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Protein ubiquitination in postsynaptic densities after hypoxia in rat neostriatum is blocked by hypothermia

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ABSTRACT

Synaptic dysfunction has been associated with neuronal cell death following hypoxia. The lack of knowledge on the mechanisms underlying this dysfunction prompted us to investigate the morphological changes in the postsynaptic densities (PSDs) induced by hypoxia. The results presented here demonstrate that PSDs of the rat neostriatum are highly modified and ubiquitinated 6 months after induction of hypoxia in a model of perinatal asphyxia. Using both two dimensional (2D) and three dimensional (3D) electron microscopic analyses of synapses stained with ethanolic phosphotungstic acid (E-PTA), we observed an increment of PSD thickness dependent on the duration and severity of the hypoxic insult. The PSDs showed clear signs of damage and intense staining for ubiquitin. These morphological and molecular changes were effectively blocked by hypothermia treatment, one of the most effective strategies for hypoxia-induced brain injury available today. Our data suggest that synaptic dysfunction following hypoxia may be caused by long-term misfolding and aggregation of proteins in the PSD.

Introduction

The ubiquitin–proteasome system (UPS) is a protein complex responsible for the degradation of misfolded proteins. Ubiquitination and protein aggregation are important factors in neuronal function and disease (McNaught et al., 2003; Ehlers, 2004; Yi and Ehlers, 2005; DeGracia and Hu, 2007; Ge et al., 2007). Although dysfunction of the UPS and protein aggregation have been implied in neuronal cell death after ischemia (Hu et al., 2000; Mengesdorf et al., 2002; Liu et al., 2006), the mechanisms responsible for neuronal damage after cerebral hypoxia are only beginning to emerge. Several lines of evidence indicate that overactivation of glutamate receptors within synapses might play a role in the damage produced by a hypoxic–ischemic insult (Choi, 1995). Although over-release of glutamate can be seen a few seconds after the hypoxic injury and rapidly returns to the control value, neuronal death can occur several days following the hypoxic episode (Kirino et al., 1984; Van de Berg et al., 2002). Hence, different types of signals may be involved in late neuronal cell death and they could be triggered at the synaptic level.

The pioneering work of Miller et al. (1964) and several other reports (Capani et al., 1997, 2003; Gisselsson et al., 2005; Clark et al., 2008; Webster et al., 2009) demonstrated that hypothermia is an effective treatment for the severe consequences of a hypoxic–ischemic insult.

We have previously demonstrated that neuronal damage in rat neostriatum after hypoxia is associated with a chain of events that includes the over production of excitatory amino acids, nitric oxide and finally an increased release of reactive oxygen species (ROS) (Capani et al., 1997, 2001, 2003). These events are well correlated with behavioral alterations (Loidl et al., 2000).

Electron microscopy observations showed alterations in neuronal subcellular organization like disaggregation of polyribosomes, abnormalities of the Golgi apparatus, edema in the oligodendrocytes and an age-related augmentation in the number of presynaptic boutons in neocortex (Kirino et al., 1984; Petito and Pulsinelli, 1984; Smith et al., 1984; Capani et al., 1997; Martone et al., 1999; Van de Berg et al., 2000; Liu et al., 2005). Recently, changes in postsynaptic density (PSD) thickness (Martone et al., 1999; Liu et al., 2004) and additional dark aggregates throughout the soma and dendrites and PSDs of post-

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ischemic dying neurons have been described (Hu et al., 2000; Liu et al., 2004).

Here, we combine two dimensional (2D) and three dimensional (3D) electron microscopy techniques, ethanolic phosphotungstic acid (E-PTA) staining, immunoelectron microscopy and Western blot analysis for ubiquitin to study the morphological and molecular modifications of PSDs in neostriatum 6 months after the induction of hypoxia. We have employed a rat model of perinatal asphyxia (PA), which reproduces clinical situations when umbilical cord circulation is altered. In this model acidosis, hypercapnia and hypoxia are present in the whole body (Lubec et al., 1997; Loidl et al., 2000). Our aims were to determine: (1) how the duration of hypoxia, i.e. the time of asphyxia exposure, correlates with alterations in PSD ultrastructure; (2) whether these changes induce a progressive accumulation of ubiquitin-protein conjugates (ubi-proteins) in PSD; and (3) whether hypothermia (HYP) treatment blocks these alterations. We have demonstrated that PSDs are highly modified and ubiquitinated dependent on the severity of the hypoxic insult. These long-term PSD alterations may be involved in the hypoxia-induced neuronal dysfunctions. In addition, we present strong evidence that hypothermia halts synaptic alterations.

Materials and methods

Animals

A total of 51 pregnant Sprague Dawley rats were obtained from the vivarium of the School of Medicine at the Universidad de Buenos Aires. At day 14 of gestation they were placed in individual cages and maintained in a temperature- (21 ± 2 °C) and humidity- ($65 \pm 5\%$) controlled environment on a 12-h light/dark cycle (lights on at 7 a.m.). The animals had ad libitum access to food (Purina chow) and tap water. One subgroup of animals ($n = 24$) were used as surrogate mothers, another subgroup ($n = 25$) were assigned to PA or PA + HYP procedures, and the remaining ($n = 2$) were the mothers of the control pups. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Buenos Aires (School of Medicine) and conducted according to principles set forth in the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All efforts were made to reduce the number of animals used and to minimize suffering.

