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pO₂ Dependent NO Production Determines OPPC Activity in Macrophages

Mary A. Robinson¹, Stephen W. Turtle¹, Cynthia M. Otto^{2,3}, and Cameron J. Koch¹

¹Department of Radiation Oncology, School of Medicine, University of Pennsylvania Philadelphia PA 19104

²Department of Clinical Studies-Philadelphia, School of Veterinary Medicine, University of Pennsylvania Philadelphia PA 19104

³Center for Sleep and Respiratory Neurobiology, University of Pennsylvania Philadelphia, PA 19104

Abstract

Stimulated macrophages produce nitric oxide (NO) via inducible nitric oxide synthase (iNOS) using molecular O₂, L-arginine, and NADPH. Exposure of macrophages to hypoxia decreases NO production within seconds suggesting substrate limitation as the mechanism. Conflicting data exist regarding the effect of pO₂ on NADPH production via the oxidative pentose phosphate cycle (OPPC). Therefore, the present studies were developed to determine whether NADPH could be limiting for NO production under hypoxia. Production of NO metabolites (NOx) and OPPC activity by RAW 264.7 cells was significantly increased by stimulation with lipopolysaccharide (LPS) and interferon γ (IFN γ) at pO₂ ranging from 0.07% to 50%. OPPC activity correlated linearly with NOx production at pO₂ > 0.13%. Increased OPPC activity by stimulated RAW 264.7 cells was significantly reduced by 1400W, an iNOS inhibitor. OPPC activity was significantly increased by concomitant treatment of stimulated RAW 264.7 cells with chemical oxidants such as hydroxy-ethyldisulfide or pimonidazole, at 0.07% and 50% O₂, without decreasing NOx production. These results are the first to investigate the effect of pO₂ on the relationship between NO production and OPPC activity, and to rule out limitations in OPPC activity as a mechanism by which NO production is decreased under hypoxia.

Keywords

NADPH; reactive nitrogen mediated stress; oxidative stress; inflammation

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Address Correspondence to: Cameron J. Koch, PhD, Department of Radiation Oncology, School of Medicine, University of Pennsylvania, Philadelphia PA 19104, Tel. 215 898-0073, Fax 215 898-0090, kochc@mail.med.upenn.edu.

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INTRODUCTION

Nitric oxide (NO) production is a key component of the macrophage response during inflammation (1, 2). Macrophages stimulated by pathogen associated molecular patterns (PAMPs) produce NO from molecular O₂, L-arginine, and NADPH via inducible nitric oxide synthase (iNOS) (3). NO production clearly depends on the partial pressure of O₂ (pO₂) (4–6), and the estimated cellular K_m O₂ (14 Torr (4) to 77 Torr (5)) is within the physiological range (5 to 71 Torr (7–13)). Systemic and/or tissue hypoxia develops during several inflammatory diseases (10, 14, 15), extending the pO₂ range for tissue macrophages to even lower levels, and potentially limiting NO production *in vivo*. Acute exposures to hypoxia have been shown to rapidly (within seconds) and reversibly decrease NO production by PAMPs stimulated macrophages without decreasing iNOS protein or assembly (6), providing evidence for the dynamic regulation of NO production by substrate limitation. While molecular O₂ is clearly limiting, an effect of hypoxia on the electron donor NADPH represents a second potential mechanism that has not been investigated in macrophages.

NADPH is constitutively produced by NADP⁺ dependent malic enzyme and isocitrate dehydrogenase. When oxidative stress is low, the ratio of NADPH:NADP⁺ is greater than 100:1. These enzymes do not have the capacity to maintain this high ratio under conditions of oxidative stress. Increased [NADP⁺] activates glucose-6-phosphate dehydrogenase (G6PD), the initial and rate limiting enzyme of the oxidative pentose phosphate cycle (OPPC) (16). Studies using G6PD mutants have demonstrated the unique role of the OPPC in maintaining adequate bio-reducing reserves (17).

An association between NADPH production by G6PD and the production of NO has previously been demonstrated in endothelial cells (18–20), an insulin-producing pancreatic beta cell line (RINm5F) (21), and macrophages (22–25). In macrophages, stimulation with PAMPs was found to elicit a parallel increase in OPPC activity (26–28) and NO production (25), by increasing G6PD activity and metabolic flux through the OPPC, rather than via the malic enzyme (29). Moreover, pharmacologic inhibition of OPPC activity or G6PD deficiency was shown to significantly impair NO production by stimulated macrophages (22–24).

