Neuroscience 144 (2007) 482–494

GLUCOCORTICOIDS EXACERBATE HYPOXIA-INDUCED EXPRESSION OF THE PRO-APOPTOTIC GENE Bnip3 IN THE DEVELOPING CORTEX

U. S. SANDAU AND R. J. HANDA*

Department of Biomedical Sciences, Colorado State University, 1617 Campus Delivery, Fort Collins, CO 80523-1617, USA

Abstract—Neonatal administration of the synthetic glucocorticoid, dexamethasone (DEX) retards brain growth, alters adult behaviors and induces cell death in the rat brain, thereby implicating glucocorticoids as developmentally neuroendangering compounds. Glucocorticoids also increase expression of pro-apoptotic Bcl-2 family members and exacerbate expression of hypoxic responsive genes. Bnip3 is a pro-apoptotic Bcl-2 family member that is upregulated in response to hypoxia. In these studies, we investigated the interactions of glucocorticoid receptor and hypoxia in the regulation of Bnip3 mRNA in cortical neurons. Using quantitative real time reverse transcription–polymerase chain reaction, we found that DEX treatment of postnatal days 4–6 rat pups caused a significant increase in Bnip3 mRNA expression compared with vehicle controls. A significant increase in Bnip3 mRNA was also measured in primary cortical neurons 72 h after treatment with RU28362, a glucocorticoid receptor selective agonist. In primary cortical neurons, hypoxia increased Bnip3 mRNA expression and this was exacerbated with RU28362 treatment. To elucidate the mechanism of glucocorticoid- and hypoxia-mediated regulation of Bnip3 transcription, a Bnip3 promoter–luciferase reporter construct was utilized in primary cortical neurons. Upregulation of the Bnip3 promoter was mediated by a single glucocorticoid response element and a hypoxic response element. Bnip3 overexpression in primary cortical neurons significantly increased cell death, which is dependent on the Bnip3 transmembrane domain. However, despite the increased expression of Bnip3 following glucocorticoid and hypoxia treatment, corresponding decreases in cell survival were minimal. These studies identify a novel pathway in the developing cortex through which glucocorticoids may enhance a metabolic insult, such as hypoxia.

Key words: Bcl-2, glucocorticoid receptor, dexamethasone, primary cortical neurons, postnatal, apoptosis.

*Corresponding author. Tel: +1-970-491-7130; fax: +1-970-491-7907.
E-mail address: Robert.handa@colostate.edu (R. J. Handa).

Abbreviations: BH3, Bcl-2 homology domain 3; Bnip3ΔGRE2-luc, Bnip3 promoter with a deletion of the GRE2 site; Bnip3ΔTM, Bnip3 protein with a deletion of transmembrane domain; DEX, dexamethasone; DIV, days *in vitro*; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GRE1-luc, Bnip3 promoter with isolated putative GRE1 site; GRE2-luc, Bnip3 promoter with isolated putative GRE2 site; GRE3-luc, Bnip3 promoter with isolated putative GRE3 site; HIF-1, hypoxia inducible factor-1; HRE, hypoxic response element; ISH, *in situ* hybridization; LDH, lactate dehydrogenase; MR, mineralocorticoid receptor; mutHRE luc, Bnip3 promoter with a mutated HRE site; PND, postnatal day; RLU, relative light units; RT-PCR, reverse-transcription–polymerase chain reaction; TM, transmembrane domain.

Corticosterone, the endogenous glucocorticoid secreted by the rat adrenal gland, binds with high affinity to two different receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Reagan and McEwen, 1997). MR activation is associated with a neuroprotective phenotype (Almeida et al., 2000), whereas GR activation is implicated in the induction of an endangered neural phenotype (Sapolsky et al., 1986; Reagan and McEwen, 1997). For example, in the adult, GR activation by the synthetic glucocorticoid dexamethasone (DEX) reduces anterior cingulate cortex volume through dendritic atrophy (Cerqueira et al., 2005a,b).

Elevated glucocorticoid levels also endanger cells in the neonatal brain as evidenced by decreased hippocampal volume (Coe et al., 2003) and retarded overall brain growth and neurological development (Flagel et al., 2002). Postnatal DEX treatment decreases both the number and size of cortical neurons (Kreider et al., 2006), which persists into adulthood as these rats show altered social behavior and learning and memory deficits (Kamphuis et al., 2004; Neal et al., 2004).

