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N-methyl-D-aspartate receptor subtype 3A promotes apoptosis in developing mouse brain exposed to hyperoxia

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Abstract

In the present study, 7 day postnatal C57/BL6 wild-type mice (hyperoxia group) and 7 day postnatal N-methyl-D-aspartate receptor subtype 3A knockout mice (NR3A KO group) were exposed to 75% oxygen and 15% nitrogen in a closed container for 5 days. Wild-type mice raised in normoxia served as controls. TdT-mediated dUTP nick end labeling (TUNEL)/neuron-specific nuclear protein (NeuN) and 5-bromo-2'-deoxyuridine (BrdU)/NeuN immunofluorescence staining showed that the number of apoptotic cells and the number of proliferative cells in the dentate subgranular zone significantly increased in the hyperoxia group compared with the control group. However, in the same hyperoxia environment, the number of apoptotic cells and the number of proliferative cells significantly decreased in the NR3A KO group compared with hyperoxia group. $TUNEL^+/NeuN^+$ and $BrdU^+/NeuN^+$ cells were observed in the NR3A KO and the hyperoxia groups. These results demonstrated that the NR3A gene can promote cell apoptosis and mediate the potential damage in the developing brain induced by exposure to non-physiologically high concentrations of oxygen.

Key Words: N-methyl-D-aspartate receptor subtype 3A; apoptosis; cell proliferation; hyperoxia; developing brain; nerve cells; mouse; neurobiology; neural regeneration

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INTRODUCTION

Treatment with high concentrations of oxygen is a main approach for hypoxic diseases in neonatal and premature infants. Long-term hyperoxia inhalation can induce retinopathy and bronchopulmonary dysplasia in premature infants[1-2]. Increasing studies have focused on the harmful effects of hyperoxia exposure to the developing brain. Oxygen toxicity may result in premature brain injury and neurological deficits[3-4]. In addition, hyperoxia can induce cell death in the developing brain[5-8]. Oxidative stress plays an important role in oxygen toxicity-induced brain injury, and reactive oxygen species mediate apoptotic cytodegeneration[9].

The tolerance to oxidative stress is significantly lower in fetuses and infants compared with adults because their anti-oxidation system is not yet mature. Recent evidence indicates that N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic activity can enhance neuronal anti-oxidative ability[10]. Blocking NMDARs can induce cell apoptosis or degeneration during brain development, but these effects are confined to postnatal 10–14 days in rodents[11]. There are three subtypes of NMDAR. The NMDAR subtype 3 has been recently confirmed as a member of NMDAR family. The NMDAR subtype 3A (NR3A) can negatively regulate NMDAR function[12].

Based on these results, NMDAR activity may protect the brain against injury induced by non-physiologically high concentrations of oxygen in the developing brain. NR3A, the negative regulator of NMDAR which are involved in neural development, may play a role in this process. The present study compared the influence of hyperoxia exposure on 7 day postnatal (P7) wild-type mice and NR3A knock out (NR3A KO) mice to investigate the possible mechanism by which NR3A mediates oxygen toxicity-induced cell apoptosis.

RESULTS

Quantitative analysis of experimental animals

A total of 17 mice were randomly assigned to three groups: hyperoxia group (C57/BL6 + hyperoxia; *n* = 6), NR3A KO group (NR3A KO + hyperoxia; *n* = 6), and control group

(C57/BL6 + normoxia; *n* = 5). All 17 mice were included in the final analysis.

The number of apoptotic cells in brain tissues of NR3A KO mice exposed to hyperoxia (Table 1, Figure 1)

Table 1 Comparison of the number of TUNEL/BrdU-positive cells among groups

Group	*n*	TUNEL$^+$ cells/ 4-fold field of view	BrdU$^+$ cells/10-fold field of view
Hyperoxia	6	240.04±25.82	59.17±10.68
NMDA receptor 3A knock out	6	186.64±21.34[a]	50.94±8.86[a]
Control	5	182.10±17.03[a]	49.34±8.69[a]

[a]*P* < 0.01, *vs.* hyperoxia group. The data are expressed as the mean ± SD. The sample mean was compared using one-way analysis of variance. The paired comparison between two groups was performed using the least significant difference *t*-test. NMDA: N-methyl-D-aspartate. TUNEL: TdT-mediated dUTP nick end labeling; BrdU: 5-bromo-2'-deoxyuridine.

