Oxygen tension limits nitric oxide synthesis by activated macrophages

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Previous studies have established that constitutive calcium-dependent ('low-output') nitric oxide synthase (NOS) is regulated by oxygen tension. We have investigated the role of oxygen tension in the synthesis of NO by the 'high-output' calcium-independent NOS in activated macrophages. Hypoxia increased macrophage NOS gene expression in the presence of one additional activator, such as lipopolysaccharide or interferon-γ, but not in the presence of both. Hypoxia markedly reduced the synthesis of NO by activated macrophages (as measured by accumulation of nitrite and citrulline), such that, at 1 % oxygen tension, NO accumulation was reduced by 80–90 %. The apparent K_m for oxygen calculated from cells exposed to a range of oxygen tensions was found to be 10.8 %, or 137 μM, O_2 This value is considerably higher than the oxygen tension in tissues, and is virtually identical to that reported recently for purified recombinant macrophage NOS. The decrease in NO synthesis did not appear to be due to diminished arginine or cofactor availability, since arginine transport and NO synthesis during recovery in normoxia were normal. Analysis of NO synthesis during hypoxia as a function of extracellular arginine indicated that an altered V_{max}, but not K_m^{Arg}, accounted for the observed decrease in NO synthesis. We conclude that oxygen tension regulates the synthesis of NO in macrophages by a mechanism similar to that described previously for the calcium-dependent low-output NOS. Our data suggest that oxygen tension may be an important physiological regulator of macrophage NO synthesis *in vivo*.

Key words: arginine transport, mouse, nitric oxide synthase (NOS), oxygen, regulation.

INTRODUCTION

Nitric oxide (NO) is an important biological gas with many diverse functions. It is synthesized by a family of enzymes called the NO synthases (NOSs), which are both induced and constitutively expressed in various tissues. Three isoforms have been characterized to date, which possess important structural and functional differences. Neuronal (type I) and endothelial (type III) NOSs are activated by changes in intracellular calcium. The latter isoform is acylated in the N-terminus and thus is associated with membranes in caveolae. The third isoform, first isolated from activated macrophages (type II, also known as inducible NOS or iNOS), is independent of intracellular calcium and is regulated by many factors via transcriptional activation. Each isoform catalyses the five-electron oxidation of arginine to form citrulline and NO, a reaction requiring five cofactors and two co-substrates, NADPH and dioxygen (for reviews see [1–6]).

The synthesis of NO by activated macrophages is regulated by many factors. Endotoxin and interferon-γ (IFN-γ) are the two most important immunologically relevant activators/enhancers of NO synthesis. On the other hand, glucocorticoids and transforming growth factor-β are potent inhibitors of NO synthesis by macrophages [7–12]. The availability of the substrate, arginine, has also been suggested to be a mechanism of regulating NO synthesis *in vivo*, especially since arginine appears to be depleted in regions of macrophage activity [13,14]. This would seem unlikely, however, given that the K_m^{Arg} for all isoforms of NOS is < 10 μM, a concentration well below that thought to exist in cells. A resolution to this paradox in endothelial cells has been suggested [15].

The co-substrate, dioxygen, has received much less attention as a factor that may limit the synthesis of NO. In fact, reduced oxygen tension (hypoxia) has been shown to increase the expression of the genes encoding both the endothelial and macrophage NOS isoforms [16,17]. Moreover, functional hypoxia response elements have been identified in the promoter of the mouse macrophage NOS gene [16,18] which are identical to those found in the erythropoietin gene [19]. Interestingly, although the NOS gene appears to be activated by hypoxia, NO synthesis may not be increased under these conditions [16]. We have investigated the role of oxygen tension in the synthesis of NO by activated macrophages. We show that an oxygen tension similar to that present in tissues limits the synthesis of NO by more than 50 %, and that this condition does not substantially affect arginine availability. Our data suggest that oxygen may limit (regulate) the synthesis of NO by macrophages *in vivo*.

EXPERIMENTAL

Cell cultures

The murine macrophage cell line RAW 264.7 was obtained from American Type Cell Culture (Bethesda, MD, U.S.A.). Primary mouse macrophages were obtained from BALB/c mice by peritoneal lavage 4 days after the intraperitoneal administration of 1 ml of 4 % thioglycollate. Elicited cells were allowed to attach in complete medium for 3 h of incubation (37 °C; air/5 % CO_2), after which time the medium was changed and cells were continued in culture for 18 h prior to experiments. Cells were incubated at 37 °C with 5 % CO_2 in RPMI medium supplemented with 10 % (v/v) fetal calf serum (Hyclone, Logan, UT, U.S.A.), 100 μg/ml streptomycin and 100 units/ml penicillin.

Cells were maintained under various oxygen tensions by using a glass desiccator (210 mm internal diam.) equipped with a modified top (19 mm-thick Plexiglas) with inlet and outlet gas ports. The top was sealed using silicon sealant and six clamps to form an airtight seal. The end of the outlet tubing outside the

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; IFN-γ, interferon-γ; LPS, lipopolysaccharide; NOS, nitric oxide synthase; iNOS, inducible NOS.

