. Introduction

Although there are no known physiological and direct regulators of mitochondrial respiration and its affinity for oxygen [1], we and others have shown that nitric oxide (NO) reversibly inhibits mitochondrial and cellular respiration by inhibiting cytochrome oxidase in competition with oxygen [2–6]. This suggests that NO may act as a physiological or pathological regulator of mitochondrial respiration and its affinity for oxygen [7]. Furthermore, it is possible that NO, which is a highly diffusible molecule in biological systems, may regulate respiration in cells other than those that generate it. To test this hypothesis, we have now co-incubated cells generating NO with target cells, and have measured their rates of cellular respiration.

2. Materials and methods

Non-activated macrophages (J774 murine macrophage cell line ATCC TIB 67) were maintained in suspension culture in RPMI 1640 with 25 mM HEPES, 10% foetal calf serum, and 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin, as in [8]. Only cells in the log phase of growth and with >90% viability (assayed by trypan blue exclusion) were used for experiments. Activated

macrophages were prepared by resuspending the cells at 2.5×10^5 cells/ml in Dulbecco's Modified Eagle Medium (DMEM) with interferon- γ (murine 50 units/ml) and endotoxin (W S Typhosa 0901, Difco, 10 μ g/ml). The cells were used after 18 h in stirrer culture, and nitrite formation was assayed by the Griess reaction, as in [8]. The concentration of nitrite was between 15 and 50 μ M in the activated cultures, and below detection (<1 μ M) in the non-activated cultures. Viability of the activated cells was between 45 and 70%. Fibroblasts (mouse C34/AN connective tissue cell line L-929, ECACC no. 85011425) were cultured in tissue culture flasks in RPMI 1640, 2 mM L-glutamine, 10% foetal calf serum, plus penicillin and streptomycin. Cells in the log phase of growth were trypsinized off the plate, and resuspended in the same incubation medium as that used for NO and oxygen measurements. Viability was >95%.

Cells were incubated in a stirred, gas-tight vessel maintained at 37°C. The incubation medium contained 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1 mM CaCl, 25 mM HEPES and 20 mM glucose, pH 7.4. The vessel contained both an oxygen electrode (Rank Brothers, Bottisham) and an NO electrode (World Precision Instruments), thus simultaneously measuring O_2 and NO concentration, as in [3]. The NO electrode was calibrated by adding aliquots of NO-saturated water (2 mM NO) as in [3]. The respiration rate of cells was calculated from the rate of oxygen consumption, assuming that air saturated medium contained 0.2 mM O_2 at 37°C, as in [3].

In experiments designed to assess whether the site of inhibition of respiration by NO was cytochrome oxidase, the specific inhibitor of respiratory complex III, myxothiazol [9] was used to prevent the supply of electrons to cytochrome oxidase from the rest of the respiratory chain. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, was used to remove any effect of ATP turnover on cytochrome oxidase activity. Ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD) were used to supply electrons specifically to cytochrome oxidase within the cells.

To determine whether the effect of NO on cellular respiration is mediated by the soluble guanylyl cyclase, the effect of a specific inhibitor of this enzyme, 1H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxaline-1-one (ODQ), was investigated on the respiration of fibroblasts.

RPMI, DMEM, penicillin, streptomycin and glutamine were from Gibco-BRL. Interferon- γ was obtained from Insight Biotechnology, foetal calf serum from Harlan Sera-lab and endotoxin from Difco Laboratories. All other reagents were from Sigma.

