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Akt pathway activation and increased neuropeptide Y mRNA expression in the rat hippocampus: Implications for seizure blockade

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ABSTRACT

The aim of this study was to analyze the expression of survival-related molecules such Akt and integrin-linked kinase (ILK) to evaluate Akt pathway activation in epileptogenesis process. Furthermore, was also investigated the mRNA expression of neuropeptide Y, a considered antiepileptic neuropeptide, in the pilocarpine-induced epilepsy. Male Wistar rats were submitted to the pilocarpine model of epilepsy. Hippocampi were removed 6 h (acute phase), 12 h (late acute), 5d (silent) and 60d (chronic) after status epilepticus (SE) onset, and from animals that received pilocarpine but did not develop SE (partial group). Hippocampi collected were used to specify mRNA expression using Real-Time PCR. Immunohistochemistry assay was employed to place ILK distribution in the hippocampus and Western blot technique was used to determine Akt activation level. A decrease in ILK mRNA content was found during acute (0.39 ± 0.03) and chronic (0.48 ± 0.06) periods when compared to control group (0.87 ± 0.10). Protein levels of ILK were also diminished during both periods. Partial group showed increased ILK mRNA expression (0.80 ± 0.06) when compared with animals in the acute stage. Silent group had ILK mRNA and immunoreactivity similar to control group. Western blot assay showed an augmentation in Akt activation in silent period (0.52 ± 0.03) in comparison with control group (0.44 ± 0.01). Neuropeptide Y mRNA expression increased in the partial group (1.67 ± 0.22) and in the silent phase (1.45 ± 0.29) when compared to control group (0.36 ± 0.12). Results suggest that neuropeptide Y (as anticonvulsant) might act in protective mechanisms occurred during epileptic phenomena. Together with ILK expression and Akt activation, these molecules could be involved in hippocampal neuroprotection in epilepsy.

1. Introduction

Integrin receptors are heterodimeric membrane receptors consisting of α- and β-chains. Integrins receptors can assume a wide range of functions beyond their role mediating the link between extracellular matrix and the cytoskeleton. By controlling cell spreading, differentiation, proliferation and survival, integrins can act in the physiology of non-neuronal cells (Clark and Brugge, 1995).

In the central nervous system, integrin receptors may participate in numerous processes. Activation of integrin receptors in growth cones may promote neurite outgrowth and mediate pathfinding during development (Weaver et al., 1995; Jones, 1996), while integrin signaling in synaptic terminals may be involved in processes such as long-term potentiation (Staubli et al., 1998). Sethi et al. (1999) showed that integrins can also prevent the death of non-neuronal cells, while Gary and Mattson (2001) demonstrated the same role in neurons.

Although integrins lack intrinsic enzymatic activity, they can activate a wide range of intracellular signaling pathways. After integrin activation, there is a recruitment of some signaling molecules, such as kinases, to the cytoplasmatic tails of integrins receptors, and these kinases are the main effectors of integrins signaling transduction (Yamada and Miyamoto, 1995).

Integrin-linked kinase (ILK) is a 59 kDa, ankirin-repeat containing, serine/threonine protein kinase, that interacts with cytoplasmatic domains of integrins β1, β2 and β3 (Hannigan et al., 1996). Its 1.8-kb transcript is widely expressed in human tissues, such as placenta, heart, skeletal muscle, brain, lung, liver, pancreas and kidney (Chung et al., 1998; Huang and Wu, 1999).

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ILK has been shown to be a critical effector in a phosphoinositide 3-kinase (PI-3)-dependent signaling pathway that is downstream from both growth factor and integrin receptor activation (Dedhar et al., 1999; Wu, 1999). Stimulation of ILK, after exposure to soluble factors or fibronectin, results in activation of Akt (protein kinase B) and inhibition of glycogen synthase kinase-3 (GSK-3) (Delcommenne et al., 1998; Attwell et al., 2000; Dedhar, 2000; Persad et al., 2000).

The PI-3 kinase-dependent Akt signaling pathway has been shown to be involved in neuronal differentiation and is downstream of both integrins (Sarner et al., 2000) and growth factors such as nerve growth factor – NGF (Kaplan and Miller, 2000). ILK is a known effector within the Akt signaling pathway and has been shown to regulate migration and differentiation (Ishii et al., 2001; Wu and Dedhar, 2001).

Systemic administration of pilocarpine in rodents reproduces the main features of human temporal lobe epilepsy (TLE) (Cavalheiro et al., 1991). Pilocarpine is a muscarinic cholinergic agonist that induces limbic seizures that become secondarily generalized evolving to SE (*status epilepticus*) that lasts up to 18 h (acute period). The SE is followed by a silent "seizure-free" period and by a chronic period characterized by the presence of spontaneous recurrent seizures (Cavalheiro et al., 1994; Leite et al., 1990). Hippocampal changes following SE include an increase in glutamate mobilization (Cavalheiro, 1995; Costa et al., 2004), cell loss, gliosis (Turski et al., 1983) and mossy fiber sprouting (Mello et al., 1993).

