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Inhibition of autophagy and glycolysis by nitric oxide during hypoxia-reoxygenation impairs cellular bioenergetics and promotes cell death in primary neurons

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Abstract

Excessive nitric oxide (NO) production is known to damage mitochondrial proteins and the autophagy repair pathway and so can potentially contribute to neurotoxicity. Accordingly, we hypothesized that protection against protein damage from reactive oxygen and nitrogen species (ROS/RNS) under conditions of low oxygen by the autophagy pathway in neurons would be impaired by NO and enhance bioenergetic dysfunction. Rat primary cortical neurons maintained the same basal cellular respiration in hypoxia as normoxia, whereas NO exposed cells exhibited a gradual decrease in mitochondrial respiration in hypoxia. Upon reoxygenation, the respiration in NO treated cells did not recover to pre-hypoxic levels. Hypoxia-reoxygenation in the presence of NO was associated with inhibition of autophagy and the inability to recovery during reoxygenation was exacerbated by the inhibitor of autophagy, 3-methyladenine. The effects of hypoxia could be recapitulated by inhibiting glycolytic flux under normoxic conditions. Under both normoxic and hypoxic conditions NO exposure induced immediate stimulation of glycolysis but prolonged NO exposure, associated with irreversible inhibition of mitochondrial respiration in hypoxia, inhibited glycolysis. Importantly, we found that NO inhibited basal respiration under normoxic conditions only when glucose was absent from the media or glycolysis was inhibited by 2-deoxy-D-glucose, revealing a novel NO-dependent mechanism for the inhibition of mitochondrial respiration which is modulated by glycolysis. Taken together these data suggest an oxygen-dependent interaction between mitochondrial respiration, glycolysis and autophagy in protecting neuronal cells exposed to NO. Importantly, they indicate that mitochondrial dysfunction is intimately linked to a failure of glycolytic flux induced by exposure to NO. In addition, these studies provide new insights into understanding how autophagy and NO may play an interactive role in neuroinflammation-induced cellular damage which is pertinent to our understanding of the pathology neurodegenerative diseases in which excessive NO is generated.

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Keywords

DetaNONOate; LC3; 3-MA; mitochondria; glucose; 2-DG

Introduction

Nitric oxide (NO) is synthesized in the nervous system by all of the 3 nitric oxide synthase isoforms depending on the cell type and pathophysiology (1). Nitric oxide induces cell signaling through a number of mechanisms including vasodilatation and long term potentiation through activation of cGMP signaling, modulation of mitochondrial respiration and, through a number of other mechanisms including S-nitrosation (2). Under conditions of seizure, stroke leading to brain ischemia, and other neuronal pathologies, increased NO is produced both by iNOS present in glia and by neurons through over-stimulation of the glutamate receptor, calcium overload and activation of nNOS (3–9). A pathological role for NO in brain ischemia-reperfusion has been suggested on the basis of both experiments in genetic models of NOS ablation and the use of NOS inhibitors (10–16). Interestingly, NO-dependent inhibition of the mitochondrial respiratory chain is O₂-dependent suggesting that it would be enhanced under conditions of low O₂ tension such as occurs in ischemia. The binding of NO to cytochrome *c* oxidase is initially reversible but as a consequence of increased superoxide production can lead to peroxynitrite formation and irreversible damage to the mitochondrion and decreased levels of the electron transport chain (17–20). It has been shown in a number of studies that peroxynitrite can irreversibly modify cytosolic and mitochondrial proteins, including mitochondrial complex I subunits, and so change bioenergetic function (2;21–25). Protection against peroxynitrite dependent damage is ultimately dependent on glycolysis through the activity of the Pentose Phosphate Pathway to maintain antioxidants such as glutathione in the reduced state (21;26). Damage to the mitochondrial population is corrected by a dynamic interaction between biogenesis and removal of defective organelles by autophagy (27–32).

Autophagy is a process that uses double membrane intracellular vesicles to encircle proteins and organelles and transports these intracellular contents to the lysosomes to be degraded (29;33). This degradation process is important for the cellular response to starvation and oxidative/nitrative stress, as it helps to conserve cellular energy as well as to limit damaged proteins and organelles that amplify toxic signals (27–29;34–37). During autophagy, microtubule associated protein light chain 3 (LC3) is converted from LC3-I to LC3-II by lipidation and LC3-II is inserted into autophagosomal membranes to facilitate the autophagosomal expansion (37). Prior studies in a variety of mammalian cell lines, including HeLa cells, HEK293 cells, SH-N-SH cells, mouse embryonic fibroblasts, and rat primary cortical neurons, have shown that treatment with NO donors, or overexpression of the three NOS isoforms, resulted in decreased autophagic flux (38). These effects were independent of cGMP signaling, the canonical pathway through which NO exerts many of its biological effects, and involved blocking JNK phosphorylation of Bcl-2, increasing its interaction with Beclin-1, and activating mTOR to negatively regulate autophagy (38). These data suggested to us that autophagic dysfunction may contribute to NO toxicity under conditions of low O₂ tension, leading to bioenergetic dysfunction. Importantly, how NO affects cellular bioenergetics and autophagy in response to hypoxia-reoxygenation is unknown and is the focus of this current study.

Impairment of cellular bioenergetics frequently results in the stimulation of glycolysis for the rapid generation of ATP. Interestingly, NO-dependent inhibition of respiration has been reported to increase glycolysis in astrocytes but not in neurons, as assessed using polarographic techniques in non-adherent cells (39). This difference was suggested to be

responsible for protection against astrocyte cell death, and may be due to an innately higher amount of 6-phosphofructo-2-kinase (PFK2, the enzyme responsible for F2,6P₂ biosynthesis) in astrocytes compared to neurons (40). The effects of NO inhibition on glycolysis and cellular bioenergetics in adherent primary neurons, under normoxic or hypoxic conditions are unknown and this is important since detachment of cells leads to a cell-death dependent process known as anoikis which confounds the interpretation of data obtained with non-adherent cells (41). In the present study we tested the hypothesis that increased exposure to exogenous NO under conditions of hypoxia-reoxygenation increased bioenergetic dysfunction, inhibited glycolysis and autophagy so exacerbating cell death in primary adherent neurons.