3. Results and discussion

Macrophages in culture (murine macrophage cell line J774) were activated with interferon- γ and endotoxin for 18 h, and incubated in a gas-tight vessel containing both an NO electrode and an oxygen electrode, to monitor simultaneously concentrations of NO and of oxygen (and thus the rate of cellular respiration). Activated macrophages (10^7 cells/ml) produced a steady-state concentration of up to 1.5 μ M NO, whereas non-activated macrophages (cultured in the absence of interferon- γ and endotoxin) and fibroblasts (mouse fibroblast cell line L-929) produced no detectable NO (Table 1). Addition of activated macrophages to fibroblasts or non-activated macrophages resulted in rapid inhibition of the oxygen consumption (Figs. 1 and 2 and Table 1). Activated macro-

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Abbreviations: DMEM, Dulbecco's modified Eagle medium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; iNOS, inducible form of nitric oxide synthase; NMMA, N^G -monomethyl-L-arginine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*] quinoxaline-1-one; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine

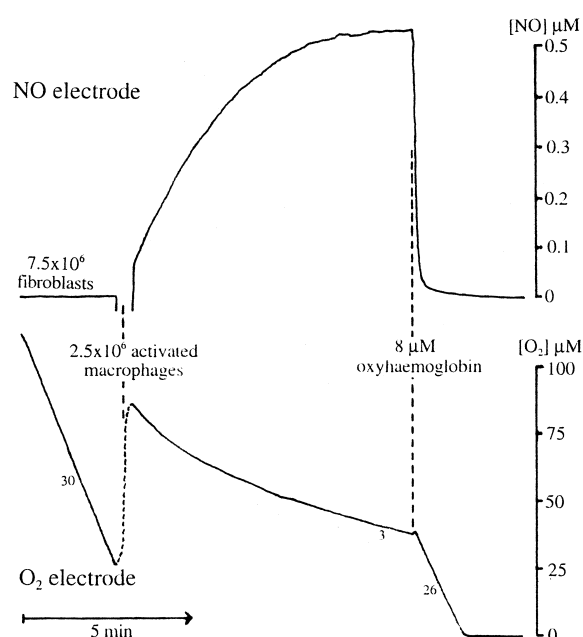


Fig. 1. Nitric oxide generated by activated macrophages causes a reversible inhibition of respiration in co-incubated fibroblasts. Fibroblasts (0.75 ml of 10^7 cells/ml) were incubated in a vessel containing NO and O_2 electrodes in order to measure simultaneously NO concentration (upper trace) and respiration rate (lower trace). Activated macrophages (0.25 ml of 10^7 viable cells/ml) were then added to the same vessel, resulting in production of NO and inhibition of respiration. The NO was then removed by adding oxyhaemoglobin ($8 \text{ } \mu\text{M}$ haem), resulting in reversal of the inhibition of respiration. The oxygen concentration in the vessel increased when the macrophages were added because the vessel was temporarily opened and because macrophage medium contained a higher initial level of oxygen. The oxygen trace levelled off at the end of the experiment because the vessel was completely depleted of oxygen. In the conditions used all the NO production is due to the macrophages, but $>90\%$ of the oxygen consumption is due to the fibroblasts. The figures next to the oxygen trace indicate the rate of oxygen consumption in the adjoining part of the trace in $\text{nmol O}_2/\text{min}/10^7$ cells. Trace representative of 3 experiments.

phages incubated alone respired at only 27% of the rate of fibroblasts and 67% of the rate of non-activated macrophages (Table 1), thus in the co-incubations where the activated macrophages constituted 25% of the cells they would contribute approximately 7% of the joint oxygen consumption with the fibroblasts and about 17% with the non-activated macrophages. Thus, as shown in Table 1, the addition of activated macrophages to fibroblasts caused the fibroblast respiration to fall by at least 75%.

In order to investigate whether the inhibition of respiration was due to NO, oxyhaemoglobin, which is known to react

rapidly with NO, was added to the co-incubations. This immediately removed NO and reversed the inhibition of respiration, although the reversal of the respiratory inhibition was possibly not complete (Fig. 1 and Table 1). Similarly, inhibition of NOS with N^G -monomethyl-L-arginine (NMMA) caused the concentration of NO to fall in parallel with a reversal of the inhibition of respiration (Fig. 2). Subsequent addition of L-arginine, the substrate for the generation of NO which competes with NMMA, increased again the generation of NO and, in parallel, re-imposed the inhibition of respiration (Fig. 2). The inhibition of respiration after addition of arginine was stronger, even though the NO concentration was lower (Fig. 2), because the oxygen concentration was low and cellular respiration was more sensitive to NO at low oxygen concentrations (see below). This is consistent with the inhibition being at cytochrome oxidase in competition with oxygen [3–5]. Addition of haemoglobin, NMMA or L-arginine at the above concentrations to L-929 cells or non-activated J774 cells alone had no detectable effect on the respiration rates ($<5\%$ change), indicating that the effect of these agents was due to their action on NO concentration rather than direct effects on respiration.