Reduction of NADP⁺ via the OPPC does not require molecular O₂. Classical studies have measured increased [NADPH] in response to short term hypoxia due to the absence of metabolically-generated oxidizing agents (30–33). More recently, however, [NADPH] was found to be significantly decreased in denuded bovine coronary arteries following brief exposure to hypoxia (~ 8 to 10 Torr, 20 minutes (34)). This effect was attributed to increased glycolytic flux, resulting in the redirection of substrate (glucose-6-phosphate) away from the OPPC. However, OPPC activity was not directly measured in these experiments. These results suggest NADPH availability may be limited during acute hypoxia in some tissues and/or cell types.

The primary goal of this study was to investigate the relationship between pO₂ (0.07% to 50%), NO production, and OPPC activity in PAMPs stimulated and unstimulated

macrophages. We chose to study RAW 264.7 cells due to the extensive literature on the pO_2 dependence of NO production in these cells (4–6), and due to their abundant and persistent NO production (35). First, we hypothesized that 4 hours of hypoxia would decrease NO production and OPPC activity without affecting iNOS concentration or iNOS dimerization. Second, we hypothesized that pO_2 would not limit the response of OPPC activity. To test this, we imposed additional oxidative stress by adding the chemicals hydroxyethylthiolsulfide (HEDS) or pimonidazole. These two compounds have substantially different modes of action. HEDS is reduced through thiol-disulfide reactions ultimately linked to maintenance of reduced glutathione (36–38), whereas pimonidazole (a 2-nitroimidazole) is reduced via the cytochrome p450 system (17). We found that these additions increased the flux of the OPPC, but did not modify NO_x production. Thus, our results suggest that pO_2 , and not NADPH availability, affects NO production by PAMPs stimulated macrophages during acute hypoxia.

METHODS

Cell Culture

RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, Utah) and 1% antibiotic/antimycotic (penicillin, streptomycin, amphotericin B; Invitrogen) for up to 10 passages. Glass dishes and vials were cleaned and baked at 420°C, then treated with 75 mM sodium carbonate (Fisher, Fair Lawn, NJ) and 15% FBS for 1 hour at 37°C, treated with 0.2% gelatin (BioRad, Richmond, CA) for 20 minutes, and dried under UV light prior to plating the cells. For experiments, 10⁶ cells were plated onto 20 mL glass vials (inner diameter ~ 24 mm), or 1.5 × 10⁶ cells were plated onto the center of 50 mm glass dishes (39). Cells were then cultured overnight with 3 mL of MEM (Invitrogen, 11095) supplemented with penicillin, streptomycin, 15% FBS, non-essential amino acids, and 1 mM pyruvate. Stimulation of RAW 264.7 cells was performed by treating cells overnight (at least 18 hours) with 1 µg/mL LPS (*E. coli* O111:B4; Sigma) and 100 U/mL CHO-derived recombinant mouse IFN γ (Cell Sciences, Canton, MA). Immediately prior to experiments, the media was replaced with 1 mL of low glucose (2 mM) MEM buffered with 25 mM HEPES (i.e. instead of sodium bicarbonate to prevent saturation of the filter paper with unlabeled CO₂ during the measurement) and containing 5% FBS, non-essential amino acids, 1% penicillin and streptomycin, and 2 mM glutamine. The glucose was labeled with ¹⁴C at either the C1 or C6 position in order to quantify oxidation by either the TCA or OPPC (see below). HEPES-buffered media pH was not affected by pO_2 or any of the other treatment conditions (data not shown). Where indicated, NO_x production was inhibited by treating cells with 100 µM N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide, dihydrochloride (1400W; Cayman Chemical, Ann Arbor, MI) in the absence of L-arginine for 1 ½ hours immediately prior to switching to low glucose media. To chemically induce oxidative stress, 2 mM hydroxyethylthiolsulfide (HEDS) or 2 mM pimonidazole (PIMO) were added to the low glucose media.