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chamber was immersed in water to prevent equilibration with atmospheric air when flow was stopped. The end of the outlet tubing inside the chamber was positioned at the bottom of the chamber, directly above the water, to facilitate efficient flushing. The chamber was flushed at the beginning of the experiment (and thereafter every 24 h) for 25 min at a flow rate of 660 ml/min. The experimental gas mixtures were obtained as bottled gas and ranged from 1% to 15% O_2, each containing 5% CO_2 and the balance N_2. The oxygen concentration was verified by measuring the outflow of gas at the end of the initial flushing period and at the end of each 24 h period using a Fyrite apparatus (Bacharach Inc., Pittsburgh, PA, U.S.A.). All media were pre-equilibrated with the specific gas mixture for at least 24 h before use. In some experiments, cells were activated with lipopolysaccharide (LPS; from *Escherichia coli*, serotype 055:B5; Sigma, St. Louis, MO, U.S.A.) and/or IFN-γ (recombinant mouse; Gibco, Rockville, MD, U.S.A.). Unless stated otherwise, all chemicals were obtained from Sigma.

Nitrite assay

NO is oxidized to a stable intermediate, nitrite, which accumulates in the medium of cells. Nitrite accumulation was used as a measure of NO synthesis, and was determined by the Griess procedure [20]. An aliquot of medium (100 μl) was mixed with an equal volume of Griess reagent in a 96-well plate. The subsequent absorbance of each well was determined using an ELISA reader equipped with a 520 nm filter. The concentration of nitrite was determined from a standard curve prepared from $NaNO_2$.

Analysis of iNOS mRNA

The isolation of total RNA was essentially as described previously [21]. Mouse iNOS cDNA was kindly provided by Dr Q. W. Xie and Dr C. Nathan (Department of Medicine, Cornell Medical School. New York, NY, U.S.A.) [22], and human β-actin cDNA was obtained from A.T.C.C. cDNA probes were biotinylated using a random primer kit (Renaissance; NEN Life Sciences, Boston, MA, U.S.A.). Hybridization and washing were conducted as described by the manufacturer.

Citrulline assay

Citrulline is also a product of the NOS reaction, and is synthesized in equimolar amounts relative to NO. We measured citrulline as an alternative measure of the synthesis of NO. Citrulline was quantified by reverse-phase HPLC. A portion of 450 μl of medium was mixed with 50 μl of 40% sulphosalicylic acid and allowed to precipitate on ice for 15 min. The supernatant was obtained by centrifugation (15000 ***g*** for 15 min) and neutralized with NaOH. A 10 μl aliquot of the sample was derivatized with *o*-phthaldialdehyde/2-mercaptoethanol as described elsewhere [23] using an automatic sample injector (Waters WiSP 710B). Samples were separated by reverse-phase chromatography using a C18 column (Phase Separations, Norwalk, CT, U.S.A.; 4.5 mm $\times$ 15 cm) and a fluorescence detector equipped with an integrator. Two solutions were used to create the gradient for the mobile phase, solvent A (100 mM sodium acetate, 9.5% methanol and 0.5% tetrahydrofuran) and solvent B (95% methanol). After sample injection and derivatization, the sample was chromatographed at 1 ml/min for 5 min with 2% B, followed by the separation gradient of 2% B to 14% B developed over a 34 min period. Purified standards were added to control medium.

Arginine transport

Arginine transport was measured as the initial rate of uptake of tracer arginine (L-[2,3,4,5-^{3}H]arginine; 60 Ci/mmol; Amersham, Piscataway, NJ, U.S.A.) in the presence of 25 μM arginine. We employed sodium-free buffers. All transport studies were conducted in 24-well culture plates. Following experimental treatment, cells were washed with 1 ml of warm transport buffer (140 mM choline, 5.9 mM KCl, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.6 mM glucose, 25 μM arginine and 10 mM Hepes, pH 7.4). A 500 μl portion of transport buffer was then added to each well, followed by 10 μl of 'working' [^{3}H]arginine (diluted from stocks in transport buffer to contain approx. 500000 d.p.m.), with constant agitation. After exactly 4 min, transport buffer was aspirated and 1 ml of ice-cold transport buffer containing 10 mM lysine was added to the well. Following an additional 1 ml rinse, 0.5 ml of 0.1 M NaOH was then added to the well to solubilize the cells. A 25 μl aliquot was removed from each well for protein determination. The remainder was transferred to 7 ml of scintillation fluid and radioactivity was determined using liquid scintillation spectrometry. Total counts were determined by adding 10 μl of working [^{3}H]arginine tracer to 500 μl of 0.1 M NaOH and then to 7 ml of scintillation fluid.

Kinetic analysis

We investigated the potential effects of reduced oxygen tension on the kinetics of nitrite production by activated macrophages at various L-arginine concentrations. Mouse macrophages (RAW 264.7) were seeded (200000 cells) into 24-well plates containing complete Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum and antibiotics. After 24 h, the medium was changed and cells were activated with 1 μg/ml LPS and 10 units/ml IFN-γ. After 24 h, cells were washed with Hanks-buffered salt solution, and then 1 ml of arginine-free DMEM ($-$ArgDMEM) containing 10% (v/v) fetal calf serum and antibiotics was added. Cells were maintained for 6 h in 1 ml of $-$ArgDMEM and at 21% or 2% O_2. Subsequently, the $-$ArgDMEM was removed and replaced with 1 ml of $-$ArgDMEM containing various concentrations of added arginine. Cells were continued in either 21% O_2 or 2% O_2 for 12 h. Nitrite accumulation thereafter was determined as described previously. Data were subjected to kinetic analysis.