In order to investigate whether NO alone could inhibit cellular respiration we added aliquots of authentic NO to the cells. Addition of $1 \text{ } \mu\text{M}$ NO to the cells (fibroblasts or non-activated macrophages) caused immediate inhibition of cellular respiration (see Fig. 2 for effect on macrophages), consistent with NO rapidly diffusing through the cell membrane and binding to cytochrome oxidase. As the NO added to the medium broke down (due to reaction with oxygen and metabolism by the cells) the inhibition of respiration was reversed (Fig. 2). This reversibility is consistent with the known reversible inhibition of cytochrome oxidase by NO [2–6,10,11]. We also tested the sensitivity of cellular respiration to NO at two different oxygen concentrations, by adding $1 \text{ } \mu\text{M}$ NO to the fibroblasts (as in Fig. 2) and determining the NO concentration at which the cellular respiration rate was inhibited by half. The concentration of NO required to inhibit cellular respiration rate by half was $0.26 \text{ } \mu\text{M}$ NO (mean of two experiments, range $0.09 \text{ } \mu\text{M}$) at an oxygen concentration of $105 \text{ } \mu\text{M}$ O_2 , and $0.06 \text{ } \mu\text{M}$ NO (range $0.01 \text{ } \mu\text{M}$) in two additional experiments using $30 \text{ } \mu\text{M}$ O_2 . Thus, the NO-induced inhibition of cellular respiration was competitive with oxygen, consistent with the known competitive inhibition of cytochrome oxidase [3–6].

To test whether a longer term exposure of cells to NO would still be reversible, we maintained the NO concentration in the vessel at the mean of $0.75 \text{ } \mu\text{M}$ (range $0.5\text{--}1.0 \text{ } \mu\text{M}$ NO) for 10 min by continually adding aliquots of NO. This treatment resulted in a 90% inhibition of respiration during the 10 min exposure to NO; however, following this time, when

Table 1
Activated macrophages reversibly inhibit the cellular respiration of co-incubated fibroblasts

Incubation conditions	Respiration rate ($\text{nmol O}_2/\text{min}/10^7$ cells)	NO concentration (μM)
10^7 activated macrophages alone	$6.5 (\pm 0.9)$	$1.07 (\pm 0.46)$
10^7 non-activated macrophages alone	$9.7 (\pm 1.1)$	0
7.5×10^6 fibroblasts alone	$24.2 (\pm 1.5)$	0
+ 2.5×10^6 activated macrophages	$5.1 (\pm 1.5)$	$0.47 (\pm 0.11)$
+ $7.5 \text{ } \mu\text{M}$ oxyhaemoglobin	$19.3 (\pm 1.8)$	0

The respiration rate and NO concentration of cells were measured as in Fig. 1. The numbers are means and standard deviations of three experiments for each condition.