pO₂ Control with 'Thin Film' Cell Culture

RAW 264.7 cells were cultured at 0.07%, 0.13%, 0.24%, 0.61%, 2%, 10%, or 50% O₂ for 4 hours at 37°C in vials or dishes contained in leak proof aluminum chambers, which enabled precise control of headspace pO₂ as previously described (31, 39–41). Briefly, glass vials were capped with a rubber stopper containing a center well (Kimble Chase, Vineland, NJ) with a 1 × 0.5 cm Whatman GF/B glass-fiber filter soaked with 100 µL 5% KOH (31). A 25G 5/8 inch needle was inserted into the stopper to enable slow gas exchange during the evacuations and pressurizations for oxygen control, while limiting gas exchange under the constant pressure conditions during the subsequent incubation (31). Vials or dishes were placed in aluminum chambers and subjected to a series of gas exchanges with N₂ or O₂ to produce the desired headspace pO₂. Chambers were warmed to 37°C and shaken continuously (64 cycles per minute) to ensure adequate gas exchange between the headspace and the media throughout the experiment (4 hours). The pO₂ in the chambers was measured at the end of the incubation period using a polarographic oxygen electrode. However, the pO₂ in the headspace of each vial was not directly accessible. Additionally, the depth of the medium layer in the vials did not conform to the “thin-layer” model that was originally developed in 50 mm glass dishes (39). Thus, in separate experiments, we added 100 µM EF5 to both dishes and vials, and assayed for EF5 adducts using flow cytometry as previously described (40, 42) in order to directly assess cellular pO₂. Note that in the experiments presented, pO₂ is defined as the percentage of oxygen in 1 atmosphere of dry gas at 37°C (i.e. 100% = 760 mm of Hg).

NO_x Measurement

Nitrite, nitrate, and nitrosothiols (NO_x) were measured in media or cell lysates by injecting 20 µL of sample into a reaction chamber containing a VCl₃/HCl mixture (0.4 g VCl₃ in 50 mL 1 N HCl) heated to 90°C. The resulting NO was continuously flushed with helium into a Sievers Nitric Oxide Analyzer 280i (GE Analytical Instruments, Boulder, CO) for reaction with ozone and measurement via chemiluminescence. Quantification was performed by comparison to standards prepared with NaNO₂.

Electrophoresis and Immunoblotting

Cell lysates were prepared by washing cell monolayers with ice cold cell rinse (6.8 g/L NaCl, 400 mg/L KCl, 122 mg/L NaH₂PO₄ anhydrous, 1 g/L glucose, 25 mM HEPES, pH 7.2), and then scraping cells into 0.4 mL ice cold protease-inhibitor containing hypotonic lysis buffer (1:1000 Protease Inhibitor Cocktail P8340 (Sigma-Aldrich, St. Louis, MO), 10 µM phenylmethylsulfonyl fluoride (Sigma-Aldrich) in dH₂O). Lysates were subjected to 3 freeze/thaw cycles (−70°C to 25°C). Cell lysate protein concentration was measured using the Biorad DC protein assay (Hercules, CA). Proteins (5 µg) were separated on a 7.5% Tris-HCl gel using SDS PAGE or low temperature SDS PAGE (LT SDS PAGE) as previously described (6), transferred to polyvinylidene fluoride (Immobilon™-FL 0.45 µm; Millipore, Bedford, MA), and immunoblotted for iNOS (1:2000; NOS2 M19 sc650, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). β actin (1:20,000; Monoclonal anti-β actin Clone AC-15 A5441, Sigma-Aldrich) was used as the loading control. Primary antibodies were immunocomplexed with IRDye™ 800 goat anti-rabbit or goat anti-mouse (1:10,000;

Rockland, Gilbertsville, PA). Proteins were detected, documented, and analyzed using an Odyssey Imaging System and software (LiCor Biosciences, Lincoln, NE).

OPPC and TCA Activity

OPPC activity and TCA activity were measured as previously described (Figure 1 and Supplemental Figure 1 in reference 31). RAW 264.7 cells were cultured in the presence of 2 mM glucose labeled with ^{14}C at either the C1 or C6 position at a specific activity of 200 $\mu\text{Ci}/\text{mmol}$ glucose in 1 ml of bicarbonate-free MEM. At the completion of each experiment, the vials were removed from the aluminum chambers, the needle was removed from the rubber stopper, and cellular metabolism was stopped by injection of 100 μL 6 N acetic acid into the media; the acidification step also releases CO_2 from the medium into the gas phase. $^{14}\text{CO}_2$ was collected on a 5% KOH saturated filter overnight at room temperature. The filter was removed and the $^{14}\text{CO}_2$ was counted with a Packard liquid scintillation counter. TCA activity leads to release of $^{14}\text{CO}_2$ from either the 1- ^{14}C or 6- ^{14}C position of glucose, while OPPC activity causes release of $^{14}\text{CO}_2$ only from the 1- ^{14}C position. Thus, OPPC activity was calculated using parallel vials and subtracting $^{14}\text{CO}_2$ produced in the presence of 6- ^{14}C glucose from the $^{14}\text{CO}_2$ produced in the presence of 1- ^{14}C glucose (Figure 1). Because of the use of radioactivity and the specificity of CO_2 capture, this method is extremely sensitive (nmol range) and specific. It has the additional advantage that the “background” activity due to the TCA cycle is very low compared to that due to the OPPC (see Table 1).