RESULTS

iNOS gene expression during hypoxia

The effect of hypoxia on the expression of the iNOS gene in activated RAW 264.7 cells and the combined effects of LPS and IFN-γ under these conditions have not been examined previously. We evaluated the effect of an oxygen tension of 1% on cells which were activated with LPS (1 μg/ml), IFN-γ (50 units/ml) or the combination of both. Cells were exposed to 1% oxygen at the time of activation. The accumulation of iNOS mRNA relative to β-actin mRNA was considerably greater in cells exposed to 1% O_2 when cells were treated with either LPS or IFN-γ (Figure 1). Cells treated with the combination of inducers, however, showed no hypoxia-induced enhancement of iNOS mRNA. This latter result was confirmed in an additional experiment investigating the restoration of oxygen tension (see Figure 5B). These results indicate that RAW 264.7 cells respond similarly to ANA-1 murine macrophages [18]. Perhaps more importantly, our data indicate that hypoxia does not enhance iNOS mRNA accumulation when both LPS and IFN-γ are employed, a result not previously reported.

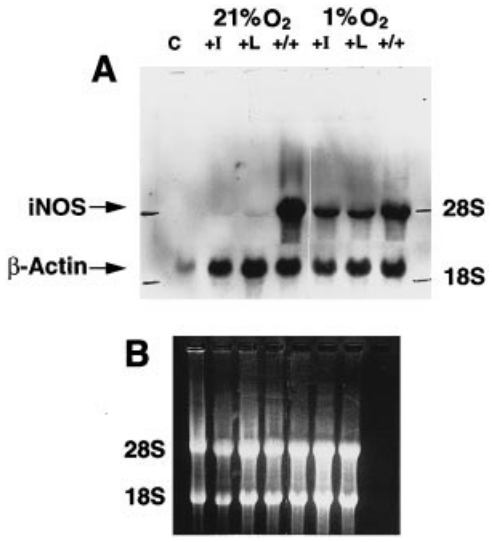

Figure 1 Effects of hypoxia (1% O_2) on the accumulation of iNOS mRNA in macrophages treated with LPS, IFN-γ or both

Mouse macrophages were exposed for 24 h to normoxia (21% O_2) or hypoxia (1% O_2) and treated with 1 μg/ml LPS (+L), 50 units/ml IFN-γ (+I) or both LPS and IFN-γ (+/+). Total RNA was isolated and analysed for iNOS and β-actin mRNAs. The figure shows one of three independent experiments with similar results. Densitometry determinations indicated a significantly greater abundance of iNOS mRNA in cells treated with either LPS or IFN-γ when exposed to hypoxia, but this effect was not apparent if cells were treated with both LPS and IFN-γ.

NO synthesis under conditions of hypoxia

We addressed the question of whether increased iNOS gene expression during hypoxia leads to increased NO synthesis. Previous studies with various cell types show conflicting results [24,25]. The effect of hypoxia (1% or 6% compared with a normal tension of 21% O_2) on the accumulation of nitrite by activated murine macrophage is shown in Figure 2. Cells were exposed to 1% or 6% O_2 for 24 h or 48 h prior to or immediately following (0 h) activation with LPS (1 μg/ml) or IFN-γ (10 units/ml), or the combination of activators. Cells exposed to 1% O_2 (Figure 2A) either immediately or for 24 h before activation produced markedly less nitrite than those exposed to air/5% CO_2. A similar experiment using 6% oxygen (Figure 2B) resulted in less inhibition. For example, cells treated with both LPS and IFN-γ and exposed to 6% O_2 showed an approx. 50% decrease in nitrite accumulation, compared with a decrease of > 82% in similar cells exposed to 1% O_2 (Figure 2A). Similar trends were observed in cells activated with either LPS or IFN-γ. Comparable results were obtained in additional experiments using 2% O_2 and 10 or 50 units/ml IFN-γ alone or in combination with LPS (not shown). These results clearly indicate that lowering the oxygen tension decreases nitrite accumulation in cultures of activated macrophages.

