Biomedicine & Pharmacotherapy 84 (2016) 258–263

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Anticonvulsant and neuroprotective effects of apelin-13 on pentylenetetrazole-induced seizures in male rats

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ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form 4 September 2016
Accepted 13 September 2016

Keywords:
Apelin-13
Seizure
Pentylenetetrazole
Rats

ABSTRACT

Epilepsy is a common neurological disorder with no effective treatment or cure. Neuropeptide apelin is an endogenous ligand of angiotensin receptor-like 1 (APJ). It has been shown that apelin has protective and anti-neurodegenerative properties. This study was designed to evaluate the effect of apelin-13 on pentylenetetrazole (PTZ)-induced rat model of seizure. Adult male Wistar rats were divided into the experimental groups as follows: control group receiving PTZ; apelin-treated group which received apelin-13 before PTZ; apelin + F13A-treated group which received apelin-13 plus the apelin receptor antagonist (F13A) before PTZ; apelin + naloxone group which received apelin-13 + naloxone before PTZ. Behavioral scoring was used to access seizure. The expression level of APJ was measured by western blotting. Neuronal degeneration, apoptosis and astrocyte activation were evaluated by vanadium acid fuchsin (VAF) staining and immunohistochemistry. Our data demonstrated that apelin-13 pretreatment significantly inhibited seizure threshold ($p < 0.001$) and tonic-clonic latency ($p < 0.001$) compared with the control group. In addition, PTZ-induced up-regulation of APJ was attenuated by apelin-13 treatment. Histological and immunohistochemical findings also showed that apelin-13 could protect cortical neurons against PTZ-induced neuroinflammation and apoptosis. In conclusion, apelin-13 has anticonvulsive and neuroprotective properties against PTZ-induced seizure in rats and provided a new pharmacological aspect of the neuropeptide apelin.

1. Introduction

Epilepsy is a common type of neurological disorder that greatly decreases the quality of life of individuals. It has been shown that common comorbidities in individuals with epilepsy are cognitive and behavioral impairments following seizures [1].

An imbalance between excitation and inhibition in the central nervous system (CNS) is the primary cause of epilepsy [2]. There is much evidence showing that the central nervous system (CNS) has great auto-protective and repairing mechanisms to protect itself from neural damage induced by epilepsy [3,4]. Therefore, determining these endogenous protective mechanisms will guide us in the use of new therapeutic strategies for seizure control.

It has been reported that there is an intimate relationship between neuronal stress and triggering of glial activation in epilepsy [5]. Seizure-induced glial activation has a critical role in neuronal degeneration and survival [6]. Experimental and clinical studies have shown that seizures can induce neuronal death which may contribute to epileptogenesis, impairments in cognitive function or the epilepsy phenotype [7]. Furthermore, numerous evidences have been suggested that oxidative stress and mitochondrial dysfunction have critical roles in the neurobiology of epilepsy [8].

Apelin is the endogenous ligand of APJ receptors (angiotensin receptor-like 1). Preproapelin has 77 amino acids and can be subdivided into several bioactive isoforms, such as apelin-13, -17, and -36 [9,10]. In the CNS, it has been reported that APJ mRNA is distributed in the hypothalamus, amygdala, dorsal raphe nucleus, and spinal cord [11–13].

The potential antioxidant property of apelin has been reported in different oxidative stress situations [14–16]. Surprisingly, apelin exerts a strong anti-apoptotic effect in *in vitro* and *in vivo* studies [17–19]. It has been demonstrated that the apelin–APJ system protects hippocampal and cortical neurons against excitotoxic injury [20]. In addition, apelin has an attenuating effect on the

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http://dx.doi.org/10.1016/j.biopha.2016.09.048
0753-3322/

expression of pro-inflammatory cytokines and inhibits microglia, astrocytes and other inflammatory cells [21].

LV et al. reported that apelin performs its cellular effects through the activation of opioid receptors [22]. Previous studies have confirmed that APJ forms a heterodimer with opioid receptors which leads to increased signal transduction by those receptors agonists [23].

Since the apelin-APJ system might play a neuroprotective role against some neurological disorders, the present study was designed to evaluate the anticonvulsive and neuroprotective effects of apelin-13 and its possible underlying mechanisms in a PTZ-induced seizure model in male rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300 g) were used in this study. The animals were kept at a constant temperature of 22 ± 1 °C under a 12/12 h light/dark cycle with free access to food and water. The experimental protocol was approved by the Ethics Committee of Kerman Neuroscience Research Center (EC/KNRC/92/62).

