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Research Report

Selective involvement of BH3-only Bcl-2 family members Bim and Bad in neonatal hypoxia–ischemia

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ARTICLE INFO

Article history:
Accepted 27 April 2006
Available online 15 June 2006

Keywords:
Apoptosis
BH3-only
Hypoxia–ischemia
Neonatal

Abbreviation:
HI, hypoxia–ischemia

ABSTRACT

Perinatal hypoxic–ischemic injury is a common cause of neurologic disability mediated in part by Bcl-2 family-regulated neuronal apoptosis. The Bcl-2 protein family consists of both pro- (e.g. Bax, Bad, Bid, Bim) and anti-apoptotic (e.g. Bcl-2, Bcl-X_L) proteins that regulate mitochondrial outer membrane integrity, cytochrome c release and caspase activation. Previous studies have implicated Bax as an important mediator of neuronal death in several models of brain injury, including neonatal hypoxia–ischemia (HI). In this study, we assessed the roles of several members of the pro-apoptotic BH3 domain-only Bcl-2 sub-family in an in vivo mouse model of neonatal HI. Seven-day old control and gene-disrupted mice underwent unilateral left carotid ligation followed by 45 min exposure to 8% oxygen and the extent of brain injury was assessed 2 days later. Following HI, mice deficient in Bad or Bim exhibited reduced activated caspase-3 and glial fibrillary acidic protein immunostaining in their brains compared to similarly treated control animals. Measurement of hippocampal area showed decreased parenchymal loss in both Bad- and Bim-deficient mice versus control animals. In contrast, loss of Bid, another BH3-only protein, provided no protection from neonatal HI brain injury. These results indicate that Bad and Bim are selectively involved in neuron death following neonatal HI and may be targets for therapeutic intervention.

1. Introduction

Perinatal hypoxic–ischemic (HI) injury is a common cause of neurological disability, complicating one in 1000 term births and 15% of all premature births (Volpe, 2000). Perinatal HI injury is accompanied by neurodegeneration, including features of both necrotic and apoptotic neuronal death as well as destruction of neurites connecting different neuronal populations (Ferriero, 2004). Functional recovery is typically limited and few treatments are currently available to reverse this type of brain damage. In studies with experimental animals, reducing neuronal death during and/or immediately after HI injury has been shown to markedly decrease long-term disability (Almli et al., 2000). In order to design appropriate neuroprotective interventions, it is necessary to characterize the molecular pathways involved in HI-induced neuronal injury and death.

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doi:10.1016/j.brainres.2006.04.132

Multiple cell death mediators have been reported to be activated in in vivo and in vitro models of neonatal HI injury, including various Bcl-2 family members, death receptors and caspases (Calvert and Zhang, 2005). The Bcl-2 family is a group of related proteins that play an essential role in cell death regulation (Akhtar et al., 2004). Anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1) contain three or all four Bcl-2 homology (BH) domains. The pro-apoptotic members can be further sub-divided into two groups: the multi-BH domain proteins (Bax, Bak, Bok) contain the BH1, BH2 and BH3 domains and have a 3D structure that is very similar to that of their pro-survival relatives (Suzuki et al., 2000). In contrast, the BH3-only proteins (Bad, Hrk, Bid, Bik, Bim, Bmf, Noxa and Puma) contain just the BH3 domain (Puthalakath and Strasser, 2002). Genetic and biochemical studies have shown that BH3-only proteins sense stress signals and initiate the death program whereas the Bax/Bak-like proteins function further downstream in apoptosis signaling (Huang and Strasser, 2000).

In mammals, apoptotic cell death can occur via two distinct but ultimately converging pathways: the Bcl-2 family regulated (also called 'intrinsic' or 'mitochondrial') pathway and the death receptor triggered, FADD/caspase-8-dependent (also called 'extrinsic') pathway. Specific death signals, such as trophic factor deficiency, hypoxia and DNA damage typically activate the Bcl-2-regulated pathway. In this pathway, Bax translocates from the cytosol to the mitochondrial membrane and triggers release of mitochondrial cytochrome c into the cytosol, which then promotes formation of the Apaf-1/caspase-9/cytochrome c 'apoptosome', leading to activation of the caspase cascade and apoptotic cell death (Green and Kroemer, 2004). BH3-only proteins initiate apoptosis signaling, at least in part, by binding pro-survival Bcl-2 family members, thereby preventing their interaction with Bax/Bak (Strasser et al., 2000). In the extrinsic apoptotic death pathway, the ligation of cell surface 'death receptors', such as Fas (APO-1/CD95), triggers caspase-8 activation, which proteolytically activates effector caspases, such as caspase-3, resulting in cell death. Bid is a proteolytic target for caspase-8, and its cleavage produces the truncated, potently pro-apoptotic form of Bid (tBid) that can translocate to the mitochondria and inactivate anti-apoptotic Bcl-2 family members (Green and Kroemer, 2004; Puthalakath and Strasser, 2002; Wang, 2001). Thus, Bid serves to amplify death receptor signaling via engagement of the Bcl-2-regulated apoptotic pathway.