Induction of asphyxia

On gestational day 22, 25 full-term pregnant rats were anesthetized and rendered unconscious by CO2 inhalation (Dorfman et al., 2006), rapidly decapitated and the uterus horns were isolated through an abdominal incision and placed in a water bath at 37 °C for 10 min (slight PA; 6 uterus horns from 3 dams), 15 min (moderate PA; 6 uterus horns from 3 dams), 19 min (subsevere PA; 8 uterus horns from 4 dams) and 20 min (severe PA; 24 uterus horns from 12 dams) (Bjelke et al., 1991; Van de Berg et al., 2003). Following the same procedure, other dams ($n = 3$) were hysterectomized and their uterus horns were placed in a bath at 15 °C for 20 min (hypothermia during insult group [HYP 20 min]). In this hypothermia procedure the temperature of the pups is expected to be higher than the one set for the water bath (Engidawork et al., 2001) and, in addition, we and others have previously obtained 100% survival rate with important protective effects using the same protocol (Capani et al., 1997; Loidl et al., 1997, 2000). We chose 20 min as the maximum time of PA because 21 or more minutes of PA results in a survival rate lower than 3% (Bjelke et al., 1991). Following asphyxia, the uterus horns were rapidly opened, the pups were removed, the amniotic fluid was cleaned and the pups were stimulated to breathe by performing tactile intermittent stimulation with pieces of medical wipes for a few minutes until regular breathing was established. The umbilical cord was ligated and the animals were left to recover for 1 h under a heating lamp. When their physiological conditions improved, they were given to surrogate mothers which had delivered normally within the last 24 h. The different groups of pups were marked and mixed with the surrogate mothers' normal litters. We maintained litters of 10 pups with each surrogate mother. The different groups of asphyctic animals was determined based on their different survival rate as described in Capani et al. (1997) and Loidl et al. (2000).

Post-asphyxia procedures

Adult male rats of 6 months of age ($n = 17$–21 animals per group), were anesthetized with 28% (w/v) chloral hydrate, 0.1 ml/100 g of body weight, and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4 through the abdominal aorta (Gonzalez Aguilar and De Robertis, 1963). Brains were dissected and post-fixed in the same solution for 2 h, and then immersed overnight in phosphate buffer 0.1 M, pH 7.4 containing 5% of sucrose. Coronal brain sections containing the neostriatum (40 μm and 200 μm thick) were cut on an Oxford vibratome and recovered for electron microscopic studies. Some of these sections were stained with cresyl violet according to the procedures described in Capani et al. (1997).

Stereological analysis of calbindin

Striatum was defined according to Paxinos and Watson (1986). Different lines were drawn to define the exact area to be quantified. Medially a line was drawn from the dorsal tip of the left-brain side to the top of the corpus callosum. Dorsal and lateral boundaries were defined by the corpus callosum; a line drawn from the ventral tip of the lateral ventricle to the rhinal fissure was used as a ventral boundary. Laterally a line was drawn from the ventral tip of the lateral ventricle to the corpus callosum. Anterior and posterior boundaries for the striatum were set at bregma 1.6 mm and −0.8 mm. (Schmitz and Hof, 2000).

For estimates of the total number of immunoreactive (IR) calbindin neurons, every 8th section of the brains of control ($n = 4$), PA (10 min [$n = 6$], 15 min [$n = 6$], 19 min [$n = 8$] and 20 min [$n = 8$]), and hypothermia ($n = 8$) treated animals were analyzed using the optical dissector.

The CAST-Grid software (Olympus, Denmark) was used for quantification. The IR neurons, which came into focus within approximately 450 systematically randomly spaced dissectors, were counted at a final magnification of ×3600 (distance between dissectors in mutually orthogonal directions x and y on the sections: 250 μm). The optical dissectors had a base area of 1250 μm^2. Estimated total numbers of IR neurons were calculated from the number of counted neurons and the sampling probability (Schmitz, 1998). Sampling was optimized for prevention of type II error probability due to stereological sampling. The precision of the estimated total numbers of neurons was predicted following Schmitz and Hof (2000).

Electron microscopic studies

Coronal brain sections were cut at a thickness of 200 μm with a vibratome through the level of the dorsal neostriatum and post-fixed for 1 h with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Then tissue sections from hypoxic and control animals were stained either by 1% E-PTA or applying the conventional osmium–uranium–lead method. For conventional osmium–uranium–lead staining, sections were post-fixed for 2 h in 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed in distilled water, and stained with 1% aqueous uranyl acetate overnight. The sections were then dehydrated in an ascending series of ethanol up to 100%, followed by dry acetone, and embedded in resin (Durcupan ACM, Fluka, Buchs, Switzerland). Thin sections were counterstained with lead citrate before