Continuous exposure to high concentrations of nitric oxide leads to persistent inhibition of oxygen consumption by J774 cells as well as extraction of oxygen by the extracellular medium

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Nitric oxide (NO) plays a key role in many physiological and pathophysiological events, including the control of cell respiration. Both reversible and irreversible inhibition of mitochondrial respiration have been reported following the generation of NO by cells. We have exposed the murine macrophage cell line J774 to high concentrations of NO, such as are generated in some pathological conditions, and determined their effect on oxygen consumption. We observed a persistent inhibition of respiration which was due to a redox-dependent, progressive inhibition of complex I activity. No other enzyme of the respiratory chain was inhibited in this way. At the same time, we detected a paradoxical removal of oxygen by the extracellular medium. This removal

was due to a chemical interaction between dissolved oxygen and NO-related species released from cells exposed to NO. A similar removal of oxygen by the cell supernatant also occurred following activation of cells with cytokines and bacterial products. Thus, the amounts of NO generated during pathological conditions may contribute to tissue hypoxia both by inhibiting cell respiration and by promoting removal of oxygen from the extracellular medium.

Key words: cell respiration, complex I, glutathione, nitric oxide, S-nitrosation.

INTRODUCTION

Nitric oxide (NO) is a short-lived messenger molecule which plays a key role in many physiological and pathophysiological events [1], including the ability to control cell respiration. Short-term exposure to exogenous NO has been shown to inhibit mitochondrial oxygen consumption in a reversible manner, by competing with oxygen at the level of complex IV [2]. Recently, it has been demonstrated that endogenously released NO regulates cell respiration physiologically [3,4]. The effects of long-term exposure of cells to high concentrations of NO have been investigated in less detail. When NO is generated continuously by the inducible NO synthase in cells activated with cytokines and bacterial products, all the enzymes of the respiratory chain, as well as aconitase, are inhibited in a persistent manner both in the activated cells and in cells co-incubated with them [5–9]. This effect resembles that of peroxynitrite (ONOO[−]), the product of the reaction of NO with superoxide (O₂[−]) generated during cell activation [9,10]. We have recently investigated the effects of long-term exposure to known amounts of NO. In those experiments, the exposure of cells to NO at concentrations of up to 1.5 μM resulted in a persistent decrease of respiration due to selective redox-dependent inhibition of complex I. This inhibition, which occurred without detectable production of ONOO[−], was preceded by a decrease in the concentration of GSH and is likely to be due to nitrosation of thiols relevant for the functioning of complex I. Complex IV was also inhibited, but this inhibition was always reversible on removal of NO [11].

In certain pathological conditions, such as brain ischaemia, the tissue concentration of NO to which cells may be exposed approaches 6–11 μM [12,13]. The effects of these high concentrations of NO on cell respiration have not yet been investigated.

Using the slow releasing NO donor (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium 1,2-diolate (DETA-NO), we have now studied the effects of high concentrations of NO (up to 8 μM) on the oxygen consumption of the murine macrophage cell line, J774. Under these conditions we observed a persistent inhibition of respiration which was solely due to a redox-dependent, progressive and selective inhibition of complex I activity, and similar to that observed at lower concentrations of NO [11]. At the same time, however, an increase in the removal of oxygen by the extracellular medium was observed. Interestingly, oxygen removal by the cell supernatant also occurred following activation with cytokines and bacterial products, indicating that endogenously released NO leads to similar cellular changes and that it may indeed have pathophysiological relevance. This removal of oxygen by the supernatant was due to a chemical interaction between dissolved oxygen and NO-related species released from cells exposed to NO.

