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Neuroscience Letters 276 (1999) 75–78

Neuroscience Letters

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Protective effects of C-phycocyanin against kainic acid-induced neuronal damage in rat hippocampus

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Received 21 June 1999; received in revised form 20 September 1999; accepted 20 September 1999

Abstract

The neuroprotective role of C-phycocyanin was examined in kainate-injured brains of rats. The effect of three different treatments with C-phycocyanin was studied. The incidence of neurobehavioral changes was significantly lower in animals receiving C-phycocyanin. These animals also gained significantly more weight than the animals only receiving kainic acid, whereas their weight gain did not differed significantly from controls. Equivalent results were found when the neuronal damage in the hippocampus was evaluated through changes in peripheral benzodiazepine receptors (microglial marker) and heat shock protein 27kD expression (astroglial marker). Our results are consistent with the oxygen radical scavenging properties of C-phycocyanin described elsewhere. Our findings and the virtual lack of toxicity of C-phycocyanin suggest this drug could be used to treat oxidative stress-induced neuronal injury in neurodegenerative diseases, such as Alzheimer's and Parkinson's.

Keywords: C-Phycocyanin; Kainate; Peripheral benzodiazepine receptor; Hsp27kD; Antioxidant; Glia; Neuroprotection

Kainic acid (KA), a glutamate analogue, binds to specific excitatory amino-acid receptors in central nervous system [6]. Systemic injection of KA in rats results in convulsive syndrome which is accompanied by severe neuronal damage and, in the hippocampus, a selective loss of neurons [1,2]. Thus, KA administration in rats is a widely used experimental model for human status epilepticus.

Overactivation of KA receptors increases the production of reactive oxygen species (ROS), which are mediators of oxidative damage [5]. Oxidative stress is associated with various neuro-degenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophyic lateral sclerosis [4,11]. Indeed, a recent study in transgenic mice shows that neuronal damage induced by KA is attenuated by superoxide dismutase [10]. Thus, administration of antioxidant drugs, such as lazaroids, vitamins (E or C) or melatonin, could be used to prevent neuronal cell death by oxidative mechanisms in vivo [5,13,14,16].

C-phycocyanin is a biliprotein pigment found in some blue-green algae *Spirulina* (*Arthospira*) species, which have attracted attention because of their nutritional value and medicinal properties [3]. This pigment has antioxidant properties in vitro [15] and anti-inflammatory and hepatoprotective activity in different experimental models in vivo [3,15]. These data support the view that C-phycocyanin may protect neurons against the injury induced by oxidative stress. To date, however, no study about this putative effect has been reported. Here, we examine the effect of C-phycocyanin on the neurobehavioral and neuronal damage induced in rats by KA.

Studies were conducted in compliance with the Spanish legislation on Animal Protection and the European Union Directive. Adult male Sprague–Dawley rats (250–275 g) were obtained from Harlan Ibérica (Spain). They were maintained on a 12:12 h light/dark cycles and, before treatment, they were fasted for 24 h. The animals were randomized into five groups: control group was injected i.p. with saline ($n = 6$); kainate group received saline orally and, 1 h later, KA (Sigma Chemical Co., St. Louis, MO, USA), 10 mg/kg, i.p. ($n = 6$), group A ($n = 6$) was administrated orally with C-phycocyanin (100 mg/kg) 1 h before receiving kainate (10 mg/kg, i.p.); group B ($n = 6$) received C-phycocyanin (100 mg/kg, p.o.) 24, 16 and 1 h before kainate administration (10 mg/kg, i.p.) and, finally, group C

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0304-3940/99/$ - see front matter
PII: S0304-3940(99)00792-2

received the scheduled treatment of group B and, additionally, 100 mg/kg, p.o. of C-phycocyanin 1 h after kainate administration. Both KA and C-phycocyanine were administered dissolved in saline.

Neurobehavioral changes were monitored after the first injection. Classic effects of KA administration, including 'wet dog shakes', tremors and seizures, were taken as signs of alterations in neurobehavior. In addition, changes in body weight were also measured in the different lots. One week after the KA administration, rats were killed by decapitation, brains quickly removed from the skull and their hippocampi were dissected, frozen and stored at −80°C until used.

