Hypoxia accelerates nitric oxide-dependent inhibition of mitochondrial complex I in activated macrophages

Matthew T. Frost, Qi Wang, Salvador Moncada, and Mervyn Singer

Wolfson Institute for Biomedical Research, University College London, London, United Kingdom

Submitted 27 July 2004; accepted in final form 11 October 2004

Hypoxia accelerates nitric oxide-dependent inhibition of mitochondrial complex I in activated macrophages. Am J Physiol Regul Integr Comp Physiol 288: R394–R400, 2005. First published October 14, 2004; doi:10.1152/ajpregu.00504.2004.—Excess production of nitric oxide (NO) is implicated in the development of multiple organ failure, with a putative mechanism involving direct mitochondrial inhibition, predominantly affecting complex I. The persistent effects of NO on complex I may be mediated through S-nitrosylation and/or nitration. The temporal contribution of these chemical modifications to the inhibition of respiration and the influence of concurrent hypoxia have not been previously examined. We therefore addressed these questions using J774 macrophages activated by endotoxin and interferon-γ over a 24-h period, incubated at 21% and 1% oxygen. Oxygen consumption and complex I activity fell progressively over time in the activated cells. This was largely prevented by coinubcation with the nonspecific NO synthase inhibitor 1-NN-o-(1-iminoethyl)-ornithine. Addition of glutathione ethyl ester reversed the inhibition at initial time points, suggesting an early mechanism involving nitrosylation. Thereafter, the inhibition of complex I became more persistent, coinciding with a progressive increase in mitochondrial nitration. Hypoxia accelerated the persistent inhibition of complex I, despite a reduction in the total amount of NO generated. Our results suggest that hypoxia amplified the mitochondrial inhibition induced by NO generated during inflammatory disease states.

S-nitrosylation; nitration; sepsis; respiration; oxygen

THE MECHANISMS THROUGH WHICH SEPSIS, THE SYSTEMIC INFLAMMATORY INSULT INITIATED BY A MICROORGANISM, RESULTS IN MULTIPLE ORGAN FAILURE REMAIN UNCLEAR (46). Excess production of nitric oxide (NO) is involved in the development of septic shock through its effect on vascular tone (31). However, its inhibitory effect on mitochondrial respiration may also be a potentially important pathophysiological mechanism underlying organ dysfunction. Our previous studies (7, 15) in cells exposed to exogenous NO demonstrated an early reversible inhibition of complex IV (24). Enhancement of free radical formation in activated cells may occur in septic shock (38). We have since demonstrated a direct correlation between clinical severity, NO production, complex I inhibition, and decreased ATP levels in skeletal muscle biopsies taken from septic shock patients (10) and a long-term animal model (12). This indicates an important role for NO in modulating cellular oxygen consumption and metabolism in sepsis and other systemic inflammatory states.

Peroxynitrite, formed by the reaction between NO and superoxide, inhibits complex I by S-nitrosylation (8, 9, 15) and by nitration (37). S-nitrosylation of thiol groups is a well-recognized mechanism of modification of enzyme activity, including that of complex I, and can be reversed by exposure to thioldegrading agents such as light and glutathione ethyl ester (9, 15). Specific nitrated tyrosine residues have also been identified on complex I after addition of ONOO− (37). The biological significance of S-nitrosylation and nitration remains uncertain (26), although, in a recent study, S-nitrosylation was functionally linked to the severity of endotoxic shock (30).

Hypoxia depresses mitochondrial respiration through decreased oxygen availability and can increase formation of reactive oxygen species even before reoxygenation (18, 29, 39, 45). This has been demonstrated to be due to an early reduction in the redox states of mitochondrial complexes, specifically complex IV (24). Enhancement of free radical formation in hypoxia may be highly pertinent to clinical shock states, as tissue hypoxia often coexists with sepsis, particularly in non-resuscitated patients.