MATERIALS AND METHODS

Materials

Culture media and foetal bovine serum were obtained from Gibco Laboratories (Paisley, Renfrewshire, Scotland, U.K.). Lipopolysaccharide (LPS) from Westphal *Salmonella typhosa* 0901 and interferon-γ (IFN-γ) were from Insight Biotechnology Ltd. (Wembley, Middx., U.K.). DETA-NO, a compound known to release NO with a half-life of 20 h at pH 7.4 [14], was purchased from Alexis Co. (Nottingham, U.K.). Oxyhaemoglobin (oxyHb) was prepared as described [15]. Myxothiazol, rotenone, luminol, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), β-hy-

Abbreviations used: NO, nitric oxide; ONOO[−], peroxynitrite; O₂[−], superoxide; DETA-NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium 1,2-diolate; LPS, lipopolysaccharide; IFN-γ, interferon-γ; oxyHb, oxyhaemoglobin; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; GSNO, S-nitrosoglutathione; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazide.

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droxybutyrate, 3-nitropropionic acid and all other reagents were from Sigma-Aldrich (Poole, Dorset, U.K.). Ultrafiltration membranes (centrifugal filter devices, Microcon YC-3, -10 and -30) were obtained from Millipore (Watford, U.K.).

S-Nitrosoglutathione (GSNO) was prepared by reacting GSH with sodium nitrite in citrate buffer, pH 7.2 [16]. Stock solutions (20 mM) were kept on ice in the dark and diluted in incubation buffer immediately before use.

Cell culture and preparation

J774 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.). Cells were grown in suspension using Micro Carriers modified stirrers (Techne Laboratories, Duxford, Cambs., U.K.) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies, Paisley, Renfrewshire, Scotland, U.K.), supplemented with foetal bovine serum (10 % v/v), L-glutamine (4 mM), penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37 °C in a humidified atmosphere containing 5 % CO₂. At the time of the experiments the cells were centrifuged, washed and resuspended in an incubation medium containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1 mM CaCl₂, 20 mM glucose and 25 mM HEPES, pH 7.2. Cell viability, as assessed by the Trypan Blue exclusion test, decreased only slightly with time (> 94 % at 0 h, 82 ± 4 % at 5 h). In some experiments, cells were resuspended in glucose-free medium and respiration was sustained on pyruvate (20 mM). In experiments aimed to investigate the effect of immunostimulation of cells on oxygen consumption, J774 macrophages were grown in suspension in DMEM, in the absence or presence of L-arginine. Cells were activated by the addition of LPS (10 µg/ml) and IFN-γ (50 units/ml) to the culture medium 18 h before the start of the experiment.

Measurement of oxygen consumption and NO generation

Cells suspended in the incubation medium (pH 7.2) at a density of 10⁷ cells/ml were maintained at 37 °C in the presence or absence of DETA-NO (0.5–10 mM), which was dissolved in the incubation medium 20 min before addition to the cells. The incubation vessel, which was gently agitated, was open to the atmosphere. The oxygen consumption of 1 ml samples was analysed, at the time-points indicated in the various experiments, in a gas-tight vessel maintained at 37 °C and equipped with a Clark-type oxygen electrode (Rank Brothers, Bottisham, U.K.) and an NO electrode (World Precision Instruments, Sarasota, FL, U.S.A.) connected to a chart recorder. The oxygen electrode was calibrated with air-saturated incubation medium kept at 37 °C, assuming an oxygen concentration of 200 µM; the NO electrode was calibrated by addition of known concentrations of NaNO₂ under reducing conditions (KI/H₂SO₄). The reversibility of NO-induced inhibition of respiration was assessed using the NO scavenger oxyHb (8–30 µM).