Two indirect markers of neuronal damage in the hippocampus were measured: peripheral benzodiazepine receptor (PBR), as a microglial marker, and heat shock protein 27kD (hsp27kD), which is expressed in astrocytes.

Microglial activation was measured by equilibrium binding assays to PBR according to Camins et al. [8]. Briefly, hippocampi were weighed and homogenized in 10 volumes of cold buffer (0.32 M sucrose and 0.01 M HEPES, pH 7.4) with the aid of a Kinematica Polytron. The homogenates were centrifuged at 15 000 × *g* for 30 min at 4°C. Protein content was determined by the Bradford method, using bovine serum albumin as a standard. Equilibrium binding assays were performed at 0–4°C using [^{3}H]PK 11195 (85 Ci/mmol, New England Nuclear, Wilmington, DE, USA). Assays were performed in a final volume of 0.25 ml (pH 7.4), which contained [^{3}H]PK 11195 in a concentration range of 0.5–15 nM and 100 μg of protein per assay. Binding assays of tissues from controls and C-phycocyanin-treated animals were performed in presence of 2 nM of [^{3}H]PK 11195. Non-specific binding was measured in the presence of 10 μM of cold Ro 5–4864 (Fluka Chemie AG, Germany). The specific binding was about 90%. After incubation for 120 min, samples were filtered under vacuum over Whatman GF/B glass fiber membranes soaked in 0.5% polyethyleneimine. Filters were rapidly washed with three 4-ml-aliquots of ice-cold Tris–HCl (50 mM) and placed in vials containing 10 ml of a multipurpose liquid scintillation cocktail (Biogreen-1, Reactivos Scharlau, Spain). Radioactivity was measured in a Beckman LS-1800 liquid scintillation spectrometer with an efficiency of about 40%. Saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) were analyzed by non-linear regression, using Radlig 4.0 software (Biosoft, Elsevier). Saturation data are expressed as the best fit value ± standard error mean (SEM) obtained from three to four separate experiments carried out in duplicate. Statistical analyses of the binding results and weight changes were performed by non-parametric (Kruskal–Wallis and Mann–Whitney) tests using SPSS+ software.

Astroglial activation was determined by Western immunoblotting assays [9]. In this case, hippocampal homogenates were centrifuged twice at 900 × *g* for 10 min, pellets were resuspended in Tris–HCl 0.5 M pH = 6.8 buffer, and protein content was determined by the Bradford method. Tissue samples were placed in a buffer (Tris–HCl 0.5 M pH = 6.8, glycerol 10%, SDS 5%, 2-β-mercaptoethanol 5%, bromophenol blue 0.05%) to obtain a protein concentration of 2 μg/μl. Finally, samples were denaturalized by boiling at 95–100°C for 5 min. Lysates containing equal amounts of protein, and pre-stained SDS-PAGE gel electrophoresis molecular weight standards (Bio-Rad), were separated by electrophoresis on 12% acrylamide gel using the Mini-Protean system (BioRad). Proteins were transferred to nitrocellulose sheets (Hybond-ECL, Amersham) using a Bio-Rad transblot apparatus. Membranes were blocked overnight in PBS-Tween containing 5% non-fat milk. Membranes were then incubated with a primary hsp27kD rabbit monoclonal antibody (1:1000, SPA-801, StressGen Biotechnologies). After 1 h, blots were washed thoroughly in 0.05% Tween 20 in PBS, and incubated for 1 h. with a peroxidase-conjugated antibody to rabbit hsp27kD (Amersham). Peroxidase activity was visualized using ECL Western Blot (Amersham). Immunoreactivity of β-tubuline was used as an internal marker for protein loading.

Systemic KA administration produced the well-described behavioral effects, including 'wet dog shakes', which progressed to generalized tonic-clonic seizures [1,9]. In the vehicle-treated group, KA induced significant neurobehavioral changes (Table 1) and weight loss (Table 2) with reference to control animals. In contrast, the incidence of neurobehavioral changes was significantly lower in lots receiving C-phycocyanin (Table 1), and the body-weight gain of these groups was not significantly different from control (Table 2). Moreover, groups B and C presented significantly higher body-weight gains than the KA group, whereas this gain was not significant in lot A animals receiving only one dose of C-phycocyanin.