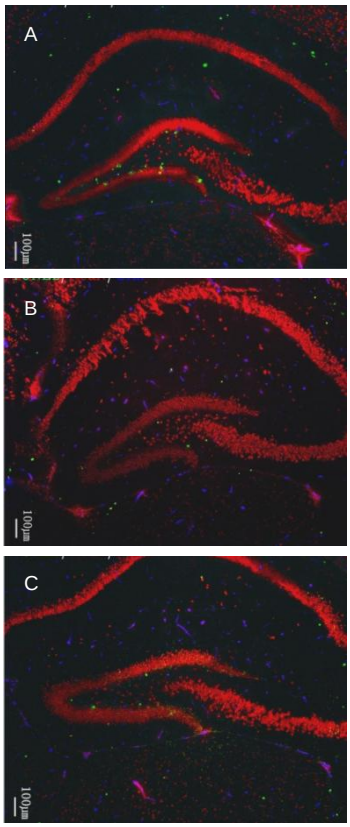

Figure 1 TdT-mediated dUTP nick end labeling (TUNEL)/neuron-specific nuclear protein (NeuN)-positive cells in the hippocampus (immunofluorescent double-labeling staining, × 4).

TUNEL-labeled apoptotic cells are green, and NeuN-labeled cells are red. The fluorochrome was Cy3.

There are more TUNEL-positive cells in the hyperoxia group (A) than in the N-methyl-D-aspartate receptor 3A knock out group (B) or the control group (C). A few TUNEL$^+$/NeuN$^+$ cells are detected.

Fluorescence double-labeling staining of TdT-mediated dUTP nick end labeling (TUNEL)/neuron-specific nuclear protein (NeuN) was used to detect cell apoptosis. Dispersed TUNEL-positive cells were observed in all three groups, but the greatest number of positive cells were found in the hyperoxia group ($P < 0.01$; Table 1). These positive cells were distributed in the hippocampus (Figure 1), cerebral cortex, thalamus, white matter and corpus striatum. No focal necrosis was observed. Co-labeling of TUNEL and NeuN was found in some cells of each of the three groups.

The number of proliferative cells in mice exposed to hyperoxia

Immunofluorescence double-labeling staining of 5-bromo-2'-deoxyuridine (BrdU)/NeuN was used to detect cell proliferation in the dentate subgranular zone. The number of BrdU-positive cells significantly increased in the hyperoxia group ($P < 0.01$; Table 1) compared with the other groups (Figure 2), and a few BrdU-positive cells migrated out of the dentate gyrus.

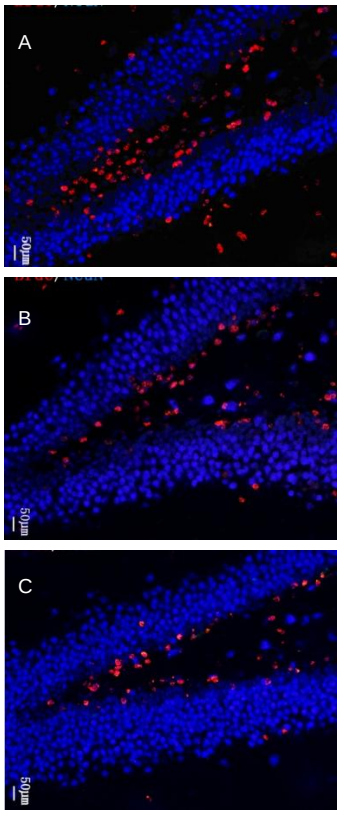

Figure 2 5-bromo-2'-deoxyuridine (BrdU)/neuron-specific nuclear protein (NeuN)-positive cells in the dentate gyrus (immunofluorescent double-label staining, × 10).

BrdU-labeled apoptotic cells are red, and the fluorochrome is Texas Red; NeuN-labeled cells are blue, and the fluorochrome is Cy5.