Data on oxygen consumption by DETA-NO-treated cells are expressed as a percentage of the oxygen uptake by control cells subjected to the same treatment in the absence of the NO donor. The oxygen consumption of control cells (1 ml) without any treatment was 27.6 ± 2.1 µM·min⁻¹. In experiments where the effect of treatment with DETA-NO (0.5–10 mM) on cells and cell supernatants were investigated separately, the cells were washed and resuspended in fresh incubation medium containing the same concentration of DETA-NO as they were exposed to during the preincubation period. Cell supernatants were obtained by spinning 1 ml of cell suspension in two consecutive centrifugations at 2500 g for 2 min and were analysed immediately. Oxygen consumption was then measured separately in both the

cells (resuspended in fresh DETA-NO-containing medium) and their supernatants. In some experiments, cell supernatants were subjected to a further treatment i.e. illumination with a visible cold light source (8 Mlx; KL 1500; Schott, Mainz, Germany), temperature change or ultrafiltration. Supernatants from LPS-activated cells were obtained by centrifugation as described above, except that here the supernatant was the original culture medium rather than the incubation medium used in other experiments.

Targets of inhibition of cellular respiration

In these experiments, inhibitors and substrates of the citric acid cycle and the mitochondrial respiratory chain were added to the cells before the addition of DETA-NO. The concentrations of various compounds were selected in preliminary experiments [11] designed as follows: oxygen consumption in untreated cell samples was followed for 4 min, and the various blockers (3-nitropropionic acid, myxothiazol, rotenone) added at increasing concentrations to select the minimally effective concentration required to reduce oxygen consumption to less than 10 % of the control. The corresponding respiratory substrates (β-hydroxybutyrate, TMPD plus ascorbate, succinate) were then added at increasing concentrations to select the one that restores respiration to at least 90 % of the level of untreated controls. To investigate whether events downstream of ATP synthesis or the glycolytic pathway were affected, control cells were either exposed for 2 min to the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 1 µM), or resuspended in glucose-free incubation medium, and respiration was sustained on pyruvate (20 mM). 3-Nitropropionic acid (0.6 mM), an inhibitor of succinate dehydrogenase, in the presence of β-hydroxybutyrate (6 mM), which supplies mitochondria with NADH via its conversion into acetoacetate, was used to investigate whether the citric acid cycle was affected. Oxygen consumption sustained by the activity of complex IV was tested in the presence of the complex III inhibitor, myxothiazol (0.5 µM). Supply of electrons to complex IV was maintained using TMPD (80 µM) plus ascorbic acid (4 mM). Oxygen consumption via complexes II and III was tested using the respiratory substrate succinate (6 mM) in the presence of the complex I inhibitor rotenone (2 µM).

Assay of complex I activity

The activity of complex I was assayed essentially as described [17]. Briefly, cells were centrifuged at 2500 g for 2 min and the pellet was freeze-thawed three times. A 20 µl aliquot of cellular homogenate was added to 1 ml of 10 mM potassium phosphate buffer (pH 8.0) containing 100 µM NADH in a 1 ml cuvette. The rate of NADH oxidation was followed at 340 nm. After recording the absorbance for 1 min, ubiquinone-1 (50 µM) was added and the stimulated rate of NADH oxidation, which was taken as complex I activity, was followed for a further 2 min. The NADH oxidation rate was calculated from the slope of absorbance decrease over time using an absorption coefficient for NADH of 6.81 mM⁻¹·cm⁻¹ at 340 nm [17].

Measurement of GSH and protein

The concentration of GSH in the supernatant of control and DETA-NO-treated cells was measured spectrophotometrically at 400 nm using the Bioxytech GSH-400 kit from Oxis International (Portland, OR, U.S.A.). Protein content was measured using a commercially available assay kit, based on the bicinchoninic acid method, and BSA as a standard (Pierce, Rockford, IL, U.S.A.).

Measurement of GSNO

The concentration of GSNO was determined using HPLC. Aliquots of the supernatants from cells exposed to DETA-NO for various lengths of time were injected into a Hewlett–Packard 1090 Series II HPLC system equipped with a LiChroSorbRP-18 column (125 mm $\times$ 4 mm) running at 1 ml/min with an isocratic eluent comprising 88% phosphate buffer (50 mM, pH 2.5)/12% methanol. Under these conditions, GSNO eluted at 2.7 min and was detected by its UV absorbance at 336 nm. Quantification was achieved by comparison with a standard curve constructed by injecting known concentrations of HPLC-grade purified GSNO under identical conditions. In order to confirm the identity of the peak at 2.7 min, samples were routinely spiked with known concentrations of authentic GSNO or its decomposed metabolite, achieved either by reaction of GSNO with 75 μ M HgCl₂ for 30 min or its illumination with high-intensity cold light (8 Mlx) for 1 h prior to analysis. In the latter two cases, the GSNO peak was completely abolished.

