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Inflammation Research

Further studies on anti-inflammatory activity of phycocyanin in some animal models of inflammation

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Abstract. *Objective*: To examine the effects of C-phycocyanin, a pigment found in blue-green algae which acts as an antioxidant in vitro and in vivo, in different animal models of inflammation.
Material: Male Sprague Dawley rats and OF_1 mice were used.
Treatments: Oedema was induced by: a) AA (0.5 mg/ear) or TPA (4 μg/ear) in the mouse ear b) carrageenan injection (0.1 mL of 1% suspension) in the rat paw (± adrenalectomy) and c) cotton pellet implantation in the rat axilla. Phycocyanin (50–300 mg/kg, p.o.) or indomethacin (1 mg/ear or 3–10 mg/kg, p.o.) as control were tested in the four animal models.
Methods: Measurement of the increase in the weight (mg) of 6 mm ear punch biopsies from treated ears were made in comparison to control ears, together with myeloperoxidase (MPO) activity as an index of neutrophil infiltration. The increase in the paw thickness (mm) was measured with a dial caliper. Cotton pellet was implanted and seven days afterwards the granuloma was removed and the dry weight was determined. Acute toxicity was studied in mice and rats. Statistics were performed using one-way analysis of variance with the Duncan Multirange test.
Results: Phycocyanin reduced significantly ($p < 0.05$) and in a dose-dependent manner ear oedema induced by AA and TPA in mice as well as carrageenan-induced rat paw oedema (both in intact and adrenalectomized animals). In the TPA test, phycocyanin also reduced MPO content. Phycocyanin also exerted an inhibitory effect in the cotton pellet granuloma test. In the acute toxicity test in rats and mice, even at the highest dose tested (3 000 mg/kg, p.o.), no toxicity was found.
Conclusions: Phycocyanin shows anti-inflammatory activity in four experimental models of inflammation. Its anti-oxidative and oxygen free radical scavenging properties may contribute, at least in part, to its anti-inflammatory activity.

Key words: C-phycocyanin – Anti-inflammatory agent – Inflammation models – Antioxidant – Indomethacin

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Introduction

C-phycocyanin is a protein-bound pigment soluble in water and found in some blue-green microalgae such as Spirulina and Arthospira, which are used in many countries as dietary supplements [1]. Phycocyanin monomers are themselves made up of two distinguishable protein subunits designated α and β, which contain at least three covalently attached bilin chromophores, open chain tetrapyrroles with no metal complex [2]. Very recently, we have discovered antioxidant and oxygen free radical scavenging effects of phycocyanin using three different radical generating systems (superoxide, hydroxyl and alkoxyl) which were detected by luminol-enhanced chemiluminescence. Phycocyanin was able to scavenge hydroxyl (OH·) and alkoxy (RO·) radicals. Furthermore, phycocyanin is able to inhibit luminol-enhanced chemiluminescence from zymosan-activated human polymorphonuclear leukocytes, microsomal lipid peroxidation induced by Fe^{+3}-ascorbic acid and the glucose-oxidase induced inflammation in mouse paw, a model of inflammatory response in which peroxide and hydroxyl radicals are involved [3].

Taking into account the former findings, we decided to test the potential anti-inflammatory activity of C-phycocyanin in other well-known animal models of inflammation.

Materials and methods

Animals

Male OF_1 mice weighing 22–25 g and male Sprague Dawley rats (175–250 g) were used in the experiments. The animals were purchased from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). The animals were housed in an environmentally ($t = 25\,°C$) and air humidity (60%) controlled room with a 12 h light-dark cycle, kept on a standard laboratory diet and drinking water ad libitum. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research (CNIC).

Reagents

Carrageenan sodium salt was purchased from BDH chemicals (Poole, UK). Arachidonic acid (AA), 12-O tetradecanoyl phorbol 13-acetate (TPA) and O-dianisidine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents of analytical grade were purchased from normal commercial sources.

