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Nitric oxide counteracts the hyperoxia-induced proliferation and pro-inflammatory responses of mouse astrocytes

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Abstract

Pre-clinical studies in the premature baboon evaluating the efficacy and potential toxicity of inhaled nitric oxide indicated a significant effect on astrocyte area density suggesting phenotypic and functional changes in astrocytes upon exposure to nitric oxide. However, the effects of nitric oxide and oxygen, the two major therapeutic gases utilized in neonatal intensive care, on astrocyte morphology and function remain vastly unknown. Herein, we report that exposure of mouse neonatal cortical astrocytes to hyperoxia results in a pro-inflammatory phenotype and increase in proliferation without significant changes in cellular morphology or levels of intermediate filament proteins. The pro-inflammatory phenotype was evident by the significant increase in cellular levels of cyclooxygenase-2 (COX-2) and the concomitant increase in prostaglandin E2 (PGE₂) secretion, a decline in the intracellular and secreted levels of apolipoprotein E (ApoE) and a significant increase in the intracellular levels of clusterin. This pro-inflammatory phenotype was not evident upon simultaneous exposure to hyperoxia and nitric oxide. These results suggest that exposure to nitric oxide in the setting of hyperoxia confers unrecognized beneficial effects by suppressing astrocytic inflammation.

Keywords

nitric oxide; oxygen; astrocytes; hyperoxia; inflammation

Introduction

Oxygen and nitric oxide are two major therapeutic agents utilized in neonatal intensive care units that have helped to improve infant survival. Although the benefits of these treatments in neonates are well documented, their potential adverse effects in the central nervous system (CNS) are largely unknown. Prior studies have suggested that oxidative stress may injure the developing brain during neonatal exposure to hyperoxia and that astrocytes may play a role in these processes (1,2). Astrocytes provide structural scaffolding for neuronal networks and participate in neurogenesis, synaptogenesis, neurotransmitter reuptake and

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release within the CNS (3–6). A typical response of astrocytes to injurious stimuli results in distinctive phenotypic changes referred to as astrogliosis. Typical astrogliosis is characterized by increased cell size, number and expression of glial fibrillary acidic protein (GFAP) (7,8). Astrogliosis also results in the elaboration and secretion of factors that can be both beneficial and detrimental to surrounding cells. For example, reactive astrocytes can produce trophic factors that are essential for tissue repair post injury as they regulate the production of protease inhibitors as well as proteases (7–10). On the other hand, reactive astrocytes are known to secrete pro-inflammatory cytokines, oxygen and nitric oxide-derived oxidants that may cause injury to other CNS cells (7,8). Astrocytes together with endothelial cells constitute the interface between circulation and the CNS and such changes in dissolved oxygen may directly influence their responses. Hyperoxic exposures have been found to raise CNS oxygen levels from 34 mmHg to 90 mmHg (11) and astrocytes are known to respond to changes in oxygen tension. Although phenotypic changes of astrocytes can significantly impact the CNS, the effect of hyperoxia on astrocyte morphology and function remains mostly unknown.

Similarly, the effects of inhaled nitric oxide in astrocyte biology remain unknown, though endogenous nitric oxide or exposure to nitric oxide donors has been shown to modulate, astrogliosis, astrocyte metabolism, respiration, glutathione levels and its own production by nitric oxide synthase 2 (12–16). In a premature baboon model, exposure to inhaled nitric oxide did not result in major morphologic changes in the CNS (17). However, analysis of the deep white matter showed that there was an increase in astrocyte density, suggesting possible phenotypic changes in astrocytes (17). Although astrocytes may not be directly exposed to nitric oxide following inhalation, they are likely exposed to nitric oxide-derived species that also convey nitric oxide bioactivity, including nitrite, a relatively stable metabolite, as well as nitric oxide carriers, such as low molecular weight and protein S-nitrosothiols, which are capable of delivering nitric oxide equivalents to target tissues and cells. Therefore, the objective of this study was to expose primary cultures of neonatal mouse astrocytes to hyperoxia, nitric oxide and the combination of both gases under defined controlled conditions in order to evaluate morphological and functional changes. The data revealed for the first time that hyperoxia induces a pro-inflammatory response in the absence of morphological changes that is absent by the simultaneous exposure to nitric oxide. The data suggest a potentially beneficial but unrecognized protective function of inhaled nitric oxide in the CNS.

