Published in final edited form as:

Neurobiol Dis. 2014 May ; 65: 172–179. doi:10.1016/j.nbd.2014.01.020.

Fetal Hypoxia Increases Vulnerability of Hypoxic-Ischemic Brain Injury in Neonatal Rats: Role of Glucocorticoid Receptors

Pablo J. Gonzalez-Rodriguez, MS*, Fuxia Xiong, MD, PhD*, Yong Li, MD, PhD, Jianjun Zhou, MD, PhD, and Lubo Zhang, PhD

Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA, 92350

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% O₂) from day 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 1₇ and 1₁₁ promoters, and reduced expression of GR exon 1₇ and 1₁₁ 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.

Keywords

hypoxia; glucocorticoids; hypoxic-ischemic brain injury; neonate

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Corresponding author: Lubo Zhang, PhD, Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University, School of Medicine, Loma Linda, CA 92350, Tel: 909-558-4325, Fax: 909-558-4029, lzhang@llu.edu.

*These two authors contribute equally to the paper.

Disclosures

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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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 (Tomalski and Johnson, 2010; Li et al., 2012). 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 (Xiong and Zhang; Weaver et al., 2005; Weaver et al., 2007; Mueller and Bale, 2008; Oberlander et al., 2008; Turner et al., 2008; Turner et al., 2010; Li et al., 2012a). 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 (Tombaugh et al., 1992; Kauffman et al., 1994; Tuor, 1995; Flavin, 1996; Tuor, 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 supra-physiological levels of glucocorticoids confer the brain protective effect to HIE challenges (Abraham et al., 2001). Herein, we present evidence of 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% O₂ from day 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% O₂ 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 hour, pups were treated with 8% O₂ for 1.5 hours. 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/minute 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 Figure I). Saline was injected as the vehicle control. The injection lasted 2 minutes and the needle was kept for additional 5 minutes 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 minutes 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 Image J 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; Méliissa 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 1 α (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 μ l 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 μ M each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM MgSO₄, SYBR Green I, 20 nM fluorescing and stabilizers. We used the following real time-PCR protocol: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, annealing for 10 seconds at appropriate temperature depending on the primer sequence, 72°C for 10 seconds. 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 1₇ promoter and SP1 at exon 1₁₁ promoter were used: 5'-agccctctgctagtgtgac-3' (Promoter-1₇-F), 5'-tttctcttctcccaggctcc-3' (Promoter-1₇-R); 5'-agtctggcgtccttttgggt-3' (Promoter-1₁₁-F), 5'-ccaatccaccctacaagccc-3' (Promoter-1₁₁-R).

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using the Chip-IT Express Kit (Active Motif), as described previously. Briefly, tissues were minced and fixed with 1% formaldehyde to crosslink and maintain the DNA/protein interactions. After the reactions were stopped with glycine, tissues were washed with PBS. Chromatin extracts were sonicated to produce DNA fragments between 200 and 600 base pairs. Antibody against Sp1, MeCP2 (Active Motif),

Egr-1 (Santa Cruz Biotechnology), and MBD1 (Abcam) were incubated with the chromatin extracts to precipitate the transcription factor/DNA complexes. Crosslinking was then reversed using a salt solution and proteins were digested with proteinase K. The antibody-pulled chromatin extracts were subjected to PCR assay using two sets of primers that flank the predicted transcription factor binding sites at GR exon 1₇ and exon 1₁₁ promoters, as described above in MeDIP. PCR products were visualized by 3% agarose Gel stained with ethidium bromide and analyzed with ImageJ software.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Experimental number (n) represents fetuses and neonates from different dams. Statistical significance ($P < 0.05$) was determined by analysis of variance (ANOVA) followed by Neuman-Keuls post hoc testing or Student's *t* test, where appropriate.