$$\text{OPPC activity} = (^{14}\text{CO}_2 \text{ from 1-}^{14}\text{C glucose}) - (^{14}\text{CO}_2 \text{ from 6-}^{14}\text{C glucose})$$

Statistics

The apparent K_m and $V_{\max}$ values were calculated by SigmaPlot Enzyme Kinetics Module 1.1 using a Michaelis-Menten non-linear analysis. Comparison of means was tested by ANOVA for the effect of pO_2 on measured values, and by two way ANOVA for the effect of pO_2 and treatment on measured values. Data presented are mean $\pm$ SD.

RESULTS

Control of Cellular pO_2

Cellular pO_2 was regulated using a modified version of the ‘Thin Film’ culture method developed in our laboratory (39). To ensure that the modified method (i.e. glass vials with rubber stopper and needle) provided the same pO_2 at the cellular level, we compared the cellular pO_2 in glass dishes with the cellular pO_2 in glass vials via the measurement of EF5 protein adducts (Figure 2). The formation of EF5-protein adducts increases as the pO_2 decreases in a quantitative manner, thus permitting an accurate measurement of cellular pO_2 because the pO_2 is constant between the gas and liquid phases (39). The pO_2 dependence of EF5 binding for RAW 264.7 cells incubated on glass dishes was similar to results from other cultured cell lines (42). Importantly, the EF5 binding for cells in glass vials closely paralleled EF5 binding for cells on glass dishes (Figure 2).

pO₂ dependence of NOx production

NOx, iNOS protein levels, and iNOS dimerization were measured after 4 hours of 'Thin Film' cell culture with the low glucose media required for the OPPC measurements. The results obtained in low glucose media were similar to previous cell culture systems (Figure 3 and references (4, 6)). Cumulative NOx released into the media by LPS and IFN γ -stimulated RAW 264.7 cells fit a Michaelis-Menten kinetic model with K_mO_2 of 0.66 ± 0.12 % (5 ± 1 Torr) and a V_{max} of 25.2 ± 1.0 nmol/10⁶ cells over 4 hours ($R^2 = 0.91$, Figure 3A). NOx measured in lysates of LPS and IFN γ -stimulated RAW 264.7 cells exposed to 50% O₂ for 4 hours was 3.5 ± 1.1 nmol/10⁶ cells (i.e. about 13% of NOx detected in the media), suggesting the majority of NOx produced were released into the media. In the absence of LPS and IFN γ stimulation, RAW 264.7 cells did not produce detectable NOx (data not shown). pO₂ did not alter iNOS protein concentration (Figure 3B) or iNOS dimerization (Figure 3C). Stimulation of RAW 264.7 cells with LPS and IFN γ decreased total protein isolated from the vials by 30%, consistent with previous reports (6, 43). pO₂ alone did not affect cell adhesion as visualized by light microscopy, or the amount of total protein isolated from the vials (data not shown).

pO₂ dependence of OPPC Activity

Unstimulated RAW 264.7 OPPC activity exhibited a biphasic response to pO₂ (Figure 4A), with a decrease between 0.07% and 2% O₂ and a 2.5 fold increase between 10% and 50% O₂. Stimulation of RAW 264.7 cells with LPS and IFN γ for 18 hours significantly increased OPPC activity at all pO₂ ($p < 0.001$). OPPC activity correlated linearly with NOx production for pO₂ greater than 0.13% O₂ in LPS and IFN γ stimulated cells (Figure 4B). Treatment of LPS and IFN γ stimulated RAW 264.7 cells with 1400W, an iNOS inhibitor, completely inhibited NOx production (Table 1), and OPPC activity decreased close to levels observed without LPS and IFN γ treatment ($p < 0.001$; Figure 4A, Table 1). 1400W also decreased OPPC activity in unstimulated RAW 264.7 cells (Table 1) and unstimulated RAW 264.7 cells treated with PIMO (data not shown) at 50% O₂, but not 2% and 0.07% O₂, suggesting possible nonspecific effects of the inhibitor at 50% O₂. Treatment of RAW 264.7 cells with LPS and IFN γ decreased TCA activity (Table 1), consistent with NOx-mediated respiratory inhibition (reviewed by (44)). Treatment with 1400W did not reverse the observed effect on TCA activity at any pO₂ (Table 1).

