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Original article

Effect of feeding with bilberry fruit on the expression pattern of αCaMKII in hippocampal neurons in normal and diabetic rats

M. Matysek[1], S. Mozel[1], R. Szalak[1], A. Zacharko-Siembida[1], K. Obszańska[2], M.B. Arciszewski[1]

[1] Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland
[2] Chair and Department of Neurosurgery, Medical University, Jaczewskiego 8, 20-090, Lublin, Poland

Abstract

αCaMKII, widely occurring in the central nervous system, plays a significant role in cognitive processes. It is well known that diabetes is a risk factor that may trigger brain atrophy, cognitive dysfunction and finally lead to memory loss. Antioxidants richly present in bilberry fruits are believed to have significant effects on diabetes-related brain dysfunctions mainly due to their abilities to modulate neurotransmitter release that lead to reduction of the negative impact of free radicals on cognitive processes. The aim of the present research was to immunohistochemically investigate the expression patterns of αCaMKII in hippocampal neurons from non-diabetic, diabetic and diabetic rats fed with an extract of bilberry fruit. The obtained results show that in comparison to the control group, in diabetic rats hippocampal neurons immunoreactive (ir) to αCaMKII were swollen and the lengths of the neuronal fibres were reduced. Further study shows that in diabetic rats fed with bilberry fruit, αCaMKII-positive nerve fibres were significantly longer when compared to the groups of diabetic and control rats. Additionally, we observed statistically significant changes in the average larger diameter of αCaMKII-ir hippocampal neurons between groups of diabetic rats (with vs. without supplement of bilberry fruit). The results of the present work suggest that antioxidants present in bilberry fruits influence the morphology of and possibly exhibit beneficial and neuroprotective effects on hippocampal neurons during diabetes. It is likely that changes in the appearance of αCaMKII-expressed hippocampal neurons may reflect the diabetes-evoked rise in Ca^{2+} level in the cerebral nerve terminals. The present research extends our knowledge of preventive mechanisms for cognitive dysfunctions occurring in the brain during diabetes.

Key words: rat, neurons, hippocampus, αCaMKII, diabetes, bilberry fruit

Correspondence to: M. Matysek, e-mail: malgorzata.matysek@up.lublin.pl, tel.: + 48 81 445 65 82

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a well-known regulator of excitatory synaptic transmission playing a role in neurotransmitter release (Colbran et al. 1989). Belonging to the serin/threonin kinases family, multifunctional CaMKII has four major subunits encoded by α, β, γ and δ genes (Tombes et al. 2003). Subunits γ and δ are generally present in various mammalian organs such as skeletal muscle (Woodgett et al. 1983), the heart (Iwasa et al. 1986), or lung (Schulman et al. 1985).

However, α and β subunits of CaMKII are mainly found in the central nervous system (CNS) (Tobimatsu and Fujisawa, 1989). αCaMKII is widely expressed in the brain, predominantly in the hippocampus, striatum, cerebral cortex, olfactory bulb and hypothalamus (Erondu and Kennedy 1985). It has been found that αCaMKII has a role in long-term potentiation (LTP), which is considered as one of the forms of synaptic plasticity (Malenka and Nicoll 1999). Additionally, αCaMKII participates in some processes associated with behavior (Hasegawa et al. 2009) and memory (Irvine et al. 2006).

The processes of learning and memory are related to the activity of the limbic system, mainly the hippocampus (El-Falougy and Benuska 2006). Several studies have shown that many structural and biochemical alterations in the brain areas are present during the course of tauopathies, which in turn may lead to dysfunction of memory (Booth et al. 2016). In CNS, several changes in the brain functions were also observed in metabolic diseases such as diabetes (Stranahan 2015). Additionally, it is well known that hippocampal neurons are sensitive to changes in glucose homeostasis (Yates et al. 2012). This may partially explain why during the course of diabetes, alterations at neurochemical and structural levels and neuronal loss are commonly noted in CNS (Hernandez-Fonseca et al. 2009). One of the methods/strategies to counteract diabetes is administration of natural substances such as berry fruits. It has been documented that bilberry is a source of multipotent antioxidants, including polyphenolic compounds, which exhibit anti-inflammatory activity, prevent neurodegeneration processes and also have antioxidant properties (Subash et al. 2014). Furthermore, recent studies provide information about the significant role of berry fruits in memory enhancement (Ramirez et al. 2005).