In juvenile rats, GR activation increases the incidence of apoptosis in the hippocampus and striatum (Hassan et al., 1996; Haynes et al., 2001). This is consistent with their ability to regulate Bcl-2 family member expression. In the juvenile rat hippocampus, DEX downregulates the anti-apoptotic genes Bcl-2 and Bcl-X_L; while upregulating the pro-apoptotic gene Bax (Almeida et al., 2000). In contrast, in the neonatal rat hippocampus, DEX does not alter Bcl-2 or Bax (Tan et al., 2002). Taken together, these data indicate that glucocorticoid regulation of apoptosis is mediated through a novel mechanism in the neonatal rat brain.

Pro and anti-apoptotic members of the Bcl-2 family initiate apoptotic events. Bnip3 is a unique pro-apoptotic member of the Bcl-2 homology domain 3 (BH3) subfamily (Boyd et al., 1994; Yasuda et al., 1998) in that Bnip3-induced cell death is independent of the BH3 domain (Ray et al., 2000), and is not prevented by Bcl-2 and Bcl-X_L overexpression (Ray et al., 2000). Bnip3 gene regulation has been predominantly investigated following hypoxia. During hypoxic events, hypoxia inducible factor-1 (HIF-1) mediates the transcription of Bnip3 via interaction with a hypoxic response element (HRE) in the promoter region (Bruick, 2000; Sowter et al., 2001). Furthermore, hypoxia induces Bnip3 expression in adult rat hippocampus, cortex and striatum which correlates with increases in cell death (Schmidt-Kastner et al., 2004; Althaus et al., 2006). Notwithstanding, Bnip3 expression in response to hypoxia, its regulation by mechanisms independent of hypoxia, or in-

0306-4522/07$30.00+0.00 © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2006.10.003

teractions between the two have not been investigated in the developing brain. Consequently, the following studies examined glucocorticoid regulation of Bnip3 during both normoxic and hypoxic conditions in cortical neurons.

EXPERIMENTAL PROCEDURES

Animals

Timed pregnant female Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at Colorado State University's laboratory animal research facility. For *in vivo* experiments that used postnatal offspring, pregnant dams were monitored on a daily basis in order to record the litter's birth date and time. The day of birth was considered as postnatal day (PND) 0. Upon birth all litters were culled into groups of 10 pups, 5 males and 5 females, and returned to the dam. Male and female neonates were s.c. injected with a daily dose of DEX (0.2 mg/kg in 100 μl safflower oil) from PND4 through 6. Controls received vehicle at the same injection regimen. Twenty-four hours after receiving the third injection (PND7) animals were halothane anesthetized and killed. For *in vitro* studies using primary cortical neurons, pregnant dams were halothane anesthetized on either gestational day 17 or 18 and the rat fetuses were delivered by cesarean section. The dam was subsequently killed by decapitation. All animal protocols were previously approved by the Colorado State University Animal Care and Use Committee and carried out in accordance with the National Institutes of Health and Institutional Animal Care and Use Guidelines. All protocols were designed to minimize both the total number of animals used and their suffering.

Primary cortical neurons

The primary cortical neurons were harvested from embryonic day 17–18 rat fetuses using protocols modified from Banker and Cowen (1977) and Brewer et al. (1993). During the dissection, brain tissue was kept in a CMF Ringer's–glucose solution (0.155 M NaCl, 5.0 mM KCl, 10.0 mM Hepes, 11.0 mM D-glucose) on ice. Dissected cortical tissue was incubated at room temperature in a solution consisting of 50% CMF Ringer's–glucose plus 50% trypsin (62,000 units/ml) for 15 min. Cortical tissue was washed once with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA [DMEM]) containing 15% fetal bovine serum then dissociated by titration in the same media. Cortical neurons were plated in Neurobasal medium without Phenol Red (Invitrogen) supplemented with 1.0× B27 (Invitrogen), 100 μg/ml penicillin–streptomycin (Invitrogen), 0.5 mM L-glutamine (Mediatach, Inc., Herndon, VA, USA) and 0.025 mM L-glutamic acid (Sigma-Aldrich Co., St. Louis, MO, USA). Primary cortical neurons were plated at a density of 5×10^5 cells/9.4 cm^2 for RNA isolation studies, 3×10^5 cells/1.9 cm^2 for MTT, lactate dehydrogenase (LDH), and Bnip3 promoter studies, and 0.3×10^5 cells/0.3 cm^2 for Bnip3 expression plasmid studies. All cultures were maintained for 3 days *in vitro* (DIV) in plating media. Experiments were begun after 4–5 DIV and concluded by 8 DIV so that results were from primary cortical neurons of an immature phenotype.

