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Research report

C-Phycocyanin protects SH-SY5Y cells from oxidative injury, rat retina from transient ischemia and rat brain mitochondria from Ca^{2+}/phosphate-induced impairment

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ABSTRACT

Oxidative stress and mitochondrial impairment are essential in the ischemic stroke cascade and eventually lead to tissue injury. C-Phycocyanin (C-PC) has previously been shown to have strong antioxidant and neuroprotective actions. In the present study, we assessed the effects of C-PC on oxidative injury induced by *tert*-butylhydroperoxide (*t*-BOOH) in SH-SY5Y neuronal cells, on transient ischemia in rat retinas, and in the calcium/phosphate-induced impairment of isolated rat brain mitochondria (RBM). In SH-SY5Y cells, *t*-BOOH induced a significant reduction of cell viability as assessed by an MTT assay, and the reduction was effectively prevented by treatment with C-PC in the low micromolar concentration range. Transient ischemia in rat retinas was induced by increasing the intraocular pressure to 120 mmHg for 45 min, which was followed by 15 min of reperfusion. This event resulted in a cell density reduction to lower than 50% in the inner nuclear layer (INL), which was significantly prevented by the intraocular pre-treatment with C-PC for 15 min. In the RBM exposed to 3 mM phosphate and/or 100 μM Ca^{2+}, C-PC prevented in the low micromolar concentration range, the mitochondrial permeability transition as assessed by mitochondrial swelling, the membrane potential dissipation, the increase of reactive oxygen species levels and the release of the pro-apoptotic cytochrome c. In addition, C-PC displayed a strong inhibitory effect against an electrochemically-generated Fenton reaction. Therefore, C-PC is a potential neuroprotective agent against ischemic stroke, resulting in reduced neuronal oxidative injury and the protection of mitochondria from impairment.

1. Introduction

Ischemic stroke remains a major cause of death and disability worldwide (Mukherjee and Patil, 2011; WHO, 2011). Although significant progress has been made to clarify the mechanisms involved in the ischemic stroke cascade, the development of new and effective treatments has been largely unsuccessful. Thrombolysis with tissue plasminogen activator (t-PA) is currently the only approved human pharmacological treatment, but it is restricted to a minority of patients due to its short therapeutic window (4.5 h) (Fonarow et al., 2011; Maiser et al., 2011). An alternative approach known as neuroprotection has been focused on protecting the neurovascular unit by blocking the main pathogenic processes that causes

Abbreviations: t-PA, tissue plasminogen activator; C-PC, C-Phycocyanin; *t*-BOOH, *tert*-butylhydroperoxide; RBM, rat brain mitochondria; DMEM, Dulbecco's Modified Eagle Medium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; IOP, intraocular pressure; INL, inner nuclear layer; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SIM, standard incubation medium; H_2DCFDA, dichlorodihydrofluorescein diacetate; DCF, dichlorofluorescein; $\Delta\Psi$, mitochondrial membrane potential; TPP^+, tetraphenylphosphonium; MPT, mitochondrial permeability transition.

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ischemic injury (Ginsberg, 2008). Reactive oxygen species (ROS) generated soon after ischemia, during reperfusion and thereafter are considered crucial mediators of ischemic injury (Saito et al., 2005). Mitochondria, an important source of ROS, contribute significantly to the elevation of these oxidants after an ischemic event (Allen and Bayraktutan, 2009). Thus, antioxidant-based neurovascular protective strategies may be potential treatments to expand the number of currently approved therapies (Jung et al., 2010).

Previous studies have demonstrated that C-Phycocyanin (C-PC), the main phycobiliprotein of cyanobacteria *Spirulina platensis*, exerts potent antioxidant, anti-inflammatory and neuroprotective actions in different experimental conditions (Romay et al., 2003). Phycobiliproteins are light-harvesting complexes composed of apo-proteins covalently attached to open chain tetrapyrrole chromophores, named phycocyanobilins. C-PC has α and β polypeptides subunits (18–20 kDa) (Pentón-Rol et al., 2011a); one phycocyanobilin is attached at cysteine 84 in the α-chain and two phycocyanobilins are attached at cysteines 84 and 155 in the β-chain (Padyana et al., 2001; Pentón-Rol et al., 2011a). Recent results from our group have shown that the administration of C-PC either prophylactically or therapeutically exhibited beneficial effects against global cerebral ischemia–reperfusion injury in gerbils (Pentón-Rol et al., 2011a). These effects were partially explained by the C-PC ability to counteract oxidative damages.