Results

Maternal hypoxia caused asymmetrical growth restriction in the fetus

There was no significant difference in the litter size between normoxia control (10.0 ± 1.6) and hypoxic (10.7 ± 0.8) groups. However, maternal hypoxia resulted in a significant decrease in body and brain weight in E21 fetuses, but a significant increase in the brain to body weight ratio (Fig. 1). In P10 pups, no significant differences in body and brain weight were observed between control and hypoxic animals, suggesting a catch-up growth of hypoxic neonates.

Maternal hypoxia increased neonatal brain vulnerability to HI injury

Maternal hypoxia significantly increased protein abundance of HIF-1 α in the fetal brain (Fig. 2), indicating tissue hypoxia in the fetal brain. This increased HIF-1 α persisted in P10 neonatal rat brains albeit in a reduced extent (Fig. 2). To evaluate the effect of fetal hypoxia on brain lesion vulnerability, brain lesion was induced by HI using the Rice-Vannucci model at P10 pups from normoxic and hypoxic animals. Prenatal hypoxia significantly increased HI-induced brain infarct size in both male and female pups (Fig. 3). To verify that this effect was not due to decreased maternal food intake associated with the hypoxic treatment, a pair-fed study of approximately 60% maternal food intake as that observed in hypoxic pregnant rats was conducted. As shown in Fig. 4, no significant differences were observed in HI-induced brain infarct size in P10 pups by maternal food restriction.

Dexamethasone reduced HI-induced neonatal brain damage

The neuroprotective effect of dexamethasone on HI-induced neonatal brain damage was examined *via* intracerebroventricular administration of dexamethasone prior to the HI treatment. Fig. 5 shows a concentration-dependent neuroprotective effect of dexamethasone on HI-induced brain injury in both male and female pups.

Maternal hypoxia down-regulated GR expression in the developing brain

The effect of maternal hypoxia on GR protein and mRNA abundance in the developing brain was shown in Fig. 6. Maternal hypoxia resulted in a significant decrease in protein and mRNA abundance of GR in brains of both female and male E21 fetuses (Fig. 6A). In P10 neonates, maternal hypoxia significantly reduced GR expression in the hippocampus (Fig. 6B).

Maternal hypoxia selectively decreased GR alternative exon 1 mRNA variants

GR gene has multiple alternative first exons that play an important role in regulating tissue specific expression of GR, and each exon 1 variant has its own promoter (Xiong and Zhang, 2013). Among the multiple exon 1s, exon 1₄ to 1₁₁ are located in the proximal region ranged from about -4.2 to -1.7 Kb upstream of exon 2, and exhibit substantial promoter activities. All exon 1 variants have unique splice donor sites and share a common exon 2 splice acceptor site, encoding a common GR protein. To investigate the effect of fetal hypoxia on the levels of specific GR exon 1 transcripts, quantitative RT-PCR assays were carried out with primers designed to amplify specific mRNA transcripts containing GR exon 1₄ to exon 1₁₁. As shown in Fig. 7A, in brains of both male and female fetuses, the abundance of exon 1₇ and 1₁₁ containing transcripts was significantly decreased by the hypoxic treatment. Other GR exon 1 mRNA variants were not changed by hypoxia. Similar reductions of exon 1₇ and 1₁₁ transcripts were also demonstrated in the hippocampus of P10 neonates (Fig. 7B).

Maternal hypoxia increased methylation of GR 1₇ and 1₁₁ promoters

Accumulating evidence indicates that prenatal stress may affect GR gene expression through epigenetic modification of promoter methylation (Mulligan et al., 2012). The effects of maternal hypoxia on DNA methylation of GR exon 1₇ and 1₁₁ promoters were determined by precipitation of methylated DNA and subsequent PCR analysis. Promoter methylation of GR exons 1₇ and 1₁₁ in the fetal brain was significantly increased by maternal hypoxia (Fig. 8).