OPPC Challenge with HEDS and PIMO

To further increase oxidative challenge, RAW 264.7 cells were treated with HEDS or PIMO, two chemical oxidants, which operate by distinct mechanisms. Both treatments significantly increased OPPC activity in RAW 264.7 cells at 0.07% O₂ and 50% O₂ ($p < 0.001$, Figure 5). Stimulation of RAW 264.7 cells with LPS and IFN γ did not alter the magnitude of the increase in OPPC activity induced by HEDS or PIMO treatment. NOx production was measured during OPPC challenge to determine whether NO production was maintained despite the additional chemical oxidant stress (i.e. to assess whether the OPPC was able to accommodate both processes). NOx production by LPS and IFN γ stimulated RAW 264.7 cells was not affected by HEDS treatment at 0.07% O₂ or 50% O₂ (Figure 6). PIMO, a nitroimidazole, was detected by the nitric oxide analyzer (2 mM PIMO in media

generated the equivalent signal of 3.6 μM NaNO_2). Even after correcting for this signal, however, NO_x measurements were significantly increased in PIMO treated LPS and $\text{IFN}\gamma$ stimulated RAW 264.7 cells at 0.07% O_2 ($p < 0.05$) and 50% O_2 ($p < 0.001$), suggesting that some metabolites of PIMO might be detected as nitrite, nitrate, or nitrosothiols (45).

DISCUSSION

Increased NO_x production and increased OPPC activity were observed over a wide range of $p\text{O}_2$ (0.07% to 50% O_2) in LPS and $\text{IFN}\gamma$ stimulated RAW 264.7 cells, consistent with previous reports of a relationship between NO production and NADPH oxidation in atmospheric O_2 (18–25). Moreover, inhibition of NO production decreased OPPC activity to near background levels at all $p\text{O}_2$ investigated, suggesting that the majority of the increased OPPC activity observed in stimulated RAW 264.7 cells was directly related to NO and/or reactive nitrogen species production. Hypoxia did not inhibit the ability of the OPPC to respond to additional chemically mediated oxidative stress induced by HEDS or PIMO, and neither compound appeared to inhibit NO_x production in stimulated cells. These results demonstrate that OPPC activity is not limiting for NO production by stimulated RAW 264.7 cells irrespective of $p\text{O}_2$.

The $p\text{O}_2$ dependence of NO_x production was well fit by a Michaelis-Menten model, and the measured $K_m\text{O}_2$ (0.66% or 5 Torr) was within the range reported previously (4–6, 46–48). The $K_m\text{O}_2$ is most consistent with our previous results using forced convection cell culture (22 Torr) (6), and our previous data from cell monolayers grown in dishes for 18 hours, after correction for iNOS activity and media depth (14 Torr) (4). The data are also consistent with measurements made by Rengasamy and Johns using RAW 264.7 cell lysates as the enzyme source in a steady state system (5 Torr) (48). Higher values have been reported using rapid equilibrium kinetics and recombinant iNOS (93 Torr (46) and 96 Torr (47)). The reasons underlying the higher apparent $K_m\text{O}_2$ measured with recombinant iNOS by rapid equilibrium kinetics are unknown, but may be due to inhibition of iNOS by NO feedback in the absence of the cellular milieu (47, 50). In any case, all of these apparent $K_m\text{O}_2$ values are within or just above the range of $p\text{O}_2$ found *in vivo* (5 to 71 Torr (7–15)), indicating that tissue $p\text{O}_2$ has the potential to significantly and rapidly affect NO production, as suggested previously (4–6, 46–48).

The $p\text{O}_2$ dependence of NO_x production was not due to changes in iNOS protein levels or iNOS dimerization, consistent with our previous studies of short-term hypoxia (40 minutes) (6). Multiple studies have documented the effects of long-term (18 to 24 hours) hypoxia on iNOS upregulation via HIF 1 α (49–51). Increased expression of iNOS protein due to concomitant exposure to LPS, $\text{IFN}\gamma$, and hypoxia, however, is reported to require incubations greater than or equal to 6 hours (52). The half-life of iNOS in atmospheric O_2 is approximately 1.6 hours (53), but the effect of hypoxia on the stability of the protein has not been investigated. In the present study, short-term hypoxia (i.e. < 4 hours) appeared to have negligible effects on the balance between transcription, translation, assembly, and degradation of iNOS.

