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Kallikrein 1 is overexpressed by astrocytes in the hippocampus of patients with refractory temporal lobe epilepsy, associated with hippocampal sclerosis

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ABSTRACT

Kallikrein 1 (hK1) is a tissue enzyme responsible for kinin release in inflammatory cascade. This study was delineated to study the distribution and the co-localization of hK1 and kinin B1 and B2 receptors with glial and/or neuronal proteins markers, in the hippocampus of patients with refractory temporal lobe epilepsy, associated with hippocampal sclerosis (TLE-HS), comparing with control tissues. Hippocampal levels of *KLK1* mRNA were also measured.

hK1, kinin B1 and B2 receptors, NeuN and GFAP were analyzed using immunohistochemistry and confocal microscopy and *KLK1* mRNA was quantified with real time PCR.

Increased expression of hK1 by astrocytes co-localized with GFAP was found, contrasting with kinin B1 and B2 receptors, which were co-localized with NeuN in the sclerotic hippocampus. In addition, *KLK1* mRNA was also up-regulated in same tissues.

These data suggest an overexpression of kallikrein–kinin system and a neuron–glia interaction in the inflammatory process present in refractory TLE-HS.

1. Introduction

Epilepsy comprises a collection of disorders whose common feature is a persistent increase in neuronal excitability. Prolonged or recurrent seizures, which are some of the important characteristics of epileptic syndrome, have been shown to correlate with several changes in brain cytoarchitecture. Among the epilepsies, temporal lobe epilepsy (TLE) is the most common chronic seizure disorder seen in adulthood. In TLE, the epileptogenic zone involves the hippocampal formation and other limbic structures. The condition features hippocampal sclerosis (HS), also known as Ammon's horn sclerosis, as the most common neuropathological finding (Babb et al., 1984) and is characterized by neuronal cell loss, mainly in the hippocampal CA1 and CA3 regions and in the dentate hilus, as well as astroglial proliferation, synaptic reorganization and mossy fiber sprouting (De Lanerolle and Lee, 2005). Denervation of the inner molecular layers, secondary to hilar cell loss, has been considered the initial stimulus for mossy fiber sprouting (Babb et al., 1991). In addition, TLE-MS results from long-lasting changes in the brain involving axonal or dendritic reorganization, dispersion of the granule cell layer or loss of inhibitory interneurons. Epileptic insults also increase cell proliferation in the subgranular layer (SGL) or subventricular zone (SVZ) known as neurogenesis (Jung et al., 2006; Parent and Lowesnstein, 1997; Jung et al., 2004).

Over the last few years, increasing evidence has suggested that activation of the immune response could be related to the upstream mechanism involved in epileptogenesis. Experimental studies in rodents show that an inflammatory reaction can enhance neuronal excitability, impair cell survival and increase

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the permeability of the blood–brain barrier to blood-borne molecules and cells (Vezzani and Granata, 2005).

A important molecules that are also involved in inflammatory processes such as kinins have also been shown to be involved in the neuronal loss brought about by excitotoxicity in TLE (Argañaraz et al., 2004; Perosa et al., 2007).

In the brain, the enzyme responsible for kinin release is known as kallikrein 1, described as a serine protease with diverse additional physiological and pathological functions. According to uniform nomenclature, the kallikrein are named *KLK* for kallikrein gene and hK for encoded proteins (Diamandis et al., 2000). Bhoola et al. (2001) provided strong evidence to indicate the presence of hK1 activity in inflammatory cells. Takano et al. (1999) found plasma kallikrein mRNA as a weak signal in cultured astrocytes but not in neurons and showed that the leptomeninges and the choroid plexus are the major sources of kininogen in rat brain. These authors also described an upregulation of kininogen, induced by prostaglandin E2 (PGE2), showing the involvement of the kallikrein–kinin system in cerebral inflammation.

Kinins, such as bradykinin (BK) and Lys-BK, are short-living peptides that are released after cleavage of their precursor kininogen. These active polypeptides exert their biological activity through different heterotrimeric G-protein coupled receptors, known as B1 and B2.

Previous work from our group has shown that kinin B1 as well as B2 receptors are upregulated in the hippocampus of human patients with TLE (Perosa et al., 2007). Furthermore, Argañaraz et al. (2004a) showed increased expression of both these receptors in neurons of the rat hippocampal formation, mainly those found in vulnerable areas such as CA1, CA3, hilus and the dentate gyrus. By employing an experimental model of epilepsy in kinin B1 knockout mice Argañaraz et al. (2004b) showed that this receptor is linked to epileptogenic events, while activation of kinin B2 receptors may have a neuroprotective function. Interestingly, the latter receptors are co-localized with NeuN, a nuclear and neuronal protein marker, in the hippocampus of rats (Argañaraz et al., 2004a). These data were obtained using pilocarpine model of experimental epilepsy.

In this context, this work was designed to study the distribution of the enzyme hK1 in the hippocampal formation of patients with TLE-HS as well as the levels of *KLK1* mRNA in the same tissues. In addition, we performed double staining experiments employing anti-NeuN and anti-kinin B1 receptor antibodies. Co-localization studies of kinin B1 and B2 receptors in the same neuron were also carried out in order to understand the localization of each component of the kallikrein–kinin system in the hippocampus of patients with TLE-HE.