Dose–response relationship

Various concentrations of oxygen were then employed to determine the range of effective oxygen tensions over which NO is synthesized by activated murine macrophages. Cells were exposed at the time of activation (1 μg/ml LPS and 10 units/ml IFN-γ) to oxygen concentrations of 1%, 2%, 4%, 6%, 12% and ambient. In each experiment, results were expressed as a percentage of those in control cells maintained under ambient

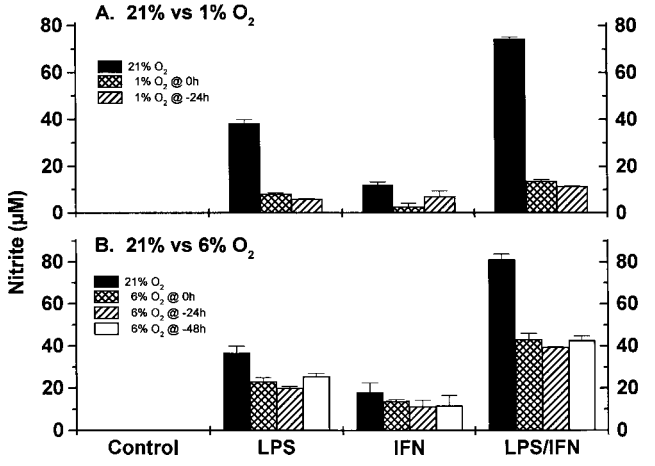

Figure 2 NO synthesis by activated macrophages during exposure to hypoxia

Mouse macrophages were exposed to hypoxia [either 1% O_2 (**A**) or 6% O_2 (**B**)] at the time of activation (0 h), 24 h before activation (−24h) or 48 h before activation (−48h). Cells were activated with either LPS (1 μg/ml) or IFN-γ (10 units/ml) or the combination of both LPS and IFN-γ. After 24 h of activation, the medium was sampled for nitrite accumulation. Results represent means ± S.D. of three observations. The experiment in each panel was repeated once for both transformed macrophages and primary macrophages (see Figure 4), with identical results.

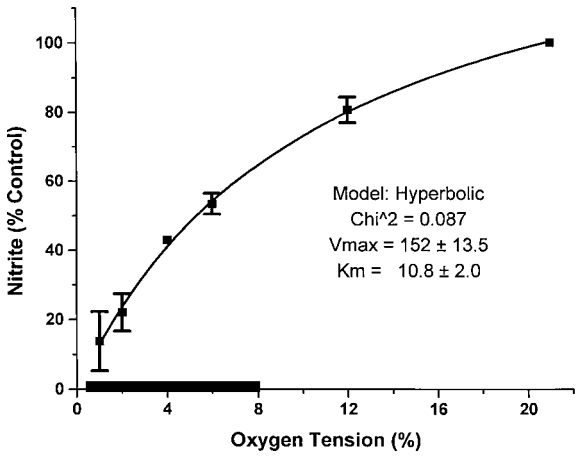

Figure 3 Effect of oxygen tension on the synthesis of NO by activated macrophages

Mouse macrophages were activated with LPS (1 μg/ml) and IFN-γ (10 units/ml) for 24 h, during which time they were exposed to various concentrations of oxygen (1, 2, 4, 6, 12 or 21%). Following 24 h of activation, medium nitrite was determined. For each experiment, nitrite accumulation for the hypoxia treatment was normalized to that of a control (21% O_2). Values represent means ± S.D. for 4–6 experiments, except for 4% O_2, which represents a single observation. Kinetic parameters for a hyperbolic response were determined: V_{max} = 152 nmol/24 h per ml and K_m = 10.8% O_2. The black bar on the *x* axis represents the range of oxygen tensions observed in various tissues [26].

conditions. The results (Figure 3) indicate a hyperbolic response to increasing concentrations of oxygen. The calculated K_m was determined from hyperbolic curve fitting to be 10.8% (64.9 Torr or 137 μM O_2). The conversions from % O_2 to μM O_2 were performed using published relationships that were obtained by measurement of actual values [24]. In addition, this analysis assumes that NO synthesis by murine macrophages could be

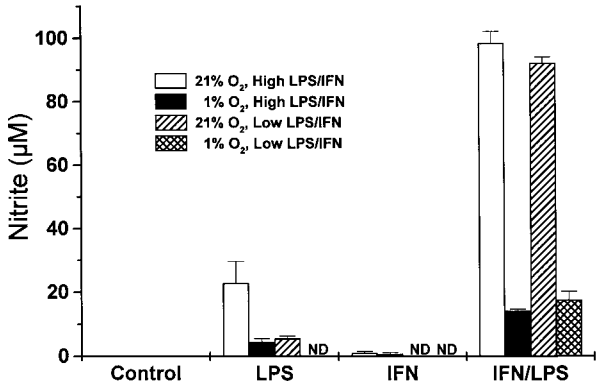

Figure 4 Effect of hypoxia on NO synthesis by activated primary macrophages

Primary macrophages were obtained from BALB/c mice and maintained in culture for 18 h prior to study. Macrophages were activated with either 'High LPS/IFN' (5 μg/ml and 20 units/ml respectively) or 'Low LPS/IFN' (1 μg/ml and 10 units/ml respectively) for 48 h, during which time they were exposed to 1% O_2. Nitrite accumulation was completely inhibited at 24 h after activation (results not shown). Values represent means $\pm$ S.D. of three observations. An additional experiment was conducted at 2% O_2 with similar results. ND, not detected.

greater than 100% at oxygen tensions greater than ambient (21%). The results indicate a considerable potential for control by oxygen tension under physiological conditions, since the oxygen tension in tissues ranges from 4% to 6% [26].