Several previous studies have investigated the effects of short term hypoxia on NADPH and/or OPPC activity (30–34), but we have found no prior studies that examined the pO_2 dependence of OPPC activity in macrophages. Interestingly, OPPC activity in unstimulated RAW 264.7 cells exhibited a biphasic pO_2 dependence in contrast to our previous work in HT1080 (human fibrosarcoma) and A549 (human lung carcinoma) cells (31). One potential source of NADP⁺ under hypoxia is the mitochondrial transhydrogenase. The decrease in mitochondrial respiration observed under severe hypoxia results in elevated glycolytic flux in order to maintain [ATP]. Hypoxic glycolysis produces lactate and NAD⁺. Mitochondrial transhydrogenase couples the reduction of NAD⁺ to the oxidation of NADPH (54). This reaction has been shown to increase under anaerobic conditions (55). We propose that the NADP⁺ produced by the mitochondrial transhydrogenase catalyzed reaction resulted in the stimulation of OPPC activity that we observed under hypoxia. While we are examining this hypothesis more thoroughly, it is important to note that the increase of OPPC activity between 2% and 0.07% for unstimulated RAW 264.7 cells under hypoxia, though statistically significant, is relatively small compared to the increase observed between 10% and 50% O_2 , and both changes are much smaller than the increase observed after LPS and IFN γ stimulation.

Stimulation of RAW 264.7 cells with LPS and IFN γ significantly increased OPPC activity at all pO_2 , thus extending previous reports for PAMPs stimulated macrophages in atmospheric O_2 (27–29, 56). These results are consistent with our results previously obtained in tumor cells (31), and conform with the classical view of decreased metabolic flux through the OPPC under hypoxia (30, 32, 33), whereby removal of O_2 leads to a reducing environment. For example, Scholz et al. measured an increase in NADPH fluorescence in rat liver within seconds of exposure to near anoxic pO_2 , and measured a new steady state within minutes (30). Treatment with the iNOS inhibitor, 1400W, significantly reversed the affect of LPS and IFN γ stimulation at all pO_2 , suggesting the increase in OPPC activity was to accommodate NO production and/or the neutralization of reactive nitrogen mediated stress. Although NADPH may also be consumed by NADPH oxidase (57), or even by iNOS to produce superoxide (58), superoxide release by LPS and IFN γ stimulated RAW 264.7 cells is reported to only occur within the first hour after stimulation (59), and to be only 6 % of the magnitude of NO production (60, 61).

As seen in Fig. 4B, OPPC activity correlated linearly with NO_x production above 0.13% O_2 , and roughly 2 molecules of CO₂ were produced for each measured molecule of NO_x. Since 2 molecules of NADPH are produced for every CO₂ molecule released, this suggests a stoichiometry of 4 molecules NADPH per molecule of NO_x. Theoretically, only 1 molecule of NADPH is required in the NO synthase reaction, but direct measurements have suggested the requirement of approximately 1.5 molecules of NADPH per NO molecule (reviewed by (58)). One possibility for this excess is that additional NADPH is required to maintain cellular redox equilibrium in the presence of reactive nitrogen and oxygen mediated stress (reviewed by (62, 63)). Our measurements suggest an even higher requirement, and another possibility for this arises from a limitation associated with our measurements: first, NO_x does not account for all NO metabolites. Second, NO released into the gas phase of the vial was not measured. Since the 'Thin Film' culture system is designed to keep the gas phase in equilibrium with the liquid phase, it is not technically possible to confine NO to the media.

Because NO production was not measured via radiolabeled L-arginine, it is impossible to calculate the amount of NO released into the gas phase. Therefore, the absolute relationship between NO, NO_x production, and NADPH consumption remains to be addressed by additional studies.

Because OPPC activity decreased with pO₂, we investigated whether OPPC activity was limited by hypoxia. HEDS and PIMO are chemical oxidants that induce oxidative stress by mechanisms that are not dependent on pO₂ (17, 36–38). Thus, these compounds were used to challenge the OPPC in RAW 264.7 cells under hypoxia. Treatment with HEDS or PIMO significantly increased OPPC activity under all conditions tested including hypoxia. To further investigate the relationship between OPPC activity and NO_x production, we measured NO_x production in LPS and IFN γ stimulated RAW 264.7 cells exposed to HEDS, and found that NO_x production was maintained despite this chemical challenge to the OPPC. Therefore, we conclude that OPPC activity is not limiting for NO production in stimulated RAW 264.7 cells.