The present study aimed at immunohistochemically evaluating the expression patterns of αCaMKII in hippocampal neurons in normal and in streptozotocin (STZ)-induced diabetic rats, and thus to determine whether the antioxidants present in the bilberry fruit may influence neuronal calcium-binding protein metabolism and potentially act as neuroprotective factor.

Materials and Methods

Animals (diabetic and non-diabetic)

Animal care protocols, experimental design and methods were reviewed and approved by the Local Ethical Committee at the University of Life Sciences in Lublin, Poland. Thirty (n=30) sexually mature male Wistar rats were used in the study. Diabetes was induced in twenty (n=20) animals by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg b. w). A freshly prepared solution of STZ was dissolved in sterile citric buffer (100 mmol/L, pH 4.5) containing 0.9% NaCl. Ten (n=10) animals received a single intraperitoneal injection of sterile citric buffer (100 mmol/L, pH 4.5) containing 0.9% NaCl. For 48 h, the animals drank 5% glucose in tap water instead of tap water.

The animals were divided into three groups (n=10): control group (group I) were fed standard rodent feed (30g/day); diabetic group (group II) were fed standard rodent feed (30g/day) and diabetic group (group III) were fed standard rodent feed supplemented (*per os*) with freeze-dried bilberry fruit (25 g/day standard feed plus 5 g/day fruit). Fresh bilberry fruit were mashed up using a Termomix TM31 (Vorwerk, Germany) for 3 min (maximal speed at ambient temperature) followed by portioning and freezing (-20°C) until use.

All rats received their respective diets for 3 months until euthanization (by overdose of CO_2).

Tissue sampling

The brains were dissected out immediately after slaughter. The brains were fixed for 12 h in cold buffered 10% formalin (pH=7.0; +4°C). The material was processed conventionally for paraffin embedding. For further immunohistochemical analyses, paraffin sections of 6μm thickness were cut and collected on SuperFrostPlus (Meznel-Glaser, Braunschweig, Germany) microscopy slides.

Immunohistochemistry and antibodies

In order to immunohistochemically stain of the slides the peroxidase- antiperoxidase method was used according to the following protocol (Szalak et al.

2015). The sections were washed in xylene (3x15min) to remove the paraffin. The preparations were then incubated in a graded series of ethyl alcohol and washed in distilled water. The slides were put in a container containing citrate buffer (pH=6.0) and heated to 97°C (3 x 7 min) in a microwave oven (800 W) in an attempt to retrieve antigen. To block endogenous peroxidase activity the sections were chilled and washed in 3% hydrogen peroxidase (20 min). The slides were then flushed in PBS (pH=7.4) twice (15 min each) and incubated in 2.5% normal horse serum (S-2012; Vector, USA) at room temperature (RT) for 20 minutes. After drying of excess of blocking serum, mouse primary antisera raised against αCaMKII (dilution 1:400; MA1-048, Thermo-Fisher Scientific) were marked and incubated overnight at 4°C. After being washed in wash buffer (2x15), the preparations were covered by anti-mouse/rabbit Ig (ImmPRESS™; MP-7500 Vector, USA), for 1 hour (RT). 3, 3'-diaminobenzidine (DAB, Vector, USA) was used for visualization of primary antisera. The working solution of DAB was applied on to the slides and the process was examined under a light microscope. The slides were washed in the distilled water. Mayer's haematoxylin (20 min) was then used for counterstain. The final stage was dehydration of the sections in ethyl alcohol, clearing in xylene, and mounting in Canadian balm and cover slipped. In the negative control, the primary antibodies were substituted with the same concentrations of appropriate non-immune IgG, to verify the antibody specificity.