Equivalent results were found when the neuronal damage in hippocampus after administration of KA was evaluated through the changes in PBR and hsp27kD. B_{max} values in the KA group (1660 ± 185 fmol/mg protein) were significantly higher than in the control group (605 ± 38 fmol/mg

Table 1
Effects of C-Phycocyanin on KA-induced neurobehavioral activities in control, Kainate group (KA group, 10 mg/kg, i.p.) and C-phycocyanin treated groups[a]

Incidence (%)	*n*	Seizures	Tremors	Wet dog shakes
Control group	6	0	0	0
KA group	6	100	100	100
A group	6	67*	67*	67*
B group	6	34**	34**	50*
C group	6	34**	34**	50*

[a] (A) C-phycocyanin (100 mg/kg, p.o.) given 1 h before KA (10 mg/kg, i.p.). (B) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA. (C) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA and, additionally, 1 h after KA administration. *$P < 0.05$, **$P < 0.01$ vs. KA values.

Table 2
Effects of C-phycocyanin on KA-induced changes in body weight, in control, Kainate group (KA group, 10 mg/kg, i.p.) and C-phycocyanin treated groups[a]

	n	Body weight (g ± SEM)		Body weight gain (%)
		Initial	End	
Control	6	197 ± 4	267 ± 4	70 ± 3
KA group	6	196 ± 3	233 ± 5	37 ± 3[#]
A group	6	194 ± 3	245 ± 7	51 ± 8
B group	6	190 ± 3	260 ± 9	70 ± 6*
C group	6	192 ± 3	254 ± 8	62 ± 8*

[a] (A) C-phycocyanin (100 mg/kg, p.o.) given 1 h before KA (10 mg/kg, i.p.). (B) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA. (C) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA and, additionally, 1 h after KA administration. Weight and percentage of body weight gain after treatment are expressed as mean ± SEM *$P < 0.05$ vs. KA values; [#]$P < 0.05$ vs. control values.

protein), and the dissociation constants for [^{3}H]PK 11195 were not significantly affected (1.81 ± 0.11 and 1.83 ± 0.13, respectively) (Fig. 1). Thus, when oral doses of C-phycocyanin were administered to animals (Fig. 2), the gliosis or neurodegenerating effect induced by KA was reversed (groups B and C) or, at least, decreased (group A). Further, hsp27kD expression was not detected by Western blot in control hippocampi, whereas strong expression was found after KA administration (Fig. 3). Meanwhile, a single dose of C-phycocyanin one hour before KA (group A) did not prevent hsp27kD expression, but the administration of C-phycocyanin several times before (group B), and before and after (lot C) KA inhibited the hsp27kD expression.

It is widely documented that non-neuronal cells can react to neuronal cell damage, developing what is known as glial activation [12]. Astroglial or microglial activation has been observed in brain as a response to several experimental situations in which neuronal injury is induced, such as ischemia [17], administration of neurotoxins [2,8] and, also, in neuro-degenerative diseases of the human brain [16]. After administration of KA to rats, microglial and astroglial activation occurs in hippocampus in the presence of neuronal degeneration [1,2,12]. PBR is found in microglia and hsp27kD is present in astroglia. Thus, increases in PBR [7,8] and expression of hsp27kD [9] are indirect signs of neuronal damage in brain in response to direct insults, including ischemic injury [17] and KA toxicity [3,8]. Here, PBR density and hsp27kD expression were used to quantify the effect of C-phycocyanin administration on the extent of neuronal damage induced in hippocampus by KA-treatment.

Our results demonstrate that C-phycocyanin prevents KA-induced behavioral and glial reactivity in the rat hippocampus, thereby suggesting a corresponding protective effect on neurons. The ability of C-phycocyanin to reduce experimental status epilepticus suggests a therapeutic efficacy of this compound in the treatment of some forms of epilepsies. KA-triggered excitotoxicity involves the production of ROS [5]. Although several mechanisms may be involved in the protective effect of C-phycocyanin against KA-induced glial activation and proliferation, this can be attributed to its free-radical scavenging and antioxidant properties. This is consistent with previous studies in rats showing that C-phycocyanin scavenges alkoxy and hydroxyl radicals and also inhibits lipid peroxidation [15]. Moreover, this antioxidant hypothesis is consistent with several studies performed with antioxidants drugs that prevented KA-induced neuronal damage [8,13,16,18].