We found that primary cortical neurons have an active basal level of autophagy, which contributes to cell survival, and is partially inhibited by NO under hypoxia-reoxygenation conditions. Surprisingly, suppression of glycolysis under normoxic conditions significantly enhances NO-dependent inhibition of mitochondrial respiration which has not been previously reported. As anticipated in response to hypoxia, NO significantly inhibited mitochondrial respiration, but this was not reversed on re-oxygenation and exacerbated either by inhibition of glycolysis or autophagy. Taken together these studies provide new insights into cellular bioenergetic and autophagic response to NO during normoxia and hypoxia-reoxygenation in neurons.

Materials and Methods

Materials

All materials for the Extracellular Flux assays were from Seahorse Biosciences. Carbonyl cyanide *p*-[trifluoromethoxy]-phenyl-hydrazone (FCCP), oligomycin, antimycin A, 3-methyladenine (3-MA), chloroquine (CQ), and 2-deoxyglucose (2-DG) were from Sigma. (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DetaNONOate) was from the Cayman Chemical Company.

Primary neuron cultures

Primary cortical neurons were obtained from embryonic day 18 (E18) embryos and data is presented from 15–20 individual preparations. All animal procedures were approved by UAB institutional IACUC. Timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were sacrificed by CO₂ inhalation and embryos were collected in a Petri dish and placed on ice. Dissections were performed in ice cold Hanks' balanced sodium salts (without Ca²⁺ and Mg²⁺). Cerebral cortices were isolated and collected in a 15 ml Falcon tube. The tissues were incubated for 15 min at 37°C with papain (Worthington). The tissues were mechanically dissociated with a fire-polished Pasteur pipette. Cells were finally concentrated by centrifugation at 25°C for 5 min at 1000 x g and resuspended in Neurobasal medium containing 2% B27 supplement (Invitrogen), 1% Pen-Strep (10,000 U/ml, 10,000 µg/ml) and 0.5 mM L-glutamine. Cell preparations were 90–95% primary neurons and plated in XF24 plates coated with 0.1 mg/ml Poly-L-Lysine (Sigma). The cultures were kept in a humid incubator (5% CO₂, 37°C) and half of the medium was changed once every 4 days. DIV7 cultures were stained with NeuN and GFAP and confirmed that 95% are neurons. All experiments used DIV7-10 cultures with similar bioenergetic profiles and sensitivity to DetaNONOate.

Measurement of mitochondrial function

To measure mitochondrial function in DIV7-10 adherent primary cortical neurons, a Seahorse Bioscience XF24 Extracellular Flux Analyzer was used. The Seahorse XF24 analyzer creates a 7 µl chamber in specialized microplates that allows for repeated measures

of O₂ and proton concentration (42;43). To allow comparison between different experiments, data are expressed as the rate of O₂ consumption in pmol/min or the rate of extracellular acidification in mpH/min, normalized to total protein in individual wells determined by the DC protein assay (BioRad). The optimal seeding density of the cells needed to obtain a measurable O₂ consumption and extracellular acidification rates (OCR and ECAR respectively) was established, and both ECAR and OCR exhibit a proportional response with cell number (data not shown). For subsequent experiments, a seeding density of 80,000 cells per well was selected to allow both potential increases and inhibition of OCR and ECAR to be assessed. Data is normalized to protein in individual wells and in the condition of hypoxia reoxygenation where cells lost from the plate after the mitochondrial function assay using parallel plates from the same neuronal preparation. We have titrated oligomycin, FCCP, and antimycin A concentrations and determined that their optimal effective concentrations to be 1 µg/ml, 1 µM and 10 µM respectively for assessment of bioenergetic function. We tested the effects of antimycin A alone, rotenone alone and antimycin A plus rotenone to assess a potential change in the non-mitochondrial respiration but found no significant differences between in any of these conditions. Thus we concluded that OCR after antimycin can be ascribed to oxygen consumption from non-mitochondrial respiratory chain sources distinct from the respiratory chain. To investigate the effects of hypoxia-reoxygenation on neuronal autophagy and survival, primary rat cortical neurons were seeded into XF24 culture plates and cultured for 7–10 days. For the purposes of these experiments we use the term normoxia to describe the condition where the cells equilibrated with air which is then hyperoxic relative to the physiological condition. However, over the conditions of changing oxygen tension used in these experiments the levels are not limiting for mitochondrial respiration. Cells were either incubated under normoxic conditions (2–4 h), or were subjected to hypoxia-reoxygenation by placing an air equilibrated plate in a hypoxia chamber (1% oxygen chamber) and allowing the plate to equilibrate with the 1% O₂ environment in the chamber over 2.5 h followed by reoxygenation for 1.5 h (Figure 1) (44). Data are presented from representative experiments repeated 3–4 times using 15–20 distinct neuronal preparations.

Assessment of cell viability

Cell viability was assessed using the Lactate Dehydrogenase (LDH) Release Assay. Following treatment, LDH activity in the medium and the cell lysates were measured and used to determine the proportion of the intracellular LDH lost to the medium.

Western blot analysis

Protein extracts were separated by SDS-PAGE and probed with respective antibodies, including: LC3 (Sigma-Aldrich), and β-actin (Sigma-Aldrich). Relative levels of proteins were quantified by densitometry using NIH Imaging software.