Statistical analysis

The results are expressed as the mean $\pm$ S.E.M. of n individual experiments. Statistical analyses were performed using the Student's t test for unpaired variables and a P value of < 0.05 was considered to denote statistical significance.

RESULTS

Effects of NO on oxygen consumption

DETA-NO diluted in the incubation medium generated increasing concentrations of NO, which reached a plateau after

Figure 1 Effect of exposure to DETA-NO on oxygen consumption by J774 cells

Cells were incubated with different concentrations of DETA-NO (0.5 mM, $\bullet$; 3 mM, $\blacksquare$; 5 mM, $\blacktriangle$; 10 mM, $\blacktriangledown$) and oxygen consumption was measured in samples taken at the times indicated. Oxygen consumption, in DETA-NO-treated cells, here and in the subsequent Figure, is reported as a percentage of that observed in control cells without DETA-NO. The rate of oxygen consumption in control cells was $13.9 \pm 1.65 \mu\text{M} \cdot \text{min}^{-1}$ ($n = 6$) and did not vary significantly throughout the experiment.

Figure 2 Effect of exposure to DETA-NO on oxygen consumption in J774 cells and in their incubation medium

(a) J774 cells were treated as in Figure 1, and at the indicated time-points cell samples were centrifuged and the pellets resuspended in fresh incubation medium containing DETA-NO at the same concentration (0.5 mM, $\bullet$; 5 mM, $\blacktriangle$; 10 mM, $\blacktriangledown$) as during the incubation. (b) Oxygen consumption in the extracellular medium obtained after separation from the cells. Results shown are from six independent experiments.

about 20 min and remained constant throughout the experiment (up to 5 h). The concentrations of NO at the plateau were 1.2 ± 0.3 , 2.8 ± 0.5 , 5.6 ± 0.6 and $7.8 \pm 0.9 \mu\text{M}$ ($n = 4$) at 0.5, 3, 5 and 10 mM DETA-NO respectively.

Exposure of cell suspensions to these concentrations of DETA-NO resulted in an immediate inhibition of oxygen consumption which, for all concentrations, was in the order of 70–80% of control respiration (Figure 1). The effect of NO was not the consequence of a non-specific toxic effect, since the cells were viable at the end of the experiments (5 h; 82 ± 4.0 and $80 \pm 5.0\%$, in controls and cells treated with 5 mM DETA-NO respectively; $n = 8$). With time, however, the initial inhibitory effect of NO decreased, becoming a stimulatory effect on oxygen consumption at higher concentrations (Figure 1). The increase in oxygen consumption occurred earlier and was more pronounced with higher concentrations of DETA-NO (Figure 1).

To investigate whether the increase in oxygen consumption was due to an action of NO on the cells, they were separated at the indicated time-points (Figure 2a) from the incubation medium by centrifugation and resuspended in fresh, DETA-NO containing medium, and their oxygen consumption was analysed immediately. As shown in Figure 2(a), oxygen consumption by the cells was always inhibited by DETA-NO and this inhibition persisted throughout the duration of the experiments. The inhibition was initially fully reversible by addition of oxyHb (8–30 μM), however, after 1 h of incubation the ability of oxyHb to reverse the inhibition decreased progressively until it became ineffective. The greater the initial concentration of

Table 1 Effects of 5 mM DETA-NO on cellular oxygen consumption using different inhibitors and substrates

Cells resuspended in incubation medium in the presence of 5 mM DETA-NO were treated with various blockers of mitochondrial respiratory complexes in the presence of specific substrates as indicated ($n = 3$ or 4). At the specified time-points, cells were resuspended in fresh medium containing the same concentrations of DETA-NO and inhibitors and substrates used during the incubation. Cellular oxygen consumption was analysed before and after the addition of oxyHb. The values are expressed as a percentage of the oxygen consumption observed in control cells that were treated with the same inhibitors and substrates but without DETA-NO.