There are significantly more BrdU-positive cells in the dentate subgranular zone in the hyperoxia group (A) compared with the N-methyl-D-aspartate receptor 3A knock out group (B) and the control group (C). A few BrdU$^+$/NeuN$^+$ cells are detected.

Some cells in all three groups were co-labeled with BrdU and NeuN.

DISCUSSION

Physiological cell apoptosis has been found in human brains from as young as a 3-month fetus to as old as 3-year children[13]. The brain of rodents is rapidly developing within 2 weeks after birth and is sensitive to non-physiological oxygen toxicity. The results from the present study showed that after P7 mice were continuously exposed to hyperoxia for 5 days, the number of apoptotic cells in brain tissues significantly increased compared with NR3A KO and control groups. These data further demonstrate that exposure of non-physiologically high concentrations of oxygen can trigger brain injury during development and accelerate cell apoptosis[5-6, 14]. To date, the mechanism of hyperoxia-induced nerve cell apoptosis remains poorly understood. Oxidative stress is highly correlated with the synthesis of reactive oxygen species, antioxidase system imbalance and growth factor expression[6, 14]. Notably, after hyperoxia-induced brain injury, caspase-3 is activated, and cell death is increased, but the anti-apoptotic Bcl-2 family expression is enhanced. These changes indicate that the hyperoxia- or hypoxia-induced cell apoptosis is triggered by different pathways[15]. A previous study showed that exposure to hyperoxia during the development period upregulated nitrogen oxide synthase in vascular endothelial cells and downregulated Cu/Zn superoxide dismutase, leading to cerebral capillary degeneration[7]. After returning to normoxia, secondary ischemia or hypoxia occurs because of the relative hypoxia, which aggravates brain injury.

However, expression levels of NMDAR subtypes and oxygen toxicity-mediated cell injury during the development period has not been reported. NMDAR extensively distribute in the mammalian central nervous system. During brain development, NMDAR regulate neuronal survival, synaptic formation and plasticity, and play an important role in learning and memory. NR3A expression peaks at P7−10 in rodents, and rapidly decreases before maintaining a low level in adults[16]. The current intensity in NMDAR-mediated cortical cells in NR3A KO mice was 2.8 times higher than wild type mice, and the dendritic spine was significantly increased in the V layer of the cerebral cortex. In addition, after NR3A transfection in NR1 cells, NR1 and NR2B cells, or NR2D cells, the current amplitude of NR3A decreased, and Ca^{2+} permeability decreased by 5 times[17-18]. NMDAR overexcitation is the main mechanism to generate oxygen-derived free radicals, but blocking NMDAR results in a large amount of cell death during brain development[12, 19]. Papadia *et al* [10] reported that NMDAR-mediated synaptic activity can enhance neuronal anti-oxidation by triggering the transcription of many molecules. First, NMDAR activation stimulates thioredoxin activity, decreases peroxidase, and increases tolerance to oxidative stress. The NMDAR antagonist memantine, which is clinically used for treating senile dementia, blocks overexciting extrasynaptic NMDAR but allows synaptic activity to be retained[10, 20] which prevents oxidative damage in newly generated neurons. For the first time, the present study investigated the effects of NR3A on brain cell apoptosis in developing mice exposed to hyperoxia. Our results indicate that neonatal NR3A KO mice exhibit a strong tolerance to oxygen toxicity injury. These results may be because the negative regulatory effects of NR3A were inhibited, which enhances NMDAR-mediated synaptic activity, increases anti-oxidation of the brain and protects nerve cells against apoptotic degeneration.

Neurogenesis is significant in brain development, neurofunctional maintenance and injury repair[21]. Neurogenesis and the regulation of cell apoptosis are critical during brain development. The present study used BrdU to observe the proliferation of neural stem cells in the dentate subgranular zone. Our results show that exposure to hyperoxia significantly increases newly generated cells. It is likely that hyperoxia activates and accelerates the proliferation of neural stem cells, which play a positive role in functional recovery and repair after brain injury.

