ELSEVIER

Int. J. Devl Neuroscience 23 (2005) 673–686

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

www.elsevier.com/locate/ijdevneu

Biphasic changes in the levels of poly(ADP-ribose) polymerase-1 and caspase 3 in the immature brain following hypoxia–ischemia

Shannon S. Martin [a], J. Regino Perez-Polo [a], Kristin M. Noppens [b], Marjorie R. Grafe [a,b,*]

[a] *University of Texas Medical Branch at Galveston, USA*
[b] *Oregon Health and Science University, USA*

Received 22 March 2005; received in revised form 24 August 2005; accepted 25 August 2005

Abstract

Poly(ADP-ribose) polymerase-1 (PARP-1) is a DNA repair-associated enzyme that has multiple roles in cell death. This study examined the involvement of PARP-1 in ischemic brain injury in the 7-day old rat, 0.5–48 h after unilateral carotid artery ligation and 2 h of 7.8% oxygen. This experimental paradigm produced a mild to moderate injury; 40–67% of animals in the ligated groups had histological evidence of neuronal death. Ipsilateral cortical injury was seen at all survival times, while mild contralateral cortical injury was seen only at the 1 h survival time. Hippocampal injury was delayed relative to the cortex and did not show a biphasic pattern. Immunohistochemical staining for PARP showed bilateral increased staining as early as 1 h post-hypoxia. PARP staining at early time periods was most intense in layer V of cortex, but did not demonstrate a pattern of cell clusters or columns. Ipsilateral PARP-1 levels quantified by western blotting showed a biphasic pattern of elevation with peaks at 0.5 and 12 h post-hypoxia. Contralateral PARP-1 levels were also elevated at 0.5 and 24 h. PARP activity as determined by immunoreactivity for poly(ADP-ribose) (PAR) was increased ipsilaterally at 0.5, 2 and 12 h survival times. Cortical caspase 3-activity was increased ipsilaterally at 6, 12, and 24 h and contralaterally at 0.5, 1, 2 and 6 h post-hypoxia.

There are three main findings in this study. First, changes in the distribution and amount of cell death correlate well with measured PARP-1 levels after hypoxia–ischemia, and both display biphasic characteristics. Second, there are significant early, transient morphological and biochemical changes in the contralateral cortex after neonatal hypoxia–ischemia due to unilateral permanent occlusion of a carotid artery followed by 2 h of systemic hypoxia. Third, variability in the responses of individual pups to hypoxia–ischemia suggests the presence of unidentified confounding factors.

Keywords: PARP-1; Hypoxia; Ischemia; Cell death; Neonatal; Rat; Brain

Hypoxic–ischemic (HI) insults to the perinatal brain contribute significantly to neonatal morbidity and mortality, particularly in premature infants. There have been multiple studies using animal models aimed at uncovering the events involved in cell death after neonatal HI. Some studies have described events that occur at the beginning of the cell death pathway, including increases in glutamate release (Silverstein et al., 1991), increased nitric oxide synthase activity (Lubec et al., 1999; Muramatsu et al., 2000) and impairment of mitochondrial function (Gilland et al., 1998b; Puka-Sundvall et al., 2001), while others have examined more downstream events including induction of caspase 3-activity (Bossenmeyer-Pourie et al., 1999; Wang et al., 2001; Gill et al., 2002), DNA damage (de Torres et al., 1997; Zhu et al., 2000), increased cytokine expression (Hagberg et al., 1996) and calpain activation (Blomgren et al., 2001). A number of studies have addressed the question of whether the mode of cell death in perinatal ischemic brain injury is apoptotic or necrotic, with many concluding that cell death occurs via both apoptosis and necrosis (Ferrer et al., 1994; Sidhu et al., 1997; Pulera et al., 1998; Martin et al., 2000; Northington et al., 2001) or that there is a spectrum of cell death modes with features of both (Portera-Cailliau et al., 1997; Puka-Sundvall et al., 2000a). One process that is involved in both apoptotic and necrotic cell death is the activation of the enzyme poly(ADP-ribose) polymerase-1 (PARP-1).