Morphometric and statistical analysis

The slides were viewed under a light microscope (Olympus BX51 light) connected to a digital camera (Olympus Color View III). From each animal approx. 30 sections immunostained for αCaMKII were analysed. Using Cell D software (Olympus) the distribution of αCaMKII neurons in the hippocampus was studied. Additionally, the larger diameter of the immunoreactivity to αCaMKII (αCaMKII-ir) neurons was arbitrarily judged and measured. Results were expressed as means ± standard deviation (SD). Statistical analysis was performed by ANOVA one-way of variance test followed by Tukey's HSD test. Probabilities of less than 5% ($p<0.05$) were considered significant.

Results

αCaMKII-ir was observed in neurons of CA1, CA2 and CA3 fields of the hippocampus in both control (I) and experimental groups (II and III). Positive αCaMKII reaction was present in neuronal cytoplasm and fibres, but was absent in nuclei (Fig. 1 A, B, C-3 A, B, C).

Group I – control. The average larger diameter of neurons in the control group in CA1, CA2 and CA3 fields of the hippocampus was respectively 8.6 ± 0.6 μm, 9.4 ± 0.8 μm and 8.8 ± 0.6 μm (Fig. 4). αCaMKII-ir hippocampal neurons were round and oval in shape (Fig. 1 A-C). Additionally, a few pyramidal neurons were also present in CA2 and CA3 fields of the hippocampus (Fig. 1 B-C). Very numerous and relatively long αCaMKII positive nerve fibres were detected in all hippocampal fields (Fig. 1A, Fig. 5).

Group II – induced diabetes. αCaMKII-ir hippocampal neurons had a larger diameter in comparison with the control group. The average diameter of neurons in group II in CA1, CA2 and CA3 fields of the hippocampus was respectively 9.9 ± 0.3 μm, 12.9 ± 1.1 μm and 13.4 ± 0.4 μm (Fig. 4). Significant differences in the average larger diameter of αCaMKII-ir neurons in all hippocampal fields were found between the control group and group II. No significant differences between homologous populations of neurons in fields CA1, CA2 and CA3 in group II were observed. αCaMKII-ir hippocampal neurons were round and oval in shape (Fig. 2 A-C). In comparison to the control group, in group II no presence of pyramidal neurons were seen. In rats with induced diabetes (but not in those from group I and III), neurons (both positive and negative to αCaMKII) were in general swollen, and reduced length of neuronal fibres was commonly observed (Fig. 2A-C, Fig. 5).

Group III – induced diabetes fed with bilberry fruit. αCaMKII-ir nerve cells were statistically smaller in size when compared to group II. The average larger diameter of neurons in field CA1, CA2 and CA3 was respectively 9.7 ± 0.1 μm, 11.6 ± 0.4 μm and 11.7 ± 0.4 μm (Fig. 4). In all hippocampal fields, significant differences in the average larger diameter of αCaMKII-ir neurons between the control and group III were observed (Fig. 4). In addition, there were significant differences between homologous populations of neurons in field CA3 of the hippocampus in group III vs. field CA3 of the hippocampus in group II. In group III, the most numerous populations of αCaMKII neurons consisted of cells round and oval in shape (Fig. 3 A-C). In the CA3 field pyramidal neurons were only occasionally found (Fig. 3 C). When compared to group II, αCaMKII positive nerve fibres found in all fields of the hippocampus of animals from group III were statistically longer, and such differences were especially visible in fields CA1 and CA2 (Fig. 3A, B, Fig. 5).