Statistical analysis

Data are reported as mean ± SEM. Comparisons between two groups were performed with unpaired Student's *t*-tests. Comparisons among multiple groups or between two groups at multiple time-points were performed by either one-way or two-way analysis of variance, as appropriate. A *p* value of less than 0.05 was considered statistically significant.

Result

The effects of NO on cellular bioenergetics under normoxia and hypoxia-reoxygenation

Mitochondrial function is susceptible to inhibition by NO and since the binding of NO to cytochrome *c* oxidase is competitive with O₂ we reasoned that the NO effects on respiration

should increase in hypoxia. To test this hypothesis cellular bioenergetics were determined in primary neurons under the conditions of normoxia and hypoxia-reoxygenation as described in Figure 1. Under normoxic conditions, the basal O₂ consumption rate (OCR) was first established before DetaNO injection at 20 min, and continued to be monitored for the next 230 min (Figure 2A). No change in basal OCR was observed for the cells over the course of the experiment with any treatments. The lack of effect of NO on basal respiration has been observed in other cells and is likely due to the fact that basal respiration is largely controlled by the availability of ADP, which is low under unstressed conditions, and not by the activity of the target for NO, cytochrome *c* oxidase (17;27;44;45). After a total of 250 min a mitochondrial profile was established by the sequential addition of inhibitors of oxidative phosphorylation as described previously (46). As expected oligomycin caused a decrease in OCR which is ascribed to ATP linked respiration and the remaining proton leak or other processes utilizing the mitochondrial proton gradient. The addition of the proton ionophore FCCP causes a rapid increase in OCR as the control of respiration by the mitochondrial proton gradient is removed. Finally, antimycin A alone or antimycin in combination with rotenone is added, to estimate the non-mitochondrial sources contributing to OCR. We found no significant effect of rotenone beyond that of antimycin (result not shown). Shown in Figure 2B is the analysis of the mitochondrial function profile. Basal, ATP linked or proton leak OCR was not affected by NO. However, NO substantially inhibited the maximal respiration and reserve capacity as has been reported previously in endothelial cells (17). There was also a small decrease of non-mitochondrial OCR after NO exposure.

The effects of NO in hypoxia were markedly different than in normoxia with increasing inhibition of respiration by NO, as indicated by the decrease in OCR, as the O₂ concentration decreased in the cell media Figure 3A and Figure 3B. As shown in Figure 1, when the plate is introduced into the hypoxia chamber the O₂ concentration slowly decreases over the subsequent 180 min at which point O₂ is reintroduced for the reoxygenation phase (44). Again, DetaNO was injected at 20 min, and the OCR monitored for the next 170 min. In the control cells a stable rate of oxygen consumption (OCR) is maintained over the period of changing O₂ concentration, indicating the O₂ has not yet become limiting to the mitochondria (Figure 3A,B). On reintroduction of O₂ a small but consistent decrease in basal OCR occurs in the control cells which did not reach significance (Figure 3A,B). After reoxygenation, sequential injection of oligomycin, FCCP and antimycin was performed to profile mitochondrial function (46). The oligomycin-inhibitable ATP-linked OCR was decreased by DetaNO after hypoxia-reoxygenation (Figure 3A,C). The addition of FCCP to stimulate maximal mitochondrial respiration revealed that in the presence of NO, profound inhibition of maximal OCR was observed, and near complete loss of reserve capacity (Figure 3A,C). There was no change of proton leak, but a slight decrease of non-mitochondrial OCR in the presence of DetaNO (Figure 3A,C).

The rate of NO release from DetaNO is not oxygen-dependent but O₂-dependent extracellular reactions and cellular metabolism may differ. However, it is unlikely that the level of exposure to NO, which increases as the oxygen decreases is significantly contributing to the differential sensitivity in normoxia compared to hypoxia-reoxygenation. We have previously shown that the level of NO is increased approximately 2 fold at the concentration of O₂ (80 μ M), which corresponds to 50% inhibition of basal respiration by NO achieved by 250 μ M DetaNO in hypoxia (44). Under normoxic conditions concentrations of 500 μ M DetaNO had no effect on basal respiration. As a further control decomposed DetaNO was injected to the equivalent concentration of 250 μ M DetaNO and found to have no effect on any bioenergetic parameters (result not shown). Taken together these data are consistent with an NO-dependent bioenergetic defect due inhibition of mitochondrial respiration which is profoundly enhanced under conditions of hypoxia which is not reversed by reoxygenation.

The effects of NO on autophagy during normoxia and hypoxia-reoxygenation

The effects of NO on neuronal autophagy flux under normoxia and hypoxia-reoxygenation conditions were assessed by incubating the neurons in the absence or presence of 250 μ M DetaNONOate (resulting in a steady state concentration of approximately 0.7 μ M NO) (17;39), which is in the range of concentrations reported for activated microglia (0.5–1 μ M) (47). In the absence of NO, hypoxia-reoxygenation compared to normoxia showed no change in the LC3-I or LC3-II levels or their ratio (Figure 4A–C). Addition of NO did not change LC3-I or LC3-II levels or the ratio under normoxic conditions. However, after hypoxia-reoxygenation, the presence of NO significantly decreased LC3-II levels and the ratio of LC3-II/LC3I compared to controls (Figure 4A, B). To test for changes in autophagic flux, CQ (40 μ M), an inhibitor of lysosomal activity and autophagy completion, resulted in the anticipated increase in the ratio of LC3-II to LC3-I under control conditions. Interestingly, the CQ-dependent increase in LC3-II was attenuated in the case of the combined treatment of NO and hypoxia-reoxygenation (Figure 4A, C) indicating an inhibition of autophagic flux by NO.