PARP-1 is an abundant DNA repair-associated enzyme that is constitutively expressed in the nuclei of eukaryotic cells. PARP-1 expression and activation occur rapidly in response to

* Corresponding author at: Department of Pathology, Oregon Health and Science University, 3181 S.W. Sam Jackson Park Road, L113 Portland, OR 97239-3098, USA. Tel.: +1 503 494 2321; fax: +1 503 494 2025.
E-mail address: grafem@ohsu.edu (M.R. Grafe).

0736-5748/$30.00
doi:10.1016/j.ijdevneu.2005.08.002

DNA damage (Zhang et al., 1995; Bursztajn et al., 2000). PARP-1 binds to sites of DNA damage, then activated PARP-1 synthesizes poly(ADP-ribose) units (PAR) from NAD+ and catalyzes the transfer of these units onto itself and other nuclear proteins, resulting in their transient inactivation and subsequent dissociation from the DNA. Activation of PARP-1 is a well-documented event in cell death (Zhang et al., 1995; Endres et al., 1997; Cosi and Marien, 1999; LaPlaca et al., 2001) and PARP inhibition has been shown to reduce infarct size in both adult and immature animals (Endres et al., 1997; Lo et al., 1998; Tokime et al., 1998; Takahashi et al., 1999; Ducrocq et al., 2000; Hagberg et al., 2004). It is likely that PARP-1 participates in cell death by multiple mechanisms (Le Rhun et al., 1998; Ha and Snyder, 2000). In response to massive levels of DNA damage, PARP-1 is over-activated and the catalytic function of PARP-1 leads to the depletion of cellular energy and eventually cell death by necrosis. In this situation, the catalytic function of PARP-1 may prevent severely damaged cells from attempting to repair themselves and surviving with high mutation frequency (Berger, 1985). The catalytic function is thought to dominate in early cell death whereas delayed cell death primarily involves PARP-1's role as a substrate for caspases (Kaufmann et al., 1993; Lazebnik et al., 1994; Nicholson et al., 1995; Tewari et al., 1995). During apoptosis, activated caspase 3 cleaves PARP-1 and inhibits the catalytic properties of PARP-1, which may, in turn, conserve the cellular energy required for apoptosis. It has been suggested that cleavage of PARP-1 by caspase 3 during apoptosis facilitates nuclear disassembly and may help to ensure the completion of the apoptotic process (Oliver et al., 1998; Herceg and Wang, 1999). Activation of PARP-1 also leads to rapid release of apoptosis-inducing factor (AIF) from the mitochondria and translocation to the nucleus (Yu et al., 2002), which then induces condensation of the chromatin and exposure of phosphatidyl serine on the outer leaflet of the plasma membrane, both considered markers of apoptosis (Susin et al., 1999; Cande et al., 2004).

The morphologic features of dying neurons in immature animals are different from those in adults, and the molecular events that occur during cell death may also differ. While many studies have examined the involvement of PARP-1 in injury paradigms in adult animals, only a few studies have examined PARP-1 in immature animals (Ducrocq et al., 2000; Joashi et al., 1999; Joly et al., 2003; Hagberg et al., 2004). Increased PARP-1 expression has also been identified in karyorrhexic neurons in newborn humans with the distinct pattern of ischemic neuronal injury termed pontosubicular necrosis (Meng et al., 2000). In the current study, we investigated the involvement of PARP-1 in the development of brain injury in postnatal day 7 rats using the Rice–Vannucci model of perinatal HI (Rice et al., 1981). We hypothesized that PARP-1 is an important mediator of cell death in the immature brain and will change in concentration and activity as cell death evolves. To address this hypothesis we examined the time course of cell death after HI and how it relates to changes in the cortical distribution of PARP, concentration of PARP-1, PARP activity, and caspase 3-activity.