Treatment	Pathways	Oxygen consumption			
		20 min	20 min + oxyHb	5 h	5 h + oxyHb
DETA-NO		8.75 $\pm$ 3.0	86.6 $\pm$ 4.4	9.0 $\pm$ 1.8	6.0 $\pm$ 5.7
DETA-NO + 3-nitropropionic acid + β -hydroxybutyrate	Complexes I–II–III	28.7 $\pm$ 3.2	99.3 $\pm$ 2.3	19.3 $\pm$ 1.2	18.0 $\pm$ 1.7
DETA-NO + myxothiazol + ascorbate/TMPD	Complex IV	21.5 $\pm$ 3.5	106.5 $\pm$ 9.5	30.0 $\pm$ 9.1	105.3 $\pm$ 15.0
DETA-NO + rotenone + succinate	Complex II–III–IV	23.7 $\pm$ 5.0	110.0 $\pm$ 4.4	4.2 $\pm$ 3.0	95.7 $\pm$ 8.9

DETA-NO, the shorter the time before oxyHb became ineffective (5 ± 0.3 , 3 ± 0.2 , 2.5 ± 0.2 and 1.5 ± 0.2 h with 0.5, 3, 5 and 10 mM DETA-NO respectively; $n = 3$). Measurements of oxygen consumption performed at the same time on the supernatant obtained after separation of the cells showed a progressive increase in oxygen consumption, that was dependent on both the concentration of DETA-NO to which cells had been originally exposed and the time of exposure (Figure 2b). The oxygen consumption in the supernatant fraction of cells treated with DETA-NO was reduced by $94 \pm 23\%$ by oxyHb; oxyHb had no effect on the supernatant of cells not treated with DETA-NO.

Targets of inhibition of cellular respiration by NO

Exposure of cells to DETA-NO (0.5–10 mM) in the presence of luminol (200 μ M) did not result in any luminol-enhanced chemiluminescence (following 1.3 and 5 h of exposure to NO; results not shown; $n = 3$), indicating that reactive oxygen intermediates were not responsible for the observed oxygen consumption.

Exposure of control cells to the protonophore FCCP (1 μ M) led to a $55.2 \pm 4.2\%$ ($n = 3$) increase in oxygen consumption. By contrast, FCCP did not have any effect on cell suspensions that had been exposed to DETA-NO (5 mM, 5 h). Incubation of cells in glucose-free, pyruvate-containing medium (20 mM) to bypass glycolysis changed neither the rate of oxygen consumption in the presence of 5 mM DETA-NO, nor the time of onset of irreversibility of the inhibitory effect of NO. Similarly, no changes in NO effects were observed when the citric acid cycle was inhibited with 3-nitropropionic acid (0.6 mM) and respiration sustained on β -hydroxybutyrate (Table 1). Thus the site of NO-induced inhibition of oxygen consumption is confined to the mitochondrial respiratory chain. Studies of potential targets of NO within the mitochondrial respiratory chain showed that inhibition of oxygen consumption occurring via complexes II–III–IV was likewise always fully reversible by oxyHb (Table 1).

Effects of NO on complex I activity

Complex I activity was measured directly in homogenates from cells treated with DETA-NO (5 mM, 5 h). Complex I activity was inhibited by DETA-NO (4.7 ± 0.6 versus 9.8 ± 1.7 pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ in control cells) to an extent similar to that observed after treatment with the complex I inhibitor, rotenone (4.6 ± 0.5 pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$). This inhibition was accompanied by a fall in the intracellular content of GSH (48 ± 1 nmol $\cdot$ mg $^{-1}$ in control cells versus 24 ± 2 nmol $\cdot$ mg $^{-1}$ in DETA-NO-treated cells (5 mM, 5 h).