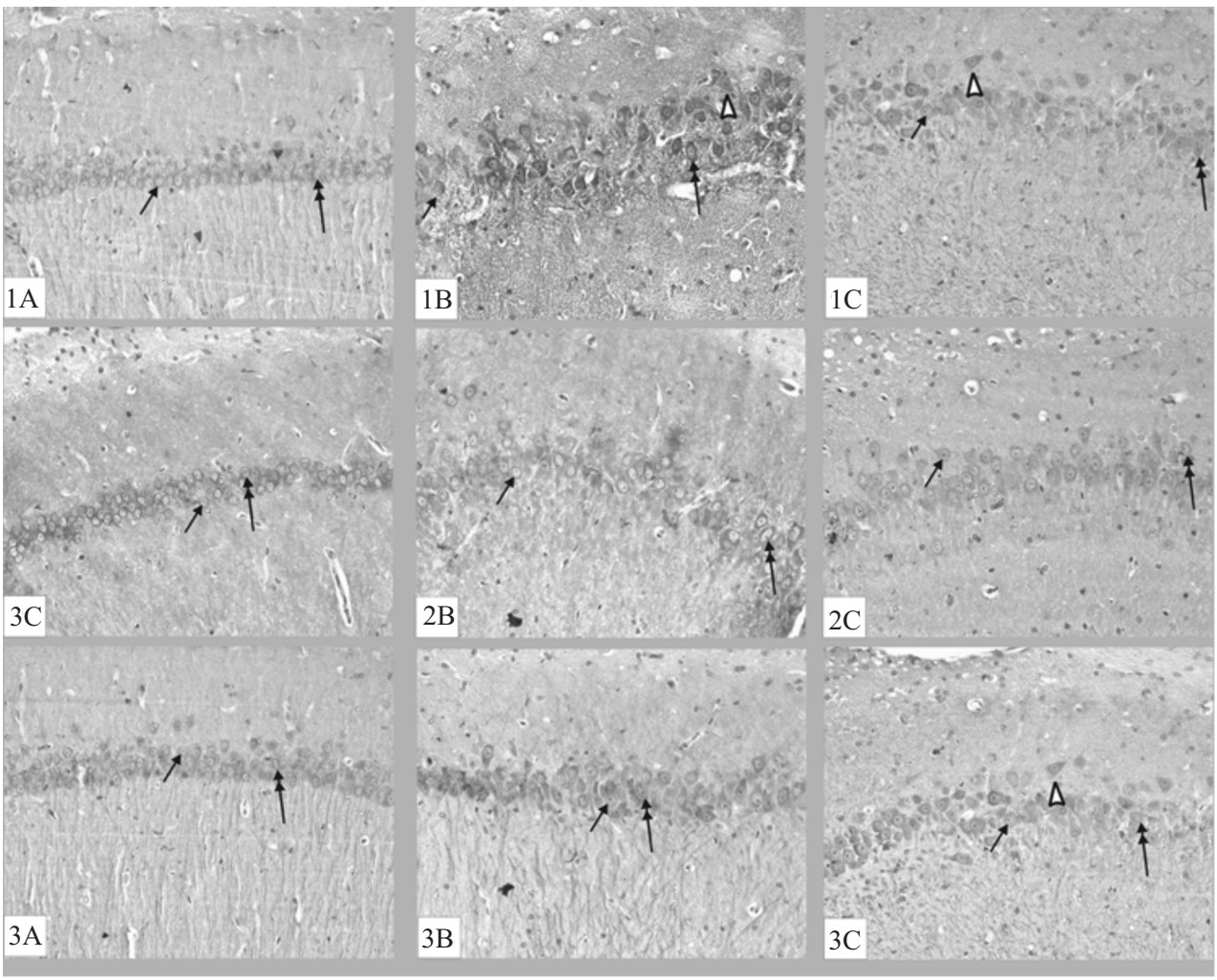

Fig. 1-3. Expression of αCaMKII in CA1 (A), CA2 (B) and CA3 (C) fields of the hippocampus of the animals from group I (1), II (2) and III (3) are presented. αCaMKII-ir neurons were classified as round (arrow), oval (double arrow) or pyramidal (arrow head) in shape. Please note in Fig. 1 and Fig. 3 the presence of αCaMKII-ir processes. x20 in (1-3).