1. Materials and methods

1.1. Hypoxia–ischemia

All procedures involving animals were conducted according to criteria approved by the Institutional Animal Care and Use Committees at the University of Texas Medical Branch and Oregon Health and Science University. Timed-pregnant Sprague–Dawley rats were purchased from a commercial breeder (Harlan), housed in individual cages, and fed a standard laboratory chow ad libidum. Offspring were delivered spontaneously and maintained with their dam; litters were culled to 10 on postnatal day 1 or 2. On postnatal day 7 (P7), HI was induced as described by Rice et al. (1981) with some modifications (Grafe, 1994). This widely used model was selected for its relative ease in studying large numbers of animals and because of the potential for long-term survival and behavioral studies (Roohey et al., 1997). The development of the P7 rat brain is similar to that of a late third gestation/term human infant brain, and specific developmental changes in susceptibility to injury are well described at this age (Dobbing and Sands, 1979; Grafe, 1994; Towfighi et al., 1997). Briefly, the rats were given halothane in air by mask inhalation for induction (4%) and maintenance of anesthesia throughout surgery (2%). The left common carotid artery was exposed through a midline incision in the neck and dually ligated with 7-0 prolene suture, then divided. The surgical procedure was performed with pups on a Deltaphase Isothermal pad (Braintree Scientific) to maintain body temperature. Four to 6 h after surgery, the rats were exposed to 7.8% oxygen at 37 °C for 2 h and then returned to the dam until euthanasia. Sham-operated rats were anesthetized, had the carotid artery isolated but not ligated, and exposed to hypoxia. Normal rats were removed from the dam for the same periods of time as the ligated and sham-operated rats and kept warm in room air.

1.2. Morphological studies

Rats were euthanized at 0.5, 1, 2, 6, 12, 24, and 48 h post-hypoxia. At each time point, 7–11 ligated and 5–8 sham-operated rats were utilized. Twelve normal rats were included in the morphological analysis. Rats were euthanized with Nembutal and perfused with saline followed by cold 4% paraformaldehyde. The brains were removed from the cranium and post-fixed in Bouin's solution. After 24 h, coronal sections of the brains were processed and embedded in paraffin. Six micrometers thick sections were made at the level of the mid-hippocampus and the tissues were stained with hematoxylin and eosin (H&E).

1.3. Quantification of injury

A defined, full-thickness (from pial surface to white matter) region of parietal cortex and all regions of the hippocampus were examined bilaterally for injury. Ischemic neurons were identified by pyknotic or karyorrhexic nuclei and hypereosinophilic cytoplasm. Ischemic injury was scored according to the following semi-quantitative scale: 0, normal (<1% of total neurons in the defined region showing ischemic change); 1, mild injury (1–10% ischemic neurons); 2, moderate injury (10–50% ischemic neurons); 3, severe injury (>50% ischemic neurons) (Tateishi et al., 1989). All sections were coded and evaluated without knowledge of treatment group. To control for scoring bias and errors, each section was scored independently by two investigators. Any differences in scores were resolved by joint review. Group injury scores were calculated as the mean score for each group.

1.4. PARP immunohistochemistry

Deparaffinized tissue sections were microwave irradiated 2 × 5 min in 0.5 mM sodium citrate buffer, pH 6.0, for retrieval of antigens, and endogenous peroxidases were quenched in a 3% hydrogen peroxide solution for 5 min. Sections were incubated with monoclonal mouse anti-human PARP (Serotec, MCA-1522) diluted 1:100 in phosphate buffered saline (PBS) containing 0.1% BSA and 0.01% Triton X-100 at room temperature for 12 min. The secondary antibody (biotinylated anti-mouse + anti-rabbit, rat absorbed; Dako LSAB2 kit) was applied to the tissue for 12 min. After washing the tissues in PBS, they were incubated with the LSAB2 peroxidase-conjugated streptavidin complex for 10 min. The reaction was visualized with 3,3′-diaminobenzidine (DAB). The