Free Radical Biology and Medicine 67 (2014) 221–234

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Caffeine protects neuronal cells against injury caused by hyperoxia in the immature brain

Stefanie Endesfelder*, Irina Zaak, Ulrike Weichelt, Christoph Bührer, Thomas Schmitz

Department of Neonatology, Charité University Medical Center, D-13353 Berlin, Germany

ARTICLE INFO

Article history:
Received 5 April 2013
Received in revised form
3 September 2013
Accepted 27 September 2013
Available online 12 October 2013

Keywords:
Postnatal neurogenesis
Hyperoxia
Methylxanthine
Oxidative stress
Preterm infants
Developmental brain
Free radicals

ABSTRACT

Caffeine administered to preterm infants has been shown to reduce rates of cerebral palsy and cognitive delay, compared to placebo. We investigated the neuroprotective potential of caffeine for the developing brain in a neonatal rat model featuring transient systemic hyperoxia. Using 6-day-old rat pups, we found that after 24 and 48 h of 80% oxygen exposure, apoptotic ($TUNEL^+$) cell numbers increased in the cortex, hippocampus, and central gray matter, but not in the hippocampus or dentate gyrus. In the dentate gyrus, high oxygen exposure led to a decrease in the number of proliferating ($Ki67^+$) cells and the number of $Ki67^+$ cells double staining for nestin (immature neurons), doublecortin (progenitors), and NeuN (mature neurons). Absolute numbers of $nestin^+$, $doublecortin^+$, and $NeuN^+$ cells also decreased after hyperoxia. This was mirrored in a decline of transcription factors expressed in immature neurons (Pax6, Sox2), progenitors (Tbr2), and mature neurons (Prox1, Tbr1). Administration of a single dose of caffeine (10 mg/kg) before high oxygen exposure almost completely prevented these effects. Our findings suggest that caffeine exerts protection for neonatal neurons exposed to high oxygen, possibly via its antioxidant capacity.

Preterm infants often suffer from neurologic impairment caused by perturbed development of the immature brain in an unphysiological, ex utero environment. Aims of current research are to design strategies effective for brain protection in these patients. Caffeine has been used as a drug for over 30 years for respiratory stimulation to prevent apnea in preterm infants [1,2]. In a large randomized placebo-controlled multicenter trial [2], a decline in cerebral palsy and neurodevelopmental impairment was achieved by administration of caffeine to preterm infants [3]. The neuroprotective properties of caffeine therapy in preterm infants have been confirmed by observational studies [2–6]. The benefits of caffeine for the brain have also been observed in animal models of Parkinson disease [7], stroke [8,9], Alzheimer disease [10], and oxygen-induced seizures [11]. In newborn rats reared under chronic hypoxia, caffeine administration was demonstrated to reduce hypomyelination and ventriculomegaly [12].

In humans, the period of fastest brain growth is observed during the last 3 months of a full-term pregnancy. In contrast, this brain growth spurt occurs between postnatal days 2 and 10 in newborn rats and mice [13,14]. Therefore, newborn rodents have been used as an experimental model in studies to investigate the mechanisms of vulnerability in the developing brain. Birth is associated with an approximately three- to fourfold sudden increase in the partial pressure of oxygen in all tissues of the body [15,16], and the immature antioxidative defense system in preterm infants is unable to handle this oxygen surge. As a result, the risk of neurological and cognitive sequelae in neonates rises with further increases in oxygen tension [17–20], such as when infants are rescued with oxygen instead of room air. A fourfold increase in the oxygen concentration in P6 rat and mouse pups for 24 to 48 h has been shown to induce apoptosis in neurons and in immature oligodendroglia [21–24], whereas mature oligodendrocytes seem to be more resistant to oxygen toxicity [25]. Two mechanisms are thought to be responsible for hyperoxia-induced injury in the immature brain: the oxidative stress response [26,27], which involves the production of reactive oxygen species, and changes in gene expression and phosphorylation of proteins that control neural cell survival during development [22,28]. Therefore, the postnatal brain and organs of immature preterm infants are vulnerable to oxygen toxicity and the consequences can be severe [20].

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DCX, doublecortin; DG, dentate gyrus; DGM, deep gray matter; FAM, 6-carboxy-fluorescein; GCL, granule cell layer; HPRT, hypoxanthine–guanine phosphoribosyltransferase; NSC, neuronal stem cell; NeuN, neuronal nuclei; 8-oxodG, 8-hydroxy-2′-deoxyguanosine; P, postnatal day; Pax6, paired box 6; PL, polymorphic layer; Prox1, prospero homeobox 1; SOX2, sex-determining region Y-box 2; SGZ, subgranular zone; SVZ, subventricular zone; TAMRA, 6-carboxy-tetramethylrhodamine; Tbr1/2, T-box brain gene 1/2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

* Corresponding author. Fax: +49 30 450 559979.
E-mail address: stefanie.endesfelder@charite.de (S. Endesfelder).

0891-5849/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.09.026

Postnatal neurogenesis in mammals, whose sequence follows the order in the adult dentate gyrus (DG) [29,30], persists in the subgranular zone (SGZ) of the DG in the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles [31–33]. Newly generated granule cell layer (GCL) neurons are integrated into the DG, a process that is tightly regulated and seems to be susceptible to perturbation by environmental factors (reviewed by [34]). Neuronal progenitor cells in the SGZ and SVZ divide slowly and are maintained in adults. Postnatal neuronal progenitor cells differentiate into precursors and give rise to more differentiated neuroblasts [31,33]. Neuroblasts in the hippocampal SGZ migrate into the GCL of the DG, differentiate, and integrate into previously established neuronal networks, where they are thought to play a role in certain forms of hippocampal-dependent learning and memory [35,36]. These neuronal differentiation stages are characterized by the expression of specific neuronal markers and relevant transcription factors [36–38]; see Fig. 1.