In summary, OPPC activity was increased following stimulation with LPS and IFN γ at all pO₂ investigated in RAW 264.7 macrophages, a response which appears to be a direct consequence of NO production. OPPC activity under conditions of chemically mediated oxidative stress (i.e. HEDS or PIMO treatment) was not limited by hypoxia, nor was it limiting for NO production under any of the conditions investigated. Finally, we conclude that O₂ substrate limitation is the primary mechanism responsible for decreased NO production by LPS and IFN γ stimulated macrophages exposed to acute hypoxia.

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LIST OF ABBREVIATIONS

G6PD	Glucose 6 phosphate dehydrogenase
HEDS	Hydroxyetheldisulfide
IFNγ	Interferon gamma
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NO_x	Nitric oxide metabolites
OPPC	Oxidative pentose phosphate cycle
PAMPS	Pathogen associated molecular patterns
PIMO	Pimonidazole
pO₂	Partial pressure of O ₂

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Figure 1.

Production of CO₂ from Glucose by the OPPC and the TCA cycle. The OPPC releases CO₂ from C1 of glucose, whereas the TCA cycle releases CO₂ symmetrically from C1 and C6 after glucose is split by glycolysis. The OPPC produces 2 molecules of NADPH per molecule of CO₂ released and ribose-5-phosphate. Ribose-5-phosphate can be further metabolized by the non-oxidative pentose cycle (NOPC). OPPC activity was calculated by subtracting the amount of ¹⁴CO₂ released when cells were incubated with 6-¹⁴C-labeled

glucose (TCA cycle activity) from the amount of $^{14}\text{CO}_2$ released when cells were incubated with 1- ^{14}C -labeled glucose (i.e. OPPC + TCA cycle activity).

Figure 2.

EF5 binding in Glass Dishes versus Vials. RAW 264.7 cells were cultured for 3 hours in the presence of 100 μ M EF5 at 0.03%, 0.14%, 0.21%, 0.59%, 1.04%, 1.91%, or 11.7% O_2 . RAW 264.7 cells were labeled with an EF5-specific Cy5 antibody and EF5 binding was measured by flow cytometry of a single cell suspension. Results across experiments were normalized to a positive control. Note that in the experiments presented, pO_2 is defined as the % of oxygen in 1 atmosphere of dry gas at 37°C (ie. 100% = 760 mm of Hg).

Figure 3.

NOx production, iNOS protein concentration, and iNOS dimerization in LPS and IFN γ stimulated RAW 264.7 cells cultured with 'Thin Film' cell culture. LPS and IFN γ stimulated RAW 264.7 cells were exposed to 0.07%, 0.13%, 0.24%, 0.61%, 2%, 10%, or 50% O₂ (balance N₂) for 4 hours using 'Thin Film' cell culture. A. Media NOx were converted to NO via reaction with vanadium chloride and measured with a Sievers Nitric Oxide Analyzer (see Methods). Data presented are mean $\pm$ SD. ($n = 3$). B, C. Representative Western blots of an SDS-PAGE gel (B) and a low temperature SDS-PAGE gel (C) for iNOS

and β actin. ($n = 3$). (R) RAW 264.7 cells grown in atmospheric O_2 . (+) RAW 264.7 cells treated with LPS and $IFN\gamma$ for at least 18 hours prior to exposure to the designated pO_2 . (–) RAW 264.7 cells not treated with LPS and $IFN\gamma$. (M) BioRad Kaleidoscope Prestained Standards (BioRad, Hercules CA).

Figure 4.

Correlation between OPPC activity and NOx production. A. RAW 264.7 cells (closed circles), LPS and IFN γ stimulated RAW 264.7 cells (open circles), or LPS and IFN γ stimulated RAW 264.7 cells pretreated with the iNOS inhibitor, 1400W at 100 μ M (open triangles) were incubated with 1-¹⁴C glucose or 6-¹⁴C glucose in parallel experiments at 0.07%, 0.13%, 0.24%, 0.61%, 2%, 10%, or 50% O₂ (balance N₂). Cumulative OPPC activity was calculated by subtracting ¹⁴CO₂ produced in the presence of 6-¹⁴C glucose (TCA Activity) from ¹⁴CO₂ produced in the presence of 1-¹⁴C glucose (OPPC Activity +

TCA Activity). OPPC activity by LPS and IFN γ stimulated RAW 264.7 cells was significantly different from RAW 264.7 cells and LPS and IFN γ stimulated RAW 264.7 cells + 1400W at all pO $_2$ ($p < 0.001$). At this concentration (100 μ M), 1400W did not completely inhibit the enhanced OPPC activity resulting from LPS and IFN γ stimulation. Data presented are mean $\pm$ SD. ($n = 4$) B. Linear regression of OPPC activity and NO $_x$ production (data shown in Figure 2). $y = [b1]x + [b0]$

Figure 5.