Materials and Methods

Astrocyte isolation

Primary cultures of mouse neonatal cortical astrocytes were obtained as described previously (9,10). All mouse studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Stokes Research Institute, Children's Hospital of Philadelphia. One or two day old, neonatal CD-1 mice (Charles River, Wilmington, MA) were anesthetized with halothane, cooled, and the brains were removed and the cortex was dissected. The cortices were washed two times with Earle's Balanced Salt Solution (EBSS; Invitrogen, Carlsbad, CA) and then placed in trypsin (0.25%) for 10 min at 37 °C. Cortices were then triturated in Minimal Essential Media (Invitrogen, Carlsbad, CA) combined with 10% FBS (Hyclone), sodium pyruvate (1 mM), L-glutamine (2 mM), D-glucose (42 mM), sodium bicarbonate (14 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), and fungizone (2.5 µg/mL). Three cortices per T-75 vent-cap flask (Corning, Corning, NY) were plated. Cortical culture mixtures were incubated in MEM for 10 days in 37 degrees and 5% CO₂ with media changes every 3–4 days. Culture flasks were then washed with cold EBSS and then underwent a shaking process for greater than 15 hours at 37 °C in order to separate astrocytes from neurons and microglia. Adherent cells were trypsinized (0.25%) and plated

on 60 mm Petri dishes (Corning) at 1.2×10^6 cells/plate (4 mL). Forty-eight hours after plating, cells were washed with EBSS and serum-free media was added to the plates.

Exposure of astrocytes to oxygen and nitric oxide

Plates were placed in the incubator at 37 °C under control conditions (21% O₂, 5% CO₂), hyperoxia (>95% O₂, 5% CO₂), nitric oxide (20 ppm, 21% O₂, 5% CO₂), nitric oxide plus hyperoxia (20 ppm, >95% O₂, 5% CO₂) (Robert's oxygen, PA- Air-Medical Grade Ultra Zero-R106A3, Medical Grade Oxygen- R5, Nitric Oxide-141 cuft, 800 ppm-M5123505087) Under these exposure conditions and considering the solubility of oxygen and nitric oxide the cells were exposed approximately to 1 mM dissolved oxygen and 45 nM nitric oxide. After the exposures, astrocyte conditioned media was pooled from a total of six plates and stored in -80 °C. The corresponding cells were also pooled among six plates after being trypsinized and centrifuged at 200 rcf for 10 minutes. Cell lysates were then stored at -80 °C. To assess viability, astrocytes were trypsinized and analyzed by trypan blue exclusion.

Biochemical and morphological analysis

Cell lysates were reconstituted in lysis buffer consisting of 20 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton, and 1 mM EGTA. After the protein concentration was determined, 30 µg was used for Western blot analysis using antibodies against GFAP (BD Pharmingen, 1:1000 dilution), Vimentin (Abcam, 1:500), PCNA (Calbiochem, 1:500), COX-2 (Cayman Chemical, 1:100), Apo E (BioDesign, 1:1000), and Apo J/Clusterin (R & D Systems, 1:1000). Astrocyte plates were fixed for immunohistochemistry with methanol followed by methanol/acetone (50:50). Plates were then washed times three with PBS-T (0.3% Triton X100) and stored at 4 °C. After blocking with bovine serum albumin (5% BSA) for 1 hour, GFAP primary antibodies (BD Pharmingen Mouse, 1:250 dilution) were added to the plates overnight followed the next day by secondary antibody (Invitrogen, Alexa Flexor, Goat Anti-Mouse antibody, 1:500). Next, nuclear stain DAPI (1:10,000) was applied. Fluorescence images at 30x magnification were obtained using Olympus IX70 inverted microscope and images were captured using the Metamorph system. Three biological replicates were analyzed in triplicate using the Image J Analysis System to quantify cell size, number, as well as number and length of processes after staining.

For astrocyte conditioned media analysis, media was first centrifuged at 4 °C and then transferred into centrifugal filters (Amicon Ultracentrifuge filters, Millipore, Bedford, MA) and was reduced to approximately 30 µl. After determining the protein concentration of the media, 15 µg of protein were used for Western blot analysis of astrocyte conditioned media using antibodies against Apo E (BioDesign, 1:1000) and Apo J/Clusterin (R & D Systems, 1:1000). Western blot quantification was performed by densitometry using Odyssey Infrared imaging software (Licor Biosciences, Lincoln, NE), with GAPDH serving as a loading control. PGE₂ levels in the conditioned media were quantified by ELISA (Cayman Chemical).