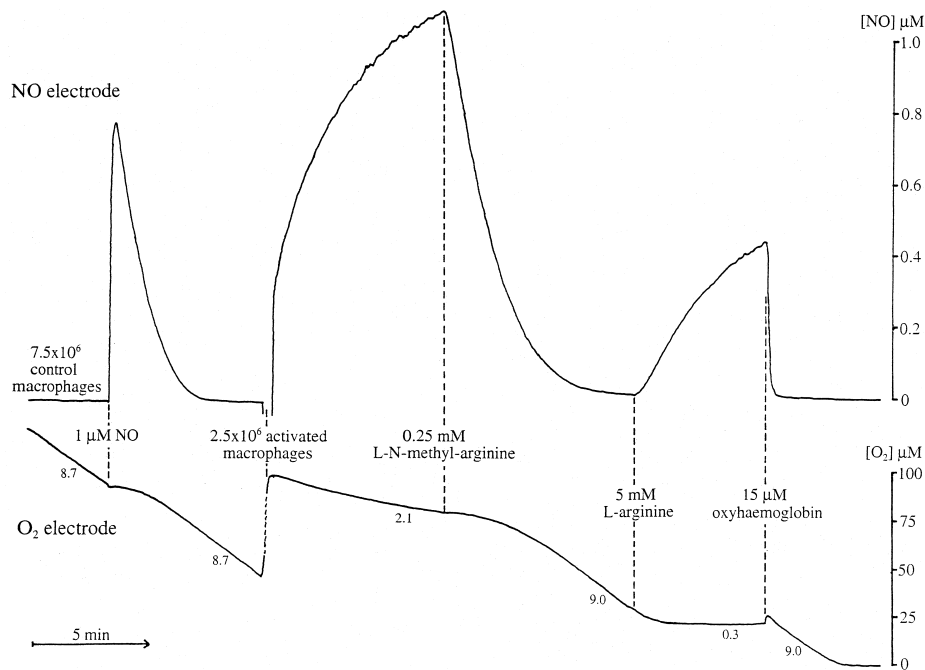


Fig. 2. The respiration of non-activated macrophages is reversibly inhibited by exogenous NO or co-incubated activated macrophages, and this inhibition is reversed by inhibition of iNOS. Non-activated macrophages (0.75 ml of 10^7 cells/ml) were incubated as in Fig. 1, and an aliquot of $1 \mu\text{M}$ NO was added, causing inhibition of respiration which was reversed as the NO broke down. Activated macrophages (0.25 ml of 10^7 cells/ml) were then added, resulting in production of NO and inhibition of respiration. This inhibition was reversed by adding 0.25 mM *N*^G-monomethyl-L-arginine (NMMA), an inhibitor of NOS. Inhibition of respiration was restored by adding 5 mM L-arginine (the substrate for NOS which reverses the action of the competitive inhibitor NMMA) and this respiratory inhibition was again reversed by adding oxyhaemoglobin to remove NO. Trace representative of 3 experiments.

the NO had broken down, the respiration rate returned to 90% of the original value. Thus, the mean respiration rates ($\text{nmol O}_2/\text{min}/10^7$ cells, standard deviation, $n=3$) of fibroblasts at $60 \mu\text{M O}_2$ were $15 (\pm 1.5)$ before addition of NO, $1.5 (\pm 0.3)$ in the presence of NO and $13.2 (\pm 1.2)$ when the NO had broken down, respectively. Thus, the inhibition of cellular respiration is fully reversible following exposure to NO for 10 min.

This inhibition of fibroblast respiration by exogenous NO (90% inhibition at $0.75 \mu\text{M}$ NO and $60 \mu\text{M O}_2$) was comparable to that observed with macrophage-derived NO (74% inhibition at $0.47 \mu\text{M}$ NO and $60 \mu\text{M O}_2$, see Table 1). This similarity in sensitivity again indicates that the inhibition of respiration induced by activated macrophages is due entirely to NO.

To investigate directly whether the inhibition of respiration by NO was at cytochrome oxidase, experiments were carried out in which oxygen consumption of the cells was entirely dependent on cytochrome oxidase alone. Addition of myxothiazol ($0.4 \mu\text{M}$) alone to either L-929 cells or non-activated J744 cells caused complete inhibition of oxygen consumption. Since myxothiazol is a specific inhibitor of mitochondrial respiration [9] this indicates that cellular oxygen consumption is due to mitochondrial respiration alone. We then added a mitochondrial uncoupler ($1 \mu\text{M}$ FCCP) to myxothiazol-treated cells to remove any effect of ATP turnover on cytochrome oxidase activity. Ascorbate ($200 \mu\text{M}$) and TMPD ($80 \mu\text{M}$) were then added to supply electrons specifically to cytochrome oxidase within the cells. Subsequent addition of NO to the L-929 cells showed that the concentration of NO required to inhibit oxygen consumption rates by half in these conditions