Maternal hypoxia reduced transcription factor binding to GR exon 1₇ and 1₁₁ promoters

To determine the functional significance of increased promoter methylation, the binding of methyl-CpG binding proteins MeCP2 and MBD1, as well as transcription factors Sp1 and Egr-1 to GR exon 1₇ and 1₁₁ promoters was determined in the fetal brain by ChIP assays. As shown in Fig. 9, maternal hypoxia significantly increased the binding of MeCP2 to both GR exon 1₇ and 1₁₁ promoters, but had no effect on the binding of MBD1 to the GR promoters. On the other hand, maternal hypoxia significantly decreased the binding of Egr-1 to exon 1₇ promoter and Sp1 to exon 1₁₁ promoter, respectively, in the fetal brain (Fig. 9).

Maternal hypoxia abrogated the neuroprotective effect of dexamethasone

The functional significance of fetal hypoxia-induced down-regulation of GR in the developing brain was determined by evaluating dexamethasone-mediated neuroprotective effect of HI-induced neonatal brain injury in the hypoxia-treated animals. As shown in Fig. 10, maternal hypoxia abolished dexamethasone-induced neuroprotective effect of HI-induced neonatal brain injury in both male and female pups.

Discussion

The present study demonstrates for the first time that fetal hypoxia increases the vulnerability of neonatal brains to hypoxic-ischemic encephalopathy (HIE) in a rat model. Intracerebroventricular administration of a synthetic glucocorticoid dexamethasone protects the neonatal brain from hypoxic-ischemic brain injury, suggesting a local and direct neuroprotective effect of GR activation in the neonatal brain. Fetal hypoxia significantly down-regulates GR expression in the developing brain and abrogates the GR-mediated neuroprotection in the neonatal brain.

In the present study, maternal hypoxia caused fetal asymmetrical growth restriction. While both body and brain weight was reduced, the brain to body weight ratio was in fact significantly increased in hypoxic animals. This hypoxia-induced growth delay in the fetus, however, was recovered by a catch-up growth in the postnatal development. Thus, in P10 pups, no significant differences in body and brain weight were observed between the control and hypoxic animals. The finding that fetal hypoxia and asymmetrical growth restriction increased the vulnerability of neonatal rat brains to HI-induced injury suggests a novel mechanism in the understanding of pathogenesis of HIE that occurs at a significantly higher rate in compromised pregnancy and fetal development. The stage of brain development in P10 neonatal rats is approximately equivalent to that in full-term human baby (Rice et al., 1981; Towfighi et al., 1991). The approach of the Rice-Vannucci model in inducing HI injury in P10 rat brains provided a means of evaluating potential brain injury caused by birth asphyxia in full-term newborns. Although there is a large body of evidence indicating a close link between fetal stress and an increased risk of cardiovascular and metabolic syndrome, stroke, and neurobehavioral pathogenesis in adolescence and/or adulthood, little is known about the effect of fetal stress on the susceptibility of neonatal HIE. Consistent with the present finding, a recent study demonstrated that maternal nicotine administration resulted in the increased vulnerability of HI-induced brain injury in neonatal rats (Li et al., 2012b). Thus, these findings provide new insights linking fetal stress and pathophysiological consequences of heightened HIE in newborns. The finding that fetal stress increased neonatal brain infarction at the macroscopic level determined by TTC staining in the present study ensures the further investigation of heightened brain injury at the microscopic level by the approaches of brain MRI imaging and immunohistochemistry and immunofluorescence, which may reveal other changes in the brain regions outside the TTC-pale areas. In addition, although our study focused on the relatively acute phase of brain injury at 48 hours after HI as it has been reported in many other studies (Hossain et al., 2004; Li et al., 2013; Zhou et al., 2010), the time course of the neurodegenerative process can differ among animals even in the same group. Thus, it is important for the future studies to assess the sustained brain injury and to test aberrant neurobehavioral function at later times. The lack of effect of maternal food restriction in the pair-fed study suggests a hypoxia-specific effect on the increased vulnerability of HI-induced brain injury in neonatal rats.