Hypoxia and nitrite accumulation in activated primary macrophages

We then addressed the possibility that hypoxia uniquely affects NO synthesis in transformed cells. We hypothesized that primary macrophages may not exhibit a similar sensitivity to hypoxia. Primary macrophages were elicited by thioglycollate administration by peritoneal lavage 4 days before cell harvest. Cells were isolated and maintained in culture for 18 h before activation and/or treatment. Cells were activated with LPS (1 or 5 μg/ml) or IFN-γ (10 or 20 units/ml) or a combination of both, and transferred to normal or hypoxic conditions. The accumulation of nitrite by cells under various conditions is shown in Figure 4. Primary cells exhibited a comparable sensitivity to hypoxia, i.e. nitrite accumulation over 24 h of activation by LPS and IFN-γ was decreased by approx. 85% in cells exposed to 2% oxygen relative to controls (21% oxygen). These results are very similar to those presented for transformed murine macrophages, and suggest that cell source (primary versus cell line) is not an important determinant in the mechanism by which NO synthesis is inhibited during hypoxia.

Effect of hypoxia on citrulline accumulation

Variations in oxygen tension could influence the formation of stable oxidation products of NO (nitrite and nitrate). As a result, levels of actual NO synthesis may be underestimated under conditions of low oxygen tension. As an alternative to nitrite accumulation, we determined the accumulation of citrulline as an independent measure of NO synthesis. Citrulline accumulates in macrophage cultures in direct proportion to NO synthesis [27] and is less likely to be directly affected by oxygen tension.

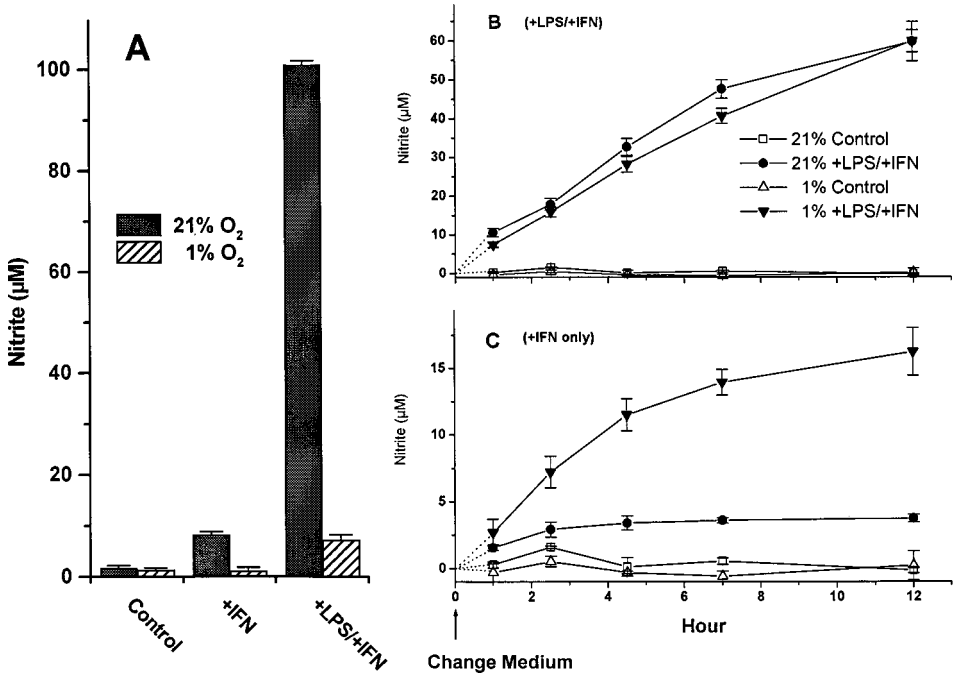

Figure 5 NO synthesis during reoxygenation of activated macrophages previously exposed to hypoxia

Mouse macrophages were activated with IFN-γ (10 units/ml) or the combination of LPS (1 μg/ml) and IFN-γ (10 units/ml) and exposed to 1% O_2 or 21% O_2 for 24 h. Nitrite analysis was then performed on samples of medium (**A**). The media for cells maintained in both normoxia and hypoxia were then changed, and the accumulation of nitrite was determined over 12 h at intervals of approx. 1 h (**B** and **C**). Data are means $\pm$ S.D. of three observations.

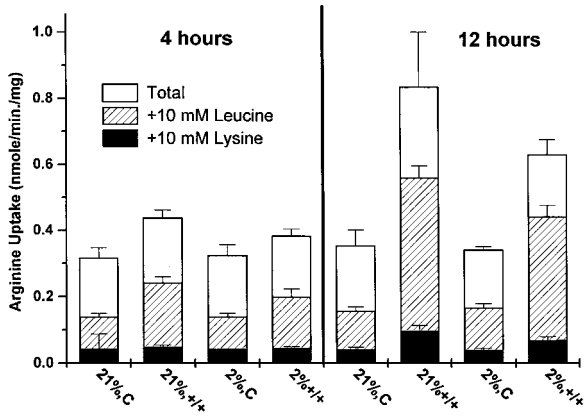

Figure 6 Arginine uptake by primary mouse macrophages following activation in both normoxia and hypoxia

Primary mouse macrophages were not activated (C) or were activated with LPS (1 μg/ml) plus IFN-γ (10 units/ml) (+/+) for 4 or 12 h, during which time they were also exposed to normoxia or hypoxia (21% or 2% O_2 respectively). Following each of these periods (4 h and 12 h), arginine transport was determined during a 4-min incubation in normoxia. The data are means $\pm$ S.D. of four observations. The total bar height represents the total sodium-independent uptake of arginine by macrophages. Hatched sections represent sodium-independent uptake by the y^+ transporter system (i.e. that not inhibited by leucine). The solid sections represent non-saturable arginine transport (i.e. not inhibited by excess lysine).