Preparation of phycocyanin extract

Phycocyanin was extracted from microalgae Arthospira maxima as described in a Cuban patent [4]. The blue powder thus obtained showed a peak in the absorption spectrum of 617 nm, which is very close to the one reported for C-phycocyanin [5].

Oedema induced by AA in mouse ear

The method described by Opas et al. [6] was followed. Mice were fasted for 18 h with free access to water and divided into groups of 7 animals. Inflammation was induced by topical application of AA (0.5 mg/20 μL acetone) to the right ear of each mouse. Left ear (control) received the vehicle. Phycocyanin (50, 100 and 200 mg/kg in water) was administered by gavage 1 h before AA. The positive control group received indomethacin, 1 mg/ear topically. Inflammation was followed for 1 h and thereafter animals were killed by cervical dislocation. A 6 mm section of ears was obtained and weighed. The swelling induced by AA was assessed as the increase in weight of ear punch of treated groups over untreated one and it was called the oedema index.

Carrageenan-induced rat paw oedema

This test was carried out as described by Boughton-Smith et al. [7]. Male Sprague Dawley rats (175–200 g) which had been fasted overnight (18 h) received phycocyanin (50, 100 and 200 mg/kg, p.o.) or indomethacin (10 mg/kg, p.o.) as positive control and they were treated at a dose volume of 5 mL/kg, 1 h before subplantar injection in the right hind paw of carrageenan (0.1 mL of 1% suspension in 0.9% saline). Paw thickness was measured from ventral to dorsal surfaces, with a dial caliper, immediately prior to carrageenan injection and also 5 h afterwards. Oedema was expressed as the increase in paw thickness (in mm) measured after carrageenan injection and compared to the pre-injection value for individual animals. Values are expressed as percent inhibition of oedema of treated animals with respect to carrageenan control group.

Adrenalectomy procedure

In order to compare the effect of phycocyanin on carrageenan-induced rat paw oedema in normal and adrenalectomized animals, eighteen rats were adrenalectomized under pentobarbital anesthesia. The dorsal part of the animals was shaved and a small incision made, 1 cm long. The adrenal glands were removed and the rats used five days after surgery. During the entire post-surgical period the rats were allowed to drink only saline. The oedema induced by carrageenan injection and treatment of the animals was performed as described above.

TPA-induced mouse ear oedema

Oedema was induced in the right ear of mice by topical application of 4 μg/ear of TPA in acetone [8]. The left ear (control) received vehicle (acetone, 20 μL). Phycocyanin was administered p.o. at doses of 100, 200 and 300 mg/kg 1 h before TPA application. Two control groups were used. One of them received saline solution p.o. and the reference group was treated with indomethacin (1 mg/ear). Six hours after TPA application, mice were killed by cervical dislocation and a 6 mm diameter disc from each ear was removed with a metal punch and weighed. The swelling induced by TPA was assessed as the increase in the weight of the right ear punch biopsy over that of the left ear and called the oedema index. Tissue samples were assessed biochemically for the neutrophil marker enzyme, myeloperoxidase (MPO), using the method of Bradley et al. [9] with minor modifications. All the ear tissue was homogenised in 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 6) containing 0.5% hexadecyl trimethylammonium bromide (HTBA) using a Polytron (Ultra-turrax T-25) homogeniser. After freeze-thawing 3 times, the samples were centrifuged at 2 500 g for 30 min at 4 °C and the resulting supernatant assayed spectrophotometrically for MPO. In brief, 40 μL of sample was mixed with 960 μL of 50-mM phosphate buffer pH 6, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured with a Spekol 220 spectrophotometer (Carl Zeiss, Germany). MPO activity data are presented as units per mg tissue. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25 °C.

Cotton pellet granuloma in rats

Autoclaved cotton pellets (50 ± 1 mg) were implanted in the axillae of rats under ether anaesthesia using the method of Swingle and Shideman [10]. Phycocyanin (50, 100 and 200 mg/kg) as well as indomethacin (3 mg/kg) and vehicle were administered daily p.o. for 7 days. Animals were killed on day 7 and the granuloma was removed and dried in an oven at 60 °C and weighed.