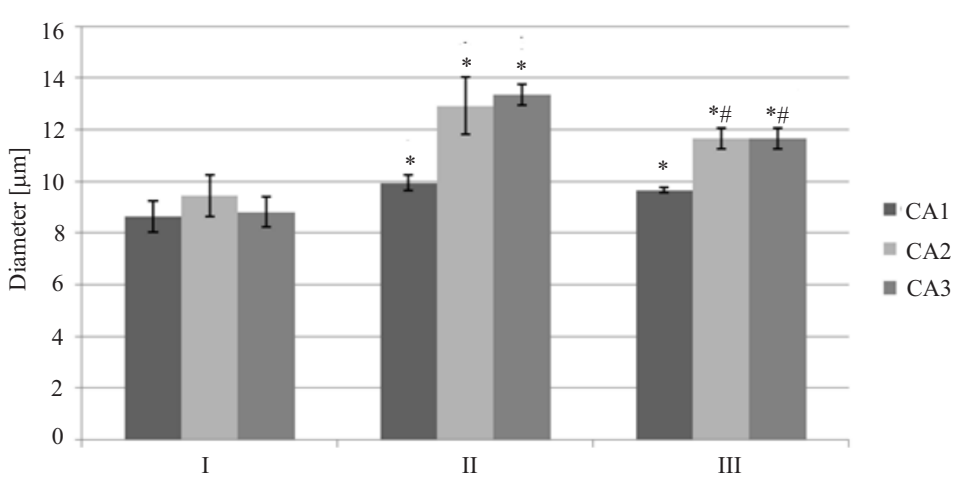

Fig. 4. Average larger diameter of αCaMKII-ir neurons detected in CA1, CA2 and CA3 fields of the hippocampus in control and experimental animals. (*) marks statistically significant differences ($p<0.05$) between homologous hippocampal neuronal populations (vs. control group; group I); (#) indicates statistically significant differences ($p<0.05$) between neuronal populations of group II and group III.

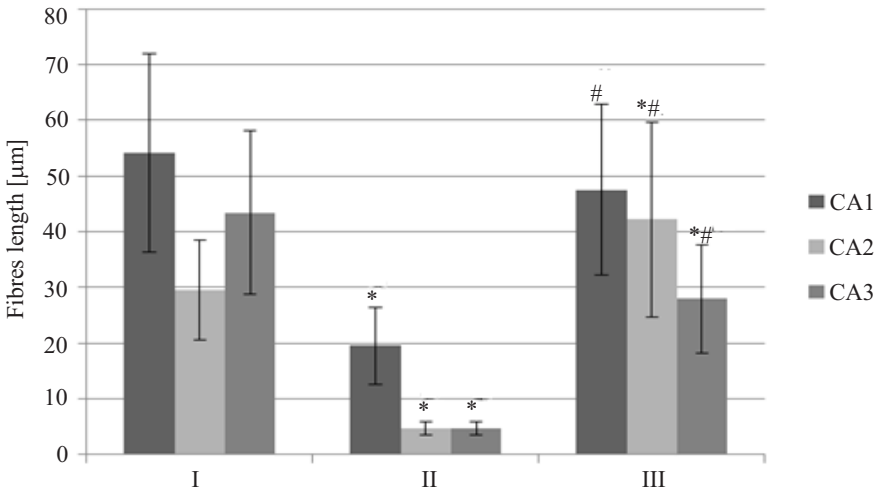

Fig. 5. Average length of αCaMKII-ir nerve fibres found in CA1, CA2 and CA3 fields of the hippocampus. Statistically significant differences between homologous populations are marked with * (vs. control) and # (diabetic rats vs. diabetic rats fed with bilberry).

Discussion

This study provides information about αCaMKII expression in the neurons of the rat hippocampus. αCaMKII positive neurons were observed in CA1, CA2, CA3 field of the hippocampus, in all three investigated groups. A positive reaction to αCaMKII was noted in the cytoplasm and dendrites of hippocampal neurons. Burgin et al. (1990) found a similar relation in the rat cerebral cortex.