In conclusion, to our knowledge, this is the first report showing that oral administration of C-phycocyanin reduces microglial and astroglial activation induced by KA. This suggests that some metabolites of this protein cross the hemato-encephalic-barrier and exert antioxidant effects in

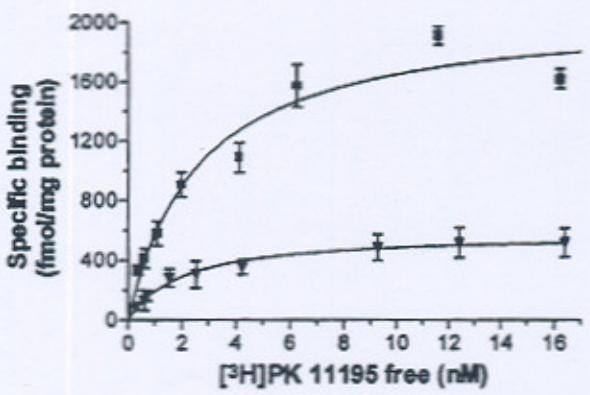

Fig. 1. Saturation analysis of [^{3}H]PK 11195 binding to hippocampal membranes from vehicle (▼)and KA (10 mg/kg, i.p.) (■) treated rats measured 1 week after administration. Each value represents the mean ± SEM of six different rats.

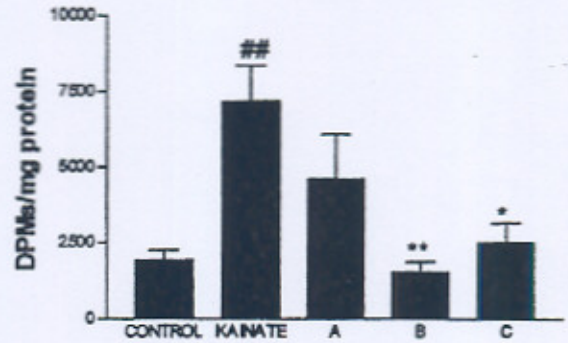

Fig. 2. Effect of different oral treatments with C-phycocyanin on Kainate (KA) toxicity. Ordinate indicates specific DPMs of [^{3}H]PK 11195 per mg protein. (A) C-phycocyanin (100 mg/kg, p.o.) given 1 h before KA (10 mg/kg, i.p.). (B) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA. (C) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA and, additionally, 1 h after KA administration. Each value represents the mean ± SEM of six different rats. *$P < 0.05$, **$P < 0.01$ vs. KA group. [##]$P < 0.005$ vs. control group.

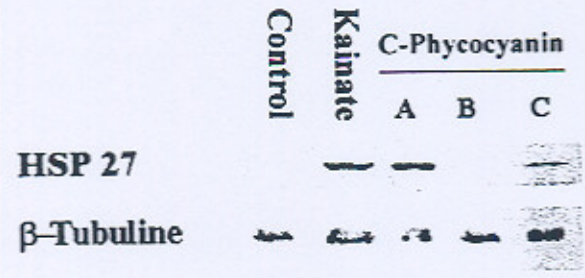

Fig. 3. Effect of different oral treatments with C-phycocyanin on hsp27kD expression. (1) Western blot hsp27kD inmunoreactivity found in control, Kainate (KA) and C-phycocyanin treated groups. (A) C-phycocyanin (100 mg/kg, p.o.) given 1 h before KA (10 mg/kg, i.p.). (B) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA. (C) C-phycocyanin (100 mg/kg, p.o.) given 24, 16 and 1 h before KA and, additionally, 1 h after KA administration. (2) Immunoreactivity of β-tubulin, which was used as an internal marker for gel loading.

the hippocampus. C-phycocyanin has very low toxicity and, in fact, it has been used as a food additive in some countries. Then, although additional studies will be needed, our results suggest that this natural product could be used in the prevention and treatment of some neurological disorders and neurodegenerative diseases associated with oxidative stress.

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