Acute toxicity in mice and rats

Mice and rats were used for the determination of lethal dose 50 (LD_{50}) by the method of Litchfield and Wilcoxon [11]. Phycocyanin extract was administered p.o. and thereafter the behaviour of animals was observed. Mortality was recorded during the 14 days after phycocyanin administration. Body weight of each animal was determined throughout the experiment. Histopathological studies were performed on surviving animals treated with the highest dose of phycocyanin used (3 g/kg, p.o.).

Statistical analysis

Data are presented as means ± standard deviation. Mean differences between groups were compared by one way analysis of variance (ANOVA) with the Duncan Multirange test. The level of statistical significance was taken as $p < 0.05$. The ED_{50} was calculated using a GraphPad InPlot software (GraphPad Software Inc., version 4.03, 1992).

Results

Effect of phycocyanin on oedema induced by AA in mouse ear

Phycocyanin (50, 100 and 200 mg/kg, p.o.) significantly ($p < 0.05$) inhibited ear oedema induced by AA in mice. The effect of phycocyanin was dose-dependent. Effective dose fifty (ED_{50}) value of phycocyanin was 66.1 mg/kg. Indomethacin also significantly ($p < 0.05$) reduced the oedema index (Table 1).

Effect of phycocyanin on carrageenan-induced rat paw oedema in normal and adrenalectomized rats

The subplantar injection of carrageenan in control rats induced an increase in paw thickness (4.0 ± 0.07 mm) over 5 h. Pre-treatment with phycocyanin (50, 100 and 200 mg/kg, p.o.) 1 h before carrageenan inhibited oedema in a dose-dependent fashion. Indomethacin (10 mg/kg, p.o.) also inhibited the increase in paw thickness induced by carrageenan (Table 2). In adrenalectomized rats phycocyanin (100 and 200 mg/kg, p.o.) also inhibited carrageenan induced paw oedema to the same extent as that in intact rats when otherwise identical treatment regimes were used (Table 2).

Table 1. Effect of phycocyanin on oedema induced by AA in mouse ear.

Treatment		Oedema index (mg) mean ± SD	Inhibition (%)
AA	0.5 mg/ear	6.3 ± 0.98	–
Phycocyanin	50 mg/kg	3.3 ± 0.59*	47.61
	100 mg/kg	2.5 ± 0.92*	60.31
	200 mg/kg	2.1 ± 0.8*	66.60
Indomethacin	1 mg/ear	1.1 ± 0.24**	82.53

Oedema index was assessed as the increase in the weight of the right ear punch biopsy over that of the left ear. The oedema was measured 1 h after AA application.
* $p < 0.05$, ** $p < 0.01$. Statistical significance with respect to AA. $n = 7$ animals per group.

Table 2. Effect of phycocyanin on carrageenan-induced paw oedema in normal and adrenalectomized rats.

Treatment		Inhibition of oedema (%)	
		normal rats	adrenalectomized rats
Indomethacin	10 mg/kg	49.4*	–
Phycocyanin	50 mg/kg	16.4*	–
	100 mg/kg	26.5*	26.2*
	200 mg/kg	43.7*	41.8*

Values are expressed as percent inhibition of oedema index of treated animals with respect to carrageenan control group.
* $p < 0.05$. Statistical significance with respect to carrageenan group. Rats were adrenalectomized five days before the assay was performed. $n = 7$ animals per group.

Effect of phycocyanin on TPA-induced ear oedema in mice

Phycocyanin (100, 200 and 300 mg/kg, p.o.) inhibited significantly ($p < 0.05$) both, ear oedema and neutrophil infiltration marker, MPO induced by TPA. Doses below 100 mg/kg, p.o. did not inhibit oedema significantly. Indomethacin (1 mg/ear) also inhibited significantly ($p < 0.05$) the inflammatory response in this model (Table 3).