The effects of 3-MA on cellular bioenergetics during normoxia and hypoxia-reoxygenation

To determine whether autophagy plays a detrimental or protective role in cell survival in response to NO and hypoxia-reoxygenation, a parallel series of experiments were performed using the inhibitor of autophagy 3-methyladenine (3-MA). We found that 3-MA at 10 mM decreased LC3-II levels both in the absence and in the presence of NO, and under both normoxic (Figure 5A,B) and hypoxia-reoxygenation conditions (Figure 5C,D).

To test whether inhibition of autophagy by 3-MA resembles or exacerbates any of the NO effects on cellular bioenergetics, we measured cellular bioenergetics in response to NO, with and without pre-incubation with 3-MA 30 min before bioenergetic analysis. Shown in Figure 6A,B, basal, ATP linked, or proton leak OCR was not affected by 3-MA. After addition of FCCP, the maximal respiration and bioenergetic reserve capacity were unchanged by 3-MA. 3-MA alone did not cause neuronal cell death and did not cause a decrease of cell death in combination with DetaNO (Figure 6C). For experiments under hypoxia-reoxygenation conditions, we also added 3-MA or medium to the cells 30 min before placing them into the XF24 analyzer in the hypoxia chamber. In the presence of 3-MA a similar profile was observed without a significant decrease in basal OCR compared to control (Figure 7A,B). 3-MA did not change NO effects on OCR during hypoxia, but significantly exacerbated the effects of NO on reoxygenation recovery (Figure 7A,B). In addition, 3-MA did not exacerbate the DetaNO effect on ATP linked OCR, but further decreased maximal respiration and reserve capacity compared to DetaNO alone (Figure 7A,C). There was also a further decrease of proton leak and non-mitochondrial OCR by 3-MA in combination with DetaNO (Figure 7A,C). Taken together these data are consistent with an NO-dependent bioenergetic defect due inhibition of mitochondrial respiration which was exacerbated by 3-MA. The effects of 3-MA on cell death were also determined alone and in combination with DetaNO (Figure 7D). There was no significant change increase in cell death with 3-MA in the absence or presence of NO at this early time point indicating that changes in bioenergetics are not due to overt cell loss.

Effects of NO on glycolysis during hypoxia and hypoxia-reoxygenation

It has been reported that NO can both promote and inhibit glycolysis in primary neurons, so it is not clear what the response would be under conditions of lower O₂ tension (3). We hypothesized that the bioenergetic defect mediated by NO during hypoxia would most likely lead to stimulation of glycolysis due to the Pasteur effect and if inhibited would be detrimental. To test whether this is occurring we first measured OCR to determine the effects of the inhibitor of glycolysis 2-deoxy-D glucose (2-DG) on mitochondrial function.

Under normoxic conditions 2-DG had no effect on basal mitochondrial parameters (Figure 8A). As described in Figure 6, NO inhibited maximal OCR without affecting basal respiration. In this experiment, NO also had a minor effect on ATP-linked OCR, but has no effect on non-mitochondrial OCR. The combined treatment of NO with 2-DG did not change basal OCR for the first 120 min after which a progressive decrease in basal OCR was observed followed by a profound suppression of maximal respiration and reserve capacity at the end of the experiment (Figure 8B).

In the same experiment the extracellular acidification rate (ECAR) was measured and is shown in Figure 8C. The basal ECAR in control cells was stable over the 3 h period of measurement and was increased by the addition of oligomycin as expected. That this increase in ECAR was due to glycolysis was confirmed by the addition of 2-DG which inhibited both basal and oligomycin stimulated ECAR. The ECAR component not-inhibitable by 2-DG (approximately 30%) we assume to be acidification processes which are not glycolytic such as carbon dioxide production from the mitochondria. Addition of NO to the cells immediately stimulated ECAR and a stable rate was established after 20 min identical to that for oligomycin to control cells which is then likely the maximal glycolytic rate obtainable by these cells. In the presence of both NO and 2-DG, after a transitory stimulation, ECAR was progressively inhibited further supporting the conclusion that NO is stimulating glycolysis. 2-DG alone did not cause neuronal cell death (Figure 8D), but decreased cell viability approximately 30% in combination with DetaNO. However, this cannot explain the changes in the bioenergetics through loss of cells since the basal oxygen measurement normalized to protein prior to the addition of DetaNO is the same for all groups (Figure 8A).

As an additional assessment of the contribution of the interactions of NO with glucose metabolism to avoid off-target effects of 2-DG, we performed parallel experiments in the absence of glucose. As shown in Figure 9A basal OCR was stable but decreased approximately 30% compared to control cells in the presence of 5 mM glucose (Figure 8A). On addition of FCCP maximal respiration failed to increase above basal levels indicating that the neurons were dependent on glycolysis to provide substrates for maximal mitochondrial respiration. In the presence of NO in glucose-free media a progressive inhibition of OCR occurred without the lag phase observed with 2-DG (Figure 9A). In the glucose-free media basal ECAR fell by approximately 40% compared to control with glucose (Figure 8C) and remained stable for 220 min (Figure 9C). NO in the absence of glucose induced a significant time-dependent decrease of ECAR and a complete loss of the oligomycin-stimulated ECAR (Figure 9C). Glucose deprivation decreased neuronal cell survival (to 90%), and decreased cell viability in combination with DetaNO (to approximately 65%) (Figure 9D).

In the next series of experiments we repeated the exposure to 2-DG under hypoxia-reoxygenation conditions and measured cellular bioenergetics (Figure 10). While NO exposure significantly inhibited respiration during hypoxia which only partially recovered after reoxygenation, 2-DG alone had no significant effect on the basal OCR, or reoxygenation damage (Figure 10A,B). The combined effects of 2-DG and NO significantly attenuated the recovery during reoxygenation (Figure 10A,B). In addition, 2-DG in combination with NO further decreased ATP-linked, proton leak, maximal, and reserve capacity OCR compared to NO alone (Figure 10A,C). ECAR in control cells under hypoxia-reoxygenation conditions remained constant and exhibited modest stimulation on the addition of oligomycin, similar to that found in normoxia (Figure 8C). Exposure of the cells to NO under hypoxia resulted in an initial stimulation of ECAR which, however, could not be sustained and had decreased to control levels after approximately 150 min and was no longer responsive to oligomycin. The combined effects of 2-DG and NO suppressed ECAR

and suppressed the stimulatory effect of NO throughout the experiment (Figure 10D), concurrent with the loss of respiratory capacity shown in Figure 10A. Under hypoxia-reoxygenation conditions, 2-DG alone did not cause neuronal cell death, but decreased cell viability in combination with DetaNO (to ~80%) (Figure 10E).