Analysis by HPLC of total citrulline in culture medium from activated murine macrophages showed it to be similarly decreased by low oxygen tension. Direct quantification of citrulline accumulation over 24 h in activated-cell medium showed an approx. 80% decrease when compared with controls (0.625 ± 0.34 compared with 4.22 ± 0.44 relative area units). These data support the conclusion that induced citrulline synthesis (and hence NO synthesis) is, in fact, decreased under conditions of reduced oxygen tension.

Functional viability of macrophages exposed to hypoxia

In order to assess the viability of activated macrophages exposed to hypoxia, we conducted experiments to evaluate NO synthesis in cells immediately upon reoxygenation. We reasoned that, if hypoxia were lowering metabolic activity and perhaps substrate/cofactor (NADPH, tetrahydrobiopterin, haem, etc.) availability, cells would be compromised immediately after exposure to hypoxia. Our data do not support such a contention. Cells exposed to normal oxygen tension immediately after hypoxia showed no difference in their response when compared with cells cultured continuously in ambient air/5% CO_2 (Figures 5B and 5C). In fact, cells exposed to hypoxia and treated with IFN-γ only (Figure 5C) showed an elevated synthesis of nitrite during reoxygenation when compared with similarly treated controls. This latter result is consistent with elevated iNOS mRNA abundance (and presumably iNOS enzyme) in cells exposed to IFN-γ and hypoxia (see Figure 1). In summary, these data suggest that cells exposed to hypoxia (2% O_2) do not appear to be compromised metabolically, and that substrate availability does not appear to limit NO synthesis. We have also measured the growth of these transformed cells under conditions of 2% O_2, and have found no effect of this oxygen tension on cell

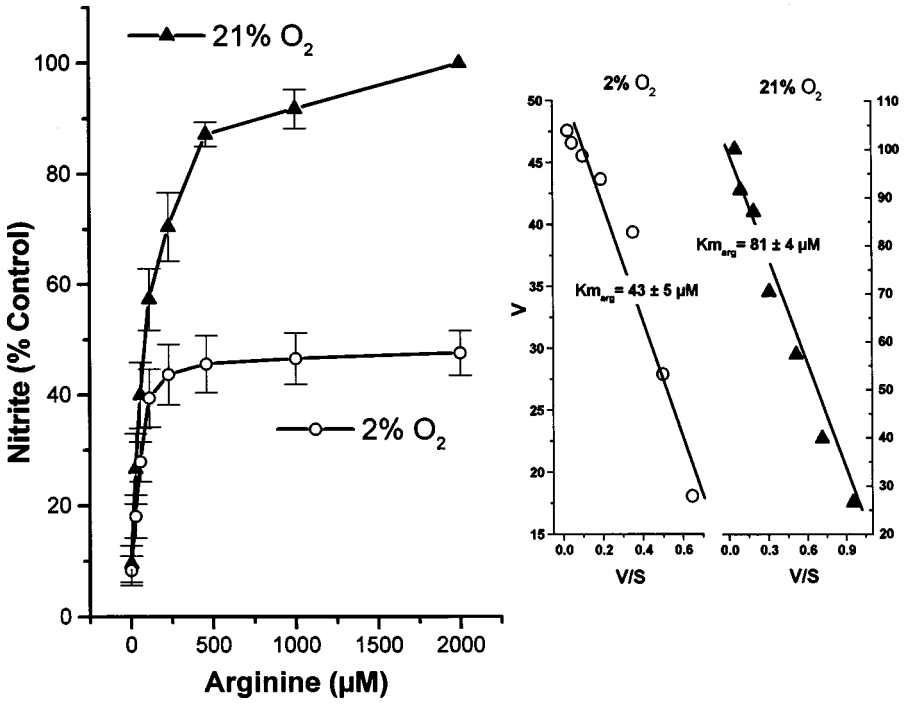

Figure 7 Kinetic analysis of the relationship between NO synthesis and extracellular arginine for activated macrophages exposed to hypoxia

Mouse macrophages were activated with LPS (1 μg/ml) and IFN-γ (10 units/ml) for 24 h in normoxia. At the end of this period, the medium was changed and various concentrations of arginine were added, and the cells were placed under conditions of either normoxia (21% O_2) or hypoxia (2% O_2) for 4–6 h in medium containing no arginine. The medium was then changed and various concentrations of arginine were added, and cells were incubated under experimental conditions for 12 h. The data are means $\pm$ S.D. of three independent experiments. The left panel shows nitrite accumulation for cells exposed to normoxia (21% O_2) or hypoxia (2% O_2). The right panel represents an Eadie–Hofstee plot of the data, showing calculated V_{max} and K_m^{Arg} values for both conditions.

growth over 4 days when compared with that observed under normoxic conditions (results not shown).