Statistical Analysis

Because of the limited space in the exposure chamber only one experimental condition and corresponding controls were analyzed at a time. Each treatment condition was replicated at least in three different biological replicates. Each measurement from the different biological replicate was performed in triplicate. Since each exposure condition had a corresponding room air control group statistical significance was determined by paired t-tests (performed using GraphPad Prism 4.00 GraphPad, San Diego, CA).

Results

Astrocyte Viability, Morphology and Proliferation

Cells exposed to hyperoxia for 48 hours exhibited a statistically significant decrease in viability ($72 \pm 5\%$ viable post exposure) compared to the room air control cells ($86 \pm 6\%$). No differences in viability were noted upon nitric oxide exposure alone or when cells were exposed to nitric oxide plus hyperoxia as compared to the corresponding room air controls (Table 1). Under these defined exposure conditions alterations in astrocyte morphological and molecular responses were investigated by quantitative morphometric evaluation. Astrocyte cell size, cell number per high power field, process length, and process number per high power field was quantified as described previously (9). After quantifying the above parameters in equal number of cells, exposure to hyperoxia, nitric oxide or the combination of both gases did not significantly alter cellular morphology as compared to the corresponding room air controls (Table 1). Immunohistochemical and quantitative western blot analysis using antibodies against glial fibrillary acidic protein (GFAP), a typical member of the intermediate filament family of proteins, failed to reveal significant differences after gas exposures (Figure 1A–C). Consistent with this, there were no significant differences in the levels of GFAP and vimentin, another intermediate filament protein, as quantified by western analysis after exposure to the different gases (Figure 1D).

Exposure to hyperoxia increased levels of the proliferation marker PCNA over the 48 h of exposure while nitric oxide decreased PCNA levels when compared to the corresponding room air control cells (Figure 1E). The levels of PCNA were no different than the room air control when the two gases were combined suggesting that nitric oxide prevented the hyperoxia-induced increase in PCNA levels (Figure 1E).

Hyperoxia-induced pro-Inflammatory phenotype of astrocytes

Protein levels of COX-2 significantly increased with exposure to hyperoxia as compared to the matching control cells (Figure 2A). The levels of COX-2 were not different with exposure of astrocytes to nitric oxide or to nitric oxide and hyperoxia simultaneously (Figure 2A). The increased protein levels of COX-2 were accompanied by a concomitant increase in the levels of PGE₂ released in the astrocyte conditioned media upon exposure to hyperoxia (Figure 2B). The levels of PGE₂ in the ACM were increased by 3-fold after 24 hours and 4-fold after 48 hours of exposure to hyperoxia as compared to room air exposed controls (Figure 2B). A similar time dependent increase in PGE₂ release was observed after 24 and 72 hours of exposure of astrocytes to inflammatory stimuli (9). Exposure to hyperoxia for 24 hours did not induce significant loss of astrocytes and had no effect on cellular morphology and expression of GFAP. PGE₂ levels were no different than controls in cells exposed for 48 hours to nitric oxide under normoxia or concurrently with hyperoxia (Figure 2B).

Effects of Hyperoxia and Nitric oxide in Astrocyte protein secretion

In addition to COX-2 and PGE₂ secretion, pro-inflammatory stimuli have been also found to modulate protein expression and protein secretion in astrocytes (9,18–20). Two of the most abundantly expressed astrocyte proteins are Apo E and clusterin (9,10). The intracellular levels and secretion of Apo E and clusterin are regulated by inflammation (9,10). After 48 hours of hyperoxia, both intracellular and secreted Apo E levels were significantly lower as compared to the corresponding controls (Figure 3). The intracellular levels of Apo E as well as the amount of protein secreted were not different than air exposed controls in cells exposed to nitric oxide or to hyperoxia plus nitric oxide (Figure 3). The intracellular levels of clusterin increased with hyperoxia exposure as compared controls (Figure 4A). The intracellular levels of clusterin were not different than the corresponding controls in cells

exposed to nitric oxide or nitric oxide plus hyperoxia (Figure 4A). No differences in the secreted levels of clusterin were quantified for all three exposures (Figure 4B).