Although caffeine is currently one of the 10 most used drugs in neonatology, its effect on the immature brain is largely underinvestigated. The results of our study in rats showed that a single dose of caffeine was efficient to protect neurons in the brain against injury caused by oxygen toxicity, highlighting caffeine as a promising drug for neuroprotection in preterm infants.

Materials and methods

Animals and drug administration

Six-day-old (P6) Wistar rats from timed-pregnant dams (Charité-Universitätsmedizin, Berlin, Germany) were divided into four biological groups: (1) normoxia (FiO_2 21%, room air) and 0.9% NaCl solution intraperitoneally (ip), (2) normoxia and caffeine (10 mg/kg body wt, ip; Sigma–Aldrich, Steinheim, Germany), (3) hyperoxia (FiO_2 80%; OxyCycler BioSpherix, Lacona, NY, USA) and 0.9% NaCl solution (ip), and (4) hyperoxia and caffeine (10 mg/kg body wt, ip; Sigma–Aldrich). Pups were treated once with either saline or caffeine at the beginning of normoxia or hyperoxia exposure lasting for 24 h (P7) or 48 h (P8). The animals were weighed before the caffeine or vehicle administration at the beginning of the experiment, and the caffeine and vehicle dosages were adjusted according to the body weight measurements. For both conditions, pups were kept with their dam, and for the 48-h pups, nursing dams were switched every 24 h between the normoxic and the hyperoxic chambers to provide equal nutrition to each litter. All procedures were approved by the state animal welfare authorities (LAGeSo G-0307/09) and followed institutional guidelines.

Tissue preparation

At 24 and 48 h of oxygen exposure, depending on condition, pups were anesthetized with an ip injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Pups were perfused with normal saline (pH 7.4) for molecular analysis and with 4% paraformaldehyde at pH 7.4 for immunohistochemical analysis. After decapitation, the olfactory bulb and cerebellum were removed. For molecular studies, brain hemispheres were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. For immunohistochemical studies, brains were postfixed at 4 °C for 3 days, embedded in paraffin, and processed for histological staining.

RNA extraction and real-time PCR

Total RNA was isolated by acidic phenol/chloroform extraction (peqGOLD RNApure; PEQLAB Biotechnologie, Erlangen, Germany), and 2 μg of RNA was reverse transcribed. The PCR products of *Pax6, SOX2, Tbr2, Prox1, Tbr1,* and *HPRT* were quantified in real time, using dye-labeled fluorogenic reporter oligonucleotide probes with the sequences summarized in Table 1. The probes were labeled at the 5′ end with the reporter dye 6-carboxy-fluorescein (FAM) and at the 3′ end with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA). The FAM spectral data were collected from reactions carried out in separate tubes using the same stock of cDNA to avoid spectral overlap between FAM/TAMRA and limitations of reagents. PCR and detection were performed in triplicate and repeated two times for each sample in 11 μl reaction mix, which contained 5 μl of 2 × KAPA PROBE FAST qPCR Mastermix (PEQLAB Biotechnologie), 2.5 μl of 1.25 μM oligonucleotide mix, 0.5 μl (0.5 μM) of probe (BioTeZ, Berlin, Germany), and 3 to 17 ng of cDNA template with *HPRT* used as an internal reference. The PCR amplification was performed in 96-well optical reaction plates for 40 cycles with each cycle at 94 °C for 15 s and 60 °C for 1 min. The expression of target genes was analyzed with the StepOnePlus real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) according to the $2^{-\Delta\Delta CT}$ method [39].

Immunoblotting

Snap-frozen brain tissues were homogenized in radioimmunoprecipitation assay buffer (Sigma–Aldrich) with complete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at 3000*g* (4 °C) for 10 min, and the microsomal fraction was subsequently centrifuged at 17,000*g* (4 °C) for 20 min. After collecting the supernatant, protein concentrations were determined using the

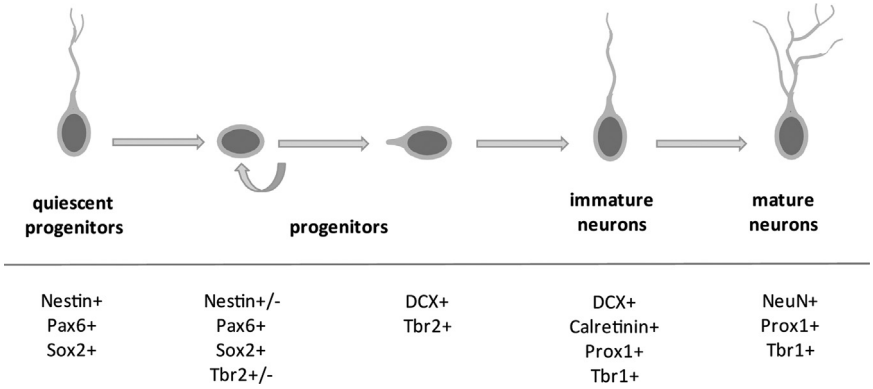

Fig. 1. Maturation of neuronal progenitors in the postnatal hippocampus. Shown are the stages of neurogenesis with expression patterns of selective markers relevant for neural lineage cell development (modified from [36,56,57]). Presented are only transcription factors and neuronal markers relevant for neural lineage cell development: Pax6, paired box 6; SOX2, sex-determining region Y-box2; Tbr1/2, T-box brain gene 1/2; DCX, doublecortin; and Prox1, prospero homeobox 1.