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Research Paper

Nitric oxide induces hypoxia ischemic injury in the neonatal brain via the disruption of neuronal iron metabolism

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ABSTRACT

We have recently shown that increased hydrogen peroxide (H_2O_2) generation is involved in hypoxia–ischemia (HI)-mediated neonatal brain injury. H_2O_2 can react with free iron to form the hydroxyl radical, through Fenton Chemistry. Thus, the objective of this study was to determine if there was a role for the hydroxyl radical in neonatal HI brain injury and to elucidate the underlying mechanisms. Our data demonstrate that HI increases the deposition of free iron and hydroxyl radical formation, in both P7 hippocampal slice cultures exposed to oxygen–glucose deprivation (OGD), and the neonatal rat exposed to HI. Both these processes were found to be nitric oxide (NO) dependent. Further analysis demonstrated that the NO-dependent increase in iron deposition was mediated through increased transferrin receptor expression and a decrease in ferritin expression. This was correlated with a reduction in aconitase activity. Both NO inhibition and iron scavenging, using deferoxamine administration, reduced hydroxyl radical levels and neuronal cell death. In conclusion, our results suggest that increased NO generation leads to neuronal cell death during neonatal HI, at least in part, by altering iron homeostasis and hydroxyl radical generation.

Introduction

Neonatal hypoxia–ischemia (HI) remains an important cause of acute mortality and chronic morbidity in infants and children. The neurologic consequences of injury include mental retardation, epilepsy, cerebral palsy, and blindness [1]. Despite progress in obstetric and neonatal care, the current clinical treatments for neonatal HI brain injury are mainly supportive. The mechanisms underlying the brain injury associated with HI is only partly understood. Although increasing evidence indicates that free radicals and reactive oxygen species are important mediators of ischemic neuronal death [2–4], little is known regarding their specific cellular sources and how they are regulated. Our previous studies have shown that there is increased superoxide and hydrogen peroxide (H_2O_2) production in the HI-exposed neonatal brain [5,6] and this is mediated at least in part via NADPH oxidase brain [5]. The neonatal brain also has a low with antioxidant capacity, including limited GPx activity [7,8] and increasing GPx activity is neuroprotective [5]. The iron-catalyzed formation of hydroxyl radical from H_2O_2 is also recognized as a potent oxidant that can cause cell damage. However, its role in neonatal HI brain injury is unresolved. The neonatal brain accumulates more ferrous and ferric iron than older animals [9–11]. Iron uptake in the neonatal rat brain is also high, reaching a peak during the first 2 weeks [12] and provides a reservoir for brain cellular development. Thus, it also makes the neonatal brain more vulnerable to oxidative stress, as iron can catalyze the Fenton reaction in which H_2O_2 is converted into highly reactive and toxic hydroxyl radicals causing cell death [13].

The free radical, nitric oxide (NO) is well recognized as a physiological mediator in the brain and plays important roles in long-term potentiation (LTP), synaptic plasticity and activity-dependent modification of neural networks [14–16]. NO is enzymatically generated from the conversion of L-arginine and oxygen by various forms of NOS, all three NOS isoforms (eNOS, nNOS and iNOS) are potential sources of NO in the brain. However, the mechanisms by which elevated levels of NO leads to neuronal cell death in the HI brain are still unclear. Interestingly, there are data suggesting that NO can modulate cellular iron metabolism through its ability to inactivate cellular aconitase [17,18] and activate the iron regulatory protein-1 [19] altering the homeostasis that exists between iron uptake and cellular storage to favor uptake.

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The aim of the present study was to investigate the potential role of increased hydroxyl radical generation in HI-mediated neonatal brain injury and to elucidate the involvement of NO. Using both an in vitro hippocampal slice culture model and an in vivo model of neonatal HI we demonstrated that an NO-dependent increase in hydroxyl radical generation plays an important role in the neuronal cell death associated with neonatal HI and this is mediated via a disruption in iron homeostasis.

Materials and methods

Hippocampal slice culture and OGD exposure

Neonatal rats (Sprague–Dawley, Charles River, Wilmington, MA, USA) at postnatal day 7 (P7) were decapitated and the hippocampi dissected under sterile conditions. Each hippocampus was sliced into 400 μm slices using a Mcilwain tissue chopper (Science Products GmbH, Switzerland). Slices were then cultured on permeable membrane Millicell inserts (Millipore, Billerica, MA, USA) (0.4 μm pore size) in six well plates for 6 days at 37 °C in 5% CO_2 as previously described [5,6]. Twenty-four hours before exposure to OGD the culture medium was changed to neurobasal-A and B27 supplement without antioxidants. Just prior to OGD, a sucrose balanced salt solution (SBSS) (120 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 20 mM HEPES, 25 mM sucrose, pH of 7.3) was infused for 1 h with 5% CO_2 and 10 l/h nitrogen gas. The inserts were then transferred into deoxygenated SBSS and placed in a ProOxC system chamber with oxygen controller (BioSpherix, NY, USA) and exposed to 0.1% O_2, 5% CO_2, 94.4% nitrogen for 90 min at 37 °C. The slices were then returned to oxygenated serum-free neurobasal medium with B27 supplement. The NOS inhibitor, L-NAME [Nω-Nitro-L-arginine methyl ester hydrochloride] (100 μM, Sigma-Aldrich, St. Louis, USA) were dissolved in DMSO and added to the medium 2 h before OGD. Control experiments contained the equivalent amount of DMSO that did not exceed 0.2% (v/v). The iron chelator, Deferoxamine (100 μM, Sigma-Aldrich, St. Louis, USA) were dissolved in autoclaved distilled water and added to the medium 2 h before OGD. The hippocampal slice cultures were harvested at 4 h after OGD for further investigation. All protocols were approved by the Institutional Animal Care Committee at Georgia Regents University.