We have therefore studied the temporal effects of endogenous NO and the influence of hypoxia on activated macrophages over a 24-h period, measuring mitochondrial respiration, complex I activity, ONOO− production, S-nitrosylation, and tyrosine nitration.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma Aldrich (Poole, Dorset, UK), except N-(tert-butylidemethylsilyl)-N-methyltrifluoroacetamide and ethyl heptafluorobutyrate (Fluorochrom, Old Glossop, Derbyshire, UK) and [13C5]tyrosine (Cambridge Isotope Labs, Andover, MA). L-N[15N]-o-(1-iminoethyl)-ornithine (t-NIO) was purchased from Alexis (Bingham, Nottingham, UK), and recombinant murine IFN-γ was from Insight Biotechnology (Wembley, Middlesex, UK). Culture medium and fetal bovine serum were obtained from Gibco (Paisley, Renfrewshire, Scotland).

Cell culture. J774 macrophages obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) were grown in suspension with the use of MicroCarriers modified stirrers (Techne Laboratories, Duxford, UK) in DMEM (GIBCO) supplemented with fetal bovine serum (10% vol/vol), l-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C in a humidified atmosphere containing 5% CO2, 74% N2, and 2% O2. For the hypoxia studies, cells were incubated in a humidified atmosphere containing 5% CO2, 94% N2, and 1% O2. By necessity, measurements were undertaken in a room air environment, although subsequent studies in which cell preparation (washing, harvesting, resuspension, lysis, and so forth) was performed in a hypoxia tent produced similar results (unpublished observations). Salmonella ty-
phosphate endotoxin LPS (10 μg/ml), IFN-γ (50 U/ml), and, in some experiments, the nonspecific NO synthase inhibitor l-NIO (1 mM) were added to the medium immediately before addition to the cells. Cell viability was assessed at 24 and 48 h by Trypan blue exclusion.

**Cell respiration (oxygen consumption).** Cells were suspended in the incubation medium (pH 7.2) at a density of 10^7 cells/ml and maintained at 37°C. LPS (10 μg/ml) and IFN-γ (50 U/ml), with and without l-NIO (1 mM), in both 1% (10 μM) and 21% (210 μM) O_2 environments were added in separate experiments. At specified time points (0, 4, 5, 6, 12, and 24 h), 1 ml was removed, centrifuged (at 3,000 g for 5 min), and resuspended in 1 ml of fresh cell culture medium. Oxygen consumption was analyzed in a gas-tight vessel maintained at 37°C, equipped with a Clark-type oxygen electrode (Rank Brothers, Bottisham, UK). This was assessed by the rate of fall in partial pressure of oxygen (P_(O2)) and subsequently corrected for protein. The oxygen electrode was precalibrated with air-saturated incubation media kept at 37°C, assuming an oxygen concentration of 210 μM. The rates of oxygen consumption were corrected for the drift experienced by the Clark electrode. To confirm that the oxygen consumption was mitochondrial, the complex III inhibitor myxothiazol (0.5 μM) was added at the end of each experiment when the P_(O2) was ~50 μM, or after 10 min. This produced >95% inhibition.

**Complex I activity.** The activity of complex I was assayed as described by Ragan et al. (41). Briefly, cells were plated at a density of 10^4 per 5 ml on tissue culture plates (150 × 25-mm plates; Marathon, London, UK) were washed at the appropriate time point, scraped with 0.5 ml of 50 mM Tris-HCl (pH 7.5) containing 0.1 mM DTT, 0.2 mM EDTA, and 10 μg/ml of a protease inhibitor cocktail (leupeptin, aprotinin, and antipain), centrifuged at 3,000 g for 5 min, washed, and stored at −80°C until analysis. The pellet was freeze thawed, and cellular homogenate (20 μl, 0.3 mg) was added to 1 ml of 10 mM potassium phosphate buffer, pH 8.0, containing 100 μM NADH in a 1-ml cuvette at 37°C. The rate of NADH oxidation was followed at 340 nm in a UV spectrophotometer (Uvikon-XS, Biotek, Loughborough, UK). After absorbance was recorded for 1 min, 5 μl of 10 mM ubiquinone-1 were added, and the stimulated rate of NADH oxidation (taken as complex I activity) was followed for 2 min more. The NADH oxidation rate was calculated from the slope of absorbance decrease over time using an extinction coefficient for NADH of 6.81 mM^-1 cm^-1 at 340 nm.

In some experiments, after the activation of the cells and the cellular respiration were measured, the sample was split in two; one was assayed for complex I activity immediately, whereas the other was incubated with glutathione ethyl ester (2 mM GSH ethyl ester) for 2 h before the assay.