To investigate the nature of complex I inhibition by NO, cells were either treated with the membrane-permeant GSH analogue, GSH ethyl ester (2 mM) or exposed to light. GSH ethyl ester and light treatment were applied either during the incubation with DETA-NO or when inhibition of respiration had become persistent (i.e. non-reversible by oxyHb). Both GSH ethyl ester and light, when present throughout the incubation, delayed the onset of persistent inhibition of oxygen consumption. Interestingly, in the presence of GSH ethyl ester, inhibition of oxygen consumption by DETA-NO was reduced by $17 \pm 3.0\%$ ($n = 4$). Furthermore, in those cells treated for 5 h with DETA-NO only, GSH ethyl ester and light partially restored the ability of oxyHb to reverse inhibition ($78 \pm 4.0\%$ and $74 \pm 8.0\%$ recovery of activity with GSH ethyl ester and light respectively; $n = 3$).

Effects of exogenous NO on extracellular oxygen consumption

In order to investigate the mechanism underlying the paradoxical increase in oxygen consumption by the supernatant, further experiments focused on the biochemical characteristics of this phenomenon.

No oxygen consumption was observed in the supernatant fraction of cell suspensions treated with decomposed DETA-NO (0.5–10 mM) or in incubation medium treated with DETA-NO in the absence of cells for up to 5 h. Thus, the oxygen consumption by the supernatant requires the presence of (a) factor(s) released by intact cells when exposed to stress.

Ultrafiltration of the supernatants using filters with different exclusion sizes showed that filtration through either a 3 or a 10 kDa membrane resulted in a $25 \pm 1.50\%$ ($n = 2$) loss of oxygen consumption whereas filtration through a 30 kDa membrane abolished it ($98 \pm 1.20\%$; $n = 2$). These results indicate that the oxygen-removing activity requires the presence of a compound with a molecular mass between 10 and 30 kDa. Protein measurements revealed the presence of relatively large concentrations of proteins in the supernatant (at 0 h, 0.31 ± 0.03 and 0.40 ± 0.03 μ g/ μ l from the control cells and 5 mM DETA-NO-treated cells respectively; at 3 h, 0.48 ± 0.09 μ g/ μ l and 1.20 ± 0.31 μ g/ μ l in the supernatant of control cells and 5 mM DETA-NO-treated cells respectively; $n = 4$). Ultrafiltration of the supernatant using filters with different exclusions showed that $88 \pm 2.1\%$ ($n = 2$) of the proteins present in the supernatant of cells exposed to 5 mM DETA-NO for 3 h had a molecular mass between 10 and 30 kDa.

The oxygen consumption by the supernatant was found to be temperature-sensitive, as storage of the supernatant at 37 °C for 1 h reduced oxygen consumption by $20 \pm 4.0\%$ ($n = 3$) and heating to 98 °C for 30 min completely abolished it ($99 \pm 2.0\%$;

$n = 4$). Furthermore, it was found to be photosensitive, as exposure of the supernatant to high intensity light for 1 h also abolished oxygen consumption ($98 \pm 1.9\%$; $n = 4$). These characteristics are reminiscent of *S*-nitrosothiol(s), the breakdown of which is known to be facilitated by the interaction with sulphhydryl (SH) groups of low-molecular-mass thiols such as GSH [16].