Arginine transport is not affected by hypoxia

Several reports have indicated that arginine transport in endothelial cells is decreased by hypoxia [28,29]. Since extracellular arginine is essential in the synthesis of NO, at least for macrophages [30], we questioned whether arginine transport might be decreased under our conditions of hypoxia in activated macrophages, thereby reducing the availability of arginine for NO synthesis. We analysed arginine transport using near-physiological concentrations of tracer arginine (25 μM) at two different times after activation with both LPS and IFN-γ. The results (Figure 6) indicate that activation resulted in a 2.35-fold increase in arginine transport by 12 h after activation in control cells. The increase exhibited by cells exposed to 2 % O_2 was slightly less (1.85-fold), but the absolute magnitude seemed to vary somewhat. This experiment was repeated two times with essentially the same results. We concluded that the small difference in the rate of arginine transport, especially that accounted for by the CAT2 transporter (i.e. not inhibited by leucine; hatched segments in Figure 6), is unlikely to account for the profound difference in NO synthesis (80–90 % decline) seen under these conditions.

Kinetics of NO synthesis during hypoxia

Our final question concerned the kinetics of NO synthesis, and whether oxygen alters (increases) the apparent K_m of the system (iNOS, Arg transporter, etc.) for arginine. Since arginine transporter abundance appeared to be minimally affected by hypoxia, other mechanisms, such as an altered apparent affinity for arginine (for either the transporter or iNOS), were investigated. In these experiments, macrophages were activated prior to exposure to hypoxia (2 % O_2) to increase the amount of NOS present. The concentration of extracellular arginine was then varied and NO synthesis was assessed over a 12 h period. A similar approach has been used to evaluate arginine transport characteristics in brain astrocytes and the effect of cytokines [31]. The kinetic analysis of NO synthesis is shown in Figure 7. Results were expressed as a percentage of those in control cells (21 % O_2) to facilitate a comparison of three experiments. The exposure of activated macrophages to hypoxia (2 % O_2) decreased the V_{max} considerably (by approx. 50 %). However, the apparent K_m for arginine was not significantly higher as hypothesized, but was somewhat lower, suggesting that the decrease in NO synthesis observed during hypoxia was not the consequence of an alteration in this kinetic parameter. Other experiments using 6 % O_2 led to similar conclusions (results not shown).

DISCUSSION

The results of the present study clearly demonstrate that hypoxia markedly affects the synthesis of NO by activated macrophages. Macrophage iNOS gene expression is increased by hypoxia in the presence of an additional stimulus (LPS or IFN-γ), but not in the presence of both. Moreover, our data indicate that oxygen tensions that are normally present in tissues (4–6 %) appear to limit the synthesis of NO by at least 50 % (relative to ambient conditions). Our preliminary assessment of the mechanism appears to eliminate arginine availability (transport) as a site of control. Arginine transport via the y^+ basic transporter does not appear to be affected significantly by hypoxia, although research with endothelial cells suggests that it may be a target of hypoxia [28,29]. Our overall conclusion is that oxygen availability clearly modulates the synthesis of NO by macrophages and that it may be the limiting factor *in vivo*.

In the present study we have focused primarily on the macrophage iNOS isoform. Other reports have suggested that oxygen may limit the synthesis of NO by the constitutively expressed calcium-dependent NOS isoforms. The most important difference between these enzymes is the quantity of NO produced – micromolar and subnanomolar respectively. The first report of a role for oxygen tension in NO synthesis by the calcium-dependent isoform was by Kim et al. [32]. These workers reported that oxygen may be a rate-limiting factor for NO production in the penile corpus cavernosum. The model they proposed suggested that oxygen tension in venous blood is insufficient to support NO synthesis, and that vasodilatation is initiated by the parasympathetic stimulation of intracavernosal blood flow. In this 'oxygen-enhanced' environment, additional NO is synthesized and mediates smooth muscle relaxation and erection. Earlier, Rengasamy and Johns [33], using bovine cerebellum homogenates as a source of NOS, found that hypoxia (28 mmHg O_2) lowered the V_{max} for arginine by approx. 50 %. They suggested that hypoxia inhibits NOS activity 'primarily through depletion of oxygen, one of the substrates for the enzyme'. These studies have highlighted the importance of oxygen availability in the synthesis of NO by the 'low-output' calcium-dependent NOS isoform.