**Nitrile.** We measured nitrile as a marker of NO production using the Griess method. Briefly, 80 μl of assay buffer containing Griess reagent (0.5% sulfanilamide, 0.05% naphthylethylene-diamine dihydrochloride, and 2.5% H_3PO_4) were added to 200 μl of cell culture medium, and the absorbance (A_540-A_620) was read on a Molecular Probes DAF-FM diacetate and dihydrodorhodamine 123 (DHFR123; Cambridge Biosciences, Cambridge, UK). Cells plated at a density of 10^5 per well (200 μl) in a 96-well plate were exposed to LPS (10 μg/ml) and IFN-γ (50 U/ml) in 21% or 1% O_2. At the appropriate time point, the medium was changed to Hanks’ solution, supplemented with 20 mM glucose, 0.5 mM arginine, and either 10 μM DAF-FM or 5 μM DHFR123. The cells were incubated for another 30 min with the respective probes and then excited at 495 nm, which emits at 515 nm for DAF-FM, or excite at 500 nm, which emits at 530 nm for DHFR123.

**Cellular 3-nitrotyrosine.** Nitrotyrosine levels were measured by a stable isotope dilution GC-MS method after alkaline hydrolysis, as previously described by Frost et al. (21). In brief, cell lysates were hydrolyzed in 1 ml of 4 M sodium hydroxide at 120°C overnight after the addition of 10 ng of [13C_9]nitrotyrosine as the nitrotyrosine internal standard. Samples were purified on an LC18 preparation column followed by further purification on an ENV+ cartridge (Supelco, Bellafonte, PA). The samples were then freeze dried and derivatized with heptafluorobutyrate and tertbutyldimethylsilyl ether. Samples were analyzed in the negative ion chemical ionization mode, with ammonia as the reagent gas. The initial column temperature was maintained at 180°C for 1 min and then increased to 300°C at 20°C/min over a period of 10 min for analysis. The ions m/z 518 and m/z 527 were specifically monitored for nitrotyrosine and [13C_9]nitrotyrosine, respectively. Concentrations were quantified based on the peak area of the internal standard compared with that of the sample. The lower limit of detection of nitrotyrosine in this assay is 1 pg (21).

**Cellular fractionation.** After treatment with LPS (10 μg/ml) and IFN-γ (50 U/ml) at 21% and 1% O_2 over a period of 24 h, J774 cells were pelleted (3000 g, 10 min), washed in PBS, and stored at −80°C until analysis. Cellular fractionation was carried out by following the method of Rickwood et al. (42). Briefly, the cell pellet was resuspended in pH 7.4 isolation buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2, 1 mM sodium EDTA, 1 mM sodium EGTA, 10 μM leupeptin, and 10 μM aprotinin) in 250 ml of sucrose. After homogenization (40 strokes, glass homogenizer), the cell membrane and nuclei were pelleted by centrifugation (2,500 g, 4°C). The resulting supernatant was removed, and the mitochondria were pelleted by centrifugation (12,000 g for 30 min), leaving the cytosolic fractions in the supernatant. The three fractions were then freeze dried and stored at −80°C until subsequent GC-MS analysis for nitration.

**Contribution of NO synthase.** Cells were coincubated with l-NIO for 24 h to investigate whether changes observed on activation by LPS or IFN-γ were NO synthase dependent.

To investigate whether the basal level of nitration was related to NO synthase, cells plated in six-well plates at a density of 10^5 cells/ml were left overnight to adhere and then incubated for 7 days with l-NIO. The medium was replaced every 2 days with fresh medium containing fresh l-NIO. Confirmation of continued l-NIO activity was performed as described by Moss et al. (35).

**Protein.** The protein content in the samples of all assays was measured with a bicinchoninic acid-based procedure (Pierce, Rockford, IL).

**Statistical analysis.** Repeated measures ANOVA was performed for overall statistical comparison between groups using StatView SE software for Macintosh. One-way repeated measures ANOVA with post hoc paired least-significant difference testing was used for assessing temporal change, and factorial analysis was used for intergroup comparisons. A P value of <0.05 was considered statistically significant.

