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Neuroglobin is up-regulated in the cerebellum of pups exposed to maternal epileptic seizures

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ABSTRACT

To evaluate a potential insult in the cerebellum of pups exposed to maternal epileptic seizures during intrauterine life, female rats were subjected to pilocarpine-induced epilepsy. Pups from different litters were sacrificed at 1, 3, 7 and 14 post-natal days (PN) and neuroglobin (Ngb) and gliosis were analyzed in the cerebellum by Western blotting (WB) and RT-PCR. ^{14}C-L-leucine-[^{14}C-Leu] incorporation was used to analyze protein synthesis at PN1. Nitric Oxide (NO) and thiobarbituric acid-reactive substances (TBARS) levels were also measured. Pups from naive mothers were used as controls. The mRNA level of Ngb was increased in experimental animals at PN1 ($^{**}p \leq 0.001$) and PN3 ($^{**}p \leq 0.001$), at PN7 ($^{***}p \leq 0.0001$) and at PN14 ($^{**}p \leq 0.001$) compared to the respective controls. The protein level of Ngb increased significantly in the experimental pups at PN1 ($^{*}p \leq 0.05$) and at PN3 ($^{**}p \leq 0.001$), when compared to the control pups at PN1 and PN3. At PN7 and PN14 no difference was found. The mRNA level of GFAP increased significantly about two times at PN3 ($^{*}p \leq 0.05$) and PN7 ($^{*}p \leq 0.05$) in the experimental pups when compared to the respective controls, but was unchanged in the other studied ages. Data showed that experimental pups at PN1 exhibited reduced (about 2 times, $^{*}p \leq 0.05$) total protein synthesis in the cerebellum when compared to control. No differences were found in the NO and TBARS levels. Our data support the hypothesis that an up-regulation of Ngb could be a compensatory mechanism in response to the hypoxic–ischemic insults caused by seizures in pups during intrauterine life.

1. Introduction

Hypoxia–ischemia is a common cause of fetal and neonatal brain damage. For this reason, the developmental stage at which a hypoxic–ischemic insult occurs is of great importance.

Many studies have investigated the brain changes from hypoxic–ischemic processes (Volpe, 1995; Golan et al., 2004; Bhat et al., 2005). During pregnancy the occurrence of hypoxic–ischemic insults during the early phases of gestation cause toxic effects on the white matter leading to a spastic form of cerebral palsy (Volpe, 2003), death of neurons including Purkinje cells in the cerebellum, pyramidal cells in the hippocampus and cortical neurons (Rees et al., 1999) and in late gestation result in neuronal death in the cerebral cortex and striatum (Loeliger et al., 2003).

Ischemia causes multiple pathways and cascades of biochemical, molecular and electrophysiological events to interact, resulting in cell death. Excitotoxicity by high glutamate release and N-methyl-D-aspartate (NMDA) activation is one of the mechanisms activated by ischemia (Choi and Rothman, 1990). The stimulation of NMDA receptors leads to an influx of calcium, which can activate the neuronal nitric oxide synthase (nNOS) resulting in a high level of NO (nitric oxide) production in the cytoplasm. High levels of NO can promote the generation of superoxide anion (O_2^-) in the mitochondria which, in the presence of NO, can form peroxynitrite ($ONOO^-$) that is harmful to deoxyribonucleic acid (DNA) (Szabó and Dawson, 1998; Pieper et al., 1999). This cascade can trigger the activation of poly (ADP-ribose) polymerase 1 (PARP-1), which reduces the generation of adenosine triphosphate (ATP) and ultimately contributes to cell death (Eguchi et al., 1999).

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Reports have shown that neuroglobin (Ngb), a recently discovered tissue globin, has a high affinity with oxygen and may be involved in a potentially protective role against hypoxia and oxidative stress (Sun et al., 2001, 2003; Mammen et al., 2002). Ngb is supposedly involved in O_2 (oxygen) storage although their physiological role remains uncertain (Burmester et al., 2000; Fago et al., 2004), and is widely and specifically expressed in neurons of central and peripheral nervous systems, in the retina in endocrine cells (Mammen et al., 2002; Reuss et al., 2002; Schmidt et al., 2003; Wystub et al., 2003). Recent reports have showed the presence of Ngb in glial cells (Mitz et al., 2009; DellaValle et al., 2010).