Experiments with gene-disrupted mice have uncovered important roles for specific Bcl-2 family members in both neonatal and adult HI injury. For example, decreased cell death is observed in Bax-deficient neonatal animals relative to wild-type littermates following exposure to HI injury (Gibson et al., 2001). In contrast, loss of Bcl-2 increased infarction size in a murine adult stroke model (Hata et al., 1999), whereas over-expression of Bcl-2 (Lawrence et al., 1997; Zhao et al., 2003) or Bcl-X_L(Parsadanian et al., 1998) was neuroprotective. In order to better understand the regulation of neonatal HI neuron death, it is important to determine the role of the BH3-only Bcl-2 family members upstream of Bax. We systematically compared the role of Bad, Bim and Bid in neonatal HI using gene-targeted mice. We found that loss of Bad or Bim attenuates neonatal HI-induced neuronal death whereas Bid deficiency had no impact, indicating that BH3-only family members selectively trigger neurodegeneration following neonatal HI.

2. Results

2.1. *Time course of neonatal HI-induced hippocampal injury*

We first characterized the Rice–Vannucci neonatal HI model (Rice et al., 1981) using several methods. To identify a time point early enough to assess apoptotic mediators yet late enough to ensure a reproducible and quantitative measurement of HI injury, we determined the time course of HI injury in C57BL6/J mice with respect to nuclear alterations as well as cleaved (activated) caspase-3 immunoreactivity in neurons and reactive gliosis (GFAP immunoreactivity). Animals were sacrificed at 0 h, 3 h, 6 h, 12 h, 1 day, 2 days, 4 days or 7 days following HI. Our preliminary studies of neonatal HI in C57BL6/J animals confirmed prior reports of relatively selective hippocampal injury (Sheldon et al., 1998) so we used the hippocampus as the focus of our analysis. Representative photomicrographs of evolving hippocampal injury from 0 h–7 days are shown in Fig. 1. Animals exposed to HI and immediately sacrificed had no increase in nuclear pyknosis, cleaved caspase-3 or GFAP immunoreactivity compared to untreated animals (0 h, Fig. 1, top row). Condensed nuclei were detectable by cresyl violet staining within the CA1 region of the hippocampus as early as 3–6 h following neonatal HI and persisted for 2 days or more (first column). One week following HI, only rare pyknotic nuclei were identified, although the pyramidal cell layer was clearly thinned, indicating extensive neuron loss.

Robust cleaved caspase-3 immunoreactivity (Fig. 1, middle column) was seen within the ipsilateral hippocampal pyramidal layer as early as 3 h following HI (data not shown), peaked between 6 and 24 h after HI and persisted up to 4 days. Scattered cleaved caspase-3-like immunoreactivity was also observed in the dentate gyrus and striatum and in the cortex adjacent to the hippocampus, especially in animals with more pronounced infarction (data not shown). The temporal pattern of nuclear pyknosis was similar to that of cleaved caspase-3 with maximal pyknosis between 6 and 24 h after HI. No increases in cleaved caspase-3 immunoreactivity were observed in the non-lesioned hemisphere at any time point (data not shown).

We also assessed GFAP staining as a measure of reactive gliosis following neonatal HI. Detectable increases in GFAP immunoreactivity were observed in the ipsilateral hippocampus as early as 1 day following HI (Fig. 1, third column) and GFAP staining increased steadily 2–7 days following HI. By 2 days following HI, increased GFAP immunoreactivity was also seen in the ipsilateral striatum and cortex overlying the injured hippocampus (data not shown).

The combined assessment of nuclear pyknosis, cleaved caspase-3 immunoreactivity and reactive gliosis enabled detection of HI-induced hippocampal injury reliably at 2 days following HI. The time course for changes in each of these parameters is depicted graphically in Fig. 2A. In Fig. 2B, the time course of hippocampal area loss is shown. Two days following injury, a significant increase in ipsilateral