Although the mechanisms underlying fetal stress-induced increase in neonate brain HI injury remain largely elusive, the present study demonstrated that intracerebroventricular administration of dexamethasone produced a concentration-dependent decrease of HI-induced brain injury in both male and female neonatal rats, suggesting a neuroprotective

effect of local and direct activation of GR in neonatal brains. Dexamethasone is a synthetic glucocorticoid that provides protection against hypoxic ischemic brain damage (Tuor et al., 1997). The present finding is consistent with the previous studies showing a dose-dependent inhibition of hypoxic-ischemic brain injury in neonatal rats by dexamethasone (Barks et al., 1991; Feng et al., 2011). More importantly, the present study demonstrated that fetal hypoxia abrogated the dexamethasone-induced neuroprotective effect in the neonatal brain, suggesting a possible mechanism of GR in fetal stress-mediated increase in vulnerability of HIE in the neonate.

The loss of dexamethasone-mediated neuroprotection in the neonate in hypoxic animals is likely due to the down-regulation of GR expression in the developing brain. The GR activity in regulating tissue and cell function relies on a tight control of its expression levels. In both humans and rodents the regulation of GR levels occurs almost exclusively at the transcriptional level. Early-life environmental events play a key role in programming of tissue GR levels (O'Donnell et al., 1994; Liu et al., 1997; Bertram et al., 2001; Weaver et al., 2004). In the present study, GR protein and mRNA abundance in fetal and neonatal brains was significantly decreased by fetal hypoxia. This is consistent with the previous finding that fetal hypoxia resulted in a significant decrease in GR expression in fetal and neonatal hearts (Xue et al., 2011). These findings suggest that the down-regulation of GR may be a common response to fetal stress among tissues. Human and rat GR genes show a high similarity and both have 11 un-translated exon 1s, all of which have unique splice donor sites and share a common exon 2 splice acceptor site, encoding a common GR protein (McCormick et al., 2000; Turner and Muller, 2005; Turner et al., 2006). Similar to human GR gene, in rats, 8 of the alternate exon 1s, 1₄ to 1₁₁, are located in the proximal region upstream of exon 2, and exhibit substantial promoter activities (McCormick et al., 2000). Whereas the mechanisms regulating the differential usage of these first exons in different tissues and individuals, and the role of the 5' un-translated region in splicing of the coding exons remain poorly understood, multiple first exons plus modulation of alternative first exon activity by their promoters represent the complexity orchestrated by tissue-specific transcription factors in programming of GR expression patterns in a tissue-specific manner. In the present study, we found that fetal hypoxia resulted in a significant decrease in expression levels of GR mRNA variants containing exon 1₇ and 1₁₁ in fetal brains, which sustained in the hippocampus of P10 neonatal brains. Whereas exon 1₇ has been shown to play an important role in regulating hippocampus GR expression and in "resetting" the HPA axis sensitivity resulting from maternal care and neonatal handling (McCormick et al., 2000; Oberlander et al., 2008), fetal stress-mediated modulation of exon 1₁₁ expression in regulating GR expression in the brain has not been reported. Indeed, a previous study suggested that neonatal handling had no significant effect on the expression of exon 1₁₁ in rat hippocampus (McCormick et al., 2000). Thus, the present finding of fetal hypoxia-induced down-regulation of exon 1₁₁ demonstrates the complexity of GR exon 1s involved in stress-specific and developmental age-specific regulation of GR expression in the developing brain, and suggests a novel mechanism of exon 1₁₁ in regulating brain GR expression patterns caused by fetal hypoxia. While maternal food restriction in the pair-fed study showed that growth restriction alone did not modify neonatal brain vulnerability to HI-injury, its effect on GR expression in the brain remains to be determined.