In the present study, we characterized the *in vitro* protective effects of C-PC against *tert*-butylhydroperoxide (*t*-BOOH)-induced oxidative injury in the SH-SY5Y human neuroblastoma cell line and *in vivo* against transient ischemia in rat retinas. The effects of C-PC were also examined in isolated rat brain mitochondria (RBM) with a Ca^{2+}/phosphate overload, a condition that mimics the mitotoxic environment that results from an ischemic injury.

2. Materials and methods

2.1. Reagents and compounds

All chemicals used were of the highest grade available and purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified. C-PC was purified by ionic exchange, as previously described (Pentón-Rol et al., 2011a). An A_{620}/A_{280} absorbance ratio of 4.58 was obtained, indicative of higher than 90% purity (Patil and Raghavarao, 2007). C-PC was dissolved in sterile phosphate-buffered saline (PBS) pH 7.2, filtered through a 0.2 μm syringe filter under sterile conditions (Sartorius Minisart®, Germany) and stored in the dark at 4 °C until use.

2.2. Animals

Rats were obtained from the Animal Breeding Center at the Ribeirão Preto Campus, University of São Paulo (USP). The animals were maintained under standard laboratory conditions (60% humidity, 22 ± 1 °C, and 12 h light/darkness cycle) with free access to food and water. All procedures were performed in compliance with the USP guidelines and with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for animal experimentation.

2.3. Cell culture

SH-SY5Y human neuroblastoma cells (Rio de Janeiro Cell Bank, Brazil) were expanded in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 100 IU/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B at 37 °C in 5% CO_2/95% air. The medium was changed every two days, and the cells were subcultured every four days by trypsinization and re-plated in T-25 or T-75 flasks (5 or 15 mL final volume, respectively) (BD Falcon, USA) at 4×10^5 cells/mL.

2.4. Cell treatment and viability assay

SH-SY5Y cells between passages three and seven were cultured in quadruplicate in 96 wells plates (BD Falcon, USA) at 4×10^4 cells/200 μL/well for 24 h. The cells were incubated with freshly prepared *t*-BOOH in complete medium at different concentrations for 6 h, or pre-treated with C-PC for 24 h followed by the incubation with fresh medium containing C-PC and 25 μM *t*-BOOH for another 6 h. Cell viability was measured by the quantity of blue formazan products obtained from the colorless 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) by mitochondrial dehydrogenases, which are only active in viable cells. After the incubation time, the cells were gently washed with sterile phosphate buffered saline (PBS) pH 7.2 (200 μL/well), and MTT (freshly prepared at 0.5 mg/mL in serum-free medium) was added at 200 μL/well and incubated for 4 h at 37 °C. After MTT withdrawal, 200 μL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals, and the absorbance of each well was measured at 570 nm in a microplate reader (Varian Cary 50, Australia). The results were expressed as the percent of absorbance relative to the undamaged control cells.

2.5. Induction of transient retinal ischemia

A transient ischemia in the rat retina was induced as previously described, with some modifications (Beleboni et al., 2006). Male Wistar rats weighing 250–300 g at the time of surgery ($n = 5$ per group) were anesthetized with 450 mg/Kg i.p. urethane (Acrôs Organic, USA) (Meyer, 2008), and the anterior chamber of the left eye was cannulated with a 27-gauge needle attached to a manometer, a pump and an air reservoir. The intraocular pressure (IOP) was raised to 120 mmHg for 45 min. Afterwards the needle was withdrawn, and the IOP was normalized for a 15 min reperfusion period. The drug was intraocularly injected 15 min prior to the ischemic insult. The vehicle group was injected with sterile PBS at pH 7.2. The right-side, untouched retinas served as control ($n = 10$).