2. Materials and methods

All experiments were performed with approval from the Institutional Ethics committee of the Universidade Federal de São Paulo (UNIFESP). Surgical specimens from patients with intractable epilepsy were submitted to standard cortico-amygdalo-hippocampectomy at Hospital São Paulo (UNIPETE-UNIFESP-EPM, Brazil). All cases showing neoplasm, vascular malformations, post-traumatic and ischemic lesions on preoperative MRI were excluded. Selected patients ($n = 12$) underwent detailed anamnesis, video-EEG recordings and MRI studies. The age of patients with epilepsy varied from 19 to 64 years (29.7 ± 5 years). The drugs used by these patients for seizure control included: carbamazepine, phenobarbital, diphenyl-hydantoin, clobazam, depakene and topiramate. Control tissues were obtained from autopsies (less than 5 h postmortem) and the age of the subjects at death varied from 28 to 64 years (47.25 ± 18 years) ($n = 12$). Control hippocampi were obtained from brains showing no evidence of pathology on the basis of gross and routine histological examination. These tissues were retrieved by a pathologist from the Pathological Anatomy Department at INCOR, FMUSP, especially trained for this purpose by the neurosurgeon, responsible for the surgery of patients with epilepsy. Using this procedure, similar hippocampal areas from epileptic patients and autopsied subjects could be compared. All patients and families of autopsy subjects had signed a consent term authorizing tissue use in this study.

2.1. Nissl staining and immunohistochemistry

Brain tissues removed during surgery or autopsy were rapidly immersed in (pH = 7.4) 1% paraformaldehyde, for 24 h at 4 °C. Collected hippocampi were sliced in 0.5 cm sections through their longitudinal axis. All tissues were then dehydrated through an ethanol series, cleared in 100% xylene and embedded in paraffin. Three micrometer sections were cut from the paraffin-embedded tissue on a microtome (Leica) and mounted on silane-coated slides. After dewaxing, sections from the hippocampus were submitted to classical Nissl staining (NS). Cresyl violet staining was done to assess the orientation of the specimen and to check the localization and the extent of the lesion. Adjacent sections were selected for immunohistochemistry ($n = 3$). During this procedure Engels's scale and sclerosis types were defined as shown in Table 1.

Sections were dewaxed using 100% xylol and endogenous peroxidases were blocked with 3% H_2O_2 for 15 min. After this procedure, the slices were submitted to 10 min of heating in a microwave oven (control and sclerotic hippocampus). Tissues were blocked using 5% bovine serum albumin for 90 min and incubated in a humid chamber with primary monoclonal anti-human Kallikrein 1 (1:50; R&D Systems, Minneapolis, USA) for 48 h in the presence of 1% bovine serum albumin. Following three washes in phosphate-buffered saline (PBS), sections were incubated with the secondary antibody (anti-mouse 1, 1:50; Calbiochem, Merck KGaA, Darmstadt, Germany). Immunodetection was performed using the Vectastain ABC Elite Kit (Vector Burlingame, CA, USA) and the antigen–antibody complex was visualized using diaminobenzidine in PBS and H_2O_2 (1 μl/ml). Tissue sections were mounted on glass slides and the material was examined with a microscope using bright-field illumination. Each group (control and sclerotic hippocampi) was analyzed by three blind subjects.

2.2. Double labeling immunofluorescence procedure

As the immunohistochemistry procedure showed the expression of hK1 in cells with the shape of astrocytes we also performed a double labeling procedure to co-localize hK1 and GFAP.

In order to verify the expression of the neuronal protein NeuN and the immunoreactivity for kinin B1 or B2 receptors in control tissue and hippocampi from patients with TLE we employed a double labeling immunofluorescence protocol. In this protocol we also aimed to verify whether kinin B1 and B2 receptors could be localized in the same cell.

The tissues were prepared as follows: first, they were dissected out and placed in 5% formaldehyde fixative solution. Forty-four hours later, hippocampi were dehydrated and embedded in paraffin. Three micrometer sections were cut on a microtome and collected onto poly-L-lysine (Sigma)-coated glass slides; the paraffin was then removed from sections using 100% xylol and they were rehydrated by sequential immersion in 100% ethanol, 95% ethanol, 50% ethanol and

Table 1
Clinic features of patients. Engel scale—control seizures classification from Engel et al. (1993) and sclerosis type according to Blumcke et al. (2007).

No	Sex	Age	Initial event	Age of begin	Duration/years	Side	Temporal polo hypersignal	Engel scale	Sclerosis type
1	M	28	–	25	3	L	–	IIIA	IA
2	M	28	+	14	14	R	–	IA	IB
3	F	29	+	24	5	L	–	IA	IB
4	F	31	+	15	16	R	–	IVA	–
5	F	19	–	8	11	L	+	IA	IA
6	F	20	–	15	5	L	–	IIIA	AT
7	F	30	–	5	25	L	+	IIIB	IA
8	M	32	–	7	25	L	–	IB	IA
9	F	26	–	21	5	L	–	IA	–
10	F	30	–	8	22	R	+	IIIA	IA
11	F	35	+	7	28	R	–	IA	–
12	F	31	+	0,8	30	R	–	IA	IB