Glucose metabolism and autophagic flux

We next examined the impact of 2-DG and glucose deprivation on autophagic activities. As shown in Figure 11A,B, under normoxic conditions, neither DetaNO nor 2-DG changed LC3II/LC3I (Figure 11C). Autophagic flux experiments showed however, the combined treatment with 2-DG+DetaNO significantly attenuated autophagic flux (Figure 11D). As Figure 8D shows that 2-DG+DetaNO under normoxia induced cell death, that these conditions also attenuated autophagic flux is consistent with a detrimental effect of inhibition of autophagy. Interestingly, minus glucose alone, as well as cells exposed to DetaNO in the absence of glucose, also inhibit autophagic flux (Figure 12), consistent with an increase of cell death as shown in Figure 9D. Under this condition, mitochondrial reserve capacity is also abolished (Figure 9A,B, control), compared to the normal condition of 5 mM glucose (Figure 8A,B and Figure 6A,B, control). Under hypoxia-reoxygenation, DetaNO decreased autophagic flux (Figure 13), consistent with a decreased cell survival (Figure 10E). 2-DG+DetaNO did not further decrease autophagic flux (Figure 13) and did not further decrease cell survival (Figure 10E).

Discussion

The potential impact of high levels of NO in the nervous system has long been recognized and it has been clearly demonstrated that in neurons NO inhibits oxidative phosphorylation, collapses the mitochondrial membrane potential and ultimately promotes apoptosis (3;39). This effects have been directly linked to inhibition of respiratory complexes notably complexes I and IV. Other studies have also demonstrated an effect of NO on autophagy under normoxic conditions (38). We have recently demonstrated that exposure of endothelial cells to low fluxes of NO stimulates glycolysis without affecting basal respiration (17). The potential interaction between cellular bioenergetics, glycolysis, NO and autophagy has not been investigated in primary neurons under either normoxic conditions or hypoxia-reoxygenation. This is of potential importance in the pathophysiology of neurodegenerative diseases in which blood supply to the brain is compromised and excessive NO is produced. In addition, since the response of the cell to NO is highly dependent on the prevailing O₂ concentration, as has been shown for NO-dependent inhibition of cytochrome c oxidase, we reasoned the NO-dependent effects on autophagy may also be O₂ dependent (2). Previous studies have reported that under normoxic conditions, persistent chronic exposure to high levels of NO over 24 hours significantly inhibited autophagic flux which are also conditions that can suppress the levels of mitochondrial proteins (17;38). It was then of interest to determine the effects of lower levels of NO for shorter periods of time under conditions where O₂ tension is also decreased.

In support of our hypothesis that the effects of NO on autophagy were O₂ dependent, we found that NO exerted a partial inhibition of autophagic flux only in response to hypoxia-reoxygenation (Figure 4). These data indicate that the effects of NO on autophagy are enhanced as the O₂ tension decreases revealing an unexpected sensitivity of the autophagic response to O₂. The mechanisms that underlie this sensitivity are not clear but could involve an enhanced generation of peroxynitrite capable of inhibiting redox sensitive proteins in the autophagic cascade. Alternatively, it could involve a retrograde signaling pathway dependent on NO-mediated inhibition of cytochrome c oxidase or complex I. Since cell

viability was further decreased in the presence of NO in response to hypoxia-reoxygenation, it suggested to us that autophagy could be protective.

The primary effect of NO on cellular bioenergetics in control cells was to partially inhibit maximal respiration but not basal respiration. The lack of effect of NO under basal conditions is perhaps surprising, but in this condition the rate of respiration in the cells is largely controlled by the availability of ADP as evidenced by the substantial effect of oligomycin in decreasing OCR (Figure 2A,B). The capacity of the electron transport chain, including cytochrome *c* oxidase, is far in excess of the basal requirements and inhibition of respiration by NO by approximately 20% will therefore have no measurable impact on basal respiration. In contrast, on addition of FCCP, ADP is no longer a factor in controlling respiration and now the inhibition of electron transport by NO is evident. These data are consistent with studies in endothelial cells and hepatocytes in culture and isolated mitochondria with both NO and NO donors (17;44;45;48). As shown in Figure 3 NO exposure under conditions where the O₂ concentration is decreasing results in a progressive inhibition of respiration. This is consistent with the onset of a bioenergetic deficit as the O₂ level decreases so requiring the mitochondria to function at a higher rate. This would result in cytochrome *c* oxidase having a greater control over respiration and as such respiration is more sensitive to inhibition by NO.

Using 3-MA as an inhibitor of autophagy further supports the idea that autophagy plays a protective role in response to hypoxia-reoxygenation damage. Under normoxia, 3-MA in combination with NO decreased cell viability, without a significant change in mitochondrial function at either basal or maximal levels (Figure 6) suggesting that in this condition autophagy may be signaling to the cell death pathway through mechanisms that do not involve bioenergetics. Under hypoxia-reoxygenation, complete inhibition of autophagy by 3-MA in the presence of NO further decreased viability consistent with an increased demand on the autophagic system under conditions of NO exposure and low O₂ tension. In hypoxia-reoxygenation experiments, 3-MA had a small effect on mitochondrial reserve capacity on its own, while exacerbating the inhibitory effect of NO after reoxygenation. Taken together these data further support the concept that autophagy is playing a protective role in response to hypoxia-reoxygenation in the presence of NO. Interestingly, we found that under conditions when autophagic flux is inhibited through impairment of glycolysis cell survival was decreased. This further supports a role of glycolytic dependent activation of autophagy in protecting against cell death.