2.6. Histological assessment

After the reperfusion period, the animals were sacrificed, and both eyes were rapidly enucleated, placed in the fixation solution (25.5 mL ethanol 80%, 3 mL formaldehyde 37% and 1.5 mL glacial acetic acid) for 24 h and transferred to 80% ethanol for another 4 days (Krinke, 2000). The dissected retina was dehydrated in graded ethanol and xylene and embedded in paraffin. Retinas were sectioned at 5 μm, approximately 1 mm from the optic nerve, and stained with hematoxylin and eosin. For each experimental group, five microscopic fields (magnification 400×) of one sagittal section of a retina were captured by a digital camera (DFC300 FX, DM 5000 B microscope, Q-Win software, Leica Microsystems, Germany). The number of viable cells, those that were not eosinophilic, was manually counted in the established areas (200 μm × 50 μm) of the inner nuclear layer (INL) using ImageJ 1.41 software (National Institutes of Health, USA). Cells showing atrophy, shrinkage, nuclear pyknosis, dark cytoplasmic coloration or ambient empty spaces were considered damaged (Beleboni et al., 2006). The results were expressed as a percentage of the number of viable cells in the control retinas.

2.7. Isolation of rat brain mitochondria

Mitochondria were isolated as previously described, with minor modifications (Mirandola et al., 2010). Briefly, two male Wistar rats weighing 200–240 g were sacrificed by decapitation, and their brains were rapidly removed and placed in 10 mL of ice-cold isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1% de-fatted bovine serum albumin (BSA), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); pH 7.2). The olfactory bulb, cerebellum and underlying regions were discarded, and the remaining forebrains were split using surgical scissors and washed twice in isolation buffer. The brain tissue was manually homogenized in 20 mL of the isolation buffer using a glass Dounce homogenizer (20–25 strokes). The homogenate was centrifuged at 4 °C (2000 × *g*, 3 min) in a Hitachi RT15A5 rotor (Japan), and the resulting supernatant was further centrifuged (12,000 × *g*, 8 min). The pellet was gently resuspended in 10 mL of isolation buffer containing 20 μL of 10% digitonin (to induce synaptosomal lysis) using a fine-tipped brush and centrifuged at 12,000 × *g* for 10 min. The dark pellet was resuspended in 10 mL of isolation medium without EGTA and BSA and centrifuged again (12,000 × *g*, 10 min), and the resulting pellet was resuspended in 200 μL of standard incubation medium (SIM: 225 mM mannitol, 75 mM sucrose, and 10 mM K^+HEPES; pH 7.2). The total protein concentration was determined by the Biuret method with BSA as a standard and was approximately 30–50 mg/mL for each preparation. All procedures were performed at 4 °C.

2.8. Mitochondrial assays

The experiments using isolated RBM were performed at 30 °C with continuous magnetic stirring in the SIM described above. The respiratory control ratio (state 3/state 4 respiratory rates) was over 4; this ratio was determined after monitoring oxygen consumption with an oxygraph equipped with a Clark-type oxygen electrode (Hansatech Instruments Ltd., UK) and with 1 mg/mL of RBM plus 5 mM succinate, 2 μM rotenone, 800 μM ADP and 2 mM KH_2PO_4. Oligomycin at 1 μg/mL was used at state 4. Mitochondrial swelling was estimated from the decrease in the apparent absorbance at 540 nm using a Model U-2910 Hitachi spectrophotometer (Japan). ROS generation in RBM was monitored using 2 μM of dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Life Technology Co., USA). The changes in the fluorescence of the DCF product were monitored using an F-4500 fluorescence spectrophotometer (Hitachi, Japan) at 503/529 nm excitation/emission wavelengths, and data were expressed as differences with respect to the initial value (Esposti, 2002; Rodrigues et al., 2011). The mitochondrial membrane potential ($\Delta\Psi$) was estimated by continuously monitoring the concentration of the lipophilic cation tetraphenylphosphonium chloride (TPP^+) in the extramitochondrial medium (Ross et al., 2005) with a TPP^+ selective electrode (World Precision Instruments Inc., USA)