Discussion

Improved outcomes following the use of inhaled nitric oxide in infants with significant respiratory disease and pulmonary hypertension have been documented (21–25). Improved developmental outcomes in premature infants treated with inhaled nitric oxide (23) and a significant decrease in large intraventricular hemorrhage and periventricular leukomalacia in prematurely born infants treated with inhaled nitric oxide has been also reported (25). Moreover, a two year follow-up from a randomized, controlled trial showed that nitric oxide did not adversely impact neurodevelopmental outcomes in preterm infants (26). Despite these encouraging data, the beneficial functions of nitric oxide on the brain remain mostly unknown. Moreover, nitric oxide is often administered together with hyperoxia and the effects of hyperoxia with or without nitric oxide in the CNS are also unknown. In this study we examined the effects of hyperoxia with or without nitric oxide on primary astrocyte cultures derived from neonatal mice. The primary motivation for studying astrocytes was derived from the examination of pre-term baboon brains that were exposed to nitric oxide that revealed apparent changes in astrocyte area density upon exposure to nitric oxide (20). The functionality of astrocytes in the CNS is expanding to include significant contributions in neuronal development, extracellular matrix maintenance, and response to injury (3–6). Utilizing a previously defined astrocyte cell culture model (9,10) and controlled exposure conditions to oxygen and nitric oxide, the data reveal for the first time that hyperoxia induces a pro-inflammatory and proliferative response that is suppressed by exposure to nitric oxide. Astrocyte responses to CNS injury include increased expression of GFAP, hypertrophy, and/or hyperplasia (7). Specifically, in response to typical inflammatory mediators, astrocytes elaborate PGE₂, pro-inflammatory cytokines and chemokines, as well as undergo transcriptional changes that lead to increased expression of proteins that coordinate immune function (9,10, 27–30). Exposure to hyperoxia resulted in a similar pro-inflammatory phenotype characterized by elevated expression of COX-2 and increased release of PGE₂. Moreover, hyperoxia induced a significant increase in the proliferation marker PCNA and the expression of the secretory glycoprotein clusterin. Increased expression of clusterin has been documented in response to brain ischemic and neurodegenerative injuries (31–34). Clusterin-mediated activation of mitogen-activated protein kinase was documented to regulate primary astrocyte proliferation in culture suggesting that the hyperoxia-induced increase in clusterin levels may be responsible for the observed astrocyte proliferation (34). Hyperoxia also significantly lowered the levels of both expressed and secreted ApoE. In the CNS, Apo E is involved in neuron development, synaptogenesis and repair (4,20,35) whereas the presence of the Apo E4 allele has been associated with cerebral palsy and neurodegenerative diseases (36,37). Secreted ApoE has been considered as the major lipid carrier protein in the CNS facilitating neuronal development and repair after brain injury (4,36). Thus the hyperoxia-induced decline in ApoE levels both intracellularly and extracellularly may have adverse effects in the developing brain. The documented changes in the expression and secretion of ApoE and clusterin are likely the result of the pro-inflammatory phenotype since similar changes in these proteins have been reported for astrocytes challenged with endotoxin (18–20). Overall, the data report the acquisition of a pro-inflammatory phenotype in mouse astrocytes following hyperoxia exposure, which may be alarming since a growing body of evidence suggests that prenatal and postnatal exposure to inflammation adversely impacts neurodevelopmental outcomes in infants (38–40).

Exposure of astrocytes to nitric oxide has been shown to regulate mitochondrial and metabolic function, as well as glutathione levels and metabolism (12–14). The metabolic

regulation and more critically the trafficking of glutathione and glutathione synthesis precursors have been considered as critical mechanisms by which astrocytes provide neuroprotection (13,14,15). Recent data also indicated that inhaled nitric oxide protect the neonatal rat brain from excitotoxic neuron death (41). The data herein indicate a potential anti-inflammatory function as another possible neuroprotective function of nitric oxide in CNS. Despite the observed beneficial effects of nitric oxide, the study has some limitations. The major limitation relates to the use of astrocytes in culture which includes cells that have acquired a reactive phenotype during the isolation procedure and culture conditions (3). Despite this limitation the isolated cultured model provided a defined system to study the effects of hyperoxia and nitric oxide exposures and documented unforeseen pro-inflammatory phenotypic changes resulting from high oxygen exposure and reversal of the phenotype by simultaneous exposure to nitric oxide. Another limitation relates to the levels of oxygen and nitric oxide delivered to cells. Astrocytes were exposed to 20 ppm nitric oxide gas which approximates a steady state concentration of 45 nM nitric oxide dissolved in cell media and cells. This concentration of nitric oxide could be achieved in the brain of baboons and rodents upon exposure to inhaled nitric oxide (41,42). We have reported increased circulating levels of low and high molecular mass S-nitrosothiols, which can deliver nitric oxide in all major organs, upon inhalation of nitric oxide (42,43). Recognizing the potential limitations, the data support clinical efforts to limit neonatal exposure to hyperoxia and provide a novel mechanism by which nitric oxide may counter the effects of hyperoxia.