In cells exposed to DETA-NO (5 mM, 3 h), both GSH and GSNO were detected at concentrations ranging from 15 to 200 μM ($107.5 \pm 16.3 \mu\text{M}$; $n = 4$) and 5 to 23 μM ($14.6 \pm 2.6 \mu\text{M}$; $n = 8$) respectively. We tested the effect of the addition of GSH and GSNO on the supernatant. GSH (0.5 mM) gave an immediate increase ($87 \pm 2.2\%$; $n = 2$) in oxygen consumption in the supernatant of cells treated with DETA-NO (5 mM, 3 h) and increased the concentration of NO already present in the supernatant by $1.4 \pm 0.2 \mu\text{M}$. The addition of the same concentration of thiol to the supernatant of control cells was without any effect. Likewise, addition of GSH to the DETA-NO-containing incubation medium in the absence of cells did not lead to oxygen consumption or NO release. Addition of GSNO (0.01–0.1 mM) to either the incubation medium or the supernatants from DETA-NO-treated and control cells did not increase oxygen consumption to an appreciable extent (results not shown). Addition of GSH (0.5 mM) together with GSNO (0.03 mM) to the incubation medium produced only a small increase ($6 \pm 1.0\%$; $n = 3$) in oxygen consumption. Thus the formation and subsequent breakdown of GSNO cannot account for the observed increase in oxygen consumption stimulated by GSH.

Effects of endogenous NO on extracellular oxygen consumption

In order to investigate whether the effects observed with the high concentrations of DETA-NO could occur in pathophysiological conditions when NO is generated by the inducible NO synthase, J774 cells were activated for 18 h with IFN- γ (50 units/ml) and LPS (10 $\mu\text{g/ml}$) in the presence or absence of the NO synthase substrate, L-arginine. Oxygen consumption by the culture medium was then measured. The supernatant obtained from activated J774 cells showed an oxygen consumption which was absent from control cells. This oxygen consumption had the same properties as that observed with 5 mM DETA-NO. Thus ultrafiltration of the supernatant with either 3 or 10 kDa membranes resulted in a $22 \pm 1.4\%$ ($n = 2$) loss of oxygen consumption, whereas filtration over a 30 kDa membrane completely abolished it ($98 \pm 2.1\%$; $n = 2$). As with the supernatants of DETA-NO-treated cells, the oxygen consumption after the treatment of cells with LPS/IFN- γ was found to be sensitive to temperature and light. Moreover, oxygen consumption increased markedly on addition of GSH, albeit to a lesser extent than observed with 5 mM DETA-NO. This phenomenon was dependent on the generation of endogenous NO, since cells activated in L-arginine-free conditions did not show any increase in oxygen consumption.

DISCUSSION

In cells exposed to cytokines and bacterial products, several enzymes involved in oxygen consumption and energy generation become inhibited with time [5,8,18,19]. This phenomenon has been ascribed to the generation of NO [20,21]; however, so far it is not clear whether this is due to NO itself or to the generation of other species such as ONOO $^-$ which results from the interaction of NO with O $_2^-$ [22]. Both NO and ONOO $^-$ have been shown to inhibit enzymes in the respiratory chain and tricar-

boxylic acid cycle [5,8,18,19], but while the NO effects are more selective for complex IV, ONOO $^-$ induces irreversible inhibition and damage of several complexes [19]. Our results have indicated that NO (up to 1.5 μM) inhibits respiration, acting selectively and in a temporal sequence on complex IV and/or then complex I, while none of the other enzymes of the respiratory chain are affected [11].

There are pathological conditions, such as brain ischaemia, that may lead to generation of higher concentrations of NO (6–11 μM) [12,13]. The effects of NO on the mitochondrial respiratory chain under these conditions have not been investigated. Using concentrations of DETA-NO that produce NO concentrations of up to 8 μM , we have now demonstrated that the only enzymes of the respiratory chain affected by NO were complexes IV and I. Inhibition of complex IV was always reversible whereas the progressive, persistent inhibition of respiration induced by NO was due to inhibition solely of complex I. This was confirmed by direct measurement of the activity of the enzyme. Thus even at such high concentrations, the action of NO is still confined to these two enzymes and occurs in a defined temporal sequence.