**RESULTS**

Activation of J774 macrophages with LPS and IFN-γ resulted in progressive increases in NO (measured as DAF-FM fluorescence) and ONOO− (measured as DHFR123 fluorescence) over a 24-h period (both P < 0.05) (Figs. 1 A and B).
NO and ONOO\(^{-}\) were significantly higher in cells incubated in 21% O\(_2\) compared with 1% O\(_2\). This was also reflected by the progressive increase in nitrite measured over 24 h (Fig. 1C, \(P < 0.05\) compared with both nonactivated controls and activated cells in 1% O\(_2\)). Coincubation with L-NIO greatly reduced the accumulation of nitrite (Fig. 1C, \(P < 0.05\)).

At 24 h, cell viability, assessed by Trypan blue exclusion, did not alter significantly under any of the experimental conditions, although cell viability fell at 48 h, being >30%, >50%, and >80% in activated hypoxic, activated normoxic, and nonactivated hypoxic cells, respectively.

Complex I activity in nonactivated cells incubated in either 21% or 1% O\(_2\) showed no change over 24 h, whereas oxygen consumption was significantly reduced in the 1% O\(_2\) environment (\(P < 0.05\)) (Fig. 2). In activated cells, there was no detectable change in either oxygen consumption or complex I activity over the first 5 h, after which there was a progressive 70–80% fall (\(P < 0.05\)) in both oxygen consumption and complex I activity, which was closely correlated (Fig. 3). The decrease occurred earlier in the 1% O\(_2\) group (6 h, \(P < 0.05\) vs. 21% O\(_2\)), but no further change was observed after 12 h. A similar degree of inhibition was observed in both oxygen environments at 24 h (Fig. 2). Coincubation with L-NIO reversed the fall in both oxygen consumption and complex I activity by \(\sim 65–80\%\) (\(P < 0.05\)) at both oxygen concentrations. Incubation of activated macrophages with GSH ethyl ester for 2 h in non-LPS- or cytokine-containing medium resulted in a partial reversal of the fall in oxygen consumption and complex I activity (\(P < 0.05\)). This was more effective at earlier time points; e.g., at 21% O\(_2\) there was a 60% reversal of...
inhibition at 12 h compared with a 15% reduction at 24 h. At 12 h, the reversibility of complex I by GSH ethyl ester was significantly less in the activated cells incubated in 1% O2 compared with those in room air.

A low basal concentration of total cell tyrosine nitration (measured by GC-MS) was detected in nonactivated cells, and this remained unchanged over 24 h (0.6 ± 0.3 ng/mg protein) in both normoxia and hypoxia. This low concentration was not affected by 7 days of coincubation with l-NIO (data not shown), suggesting that basal nitration may be independent of NO synthase. However, in the activated cells, tyrosine nitration increased progressively over time (Fig. 4). As with nitrite, coincubation with l-NIO significantly reduced tyrosine nitration by 80–90% in both the 21% and 1% O2 environments. Although initially accelerated in the low oxygen environment, tyrosine nitration was twofold higher in the normoxic cells by 24 h (both *P < 0.05). This early rise seen with hypoxia was due to increased nitration within the mitochondrial fraction (Fig. 5). Tyrosine nitration also increased significantly in the cytosolic fraction but not in the membrane or nuclear fraction (data not shown).

iNOS protein could not be detected in nonactivated cells incubated in either 21% or 1% O2 over the 24-h period (Fig. 6). However, there was a progressive increase in iNOS protein expression in the activated macrophages, peaking at 12 h. iNOS protein expression was greater in activated cells maintained at 21% than at 1% O2. Coincubation with l-NIO markedly attenuated the fall in iNOS protein at 24 h.

**DISCUSSION**

Our results show that NO generated via the NO synthase pathway by LPS- and IFN-γ-activated macrophages modulates the activity of complex I of the mitochondrial respiratory chain. This inhibitory effect increases over time and is accelerated by a low oxygen environment, despite less NO and ONOO− being generated. The degree of reversibility of complex I inhibition by GSH ethyl ester falls as mitochondrial nitration increases, indicative of an initial S-nitrosylation followed by progressive tyrosine nitration of the complex. The direct relationship found between cellular oxygen consumption and complex I activity across the different conditions tested (cytokine activation and/or hypoxia) emphasizes the importance of this particular enzyme as a rate-limiting component of mitochondrial respiration in pathological states.