An important metabolic response to hypoxia is the activation of glycolysis to provide a rapid source of ATP. As shown in Figure 8C a stable rate of extracellular acidification was readily detectable (0.8 ± 0.02 mpH/min/ μ g protein) in primary neurons which was stimulated approximately 2 fold on exposure to oligomycin and inhibited approximately 50% by 2-DG. Glucose deprivation further inhibited ECAR to 0.5 ± 0.02 mpH/min/ μ g protein from which we conclude that in neurons 40% of the basal ECAR rate can be attributed to glycolysis which is inhibitable by 2-DG. Although 2-DG and glucose deprivation stress differ in several important ways they had similar effects in enhancing the NO-dependent inhibition of respiration. Together, these data indicate that primary neurons have an active glycolytic pathway which is stimulated by mitochondrial inhibition and plays a regulatory role in autophagy. Exposure to NO under normoxic conditions resulted in a rapid stimulation of a 2-DG inhibitable ECAR to the same level found in response to oligomycin (Figure 8A). Under these normoxic conditions 2-DG did not significantly suppress maximal respiration which is consistent with a partial inhibition of glycolysis. In the presence of NO bioenergetic reserve capacity was decreased under normoxic conditions and this was further depressed in the presence of 2-DG or the absence of glucose (Figure 8,10).

The damage to the mitochondrial function in the presence of NO and impairment of glycolysis has been previously ascribed to decreased flux through the pentose phosphate pathway and the failure to provide sufficient NADPH for effective function of the glutathione antioxidant pathway (49). However, this seems unlikely to be making a major contribution in this setting since 2-DG is a substrate for the first step of the pentose cycle yielding the regeneration of one molecule of NADPH from NADP⁺ which could be used to decompose H₂O₂ through GSH/GPX or TRX/PRX metabolism (50;51). In mixed neuronal-glial cell cultures it has been shown that NO generated by nNOS sensitizes the cells to hypoxic cell death and this is further enhanced by the inhibitor of glycolysis, 2-DG (52). It has also been suggested that inhibition of mitochondrial respiration by NO alone is not sufficient to promote cell death and that inhibition of glycolysis is also required and this is now supported by the effects on cellular bioenergetics (52). Based upon the Pasteur effect it would be predicted that inhibition of mitochondrial respiration by NO will stimulate glycolysis and this has been reported in astrocytes but not in suspended primary neurons due to a lower level of the enzyme 6-phosphofructo-1-kinase in neurons (40). In endothelial cells we have recently shown that exogenous NO also stimulates glycolysis but under conditions in which basal cellular mitochondrial respiration is not inhibited, suggesting a more direct effect on the regulation of glycolysis (17;45). Other studies have shown that NO, specifically S-nitrosothiols, can inhibit glycolysis through S-nitrosation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (45;53–55) or alternatively lead to activation through either stimulation of glucose uptake into the cell or activation of glucose-6-phosphate dehydrogenase (49). It has also been shown that S-nitrosation of GAPDH promotes its interaction with Siah, translocation to the nucleus, and induction of apoptosis (56). Since bioenergetic function was significantly compromised by the absence of glucose or 2-DG in both the normoxic and hypoxia-reoxygenation conditions it suggests that functional glucose-dependent metabolism is essential to maintain mitochondrial function and autophagy in the presence of high fluxes of NO.

In summary, in this study we have found a complex cellular bioenergetic, glycolytic and autophagic response to NO under normoxia versus hypoxia-reoxygenation conditions. Importantly, NO-dependent effects on autophagic and glycolytic pathways are clearly highly dependent on O₂ tension. Under conditions of lower O₂ tension NO inhibited hypoxia-reoxygenation-induced autophagy, and concurrently stimulated glycolysis. Further inhibition of autophagy or glycolysis exacerbated NO effects on mitochondrial respiration and cell death. We conclude that both autophagy and glycolysis play an important role in cell survival in response to excess NO under conditions of changing O₂ levels.

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Abbreviations

2-DG	2-deoxyglucose
3-MA	3-methyladenine
CQ	chloroquine
DetaNONOate, DetaNO	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate
ECAR	extracellular acidification rate

FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine
NO	nitric oxide
OCR	oxygen consumption rate

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In primary neurons NO induces mitochondrial dysfunction under hypoxic conditions.

NO inhibits protective autophagy during hypoxia-reoxygenation

NO induces immediate stimulation of glycolysis in hypoxia and normoxia

Prolonged NO exposure under hypoxia inhibited glycolysis

Inhibition of glycolysis inhibits autophagy and damages mitochondria

Figure 1. Model of hypoxia-reoxygenation for primary neurons

An air equilibrated seahorse plate was placed in a hypoxia chamber in which O_2 was established at 1 % with Argon. The O_2 tension in the plate was then measured every 8 min over the next 160–180 min and the O_2 concentration determined and is shown compared to an instrument maintained under normoxic conditions. After 160 min the chamber was opened and air reintroduced for the reoxygenation phase over which time O_2 levels were monitored for a further 90–110 min.

Figure 2. Effects of DetaNO on oxygen consumption rate (OCR) of primary cortical neurons under normoxia

Cells were seeded at 80,000 cells per well, into specialized 24-well Seahorse Biosciences V7 microplates then bioenergetic function was assessed using the Seahorse XF24 Analyzer. The effects of DetaNONOate (250 μM) were evaluated under normoxia. ATP synthase inhibitor oligomycin (O, 1 μg/ml), uncoupler FCCP (F, 1 μM) and complex III inhibitor antimycin A (A, 10 μM) were injected at the times indicated to determine different parameters of mitochondrial function. (A) OCR measured over time. (B) Bioenergetic profile parameters. OCRs were normalized to protein. Data = mean ± SEM, n=4–5 per group. *p<0.05 compared to control.