Experiments that involve RNA isolation and cell death as a result of glucocorticoid and hypoxia treatment were conducted in cells grown in Neurobasal media without Phenol Red, and supplemented with 0.1× B27, 100 μg/ml penicillin–streptomycin and 0.5 mM L-glutamine. Transfection studies using the Bnip3 promoter constructs were conducted in cells grown in the above media minus penicillin–streptomycin. The B27 was diluted in order to obtain a final concentration of 2.8 nM corticosterone in the treatment media. Primary cortical neurons treated with glucocorticoids received either vehicle (0.005% of 95% EtOH in the previously described media) or doses of the serially diluted GR agonist RU28362 (0.1, 1.0, 5.0 or 10.0 nM; gift from Roussel-UCLAF, Romainville, France). Throughout the experiment cultures were maintained at 37 °C in ambient air infused with 5% CO_2, or in Modulator Incubator Chambers (Billups-Rothenberg Inc., Del Mar, CA, USA) filled with 20% O_2, 5% CO_2, N_2 balanced (normoxic) or 1% O_2, 5% CO_2, N_2 balanced (hypoxic) for either 48 or 72 h. Transfection of primary cortical neurons with the Bnip3 and Bnip3 protein with a deletion of transmembrane domain (Bnip3ΔTM) expression plasmids was conducted in Neurobasal medium minus Phenol Red, supplemented with 1× B27, and 0.5 mM L-glutamine.

Bnip3 cDNA and expression plasmids

A Bnip3 cDNA plasmid was designed for use in *in situ* hybridization (ISH) studies and for generating the Bnip3 cDNA standards for real time reverse transcription–polymerase chain reaction (RT-PCR). The Bnip3 cDNA plasmid was designed as previously described by Sandau and Handa (2006). The Bnip3 and Bnip3ΔTM expression plasmids were kindly provided by Dr. Don Dubik (Manitoba Institute of Cell Research, Winnipeg, MB, Canada). The expression plasmids were synthesized as previously described (Chen et al., 1997). The pcDNA 3.0 (Invitrogen) expression plasmid was transfected as a negative control.

Promoter reporter plasmids

The Bnip3 and Bnip3 promoter with a mutated HRE site (mutHRE luc) promoter luciferase reporter plasmids were kindly provided by Dr. Richard Bruick (University of Texas Southwestern Medical Center, Dallas, TX, USA). The promoter/reporter gene plasmids were generated as previously described (Bruick, 2000). The Bnip3 and mutHRE luc promoters were inserted into the pGL3-Basic plasmid (Promega Corporation, Madison, WI, USA). The Bnip3 promoter luciferase reporter plasmid was used to generate the GRE1 (GRE1-luc), GRE2 (GRE2-luc), GRE3 (GRE3-luc) and Bnip3ΔGRE2 promoter luciferase reporter plasmids. The locations of the three putative GREs within the Bnip3 promoter (GenBank accession nos. AF283504) are −528 to −514 (GRE1), −367 to −353 (GRE2) and −211 to −197 (GRE3). To generate the GRE1-luc and GRE2-luc plasmids, forward and reverse primer pairs were designed to individually isolate the Bnip3 promoter regions containing the putative GRE1 and GRE2 sites. The forward (5′ CAC AGG TAC CGC AGG AGG AGG TCC CCA ACC C 3′) and reverse (5′ CAC AGC TCA GCG AGC AAC ACT GAG GCG CTA GG 3′) primer pairs for amplifying GRE1 are at positions −500 and −455. The forward (5′ CAC AGG TAC CGC TCA GTG TTG CTC GCA CCT CG 3′) and reverse (3′ CAC AGC TCA GCG GGG TGG AGC CTG GTT GG 3′) primer pairs for amplifying GRE2 are at position −499 to −263. The forward primers include a 5′ *Kpn*I restriction site; while the reverse primers have a 5′ BpII restriction site. PCR amplification of GRE1 and GRE2 was conducted using the Bnip3 promoter as template. Amplified PCR product was digested with the restriction enzymes *Kpn*I and BlpI. Double digest with *Kpn*I and BlpI was also performed on the full length Bnip3 promoter to excise the three putative GREs. The size of the GRE1 and GRE2 cDNAs and digested plasmid was confirmed by 1% agarose gel electrophoresis. GRE1 and GRE2 were subsequently directional cloned into the digested Bnip3 plasmid using the Rapid DNA Ligation Kit (Roche Inst., Indianapolis, IN, USA). The GRE3-luc plasmid was generated by double digest of the full length Bnip3 promoter luciferase reporter plasmid with *Bst*XI and *Kpn*I to excise the promoter region containing the putative GRE1 and GRE2 sites. The digested plasmid was blunt end ligated with the Rapid DNA Ligation Kit. The Bnip3ΔGRE2-luc plasmid was generated by site directed mutagenesis using the QuikChangeII XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) per the manufacturer's specifications. Forward (5′ CGT GCA GGT CCC GGC TAG CCT CAA GG 3′) and reverse (5′ CCT GAG GCT AGC CGG