NO affects mitochondrial respiration by inhibiting complex IV and complex I (15). Its inhibition of complex IV is rapid in onset, reversible, and competitive with oxygen (17). By contrast, inhibition of complex I induced by exogenous NO occurs later, is dependent on oxidative stress, and is increasingly persistent (7). Addition of GSH ethyl ester or cold light reverses the early inhibition of complex I, implicating a mechanism involving S-nitrosylation (9, 15). Indeed, nitrosylation of cysteine thiols was recently confirmed as an important mechanism of NO action in endotoxic shock (30).

Riobo et al. (43) suggested that tyrosine nitration was the likely mechanism responsible for the later onset, irreversible inhibition of complex I seen with exogenously administered NO. This was specifically attributed to mitochondrial generation of superoxide and ONOO− because it could be partially prevented by superoxide dismutase and uric acid and could be mimicked by exogenous administration of ONOO−. Our present findings support the presence of a similar process following endogenous NO production. The approximate 80% reversal of the fall in oxygen consumption at 24 h using a NO synthase inhibitor emphasizes the importance of this pathway. NO itself is unlikely to be directly responsible for either

---

**Fig. 3.** Correlation of complex I activity with oxygen consumption in activated J774 macrophages at 21% and 1% oxygen. Macrophages were activated for up to 24 h at 21% oxygen (○) and 1% oxygen (●) before harvesting for measurement of oxygen consumption and maximal complex I activity.

**Fig. 4.** Tyrosine nitration by J774 macrophages activated with LPS and IFN-γ at 21% oxygen (A) and 1% oxygen (B). Macrophages were incubated in the presence of LPS and IFN-γ. At the indicated time points, the cells were harvested, and total cell nitration was assessed by GC-MS at 21% or 1% O2. †*P < 0.05 signifies overall significant differences between groups (repeated measures ANOVA). *P < 0.05, signifies significant difference between 21% and 1% O2 groups at specific time points (by factorial analysis).
S-nitrosylation or nitration, whereas ONOO$^-$, formed in the mitochondria by reaction between NO and superoxide, is capable of both (40). Murray et al. (37) recently reported high levels of complex I inhibition following ONOO$^-$ exposure. With the use of an enriched complex I preparation, they demonstrated nitration of tyrosine residues on specific subunits, in particular B14 and B15. ONOO$^-$ is considered the predominant source for mitochondrial tyrosine nitration in most cell lines, although other mechanisms could also exist because both peroxidases (13) and cytochrome c (14) can catalyze nitration of tyrosine residues.

Although the functional pathophysiological significance of protein nitration in vivo remains uncertain, increased nitration has been described in a variety of human disease states, including sepsis (6, 25). The temporal relationship we that observed between increasing levels of ONOO$^-$ and mitochondrial tyrosine nitration, the progressively persistent inhibition of complex I and oxygen consumption, and prevention by coinubcation with a NO synthase inhibitor further implicate nitration as an important mechanism of respiratory inhibition. Indeed, we have previously found reduced complex I activity (and decreased ATP levels) in muscle biopsies taken from patients in septic shock and animal models that could not be reversed by the ex vivo addition of GSH ethyl ester (10, 12). In this situation, complex I activity is likely to recover only on repair (5, 22, 27) or production of new protein (or affected subunits) (29) and/or new mitochondria, provided that cellular viability is maintained. Unlike Moss and Bates (36), who described significant cell death in macrophage and microglial cell lines after 20-h incubation with LPS and IFN-γ, we and others (19) found that cell viability was maintained at 24 h using a similar protocol. Interestingly in one of these studies, it was found that a concentration of NO that was nontoxic at 21% O$_2$ triggered cell death in fibroblasts under hypoxic (1.5% O$_2$) conditions at 48 h but not at 24 h (28).

Specific nitrated and nitrosylated complex I subunits causing loss of functional activity remain to be identified. A recent study by Elfering et al. (20) demonstrated that nitration within mitochondrial subfractions mainly affected proteins within metabolic pathways (78.4%) compared with energy transduction (16.2%) and structural functions (5.4%); however, no particular pathway had all its mitochondrial constituents nitrated.