Figure 3. Effects of DetaNO on oxygen consumption rate (OCR) of primary cortical neurons under hypoxia-reoxygenation

Cells were seeded at 80,000 cells per well, into specialized 24-well Seahorse Biosciences V7 microplates then bioenergetic function was assessed using the Seahorse XF24 Analyzer. The effects of DetaNONOate (250 μM) were evaluated under hypoxia-reoxygenation. ATP synthase inhibitor oligomycin (O, 1 μg/ml), uncoupler FCCP (F, 1 μM) and complex III inhibitor antimycin A (A, 10 μM) were injected at indicated times to determine different parameters of mitochondrial function. (A) OCR measured over time. (B) Basal OCR at 10 min before DetaNO injection, OCR at 173 min hypoxia and at 245 min reoxygenation. (C) Bioenergetic profile parameters. OCRs were normalized to protein. Data = mean ± SEM, n=4–5 per group. *p<0.05 compared to control.

Figure 4. Nitric oxide inhibits autophagy flux and exacerbates hypoxia-reoxygenation-induced cell death

Primary cortical neurons were seeded at 80,000 cells/well in Seahorse plates and cultured for 7 days. On DIV7, the Seahorse plates media were changed to XF media with or without 40 μ M chloroquine (CQ) and then placed in an XF24 Analyzer in either room air (normoxia) or 1% O_2 for 170 min before O_2 was restored (hypoxia-reoxygenation). DetaNONOate was injected into Seahorse plates to give a final concentration of 250 μ M. The neurons were exposed to DetaNO for 4 hr. N=normoxia. H=hypoxia-reoxygenation. (A) Protein extracts were collected and Western blots were performed with an anti-LC3 antibody. The Western blot of β -actin was used as a loading control. (B) Quantification for the samples without CQ. (C) Quantification for the samples with CQ. Data = mean + SEM, n=3–5. *p<0.05 compared to normoxia without DetaNO or CQ. #p<0.05 compared to hypoxia without DetaNO.

Figure 5. Effects of inhibitor of autophagy 3-methyladenine (3-MA) on autophagy under normoxia and hypoxia-reoxygenation conditions in the absence and presence of NO
Primary cortical neurons were seeded at 80,000 cells/well in Seahorse plates and cultured for 7 days. On DIV7, the Seahorse plates media were changed to XF media with or without 10 mM 3-MA and then placed in an XF24 Analyzer in either room air (normoxia) or 1% O₂ for 170 min before O₂ was restored (hypoxia-reoxygenation). DetaNONOate were injected into Seahorse plates to give a final concentration of 250 μ M. The neurons were exposed to DetaNONOate for 4 hr. (A) Protein extracts from cells under normoxia conditions were collected and Western blot was performed with an anti-LC3 antibody. Western blot of β -actin was used as a loading control. (B) Quantification for the samples under normoxia conditions. (C) Protein extracts from cells under hypoxia-reoxygenation conditions were collected and Western blot was performed with an anti-LC3 antibody. Western blot of β -actin was used as a loading control. (D) Quantification for the samples after hypoxia-reoxygenation. Data = mean + SEM, n=3–5. *p<0.05 compared to normoxia without DetaNO; †p<0.05 compared to DetaNO without 3-MA.

Figure 6. Incubation with 3-MA did not change the effects of DetaNO on oxygen consumption rate of primary cortical neurons under normoxia

Cells were seeded at 80,000 cells per well, into specialized 24-well Seahorse Biosciences V7 microplates then bioenergetic function was assessed using the Seahorse XF24 Analyzer. The effects of DetaNONOate (250 μ M) $\pm$ 3-MA (10 mM) were evaluated under normoxia. ATP synthase inhibitor oligomycin (O, 1 μ g/ml), uncoupler FCCP (F, 1 μ M) and complex III inhibitor antimycin A (A, 10 μ M) were injected at the times indicated to determine different parameters of mitochondrial function. (A) OCR measured over time. (B) Bioenergetic profile parameters. OCRs were normalized to protein. (C) Cell viability assayed by LDH release. Data = mean $\pm$ SEM, n=4–5 per group. *p<0.05 compared to control; †p<0.05 compared to 3-MA.

Figure 7. Incubation with 3-MA exacerbated the effects of DetaNO on reoxygenation recovery
Cells were seeded at 80,000 cells per well, into specialized 24-well Seahorse Biosciences V7 microplates then bioenergetic function was assessed using the Seahorse XF24 Analyzer. The effects of DetaNONOate (250 μM) ±3-MA (10 mM) were evaluated under hypoxia-reoxygenation. ATP synthase inhibitor oligomycin (O, 1 μg/ml), uncoupler FCCP (F, 1 μM) and complex III inhibitor antimycin A (A, 10 μM) were injected at indicated times to determine different parameters of mitochondrial function. (A) OCR measured over time. (B) Basal OCR at 10 min before DetaNO injection, OCR at 173 min hypoxia and at 245 min reoxygenation. (C) Bioenergetic profile parameters. OCRs were normalized to protein. (C) Cell viability assayed by LDH release. Data = mean ± SEM, n=4–5 per group. *p<0.05 compared to control, †p<0.05 compared to 3-MA, #p<0.05 compared to DetaNO.