OPPC Challenge with HEDS and PIMO. OPPC activity of RAW 264.7 cells and LPS and IFN_γ stimulated RAW 264.7 cells was measured during treatment with one of two chemical oxidants, HEDS (2 mM) or PIMO (2 mM), at 0.07% and 50% O₂ for 4 hours. (*) Significant effect of HEDS or PIMO treatment on OPPC activity versus no treatment ($p < 0.001$). Data presented are mean $\pm$ SD. ($n = 4$)

Figure 6.

NOx production during OPPC challenge with HEDS and PIMO. RAW 264.7 cells and LPS and IFN γ stimulated RAW 264.7 cells were treated with 2 mM HEDS or 2 mM PIMO during a 4 hour exposure to 0.07% or 50% O₂. Media NOx were converted to NO via reaction with vanadium chloride and measured with a Sievers Nitric Oxide Analyzer. PIMO data were corrected for the signal of PIMO in media (3.64 nmol). A statistically significant effect of HEDS or PIMO on NOx versus no treatment was calculated where indicated (* p < 0.05, ** p < 0.001). Data presented are mean $\pm$ SD. (n = 3).

Table 1

Effect of 1400W on RAW 264.7 NOx Production, OPPC activity, and TCA activity.

	%pO ₂	0.07	0.07	2	2	50	50
	1400W	-	+	-	+	-	+
NOx	NS	1.5 ± 2.1	ND	0.1 ± 1.1	ND	0.2 ± 0.3	ND
Nmol/10 ⁶ cells	S	4.9 ± 3.0 *	0.0 ± 0.5 †	15.5 ± 2.8 *	ND	26.4 ± 1.5 *	0.3 ± 1.3 †
OPPC	NS	14.7 ± 3.8 ‡	10.7 ± 1.7	7.4 ± 0.9	5.0 ± 1.9	25.1 ± 7.9 ‡	9.7 ± 1.0 §
Nmol CO ₂ /10 ⁶ cells	S	25.0 ± 2.2 ^a	13.1 ± 0.4 ^b	59.0 ± 4.8 ^a	18.8 ± 1.9 ^{a,b}	71.8 ± 7.3 ^a	27.9 ± 5.3 ^{a,b}
TCA	NS	0.3 ± 0.1	0.2 ± 0.02	1.7 ± 0.4	4.9 ± 0.4 ^c	4.1 ± 2.9	5.78 ± 2.9
Nmol CO ₂ /10 ⁶ cells	S	0.4 ± 0.1	0.3 ± 0.01	0.6 ± 0.04	0.3 ± 0.04 ^d	0.6 ± 0.04 ^d	0.3 ± 0.1 ^d

RAW 264.7 cells were stimulated (S), or not (NS), with 1 µg/mL LPS and 100 U/mL IFN γ prior to exposure to 4 hours of 0.07%, 2%, or 10% O₂ with 'Thin Film' cell culture. RAW 264.7 cells were treated with 100 µM 1400W (+), or not (-), for 1½ hours prior to 'Thin Film' cell culture. (ND) = not determined.

* Effect of LPS and IFN γ treatment on media NOx (0.07% p < 0.05; 2% and 50% p < 0.001).

† Effect of 1400W treatment on media NOx (0.07% p < 0.01, 50% p < 0.001).

‡ Effect of pO₂ on RAW 264.7 OPPC activity (0.07% and 50% > 2%, p < 0.05).

§ Effect of 1400W treatment on RAW 264.7 cells OPPC activity (p < 0.001).

^a Effect of LPS and IFN γ treatment on OPPC activity (p < 0.001).

^b Effect of 1400W treatment on OPPC activity by LPS and IFN γ stimulated RAW 264.7 cells (p < 0.001).

^c Effect of 1400W on TCA activity (p < 0.05).

^d Effect of LPS and IFN γ treatment on TCA activity (Without 1400W p < 0.01, With 1400W p < 0.001). Data presented are mean ± SD. (NOx n = 3, OPPC n = 4, TCA n = 2).