However, there are potential risks to treat hypoxic disease with high concentrations of oxygen in young children[22-23]. The present study provides evidence of cell apoptosis and a possible mechanism for this apoptosis after hyperoxic exposure in the developing brain. However, further studies should investigate the influence of hyperoxia-mediated cell injury on long-term neurological function, and the correlation between NMDAR and oxygen toxicity.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

This study was performed at the Laboratory of Pathology, the Medical University of South Carolina, USA from June 2005 to June 2006.

Materials

P7 C57/BL6 mice were provided by the Jackson Laboratories (Bar Harbor, ME, USA). The animals were housed at room temperature (21 ± 2°C) with a 12-hour light/dark cycle in the pathogen-free Laboratory Animal Center for Research at the Medical University of South Carolina. All animal experiments and surgical procedures were approved by the University Animal Research Committee and in accordance with NIH standards.

Methods

Preparation of brain tissue samples

Hyperoxia-exposed mice were placed in a closed chamber (75% oxygen and 15% nitrogen[24]; Biospherix Ltd, Redifield, NY, USA). The chamber was opened once

a day to supplement food and water. After 5 days, the mice were placed in normoxia and intraperitoneally injected with BrdU (50 mg/kg)[25-26], daily for 5 consecutive days, to label proliferative mitotic cells. The control mice were housed in normoxia. All mice were anesthetized and sacrificed at P12. The brains were rapidly harvested, embedded in OTC and stored at −80°C. Frozen serial coronal sections (10 μm thick) were prepared. The fifteenth (lateral ventricle) and the twenty-first (dentate gyrus) sections were stained according to the mouse brain atlas[25].

TUNEL/NeuN immunofluorescent double-labeling staining for cell apoptosis in brain tissues

TUNEL staining was performed as previously described[26]. Briefly, the sections were fixed, pretreated with alcohol/acetic acid and Triton X-100, and stained according to the TUNEL apoptosis kit (DeadEnd™Fluorometric TUNEL system, Promega, Madison, WI, USA). The sections were incubated with the mouse anti-NeuN (a specific marker for mature neurons) monoclonal antibody (Chemicon International, Inc., Temecula, CA, USA; 1: 400) overnight at 4°C, followed by the Cy3 rabbit anti-mouse monoclonal antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1: 500) at room temperature for 2 hours. The sections were mounted with long-acting mounting medium.

BrdU/NeuN immunofluorescent double-labeling staining for proliferative cells in the dentate gyrus

BrdU staining was performed as previously described[26]. Briefly, the sections were fixed, incubated with 2 N hydrochloric acid for 1 hour and washed with boric acid before being blocked with 1% fish gel. These sections then were incubated with a rat anti-BrdU monoclonal antibody (Abcam Inc., Cambridge, MA, USA; 1: 400) overnight at 4°C, followed by incubation with the Texas Red goat anti-rat polyclonal antibody (Jackson ImmunoResearch; 1: 500) at room temperature for 2 hours. NeuN staining was performed using the same procedures. Prior to BrdU staining, the primary antibody used was the mouse anti-NeuN monoclonal antibody (Chemicon; 1: 400) and the secondary antibody was a Cy5 goat anti-mouse monoclonal antibody (Jackson ImmunoResearch; 1: 400).

Fluorescent microscopy for cell observation and quantification

A fluorescent microscope (Olympus, Tokyo, Japan) was used to observe stained sections. Every section was viewed in its entirety to quantitate the TUNEL-labeled cells. TUNEL-positive cells in lateral ventricle sections were quantified under the 4× objective of the fluorescent microscope, and the sections of dentate gyrus were photographed under the 10× objective. Newly generated cells labeled with BrdU were also quantified.

Statistical analysis

Measurement data were expressed as the mean ± SD and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). The sample mean was compared using one-way analysis of variance. Paired comparison tests between two groups were performed using the least significant difference *t*-test. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Jimei Li collected and analyzed the experimental data, and wrote the manuscript. Zhongyang Lu collected data and designed this study. Shanping Yu revised the study and obtained funding. Osama Mohamad analyzed the data. Ling Wei obtained funding, provided technical support and guided the study.

Conflicts of interest: None declared.

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Ethical approval: This study received permission from the Ethics Committee of the Medical University of South Carolina, USA.

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