The finding that fetal hypoxia-induced brain GR down-regulation sustained in the P10 neonate suggests a permanent GR repression in the developing brain. Accumulating evidence indicates that perinatal stress affects GR gene expression mainly through epigenetic mechanisms, and increased methylation of GR promoters has been associated with early life experiences in both humans and rats (Oberlander et al., 2008; Suderman et al., 2012). As important regulators of chromosome structure and gene expression, the methyl-CpG binding proteins, MeCP2 and MBD1 can bind to 5' methylated cytosine of CpGs on DNA and suppress gene expression (Guy et al., 2011). The present study showed the binding of both MeCP2 and MBD1 to the promoters of GR exon 1₇ and 1₁₁ in the fetal brain, and the binding of MeCP2 was significantly increased by fetal hypoxia. Increased methyl-CpG binding proteins to GR promoters is likely to induce chromatin remodeling that makes local chromatin structure more condensed and less accessible to transcription factor binding (Jones et al., 1998; Nan et al., 1998; Jaenisch and Bird, 2003). Indeed, the present study demonstrated a significant decrease in the binding of Egr-1 to GR promoter 1₇ and Sp1 to GR promoter 1₁₁, respectively, providing a novel mechanism in fetal stress-induced down-regulation of GR expression in the brain at the molecular level. Among the transcription factor binding sites defined on GR promoters, the NGFI-A (Egr-1) binding site at rat promoter 1₇ and equivalent human promoter 1F has been extensively studied in perinatal programming of GR expression and subsequent disease (Weaver et al., 2004). Sp1 binding sites have also been reported to regulate GR gene expression (Suehiro et al., 2004). In agreement with the present study, previous studies demonstrated that fetal stress resulted in heightened CpG methylation and decreased Egr-1 and Sp1 binding to PKC epsilon promoter, leading to down-regulation of PKC epsilon expression in the developing heart (Patterson et al., 2010; Lawrence et al., 2011). It is thus plausible that transcription factors including Egr-1 and Sp1 and their corresponding binding sites on specific gene promoters are common targets of epigenetic regulation by perinatal environments. Although the present study focused on the epigenetic mechanism of DNA methylation in regulating GR expression levels in the developing brain, future study of immunohistochemical approach may provide additional information in GR distribution in brain regions at the neuroanatomical level and their role in regulating brain function.

The present investigation provides novel evidence that fetal hypoxia increases HI-induced brain injury in neonatal rats, providing new insights linking fetal stress and pathophysiological consequences of heightened HIE in newborns. Although it may be difficult to translate the present findings directly into humans, the possibility that fetal hypoxia may increase the risk of HIE in newborns provides a mechanistic understanding worthy of investigation in humans. This is because hypoxia is one of the most important and clinically relevant stresses to the fetus and because HIE occurs at a significantly higher rate in compromised pregnancy and fetal development. The finding of fetal stress-mediated epigenetic repression of GR in the developing brain provides a novel mechanism in the understanding of HIE at the molecular level, and may suggest new insights for the development of new therapeutic strategies that may be beneficial for the treatment of newborns with HIE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FINANCIAL SUPPORT: This work was supported in part by National Institutes of Health grants HL082779 (L.Z.) and HL083966 (L.Z.).

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Highlights

Fetal hypoxia increases neonatal brain vulnerability to hypoxic-ischemic (HI) injury.

Glucocorticoid receptor (GR) has neuroprotective effects on HI-induced brain injury.

Hypoxia induces epigenetic repression of GR in the developing brain.

Down-regulation of GR diminishes neuroprotection of HI injury in the neonatal brain.

Figure 1. Effect of maternal hypoxia on fetal (A) and neonate (B) body and brain weight
Pregnant rats were treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. Body and brain weight of E21 fetuses (male control, $n = 12$; male hypoxia, $n = 12$; female control, $n = 13$ and female hypoxia, $n = 14$) and day 10 pups (male control, $n = 12$; male hypoxia, $n = 9$; female control, $n = 9$ and female hypoxia, $n = 7$) was measured. Data are mean $\pm$ SEM. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 2. Effect of maternal hypoxia on HIF-1α protein abundance in fetal and neonatal brains
Brains were isolated from E21 fetuses and postnatal day 10 (P10) pups from pregnant rats treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. HIF-1α protein abundance was determined using Western blot analysis. Data are mean ± SEM, $n = 5$. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 3. Effect of maternal hypoxia on HI-induced brain infarct size in the neonate