Our present study represents the first direct characterization of the cellular oxygen requirement for the macrophage 'high-output' NOS isoform (iNOS). We show that, despite enhanced NOS gene activation, NO synthesis is decreased in proportion to oxygen tension over a physiologically relevant range. In fact, the K_m (10.8 % O_2 or 137 μM O_2) was considerably higher than the measured oxygen tension of 4–6 % in most tissues [26]. Our results are strikingly similar to those of Dweik et al. [34], who studied NO synthesis in a static lung model system. They demonstrated that inspired oxygen tension appeared to 'regulate' NO levels in bronchiolar gas at end-expiration. The calculated 'K_mO_2' was reported to be 192 μM, which was similar in their study to the K_mO_2 of 135 μM for purified recombinant murine iNOS. Based on these similarities and previous observations that airway epithelium expresses predominately iNOS, the authors proposed that iNOS is the mediator of the vascular response to oxygen in the lung. Other estimates of the oxygen requirement for NOS have been reported previously. They range from 400 μM [35] for purified type I NOS to 6.3 μM [36] for cytosolic extracts of induced mouse macrophages. Our direct estimates using activated macrophages in cell culture and those of Dweik et al. [34] for purified iNOS suggest that iNOS is regulated by oxygen tension in a physiologically relevant range.

Hypoxia may affect cellular respiration and metabolism, and thus decrease cellular levels of co-substrates required for NOS activity. Specifically, hypoxia appears to reduce arginine transport by the y^+ cationic amino transporter in endothelial cells [28,29]. This effect occurs within hours of exposure and is not reversed upon return to normoxia for 24 h. To our knowledge, there are no published reports of arginine transport by activated macrophages exposed to hypoxia. This is in marked contrast with the many studies that have demonstrated increased arginine transport in macrophages following activation [37–40]. It appeared to us that, since arginine uptake is increased in activated macrophages, this was a logical mechanism to explain the decrease in NO synthesis observed during hypoxia. However, two lines of evidence obtained in the present study appear to indicate that arginine availability is not compromised during hypoxia. The strongest evidence is the lack of an effect of hypoxia on arginine uptake by activated macrophages (Figure

6). The uptake observed in the presence of 10 mM leucine (without sodium) represents uptake of arginine by the y^+ cationic transporter that is found in activated macrophages as well as other cells. This particular transporter is characterized by its independence of sodium, its high affinity (K_m 100 μM) for dibasic amino acids and the ability of substrate on the opposite (trans) side of the membrane to increase transport activity [41]. Transport was increased significantly (and comparably) by activation in macrophages, regardless of the oxygen tension. The increase observed was similar to that reported previously for activated macrophages [37–39]. To explain the 80% decrease in NO synthesis at 2% O_2, we anticipated that arginine uptake would be comparably decreased. Since this clearly was not the case, we conclude that arginine transport is not the mechanism by which hypoxia lowers NO synthesis. The second line of evidence to support this conclusion is the result shown in Figure 5(B). Within 1 h of restoring normal oxygen tension, NO synthesis was restored comparably, independent of the oxygen tension to which the cells had been previously exposed. Since the effect of hypoxia on arginine uptake in endothelial cells is not restored upon reoxygenation, we surmise: (1) that the effects of hypoxia on macrophage and endothelial arginine transport are not similar, and (2) that substrate availability appears not to be compromised by hypoxia.

A final possibility tested in the present study was the effect of low O_2 tension on the kinetic characteristics of the NOS system. It seemed plausible that low oxygen tension might shift the 'requirement' of NOS for substrate, namely arginine. The most direct way of assessing this was to evaluate the kinetic relationship between NO synthesis and arginine concentration in intact cells. Stevens et al. [31] employed a similar approach to evaluate the role of extracellular arginine in NO synthesis by astroglial cells. They found hyperbolic kinetics of 'inducible NOS' activity, measured as a function of extracellular arginine, and calculated a K_m of 46 ± 3 μM. This value is very similar to that determined in the present study (Figure 7) for cells exposed to either normoxia or hypoxia (2% O_2). Granger et al. [30] also reported a K_m for arginine of approx. 73 μM in activated murine macrophages. It is noteworthy that these values are very similar to those reported for the arginine transporter, i.e. approx. 50 μM for astroglial cells [31] and approx. 100 μM for macrophages [37–40]. Overall, these results imply that NO synthesis by cells maintained in culture is determined by the availability of extracellular arginine and is dependent upon the active transport of arginine. Our results comparing the kinetic parameters of NO synthesis as a function of arginine concentration suggest that hypoxia does not lower NO synthesis by a mechanism related to a change in the apparent requirement for arginine. The measured K_m for arginine was not significantly increased by hypoxia (Figure 7). We conclude that alterations in this kinetic parameter do not appear to explain the marked change (decrease) in NO synthesis by hypoxia.

Overall, our study demonstrates directly that oxygen may be a physiological regulator of the synthesis of NO by the 'high-output' iNOS of activated macrophages. This now appears to be a general phenomenon for NOS, since others have shown that the 'low-output' calcium-dependent NOS is also regulated by oxygen tension. Our data suggest that hypoxia limits NO synthesis by directly limiting O_2 availability as a substrate, and not indirectly by affecting the supply of other necessary substrates or cofactors. It is not clear, however, why an additional mechanism (O_2 availability) exists to limit NO synthesis, especially since these conditions up-regulate the expression of the NOS gene. Perhaps NOS assumes a different role under conditions of decreased oxygen availability. Studies have clearly indicated that NOS can generate superoxide when cellular arginine levels are decreased [42]. The possibility remains that reduced oxygen tension may have a similar effect.

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