Neurobiology of Disease 65 (2014) 172–179

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Fetal hypoxia increases vulnerability of hypoxic–ischemic brain injury in neonatal rats: Role of glucocorticoid receptors ☆

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ARTICLE INFO

Article history:
Received 12 November 2013
Revised 20 January 2014
Accepted 30 January 2014
Available online 7 February 2014

Keywords:
Hypoxia
Glucocorticoids
Hypoxic–ischemic brain injury
Neonate

ABSTRACT

Gestational hypoxia is a common stress to the fetal development and increases the risk of neonatal morbidity. The present study tested the hypothesis that fetal hypoxia results in heightened brain vulnerability to hypoxic–ischemic (HI) injury in neonatal rats *via* down-regulation of glucocorticoid receptor (GR) in the developing brain. Time-dated pregnant rats were exposed to hypoxia (10.5% O_2) from days 15 to 21 of gestation. Brain HI injury was determined in day 10 pups. Maternal hypoxia resulted in asymmetric intrauterine growth restriction in the fetus. The brain HI injury was significantly increased in maternal hypoxia-treated pups as compared with the normoxia control in both males and females. Activation of brain GR by dexamethasone injection into the right lateral ventricle produced a concentration-dependent reduction of HI-induced brain injury in control pups. Maternal hypoxia significantly decreased GR mRNA and protein abundance in the fetal brain and neonatal hippocampus and abolished the dexamethasone-mediated neuroprotective effect in pup brains. This decreased GR expression was resulted from increased DNA methylation, decreased binding of transcription factors Egr-1 and Sp1 to GR gene exon 1_7 and 1_{11} promoters, and reduced expression of GR exon 1_7 and 1_{11} mRNA variants. The results demonstrate that gestational hypoxia causes epigenetic repression of GR gene expression in the developing brain resulting in the heightened brain vulnerability to HI injury in neonatal rats.

Introduction

Hypoxic–ischemic encephalopathy (HIE) is the most common cause of neonatal brain damage due to systemic asphyxia, which may occur prior, during or after birth (Douglas-Escobar and Weiss, 2012). HIE occurs in about 2% of full-term infants and close to 60% in premature newborns, and causes significant mortality and long-term neurologic sequelae, including learning disabilities, mental retardation, seizure and cerebral palsy (Graham et al., 2008; Kurinczuk et al., 2010). Although little is known about the pathogenesis of HIE, recent studies have suggested that adverse intrauterine environment may contribute to aberrant brain development (Li et al., 2012a,b; Tomalski and Johnson, 2010). Hypoxia is a common form of intrauterine stress, and the fetus may experience prolonged hypoxic stress under a variety of conditions, including pregnancy at high altitude, pregnancy with anemia, placental insufficiency, cord compression, preeclampsia, heart, lung and kidney disease, or with hemoglobinopathy. Although it has been shown that fetal hypoxia affects normal brain development and induces neurological deficits in a variety of behavioral tests in offspring (Li et al., 2012a), the effect of fetal hypoxia-mediated stress on newborn brain HIE remains elusive.

Many factors may be involved in the stress response in the developing brain. Among them, glucocorticoids are essential for the brain development and play a center role in the response to stress. The effects of glucocorticoids are mainly mediated *via* binding to glucocorticoid receptors (GRs), and GRs are highly expressed in the developing brain with dynamic and complicated ontogeny. It has been demonstrated in humans and rodents that early life environment and events are critical in programming of tissue-specific GR expression patterns, particularly in the hippocampus (Li et al., 2012a; Mueller and Bale, 2008; Oberlander et al., 2008; Turner et al., 2008, 2010; Weaver et al., 2005, 2007; Xiong and Zhang, 2013). Both neurodegenerative and neuroprotective effects of glucocorticoids have been reported (Abraham et al., 2001). Glucocorticoids have been shown to affect the vulnerability of fetal and neonatal brains to hypoxia–ischemia challenge; however, the results were inconsistent and dependent on experimental protocol, dosage, timing, animal age, strains and species (Flavin, 1996; Kauffman et al., 1994; Tombaugh et al., 1992; Tuor, 1995, 1997; Whitelaw and Thoresen, 2000). It appears that the concentration and duration of glucocorticoid treatment are the two key factors determining either detrimental or beneficial effects of glucocorticoids in the brain. Although exposure to long-term and high levels of glucocorticoids enhances neurotoxic effects in brain injury, physiological or slightly supraphysiological levels of glucocorticoids confer the brain protective effect to HIE challenges (Abraham et al., 2001). Herein, we present evidence of

☆ Financial support: This work was supported in part by the National Institutes of Health grants HL082779 (L.Z.) and HL083966 (L.Z.).

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Available online on ScienceDirect (www.sciencedirect.com).

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http://dx.doi.org/10.1016/j.nbd.2014.01.020

a novel finding that chronic fetal hypoxia down-regulates GR expression in the developing brain resulting in the increased brain susceptibility to HI injury in neonatal rats, and suggest new insights of molecular mechanisms linking fetal hypoxia to the heightened HIE vulnerability in newborns.