HI-induced brain infarction was measured in day 10 pups from pregnant rats treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. Four groups were evaluated: male control ($n = 8$), male hypoxia ($n = 20$), female control ($n = 11$) and female hypoxia ($n = 18$). Data are mean $\pm$ SEM. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 4. Effect of maternal food restriction (Pair-Fed) on HI-induced brain infarct size in the neonate

HI-induced brain infarction was measured in day 10 pups from control pregnant rats and those pair-fed with the food intake of pregnant rats treated with hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. Data are mean $\pm$ SEM. $n = 4$.

Figure 5. Effect of dexamethasone on HI-induced brain infarct size in the neonate

Dexamethasone (Dex) was injected into the right lateral ventricle in day 10 pups before HI-induced brain injury. Infarct size at the ipsilateral brain hemisphere was measured and reported as percent of the whole brain. Eight groups were evaluated: saline control (male: $n = 4$; female: $n = 6$), Dex 0.01 µg (male: $n = 10$; female: $n = 10$), Dex 0.05 µg (male: $n = 12$; female: $n = 9$) and Dex 0.1 µg (male: $n = 11$; female: $n = 12$). Data are mean $\pm$ SEM. * $P < 0.05$, Dex vs. saline control.

Figure 6. Effect of maternal hypoxia on GR protein and mRNA abundance in the fetal brain (A) and neonatal hippocampus (B)

Brains were isolated from E21 fetuses and hippocampi from day 10 pups from pregnant rats treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. GR protein and mRNA abundance was determined using Western blot analysis and quantitative real-time RT-PCR, respectively. Data are mean $\pm$ SEM, $n = 5$. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 7. Effect of maternal hypoxia on mRNA abundance of GR exon 1 variants in the fetal brain (A) and neonatal hippocampus (B)

Brains were isolated from E21 fetuses and hippocampi from day 10 pups from pregnant rats treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. mRNA abundance of GR exon 1 variants was determined by quantitative real-time RT-PCR. Data are mean $\pm$ SEM, $n = 5$. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 8. Effect of maternal hypoxia on promoter methylation of GR exon 17 and 111 in the fetal brain

Brains were isolated from E21 fetuses from pregnant rats treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. Methylation of promoter region of GR exon 17 and 111 variants was determined. Data are mean $\pm$ SEM, $n = 5$. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 9. Effect of maternal hypoxia on the binding of methyl-binding proteins and transcription factors to GR exon 17 and 11 promoters in the fetal brain

Brains were isolated from E21 fetuses from pregnant rats treated with normoxia control or hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. Binding of Sp1, Egr-1, MeCP2 and MBD1 was determined by ChIP assays. M, DNA markers. Data are mean $\pm$ SEM, $n = 5$. * $P < 0.05$, hypoxia vs. normoxia control.

Figure 10. Effect of maternal hypoxia on dexamethasone-mediated neuroprotective effect in the neonate

HI-induced brain infarction was measured in day 10 pups from pregnant rats treated with hypoxia at 10.5% O₂ from day 15 to day 21 of gestation. Dexamethasone (Dex) was injected into the right lateral ventricle before HI-induced injury. Four groups were evaluated: male control ($n = 5$), male dexamethasone ($n = 5$), female control ($n = 10$) and female dexamethasone ($n = 7$). Data are mean $\pm$ SEM.

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	ACCTGG CGG CAC GCG AGT	GCAGCCACTGAGGGCGAAGA
exon 1 ₈	GACAGTCGCCAACAGGTAA	TGAGAAGCAGCAGCCACT
exon 1 ₉	GTCAGTGCCTGGAGCCCGAG	AGCAGCCACTGAGGGCGAAG
GR	AGGTCTGAAGAGCCAAGAGTTA	TGGAAGCAGTAGGTAAGGAGAT
actin	TCAGGTCATCACTATCGGCAAT	ACTGTGTTGGCATAGAGGTCTT