was $0.22 \mu\text{M} (\pm 0.04; n=3)$ at $80 \mu\text{M O}_2$. Prior to addition of these substrates and inhibitors, when the cells were respiring on glucose, respiration was inhibited by half at $0.18 \mu\text{M NO} (\pm 0.03, n=3)$ at $80 \mu\text{M O}_2$. Since in the presence of myxothiazol, FCCP, TMPD and ascorbate the oxygen consumption is controlled by cytochrome oxidase activity alone and since in both cases the inhibition occurred at approximately the same concentrations of NO, these results suggest that the inhibition of oxygen consumption by NO in both cases is due solely to the direct inhibition of cytochrome oxidase.

To test whether the effect of NO on respiration is mediated by stimulation of guanylyl cyclase, we also measured the sensitivity of cellular respiration to NO in the presence of ODQ, a specific inhibitor of the soluble guanylyl cyclase [12,13]. Incubation of L-929 cells with $20 \mu\text{M ODQ}$ for 5 min caused no change in respiration rate and no change in the sensitivity of respiration to NO. Inhibition of respiration by half occurred at $0.17 \mu\text{M NO} (\pm 0.02, n=3)$ in the presence of ODQ, thus demonstrating that the soluble guanylyl cyclase is not involved in the inhibition of respiration by NO.

The inhibition of respiration reported here is due to low concentrations of NO itself, is reversible, is competitive with O_2 and occurs at cytochrome oxidase, either within the cells generating it or within nearby cells. In contrast, activated macrophages have previously been shown to inhibit irreversibly the respiration of co-incubated cells after long-term (about one day) incubation, and this inhibition has been attributed to irreversible inhibition of aconitase and respiratory complexes I and II [11,14,15]. This irreversible inhibition might be mediated by peroxynitrite, which is known to inhibit aconitase, respiratory complexes I and II, and other mitochondrial com-

ponents [10,11], or be mediated by nitrogen dioxide, or may be due to NO itself which may inhibit complex I after long-term incubation [16]. The macrophage-induced inhibition of cellular respiration was possibly not completely reversed by oxyhaemoglobin (Fig. 1 and Table 1), and any such irreversible inhibition might be mediated by any of the above mechanisms.

Many cell types, in response to inflammation, cytokines, endotoxins, hypoxia and/or oxidative stress express iNOS, and this has been shown to cause an initial reversible inhibition of respiration in astrocytes [17] followed by an irreversible inhibition [18], and an irreversible inhibition in a variety of other cell types [19–23]. Inhibition of respiration by NO may contribute to the cytostatic and cytotoxic effects of activated macrophages and iNOS-expressing cells generally, which have been implicated both in host defence and in a variety of pathologies such as sepsis, arthritis, ulcerative colitis, Type I diabetes and neurodegenerative disease [24].

Recently it has been shown that mitochondria may contain a mitochondrial NO synthase (mtNOS), and this NOS may be involved in regulating mitochondrial respiration [25,26]. We found that addition of oxyhaemoglobin or NMMA to L-929 or non-activated J774 cells caused no significant change in respiration rate (see above), and thus if mtNOS was present in these cells, it was not significantly regulating cellular respiration in the conditions used.

Since virtually all cellular processes depend on respiration and ATP production, it is important to distinguish between the acute, reversible inhibition of respiration, which may be a physiological process, and irreversible inhibition, which may be a pathophysiological mechanism. Current evidence indicates that NO can act as a modulator of mitochondrial respiration in physiological conditions and as a disrupter of mitochondrial activity in pathological conditions. The mechanism(s) by which NO exerts the latter actions remains to be fully elucidated.

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