The chemical modification responsible for persistent inhibition of complex I appears to be the same as that observed at lower NO concentrations, as it was reversed by addition of GSH and treatment with light. Our results show that exposure to high concentrations of NO does not lead to a widespread generation of O $_2^-$ and ONOO $^-$ in the cells, as we could not detect production of reactive oxygen species at any concentration of NO tested. Preliminary experiments have, however, suggested that nitrosation, due to the generation of ONOO $^-$, might occur in the microenvironment of complex I (N. Riobo, E. Clementi, M. Melani, A. Boveris, S. Moncada and S. J. Poderoso, unpublished work), following the generation of O $_2^-$ known to occur at this enzyme [23]. A localized generation of O $_2^-$, with subsequent formation of ONOO $^-$ in a precise location, might thus restrict the biological action of NO to a specific target and define microdomains in which its action can be predicted on the basis of the biochemical properties of the microenvironment. Our observations show that non-activated J774 cells exposed to high concentrations of NO for long periods exhibit a sequential inhibition of only two enzymes of the respiratory chain, whereas, in activated macrophages, a variety of enzymes have been reported to be inhibited [5,8,18,19]. Although in both situations the inhibition is NO-dependent, it is likely that the activated and non-activated cells provide a different biochemical environment in which NO exerts its actions, leading to different effects.

Interestingly, our experiments revealed an additional effect of NO, i.e. the extraction of oxygen by the extracellular medium, which was dependent on the time of exposure of cells to NO and on the NO concentration. This event was caused by the presence in the extracellular medium of one or more nitrosated species with an apparent molecular mass of 10 to 30 kDa. The consumption of oxygen was reduced by oxyHb, inhibited by exposure to light and temperature and increased by addition of GSH, suggesting that it may be caused by the release of NO or a NO-related species generated during the breakdown of protein nitrosothiol(s) released from the cells, and chemically interacting with the oxygen dissolved in the incubation medium.

Whether this nitrosated species in the supernatant resulted from intracellular events followed by the release of the nitrosothiol(s) into the extracellular medium, or from nitrosation of cytosolic constituents leaking out from stressed cells, has not been investigated here. However, incubating cells killed by freeze-thawing with DETA-NO for up to 5 h did not result in oxygen consumption in the extracellular medium (results not shown),

indicating that this phenomenon requires the presence of metabolically active cells.

The oxygen consumption in the supernatant may be explained in part by the breakdown of nitrosothiol(s) spontaneously, metal-catalysed or following the interaction with thiols, such as GSH, all of which are present in the extracellular medium. These reactions are accompanied by the release of either NO or nitroxyl ion (NO^-), both of which can further react with dissolved oxygen to form NO_2^- or ONOO^- respectively. The interaction of nitrosothiols with endogenous GSH can account for only part of the total effect, because the extent of oxygen consumption in supernatants where the low-molecular-mass fraction was removed by ultrafiltration was reduced by only 20–25%. The dramatic increase in oxygen consumption on addition of exogenous GSH is likely to be caused by an enhanced decomposition of nitrosothiol(s), leading to a burst of NO and possibly NO^- release and consequent oxygen consumption.

Extraction of oxygen by the extracellular medium is not restricted to cells exposed to exogenous NO, since it was also observed after cell activation with IFN- γ and LPS. In this case extracellular oxygen consumption was entirely dependent on the endogenous generation of NO, since it required the presence of L-arginine, without which these cells are unable to generate NO [24–26]. Extraction of oxygen by the medium of activated cells showed the same characteristics as observed with DETA-NO. This confirms that extracellular oxygen consumption is an event which is specifically dependent on NO and not due to other actions of DETA-NO.

Thus, following exposure to endogenous or exogenous NO for long periods, there is a decrease in oxygen utilization by cells due to two distinct but synergistic actions, one being the persistent inhibition of oxygen consumption by the mitochondria, and the other, the generation of a hypoxic microenvironment due to the extraction of oxygen by the extracellular medium. Whether such a situation occurs *in vivo* needs to be investigated, since such a synergistic deleterious effect may be responsible for the progression of pathology in some clinical conditions.

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