Effect of phycocyanin on cotton pellet granuloma in rats

Phycocyanin, 100 and 200 mg/kg, p.o., but not 50 mg/kg, significantly ($p < 0.05$) inhibited the cotton pellet test. At a dose of 200 mg/kg, the inhibitory response averaged 36%, whereas with indomethacin (3 mg/kg) it was 53% (Table 4).

Acute toxicity studies

LD_{50} values were determined by the oral administration of phycocyanin into mice and rats. The measured LD_{50} values were estimated to be greater than 3 g/kg for both species. No mortality was induced even at the highest dose of phycocyanin tested (3 g/kg p.o.). The animals were observed for 14 days and neither alterations in behaviour nor statistical differences in body weight were found between treated and non-treated animals. In the histopathological studies, no damage to organs or tissues was found.

Discussion

Currently, there is a considerable body of evidence that certain types of inflammatory tissue injury are mediated by reactive oxygen species (ROS) [12]. Oxidants such as superoxide and hydroxyl radicals, hydrogen peroxide and hypochlorous acid are formed at sites of inflammation, and

Table 3. Effect of phycocyanin on TPA-induced oedema in the mouse ear.

Treatment		Oedema index (mg)		MPO (U/mg tissue)	
		mean ± SD	inhibition (%)	mean ± SD	inhibition (%)
TPA	4 μg/ear	9.4 ± 0.99	–	4.2 ± 0.19	–
Indomethacin 1 mg/ear		0.61 ± 0.07**	93.4	0.4 ± 0.07**	89.5
Phycocyanin					
100 mg/kg		8.0 ± 0.77*	14.1	3.6 ± 0.18*	14.2
200 mg/kg		6.7 ± 0.92*	28.6	2.7 ± 0.29*	35.4
300 mg/kg		5.4 ± 0.56*	45.5	2.2 ± 0.18*	46.5

Oedema was measured 6 h after treatment with TPA. Oedema index was assessed as the increase in the weight of the right ear punch biopsy over that of the left ear.
* $p < 0.05$, ** $p < 0.01$. Statistical significance with respect to TPA group. $n = 7$ animals per group.

Table 4. Effect of phycocyanin on cotton pellet granuloma in rats.

Treatment		Granuloma weight (g) mean ± SD	Inhibition (%)
Control		0.94 ± 0.05	–
Indomethacin	3 mg/kg	0.44 ± 0.05*	53.19
Phycocyanin	50 mg/kg	0.85 ± 0.08	9.57
	100 mg/kg	0.66 ± 0.08*	29.78
	200 mg/kg	0.60 ± 0.06*	36.17

Dry weight of granuloma was measured after seven days administration of tested agents (p.o.), * $p < 0.05$. Statistical significance with respect to control. $n = 7$ animals per group.

appear to contribute to the tissue damage in some acute and chronic inflammatory diseases. It has been suggested that many anti-inflammatory drugs might exert some of their effects by scavenging oxidants, and decreasing formation of ROS by activated phagocyte [13].

We recently found that C-phycocyanin has antioxidant properties [3]. It was able to scavenge alkoxy (RO·) and hydroxyl (OH·) radicals. The second-order rate constant for reaction of phycocyanin with OH· was in the range ($10^9 - 10^{10}\,M^{-1}\,S^{-1}$) of that obtained for some NSAIDs, like indomethacin and ibuprofen. Phycocyanin was also capable of inhibiting liver microsomal lipid peroxidation induced by Fe^{+3}-ascorbic acid ($IC_{50} = 12$ mg/mL), the luminol-amplified chemiluminescent response of PMNLs stimulated with opsonised zymosan, as well as the oedema induced by glucose oxidase injection in the mouse paw.

Taking into account these findings, we decided to research the potential activity of this product as an anti-inflammatory agent. Thus, we evaluated the phycocyanin extract in some experimental models in which both ROS and AA metabolites are involved in the induction and development of the inflammatory response.