Quantification of slice culture cell death

This was carried out using propidium iodide staining, the live slice culture fluorescence images were recorded at on OGD, and 4 h after OGD groups. The evaluation of cell death was performed using a modification of the method of Cronberg et al. [20], as previously described [5,6].

Aconitase activity

Aconitase activity was measured using the Aconitase Assay Kit (Cayman Chemical, Ann Arbor, MI) [21]. Briefly, hippocampal slice cultures, or isolated rat brain tissue, were washed with ice-cold PBS and lysed in lysis buffer containing 1% Triton X-100, 20 mM Tris, pH 7.4, 100 mM NaCl, with 1 × protease inhibitor cocktail, and 1 × phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples were sonicated on crushed ice with two 10 s bursts and centrifuged at 13,000*g* for 5 min at 4 °C. Supernatants were treated according to the manufacturer's instructions and the protein content was measured. Samples were diluted 1:10 in the kinase buffer provided with the kit, and the absorbance read at 450 nm, using a microplate reader (Synergy HT, Biotek Instruments, VT, USA). Data are present as percentage to the control.

LDH cytotoxicity assay

Cytotoxicity was evaluated by quantification of lactate dehydrogenase (LDH) using a Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany) in the slice culture medium as described [5,6]. All LDH measurements were divided by the protein levels of the samples (Bradford protein assay, Bio-Rad Laboratories, CA, USA).

Histologic evaluations

Brain tissues were washed in PBS, fixed in 4% paraformaldehyde (RT, 1 h), then in 30% sucrose (RT, 1 h), embedded in O.C.T embedding medium (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) and stored at −80 °C overnight. Embedded tissues were frozen sectioned (15 μm), mounted on glass slides. Sections were analyzed for the presence of apoptotic nuclei using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) as described [5,6]. Quantification of the TUNEL stained nuclei and total nuclei was processed by Image-Pro software and presented as a percentage of total nuclei in the field as described [5,6]. Tissue iron deposition was detected in cryostat sections using the Prussian Blue Iron Stain Kit (Sigma-Aldrich, St. Louis, USA) as previously described [22,23]. Briefly, sections were incubated in distilled water with 1% potassium ferrocyanide and 1% hydrochloric acid (HCl) for 15 min. After rinsing with distilled water, sections were counterstained with neutral red. Images were then captured using an Olympus IX70 microscope (Olympus, Japan). The iron intensity (blue) was then quantified using ImageJ (NIH).

Immunoblot analyses

Tissue was homogenized in lysis buffer containing 1% Triton X-100, 20 mM Tris, pH 7.4, 100 mM NaCl, with 1 × protease inhibitor cocktail, and 1 × phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). Lysates were centrifuged at 13,000*g* for 10 min at 4 °C to precipitate the debris, and the protein content in the supernatant determined using the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). Lysate protein (20 μg/lane) was separated using 4–20% gradient gels (Bio-Red, Hercules, CA, USA) and transferred to PVDF membranes with the Trans-Blot® Turbo™ Transfer System(Bio-Red, Hercules, CA, USA). The blots were then probed with the appropriate antibody overnight at 4 °C. Primary antibodies used were anti-IRP-1 (Abcam Inc., Cambridge, MA, USA); anti-Ferritin (Abcam Inc., Cambridge, MA, USA); anti-TfR (Abcam Inc., Cambridge, MA, USA). Blots were washed in 1 × TBST (3 × 15 min) and the appropriate secondary antibodies conjugated to HRP (Sigma, St. Louis, MO, USA) were then added for 1 h at RT (Thermo Scientific, Rockford, IL, USA). After further washing in TBST (3 × 15 min) bands were visualized by chemiluminescence (West-Femto, Pierce, Rockford, IL, USA) and quantified using a Kodak Molecular Imaging System (Kodak, Rochester, NY, USA).

Measurement of hydroxyl radical levels

Hydroxyl radical production was measured using electron paramagnetic resonance (EPR) spectroscopy [24] (Miniscope MS 200, Megnet tech, Berlin, Germany). Fresh tissue homogenates protein levels were measured. Sample solutions for analysis contained 35 μl homogenate and 5 μl of spin trap, 5,5-dimethey-1-pyrroline-N-oxide (DMPO, Cayman Chemical Company, Ann Arbor, MI). Under room temperature, the spectra were obtained using, 2 mW of microwave power, 100 kHz of modulation frequence, 2.0 G of modulation amplitude a 3 min scan time. To quantify the