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Abbreviations

ACM	Astrocyte conditioned media
ApoE	apolipoprotein E
ApoJ/clusterin	apolipoprotein J
CNS	central nervous system
GFAP	glial fibrillary acidic protein
PCNA	Proliferating cell nuclear antigen
PGE₂	Prostaglandin E ₂
PVL	periventricular leukomalacia

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Figure 1.

Panel A: Representative images of astrocytes in culture after staining with anti-GFAP antibody (green) following exposure to hyperoxia, nitric oxide and hyperoxia plus nitric oxide (blue, DAPI nuclear stain). **Panel B:** representative western blots of GFAP evaluating the levels of GFAP in astrocytes after the different exposures. **Panels C, D and E:** Levels of GFAP, vimentin and proliferation marker PCNA quantified by densitometric analysis of western blots and normalized to GAPDH levels, respectively. Data is expressed as fold change (mean $\pm$ SEM) from the corresponding controls, $n=3$ for all exposure groups and controls. Open box, control cells; black box, hyperoxia exposed cells; grey box, nitric oxide exposed cells and hatched box, nitric oxide plus hyperoxia exposed cells. * $p<0.05$ compared to the corresponding room air exposed astrocytes.

Figure 2.

Panel A: Cellular COX-2 levels normalized to GAPDH and expressed as fold change from the corresponding control, n=3 for all exposure groups; values represent mean $\pm$ SEM.

Panel B: Levels of PGE₂ in the astrocyte conditioned media *p<0.05 compared to the corresponding room air exposed astrocytes, n=3 for all exposure groups. Open box, control cells; black box, hyperoxia exposed cells; grey box, nitric oxide exposed cells and hatched box, nitric oxide plus hyperoxia exposed cells.

Figure 3.

Panel A and B: Intracellular and extracellular levels of ApoE. Data is expressed as fold change from the corresponding control after normalizing to GAPDH, for the intracellular levels and to total protein for the extracellular levels * $p < 0.05$ compared to the corresponding room air exposed astrocytes, $n=3$ for all exposure groups; values represent mean $\pm$ SEM. Open box, control cells; black box, hyperoxia exposed cells; grey box, nitric oxide exposed cells and hatched box, nitric oxide plus hyperoxia exposed cells. **Insert Panel A and B:** Representative western blots using anti-ApoE antibody. Since each experimental condition had a corresponding control the western blots were performed in pairs in different gels. Lanes 1 and 2 control and hyperoxia exposed cells respectively; Lanes 3 and 4, control and nitric oxide exposed cells; and Lanes 5 and 6 cells, control and cells exposed to nitric oxide plus hyperoxia.

Figure 4.

Panels A and B: Intracellular and extracellular levels of clusterin. Data is expressed as fold change from the corresponding control after normalizing to GAPDH, for the intracellular levels and to total protein for the extracellular levels * $p < 0.05$ compared to the corresponding room air exposed astrocytes, $n=3$ for all exposure groups; values represent mean $\pm$ SEM. Open box, control cells; black box, hyperoxia exposed cells; grey box, nitric oxide exposed cells and hatched box, nitric oxide plus hyperoxia exposed cells.

Table 1

Astrocyte Viability and Quantification of Cellular Morphology

	Control	Hyperoxia	Nitric Oxide	Hyperoxia/Nitric Oxide
Viability (%)	86 ± 6	72 ± 5*	85 ± 6	87 ± 4
Cell size (μm)	46 ± 11	42 ± 8	44 ± 12	47 ± 10
Process (number/hpf)	9 ± 3	11 ± 3	8 ± 3	7 ± 2
Process length (μm)	47 ± 10	42 ± 6	45 ± 9	40 ± 7

The number of cells examined per high power field (hpf) was equal among the four different groups 54 ± 11, 56 ± 8, 58 ± 11, 49 ± 9 (mean ± std) for control, hyperoxia, nitric oxide and hyperoxia plus nitric oxide respectively.

* p<0.05 compared to the corresponding room air exposed astrocytes.