Taking into account that several animal models of epilepsy yield similar patterns of hippocampal cell death, we might presume that selective neuronal loss is one of the earliest consequences of prolonged seizure activity, commonly mediated by glutamate-induced excitotoxicity in TLE (Cavalheiro et al., 1994; Smolders et al., 1997; Khan et al., 1999; Costa et al., 2004).

Gary et al. (2003) have showed that ILK-mediated Akt activation is able to protect hippocampal neurons from apoptosis induced by glutamate. Considering that diverse epilepsy models show a neuronal loss associated with glutamate excitotoxicity (Oxbury and Whitty, 1971; Millan et al., 1993; Costa et al., 2004), we speculated that ILK participates in the control of neuronal survival in the pilocarpine model of epilepsy. Furthermore, the stimulation of glutamate receptors may be involved in triggering the neuropeptide Y (NPY) messenger RNA (mRNA) release (Vezzani and Sperk, 2004) and a strong antiepileptic effect of NPY have also been reported in numerous studies, proposing a critical role of endogenous NPY in seizure regulation by controlling neuronal excitability (Vezzani et al., 1999; DePrato Primeaux et al., 2000; Sorensen et al., 2009).

It has been demonstrated in a number of studies that expression of neuropeptides is dramatically changed, mostly increased, in animal models for epilepsy and that this occurs in specific subregions, especially in the hippocampal formation (Fetissov et al., 2003). Lurton and Cavalheiro (1997) showed pronounced NPY immunoreactivity localized within the region of the mossy fiber terminals in pilocarpine-treated animals. NPY may act as an endogenous antiepileptic agent in the hippocampus as response to seizure activity (Gruber et al., 1994).

The aim of this study was to study neuropeptide Y and ILK mRNA expression, and Akt activation levels in the hippocampus of rats submitted to the pilocarpine model of epilepsy.

2. Methods

2.1. Animal procedures

Adult male Wistar rats (230–250 g, $n = 100$) were kept under standard laboratory conditions, housed in groups of three to four per cage and maintained in controlled room temperature, humidity and a 12 h light–dark cycle with tap water and chow pellets available *ad libitum*. Animals received a single intraperitoneal dose of pilocarpine (350 mg/kg, Sigma, Saint Louis, MO). Eighty rats were injected subcutaneously with a dose of 1 mg/kg of scopolamine methylnitrate (Sigma) 30 min before pilocarpine administration to prevent peripheral cholinergic effects. A group of animals ($n = 14$) was killed 6 h after *status epilepticus* onset (6 h SE) (acute group), and another 12 h after SE onset (12 h SE) (late acute, $n = 14$). Another group ($n = 14$) was killed 5 days after SE onset, during the seizure free period (silent group). The last set of animals ($n = 14$) was killed 60 days after SE induction (chronic group), period in which the animals suffer spontaneous recurrent seizures. Rats that received pilocarpine but did not develop SE, presenting only partial seizures (partial group, $n = 14$) were killed 6 h after pilocarpine injection. Animals composing chronic group were sacrificed at least 24 h after an evident seizure during the interictal period. Nine animals died within the first 72 h after pilocarpine administration. Control groups were composed by animals sacrificed 6 h ($n = 7$), 5 ($n = 7$) and 60 days ($n = 7$) after scopolamine methylnitrate and saline injection. Hippocampi from experimental groups were dissected and used for ILK mRNA quantification by Real-Time RT-PCR ($n = 7$ per group), and to study spatial and temporal localization of ILK using immunohistochemistry ($n = 3$ per group), and Akt activation by Western blot ($n = 4$ per group). Animal experiments were performed under UNIFESP institutional approval and all ethical requirements were respected. Moreover, assisted feeding and hydration were carried out during the initial recovery period to improve general clinical state and to reduce mortality, decreasing the number of rats used.

2.2. Total RNA isolation

mRNA levels of neuropeptide Y and ILK were measured from animal groups mentioned above. Animals were killed by decapitation, brains were excised from the skull and hippocampi were quickly dissected and frozen in dry ice, before storage at −80 °C. Thawed tissues were homogenized in 1 ml of TRIzol reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated accordingly to the manufacturer's instructions.

2.3. Quantitative Real-Time RT-PCR

One microgram of total RNA was used for cDNA synthesis and Real-Time PCR gene expression analysis. Initially, contaminating DNA was removed using DNase I (Invitrogen, Carlsbad, CA) at a concentration of 1 unit/μg RNA in the presence of 20 mM Tris–HCl, pH 8.4, containing 2 mM $MgCl_2$ for 15 min at 37 °C, followed by incubation at 95 °C for 5 min for enzyme inactivation. Then, the reverse transcription (RT) was carried out in a 200 μl reaction in the presence of 50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen). The reactions conditions were: 20 °C for 10 min, 42 °C for 45 min and 95 °C for 5 min.

Reaction product was amplified by Real-Time PCR on the 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBRGreen core reaction kit (Applied Biosystems). The thermal cycling conditions were: 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Experiments were performed in triplicates for each data point. Abundance of neuropeptide Y and ILK mRNAs was quantified as relative values compared with an internal reference, GAPDH, whose abundance was believed not to change between the varying experimental conditions. Primers used for Real-Time PCR are as follows: rat neuropeptide Y (GenBank™ accession number NM_012614), forward primer 5′-TGGCCAGATACTACTCCGCT-3′