Complex I activity and oxygen consumption were affected at an earlier time point in activated macrophages maintained at 1% O$_2$, despite the fact that these cells produced lower levels of NO, ONOO$^-$, and tyrosine nitration at 24 h than cells maintained at 21% O$_2$. This was paralleled by a more rapid appearance of tyrosine nitration in the mitochondrial fraction of the cells maintained at 1% O$_2$. There is an earlier diminution in complex IV activity resulting from reduced oxygen availability in the presence of NO competing for the heme-copper active site and elevating the apparent K$_m$ for oxygen (17). It is likely that this would lead to more rapid reduction of the electron transport chain, earlier production of superoxide and ONOO$^-$, and thus faster S-nitrosylation and nitration of complex I. Although superoxide generation is dependent on O$_2$ concentration (2) and is formed in excess during reoxygenation, production arising from complex I and the ubisemiquinone site on complex III also increases in the reduced state.
which characterizes cellular hypoxia (18, 29, 39). Thus, even before reoxygenation, there is sufficient NO and superoxide present to form ONOO\(^{-}\), albeit in reduced amounts. Nevertheless, its potency in nitrating tyrosine residues and inhibiting cellular respiration is significantly enhanced in the low oxygen environment.

Unlike the findings from an ex vivo study (3), we and others (1, 19, 32, 33) did not detect any increase in NO synthesis in nonactivated cells under hypoxic conditions, yet all observed a significantly decreased generation once activation, although Daniliuc et al. (19) reported no increase in iNOS protein under similar conditions. This can be related to either a change in the level of NO synthase protein and/or NO production rates by the enzyme. We found that iNOS protein was induced in activated cells at 1% O\(_2\) but to a lesser extent than in cells exposed to 21% O\(_2\). McCormick et al. (32) also reported no enhancement of iNOS mRNA in hypoxic macrophages exposed to a combination of LPS and IFN-\(\gamma\). The large fall in NO synthesis seen during hypoxia could also be explained by an elevation in the apparent \(K_m\) for oxygen of the iNOS enzyme related to formation of a heme-NO complex (1) or by affecting the interaction between the enzyme complex and the cytoskeleton, resulting in displacement of iNOS from submembranal zones and subsequent loss of activity (19). Lack of substrate availability, altered arginine transport, and changed apparent \(K_m\) for arginine were ruled out (32). Further studies could usefully delineate the relationship between expression, protein levels, and activity of the iNOS isoform during hypoxia and/or activation.

Coincubation with l-NIO, a nonspecific NO synthase inhibitor, considerably diminished (70–80%), but did not completely abolish, the elevated levels of nitrate and tyrosine nitration in activated cells in both normoxic and hypoxic conditions. A fivefold increase in l-NIO dosage had no additional effect (unpublished observations), whereas incubation of unstimulated macrophages with l-NIO for up to 1 wk did not reduce the low basal level of tyrosine nitration. This suggests either an inability of l-NIO to inhibit NO synthase completely or the existence of non-NO synthase-dependent nitrating mechanisms, as described above (13, 14, 34). As previously reported with other NO synthase inhibitors or scavengers (4, 23, 36), we found that iNOS protein was greatly increased at 24 h in the presence of l-NIO. A similar pattern was seen at 1% O\(_2\), albeit at lower levels. This is considered to be due to the loss of negative feedback of NO upon NO synthase transcription and/or translation.

In summary, we have presented evidence indicating that hypoxia accelerates NO-dependent inhibition of complex I and mitochondrial respiration. Our new findings are of potential clinical relevance because sepsis-induced multigorgan failure is associated with mitochondrial dysfunction (10–12) and because body tissues may experience reduced oxygen tension in a variety of severe inflammatory conditions, including unsuscitated sepsis. This emphasizes the need for prompt interventions that reduce the synergistic effect of coexisting tissue hypoxia (44).

ACKNOWLEDGMENTS

We thank Annie Higgs for critical reading of this manuscript.

GRANTS

Matthew Frost is currently supported by the Medical Research Council.

REFERENCES


Downloaded from http://ajpregu.physiology.org/ on March 30, 2017