Several previous reports clearly indicated that CaMKII represents 2% of total proteins in the hippocampus (Erondu and Kennedy 1985). The presence of the alpha subunit of CaMKII in the CNS is not only observed in the rat hippocampus but also in other mammalian brain structures such as the medulla, thalamic nuclei and amygdaloid nuclei (Erondu and Kennedy 1985).

Based on the findings of Matsuo et al. (2009) it can be argued that αCaMKII has a synaptic plasticity role in the CNS. Hasegawa et al. (2009) found that αCaMKII participates in the regulation of anxiety and aggressive behavior. Phosphorylation of proteins is one of the main mechanisms of signal transduction in which αCaMKII is involved. αCaMKII is able to phosphorylate several proteins including AMPA-type glutamate receptors, microtubule-associated protein 2 (MAP-2) and thyrosine hydroksylase (Thiagarajan et al. 2002, Yamasaki et al. 2008). Yamauchi (2005) suggested that through the activation by Ca^{2+} and calmodulin, αCaMKII is capable of autophosphorylation. Therefore, once activated αCaMKII remains active even with the absence of Ca^{2+} and calmodulin. The phenomenon of autophosphorylation is essential to long-term potentiation (LTP). LTP triggered by postsynaptic Ca^{2+} influx, originally found in the hippocampus, is considered as a model for studying spatial learning and memory processes (Giese et al. 1998, Irvine et al. 2006). Several functional changes in hippocampal neuron activity have been reported during certain diseases, including Alzheimer's disease, and it is believed that these processes are connected with learning and memory disorders (Booth et al. 2016; McGowan et al. 2006). The hippocampal neurons widely express insulin receptors, which makes them sensitive to glucose and changes. The latter phenomenon may be an explanation why changes in physiological action of the hippocampal neurons are observed in the course of metabolic diseases such as diabetes (Sadeghi et al. 2016).

Diabetes adversely affects the cognitive functions via a number of factors, mainly by apoptosis of hippocampal neuronal cells (Li et al. 2002), brain atrophy (Cherbuin et al. 2012) and synthesis and release of neurotransmitters (Baptista et al. 2011). It has been previously shown that diabetes evokes an increase in the level of Ca^{2+} in the brain (Biessels et al. 2002). According to Levy et al. (1994) and Bhardwaj and Kaur (1999), the alterations in the physiological level of Ca^{2+} can lead to cellular damage and signal transduction disorders. In the present study we observed that, in the rats with induced diabetes a swelling of αCaMKI-positive as well as αCaMKII-negative neurons in the CA1, CA2, CA3 hippocampal fields was frequently noted and these effects were statistically significant when compared to the control. This is in line with a previous report demonstrating the presence of swollen neurons in the cerebellum, cerebral cortex and hypothalamus of the diabetic rat (Hernandez-Fonseca et al. 2009). We also reported that

during diabetes the presence of αCaMKII in sparse hippocampal nerve fibres can be incidentally found and we speculate that this phenomenon may be related to the reduction in the length of hippocampal dendrites triggered by the induction of diabetes by STZ (Martínez-Tellez et al. 2005).

In previous studies it has been shown that oxidative stress in developing diabetes can cause, for instance, cognitive dysfunction in the brain (Tuzcu and Baydas 2006). It was also found that antioxidants have a neuroprotective action which may explain why the supplementation of this compound is beneficial during diabetes treatment. Šavikin et al. (2009) and Torronen et al. (2013) reported that bilberry fruits are a potent source of antioxidants. In the present study, we noticed that in diabetic rats treated with the extract of bilberry fruit, a swelling of αCaMKII-ir neurons of significantly smaller size was found in CA2 as well as CA3 hippocampal fields.

In conclusion, results of the present study suggest that the antioxidants which are presented in bilberry fruits may be an efficient factor influencing the calcium metabolism of hippocampal neurons, and may have a neuroprotective effect on hippocampal neurons during the course of diabetes. Additionally, it is suggested that during the course of diabetes an increased level of αCaMKII activity may lead to a rise in Ca^{2+} level in the brain.

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