Figure 8. Effects of 2-deoxyglucose (2-DG) on mitochondrial function under normoxic conditions
Cells were seeded at 80,000 cells per well as described previously. The effects of DETAONOate (250 μ M) $\pm$ 2-DG (20 mM) or in the absence of glucose were evaluated under normoxia. Both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured. (A) OCR over time. ATP synthase inhibitor oligomycin (**O**, 1 μ g/ml), uncoupler FCCP (**F**, 1 μ M) and complex III inhibitor antimycin A (**A**, 10 μ M) were injected at indicated times to determine different parameters of mitochondrial functions. (B) Bioenergetic profile parameters. (C) ECAR over time in the same experiment. Data = mean $\pm$ SEM, normalized to protein, n=3–5 per group. (C) Cell viability assayed by LDH release. *p<0.05 compared to control; †p<0.05 compared to 2-DG; and ‡p<0.05 compared to DETA.

Figure 9. Effects of NO on mitochondrial function in glucose-free medium under normoxia
Cells were seeded at 80,000 cells per well as described above. (A) OCR over time in the absence of glucose with or without DetaNONOate exposure. Then subsequent injections of oligomycin (O, 1 μg/ml), FCCP (F, 1 μM), and antimycin A (A, 10 μM) were made to determine mitochondrial function. (B) Bioenergetic profile parameters. (C) ECAR over time in the same experiment. Data = mean ± SEM normalized to protein, n=4–5 per group. (D) Cell viability assayed by LDH release. *p<0.05 compared to control.

Figure 10. Effects of 2-deoxyglucose (2-DG) on mitochondrial functions in response to hypoxia-reoxygenation

The effects of DetaNONOate (250 μM) ± 2-DG (20 mM) were evaluated under hypoxia-reoxygenation. Both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured. (A) OCR over time. ATP synthase inhibitor oligomycin (O, 1 μg/ml), uncoupler FCCP (F, 1 μM) and complex III inhibitor antimycin A (A, 10 μM) were injected at indicated times to determine different parameters of mitochondrial functions. (B) Basal OCR at 10 min before DetaNO injection, OCR at 173 min hypoxia and at 215 min reoxygenation. (C) Bioenergetic parameters. (D) ECAR over time in the same experiment. Data = mean ± SEM, normalized to protein, n=3–5 per group. (E) Cell viability assayed by LDH release. *p<0.05 compared to control; †p<0.05 compared to 2-DG, #p<0.05 compared to NO.

Figure 11. Exposure to DetaNO in the presence of 2-DG inhibits autophagic flux in normoxia
 Primary cortical neurons were seeded at 80,000 cells/well in seahorse plates and cultured for 7 days. (A) On DIV7, the seahorse plates media were changed to XF media with or without 20 mM 2-DG and then placed in an XF24 Analyzer in room air (normoxia). DetaNONOate were injected into seahorse plates to give a final concentration of 250 μ M. The neurons were exposed to DetaNONOate for 4 hr. Protein extracts were collected and western blot was performed with an anti-LC3 antibody. β -actin was used as a loading control. (B) On DIV7, the seahorse plates media were changed to XF media with or without 20 mM 2-DG + Chloroquine (CQ) and then placed in an XF24 Analyzer in room air (normoxia). DetaNONOate were injected into seahorse plates to give a final concentration of 250 μ M. The neurons were exposed to DetaNONOate for 4 hr. Protein extracts were collected and western blot was performed with an anti-LC3 antibody. Western blot of β -actin was used as a loading control. (C) Quantification for A. (D) Quantification for B. Data = mean + SEM, n=3. *p<0.05 compared to control.

Figure 12. Glucose deprivation inhibits autophagic flux in normoxia

Primary cortical neurons were seeded at 80,000 cells/well in seahorse plates and cultured for 7 days. (A) On DIV7, the seahorse plates media were changed to XF media with or without 5 mM glucose and then placed in an XF24 Analyzer in room air (normoxia). DetaNONOate were injected into seahorse plates to give a final concentration of 250 μM. The neurons were exposed to DetaNONOate for 4 hr. Protein extracts were collected and western blot was performed with an anti-LC3 antibody. Western blot of β-actin was used as a loading control. (B) On DIV7, the seahorse plates media were changed to XF media+chloroquine (CQ), with or without 5 mM glucose and then placed in an XF24 Analyzer in room air (normoxia). DetaNONOate were injected into seahorse plates to give a final concentration of 250 μM. The neurons were exposed to DetaNONOate for 4 hr. Protein extracts were collected and western blot was performed with an anti-LC3 antibody. Western blot of β-actin was used as a loading control. (C) Quantification for A. (D) Quantification for B. Data = mean + SEM, n=3. *p<0.05 compared to glucose.

Figure 13. 2-DG did not further inhibit autophagic flux when combined with DETA-NO in hypoxia-reoxygenation

Primary cortical neurons were seeded at 80,000 cells/well in seahorse plates and cultured for 7 days. (A) On DIV7, the seahorse plates media were changed to XF media with or without 20 mM 2-DG and then placed in an XF24 Analyzer in 1% O₂ for 170 min before O₂ was restored (hypoxia-reoxygenation). DETA-NO was injected into seahorse plates to give a final concentration of 250 μ M. The neurons were exposed to DETA-NO for 4 hr. Protein extracts were collected and western blot was performed with an anti-LC3 antibody. Western blot of β -actin was used as a loading control. (B) On DIV7, the seahorse plates media were changed to XF media with or without 20 mM 2-DG + chloroquine (CQ) and then placed in an XF24 Analyzer in 1% O₂ for 170 min before O₂ was restored (hypoxia-reoxygenation). DETA-NO was injected into seahorse plates to give a final concentration of 250 μ M. The neurons were exposed to DETA-NO for 4 hr. Protein extracts were collected and western blot was performed with an anti-LC3 antibody. Western blot of β -actin was used as a loading control. (C) Quantification from A. (D) Quantification from B. Data = mean + SEM, n=3. *p<0.05 compared to control.