2.2. Materials

Pentylentetrazole (PTZ, a non-competitive antagonist of the gamma-amino butyric acid) ($GABA_A$) receptor was purchased from Abcam, UK. Apelin-13 (APJ receptor agonist) and apelin-13(F13A), the specific APJ receptor antagonist, were purchased from Sigma, USA. Naloxone hydrochloride, the classic opioid receptor antagonist, was prepared by Tolid-daru pharmaceutical company, Tehran, Iran. Antibodies were purchased from BioGenex (San Ramon, California, USA) and Santa Cruz Biotechnology (USA). Apelin-13 and apelin-13(F13A) were dissolved and aliquoted in sterile physiological saline. The aliquots were stored at −20 °C until use and were diluted before injection.

2.3. Experimental design

The animals were randomly divided into 4 experimental groups as follows (n = 7–8): control group which was received PTZ (50 mg/kg; intraperitoneally, i.p.). Apelin-13 treated groups which were received apelin-13 (1, 3, 6 μg/rat; intracerebroventricularly, i.c.v.) 15 min before PTZ administration. F13A-treated group which had apelin-13 plus F13A as an apelin receptor antagonist (0.8 μg/rat, i.c.v.) 15 min before PTZ administration. Naloxone group that had apelin-13 accompanied with naloxone hydrochloride (2 nmol/rat, i.c.v.) 15 min before PTZ administration. All drugs except PTZ were injected in a volume of 3 μl/rat using a 25 μl Hamilton micro syringe.

2.4. Surgery

For i.c.v. injection, the animals were anesthetized with ketamine and xylasine (80 and 10 mg/kg, i.p., respectively) and a stainless steel cannula was inserted stereotaxically (1.3 mm from the middle, 0.9 mm posterior to the bregma and 3.5 mm from the surface of skull) into the left ventricle. The animals spent 5 days recovery period. Confirmation of the injection site was verified by methylene blue staining.

2.5. PTZ-induced seizures

PTZ was used for inducing seizures according to the previously reported protocol [24]. At first, the minimum dose (50 mg/kg) of PTZ that induced seizures sign in the animal was determined (our pilot study). This dose was then used to screen possible antiepileptic of apelin-13. Apelin-13 alone or in accompanied with F13A and naloxone were administered 15 min before PTZ injection. Immediately, the animals were observed to find seizure signs for 30 min and graded scores were determined as follow: score 0: no seizures, score 1: eye or facial twitches, score 2: convulsive waves across the body, score 3: myo-clonic jerks or rearing, score 4: turn over onto one side position, and score 5: turn over onto back position, generalized tonic-clonic seizures, or die during the experiment period. In all behavioral trials, the animals were observed in a blind mode in which the identities of the animal groups were unknown to the investigator.

2.6. Western blot analysis of apelin receptor (APJ) expression

After behavioral studies, the animals were sacrificed under deep anesthesia using chloral hydrate (400 mg/kg) and their brains were removed from the skull and brain cortices were dissected and homogenized in ice-cold buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 1% NP-40 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, and 10 μg/ml aprotinin) and 1 mM sodium orthovanadate. The homogenate was centrifuged at 14,000 *g* for 15 min at 4 °C. The resulting supernatant was retained as the whole cell fraction. Equal amounts of protein (40 μg) were electrophoresed through 9% SDS-PAGE gel and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare Bio-Sciences Corporation, NJ, USA). After blocking for 2 h at room temperature with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.1% Tween 20), the membranes were probed with specific apelin receptor antibody (sc-33823, 1:1000, Santa Cruz Biotechnology, USA) overnight at 4 °C. After washing in TBS-T (3times for 5 min), the blots were incubated for 60 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:15,000, Santa Cruz Biotechnology). The antibodies were diluted in blocking buffer. The antibody–antigen complexes were detected using the ECL system and exposed to Lumi-Film chemiluminescent detection film (Roche, Germany).

2.7. Histological study

The dissected brains were processed for light microscopy. After fixation in formaldehyde (10% for 48 h), the specimens were dehydrated in a graded series of ethanol solutions and embedded in paraffin. Sections of 5 μm diameter were obtained from the brains (sensorimotor cortex) and stained with vanadium acid fuchsin. Neuronal damage was determined as a ratio of the number of degenerated neurons to that of total (both the surviving and degenerated neurons) in three distinct areas of the cortex subfields [25].

2.8. TUNEL immunohistochemistry

The terminal deoxynucleotidyl transferase (TdT)-mediated in situ dUTP nick end-labeling (TUNEL) assay was performed on the brain sections using the cell death detection Kit POD (Roche; Indianapolis, IN) according to the manufacturer's instructions. Briefly, 5 μm sections were deparaffinized and rehydrated. Protein digestion was completed by incubating tissue sections in 20 mg/ml proteinase K (Roche; Indianapolis, IN) for 20 min at room temperature (RT). Endogenous peroxidase was inactivated with 3% H_2O_2 in distilled water for 5 min at RT. The labeling mixture containing biotinylated dUTP in TdT enzyme buffer was added to the sections and incubated at 37 °C in a humidified chamber for 1 h. After stopping the enzymatic reaction, the brain sections were