Materials and methods

Experimental animals

Pregnant Sprague–Dawley rats were randomly divided into 2 groups: normoxic control and hypoxic treatment of 10.5% O_2 from days 15 to 21 of gestation, as described previously (Patterson et al., 2010). Given that hypoxia decreased maternal food intake by approximately 40%, a group of pregnant rats were randomized to 60% of control food intake under the normoxic condition during the same gestational period as a pair-fed control. On day 21 of pregnancy, some rats were euthanized and fetal (E21) brains were isolated. Other rats were allowed to give birth, and further studies were conducted in 10-day-old neonatal (P10) pups of both sexes. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Brain HI treatment

The Rice–Vannucci model of unilateral common carotid artery ligation followed by 8% O_2 treatment has been widely used in studies of brain damage in neonatal rats. A modified Rice–Vannucci model was conducted in P10 pups, as previously described (Li et al., 2012b). Pups were anesthetized with 2% isoflurane and the right common carotid artery was ligated. After recovery for 1 h, pups were treated with 8% O_2 for 1.5 h. To determine the role of GR in brain HI injury, a GR agonist dexamethasone (Sigma-Aldrich) was injected into the right lateral ventricle prior to the HI treatment. Pups were anesthetized and fixed on a stereotaxic apparatus (Stoelting, Wood Dale, IL). An incision was made on the skull surface and bregma was exposed. Dexamethasone was injected at a rate of 1 μl/min with a 10 μl syringe (Stoelting, Wood Dale, IL) on the right hemisphere following the coordinates relative to bregma: 2 mm posterior, 1.5 mm lateral and 3.0 mm below the skull surface (Li et al., 2012b). The needle track traced postmortem by brain MRI imaging confirmed intraventricular placement of the needle (online Supplemental Fig. 1). Saline was injected as the vehicle control. The injection lasted 2 min and the needle was kept for additional 5 min before its removal. The incision was sutured.

Measurement of brain infarct size

Forty-eight hours after the HI treatment, pups were sacrificed and brain infarct size was determined as previously described (Li et al., 2012b). Briefly, coronal slices of the brain (2 mm thick) were cut and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride monohydrate (TTC; Sigma-Aldrich) for 5 min at 37 °C and then fixed by 10% formaldehyde overnight. Each tissue slice was weighed, photographed separately and the percentage of infarction area in the ipsilateral hemisphere for each slice was traced and analyzed by ImageJ software (Version 1.40; National Institute of Health, Bethesda, MD) using the control hemisphere to minimize differences in background and adjust the optimal exposure, as it has been previously reported in many studies (Bhattacharya et al., 2013; Gerriets et al., 2004; Li et al., 2012b, 2013; Mélissa et al., 2012). The infarct size was corrected by tissue slice weight, summed for each brain, and expressed as a percentage of whole brain weight.

Western blot

GR protein abundance was determined by Western blot (Xue et al., 2011). Briefly, brains were homogenized and protein concentrations in supernatants were determined. Samples with equal amounts of proteins were separated by electrophoresis, and were probed with primary antibodies against GR or HIF-1α (Santa Cruz Biotechnology). Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody, and proteins were visualized with enhanced chemiluminescence reagents. The target protein abundance was normalized to actin.

Real-time RT-PCR

Total RNA was isolated and subjected to reverse transcription with Superscript III First-Strand Synthesis System (Invitrogen). The abundance of GR mRNA and the alternate exon 1 variants was measured with real-time PCR using iQ SYBR Green Supermix (Bio-Rad), as described previously (Meyer et al., 2009; Xiong et al., 2012). Primers used were listed in Table 1. Real-time PCR was performed in a final volume of 25 μl and each PCR reaction mixture consisted of 500 nM of primers and iQ SYBR Green Supermix containing 0.625 unit hot-start Taq polymerase, 400 μM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris–HCl, 6 mM $MgSO_4$, SYBR Green I, 20 nM fluorescing and stabilizers. We used the following real time-PCR protocol: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, annealing for 10 s at appropriate temperature depending on the primer sequence, 72 °C for 10 s. Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification. PCR was done in triplicate and threshold cycle numbers were averaged for each sample.

Methylated DNA immunoprecipitation (MeDIP)

MeDIP assays were performed with the MeDIP kit (Active Motif, Carlsbad, CA, USA), following the manufacturer's instruction. Briefly, genomic DNA was extracted from tissues and sonicated to yield fragments ranging in size from 200 to 600 base pairs. The double strand DNA fragments were denatured at 95 °C to produce single strand DNA, and a 5-methylcytosine (5-mC) antibody was then used to precipitate DNA containing 5-mC. The 5-mC enriched DNA was subjected to PCR analysis with primers flanking the GR promoter and the PCR products were visualized with 3% agarose gel stained with ethidium bromide and analyzed with ImageJ software. Two sets of primers flanking transcription factor binding sites SP1 and Egr-1 at exon 1_7 promoter and SP1 at exon 1_{11} promoter were used: 5′-AGCCCCTCTGCTAGTGTGAC-3′ (promoter-1_7-F), 5′-TTTCTCTTCTCCCAGGCTCC-3′ (promoter-1_7-R); 5′-AGTCTGGCGT CCTTTTTGGT-3′ (promoter-1_{11}-F), 5′-CCAATCCACCCTACAAGCCC-3′ (promoter-1_{11}-R).

Table 1
Primer sequences for GR and GR gene first exons.

	Forward	Reverse
Exon 1_4	AAGCAACACCGTAACACCTT	AGAAGCAGCAGCCACTGA
Exon 1_5	CATGCAACTTCCTCCGAGT	
Exon 1_7	GGAGCCTGGGAGAAGAGAAA	
Exon 1_{11}	GCCGCAGAGAACTCAACAG	
Exon 1_{10}	CACGCCGACTTGTTTATC	TCTGCTGCTTGGAATCTG
Exon 1_6	ACCTGGCGGCACGCGAGT	GCAGCCACTGAGGGCGAAGA
Exon 1_8	GACAGTCGCCAACAGGTTAA	TGAGAAGCAGCAGCCACT
Exon 1_9	GTCAGTGCCTGGAGCCCGAG	AGCAGCCACTGAGGGCGAAG
GR	AGGTCTGAAGAGCCAAGAGTTA	TGGAAGCAGTAGGTAAGGAGAT
Actin	TCAGGTCATCACTATCGGCAAT	ACTGTGTTGGCATAGAGGTCTT