Studies showed that the Ngb expression is increased by neuronal hypoxia *in vitro* and focal cerebral ischemia *in vivo*, and that neuronal survival after hypoxia is reduced by inhibiting Ngb expression and that this protein acts as an endogenous neuroprotective factor in focal cerebral (Sun et al., 2001, 2003). Ngb has been suggested to play an important role in the oxygen homeostasis of neuronal tissues (Burmester et al., 2000; Moens and Dewilde, 2000; Schmidt et al., 2003; Reuss et al., 2002), and there is evidence that Ngb promotes the survival of neuronal cells kept under hypoxic/ischemic conditions (Sun et al., 2001). Other studies reports that the Ngb no changes in hypoxic exposure (Mammen et al., 2002; Hundahl et al., 2005).

Protein synthesis is a complex processes that can be subdivided into the initiation, elongation and the termination steps. A severe form of cellular stress usually results in dysfunction of processes necessary for protein processing and folding interfering with the initiation step of translation (Ron and Harding, 2000; Schneider, 2000).

The investigation of brain injury due to hypoxia and reperfusion may be based on observations of phenomena such as prolonged suppression of protein synthesis in selective and vulnerable neurons (Krause et al., 1988; White et al., 1996, 2000). Thus, the suppression of protein synthesis is a common response to various forms of severe stress in cells, including heat stress, physical or metabolic, and viral infection of cells (Clemens, 2001; Clemens et al., 2000a,b), observed in several models of transient cerebral ischemia (Kleihues et al., 1975; Cooper et al., 1977; Burda et al., 1994).

Protein synthesis is a process that consumes a great amount of energy, accounting for 18–26% of cellular energy expenditure (Hawkins, 1991). Studies involving a variety of models of cerebral hypoxia and ischemia have shown that the circumstances that lead to energy reduction and/or their substrates in the brain causes an inhibition of cerebral protein synthesis (Blomstrand, 1970; Holstein and Myers, 1971; Kleihues and Hossman, 1971; Albrecht et al., 1972; Albrecht and Smiatek, 1975; Serra et al., 1981; Thilmann et al., 1986; Burda et al., 1994; Paschen, 2004).

Recent experimental study (Lima et al., 2010) demonstrated the presence of ischemic infarct in the placenta of epileptic female rats suggesting that seizures can cause hypoxic–ischemic insults in pups. Moreover, a previous study shows that seizures during pregnancy can affect the development of hippocampal interneurons which in turn can predispose to hyper excitability (do Vale et al., 2010). The mechanisms involved in the deleterious effect of seizures during pregnancy on pups' brains need to be elucidated. In the present study, we investigated parameters related to oxidative stress (nitric oxide and thiobarbituric acid-reactive substances, TBARS), Ngb as hypoxia sensor, glial fibrillary acidic protein (GFAP) as glial response and total protein synthesis in the cerebellum of pups which were generated under epileptic conditions, i.e., in rats presenting recurrent spontaneous seizures during pregnancy in the pilocarpine model. The cerebellum was choose because we found in previous work deficits in motor coordination in the rotarod test and increased immobility in the open-field-test in offsprings of epileptic mothers (Lima et al., 2010). Other structures were used in other experiments by our group.

2. Materials and methods

Experiments were performed after approval from the Ethical Committee of the Universidade Federal de São Paulo (EPM/UNIFESP = 1464/07). All efforts were made to minimize animal suffering according to the International Ethical Guidelines for Biomedical Research (CIOMS/OMS, 1985).

Wistar adult female rats weighing 200–250 g were housed under environmentally controlled conditions (12/12 h light/dark cycle) with free access to food and water. The *status epilepticus* (SE) was induced by administration of pilocarpine (Sigma, St. Louis, MO), a muscarinic agonist at a dose of 350 mg/kg intraperitoneally (Turski et al., 1983; Cavalheiro et al., 1987). Scopolamine methylnitrate (Sigma, 1 mg/kg s.c.) was administered subcutaneously 30 min before pilocarpine in order to prevent peripheral cholinergic effects. Treatment with pilocarpine was performed during the estrus phase of the estrous cycle, confirmed by vaginal cytology and according to this model developed in female rats (Amado and Cavalheiro, 1998).

Administration of pilocarpine in rats produces electrographic and behavioral limbic seizures and long-lasting SE (acute phase). SE is accompanied by widespread brain damage followed by a silent (seizure free) period and finally by a chronic period characterized by spontaneous recurrent seizures (Cavalheiro et al., 1987; Cavalheiro, 1995; Leite et al., 1990).