In this study, oral administration of phycocyanin extract at doses of 50–300 mg/kg led to anti-inflammatory activity in the models of inflammation tested although the phycocyanin effect varied. It was a more powerful inhibitor in the AA-induced mouse ear oedema, which is considered to be a suitable test for the detection of cyclooxygenase (COX) and/or lipoxygenase (LOX) inhibitors of AA metabolism [6]. Also, Crummey et al. [14] demonstrated that free radical scavengers and antioxidants such as butylated hydroxyanisole, α-tocopherol, retinoic acid and desferrioxamine are able to strongly inhibit AA-induced mouse ear oedema. They postulated that the marked inhibitory activity of the free radical scavengers might be due to a direct reduction of both enzymatic and non-enzymatic lipid peroxidation (and hence AA metabolism) as well as to a further reduction in the activity of cyclo-oxygenase and lipoxygenase due to their requirement for (hydro)-peroxides to stimulate enzymatic function.

Recently Maccarrone et al. [15] presented evidence that the chain-breaking antioxidants, ascorbic acid, 6-palmitoyl-ascorbic acid and trolox inhibited soybean lipoxygenase-1 in the micromolar concentration range. The inhibition was competitive, complete and reversible. They concluded that the effect of these chain-breaking antioxidants in protecting cell membranes by preventing lipid peroxidation can occur through the formation of an inactive complex with LOX, besides the trapping of free radicals.

Phycocyanin was also effective in the carrageenan-induced rat paw oedema, which has been suggested as a suitable model for the in vivo assessment of the anti-inflammatory actions of novel antioxidants and inhibitors of oxygen radical generation [7]. These authors have provided evidence that oxygen radicals play an important role in the maintenance of carrageenan paw oedema. On the other hand, the anti-inflammatory activity of phycocyanin is not dependent on corticosteroid release, because it inhibited carrageenan-induced rat paw oedema to the same extent in intact and adrenalectomized rats.

Phycocyanin inhibited, in a dose-dependent manner, oedema and MPO in the TPA test, but in contrast to its effects in AA and carrageenan tests, in the TPA-induced mouse ear oedema the inhibition was weak, especially in relation to the AA test in which almost the same percent inhibition was obtained using a six fold lower dose. According to Nishizuka [16], an inhibitory effect in the TPA test would indicate that the inhibition of the oedema could be due essentially to a blockade of protein kinase C. Our results in this test indicate that inhibition of protein kinase C by phycocyanin most probably does not occur. However, the actions of TPA certainly appear to be very complex and do not conform to the assumption that protein kinase C is the only target for TPA [17]. It has been recently shown that treatment of mouse skin with PKC promotors, such as TPA, induces formation of free radicals in vivo [18]. Therefore, pre-treatment with free radical scavengers such as phycocyanin may exert some inhibitory effect in TPA-test.

Phycocyanin showed anti-inflammatory activity, not only in the acute models of inflammation used, but also in a sub-chronic model, the cotton pellet granuloma in rats.

The standard anti-inflammatory drug, indomethacin, used as positive control in the experiments, exerted significant effects in the range 1–10 mg (1 mg/ear or 3–10- mg/kg, p.o.), whereas phycocyanin only did so at high doses of 50–300 mg/kg. However, it is well known that indomethacin has an LD_{50} of 12 mg/kg in rats and 50 mg/kg in mice p.o. and also induces many adverse effects in patients under treatment [19]. On the other hand, phycocyanin did not induce mortality in rats and mice even at the high dose of 3 g/kg, p.o. in acute toxicity experiments. No alterations in behaviour or in organs and tissues were observed.

In conclusion, oral administration of phycocyanin exerted anti-inflammatory activity in four experimental models of inflammation. Our previous and present results indicate that the anti-inflammatory action is due most probably to its antioxidative and oxygen free radical scavenger properties and perhaps to inhibitory effects on AA metabolism. Research is in progress in our laboratory in order to elucidate the mechanism of action.

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