Animals that survived to SE ($n = 18$) were continuously monitored by video for 24 h/day after the induction of SE and until the appearance of the first spontaneous seizure, which defines the onset of the chronic phase of this model. After SE, the animals received special care including hydration and fractionated diet. Surviving animals were monitored continuously to detect spontaneous seizures using a video recording system. After the first spontaneous seizure the animals were then mated and the frequency of epileptic seizures was monitored during the first, second, and third week of pregnancy. Immediately after birth, the litters were subjected to cross-fostering with untreated control mothers delivering on the same day (do Vale et al., 2010; Lima et al., 2010). Two control groups without epilepsy (control and control submitted to *cross-fostering* with the experimental hatch) were submitted the same procedure of mated and the pups birth of rats without epilepsy (control) were compared with the pups birth of rats with epilepsy (experimental). These groups were created in order to preserve the experimental hatch, due to aggressive behavior show that the epileptic rats with their pups, as reported by our group (Vale, 2007). The analysis of Ngb and GFAP was done by Western blot (WB) and Reverse transcription polymerase chain reaction (RT-PCR) in the cerebellum of pups of control rats and epileptic rats. In order to obtain enough material was necessary to make a *pool* with the cerebellum of pups. Thus for each ($n = 1$) we used three cerebella from different mothers. To perform the pool, animals with the same sex and from the same mother were used. The experiments were realized with $n = 4$ (*pool* of 12 pups of control and experimental rats sacrificed at 1, 3, 7 and 14 postnatal days (PN). To analyze NO, lipid peroxidation (TBARS) and the pattern of protein synthesis only PN1 pups were chosen for being the nearest in time to the occurrence of seizures in the uterus. Furthermore, for this study, only male pups were used.

2.1. WB protocol for Ngb and GFAP

Protein quantification of Ngb and GFAP expression were performed in the cerebellum ($n = 4$ for each age; *pool*-$n = 3$ pups/mother) by WB. Tissues were dissected and stored at −80 °C until assay. Samples were homogenized in lysis buffer with protease inhibitor cocktails (0.1 M NaCl, 0.01 M Tris–HCl pH 7.6, 0.001 M EDTA pH 8.0, 1% NP-40, 10% glycerol, 10 μM PMSF, 1 mM sodium metavanadate, 0.05 M NaF, 2 nM okadaic acid). Protein content was determined using the Lowry method (Lowry et al., 1951). The samples were diluted in Laemmli buffer and boiled for 5 min. Standard curves were performed using several protein concentrations, for each age, when in PN1, PN3, PN7 and PN14 was applied in polyacrylamide mini-gel (35 μg/μl, 25 μg/μl, 40 μg/μl and 35 μg/μl to Ngb and 35 μg/μl, 40 μg/μl, 35 μg/μl and 30 μg/μl to GFAP) respectively. A linear range was obtained to both proteins. Thus, equivalent amounts of protein was loaded on 15% polyacrylamide mini-gel to Ngb and 10% polyacrylamide mini-gel to GFAP, separated by electrophoresis and transferred to PVDF (Polyvinylidene fluoride) membrane by electroblotting (Millipore – Pore Size: 0.2 μm – ISEQ00010) to Ngb and nitrocellulose membrane (GE Healthcare – Pore Size: 0.45 μm – RPN303D) to GFAP.

Blocking was performed in 5% fetal bovine serum and 5% nonfat milk for 2 h at room temperature to Ngb and GFAP, respectively. Blots were then probed with an affinity isolated antibody, produced in rabbit, anti-Neuroglobin (1:2500, Sigma, N7162) in TBS plus 2% fetal bovine serum and antibody produced in mouse, anti-GFAP (1:1000, Dako, 20334) in TBS plus 2% nonfat milk. After overnight incubation, blots were washed in TBS (3 × 5 min) and incubated for 90 min in Goat anti-rabbit IgG, Heavy and Light Chain, Biotin Conjugate, (1:8000, Calbiochem, 401313) diluted in TBS plus 2% fetal bovine serum. Blot were washed in TBS (3 × 5 min) and incubated for 90 min in streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA). After this, the membranes were incubated with anti-β-actin peroxidase (1:3000, Sigma, A5441) for 90 min at room temperature. Blot were washed in TBS (3 × 5 min) and revealed with 3,3′-Diaminobenzidine (DAB) (Sigma, 078K8200). The rate between optical density of Ngb, GFAP and β-actin bands is presented as