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Phycobiliprotein C-Phycocyanin from *Spirulina platensis* Is Powerfully Responsible for Reducing Oxidative Stress and NADPH Oxidase Expression Induced by an Atherogenic Diet in Hamsters

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The effects of spirulina and its chromophore phycocyanin, both without bound Se or selenium-enriched, were studied on plasma cholesterol, early atherosclerosis, cardiac production of superoxide anions, and NAD(P)H oxidase expression in hamsters. Forty hamsters were divided into 5 groups of 8 and fed an atherogenic diet for 12 weeks. They received by gavage either 7.14 mL/(kg day) phycocyanin (PC), Se-rich phycocyanin (SePC), spirulina (SP) or Se-rich spirulina (SeSP) in water, or water as control. SeSP and SePC supplied 0.4 μg of Se per 100 g body weight. Plasma cholesterol and non-HDL cholesterol concentrations were lower in group consuming SePC. HDL-cholesterol was never affected. SePC significantly increased plasma antioxidant capacity by 42% compared with controls. A sparing effect in liver glutathione peroxidase (87% on average) and superoxide dismutase (56% on average) activity was observed for all the groups compared to controls. Aortic fatty streak area was significantly reduced in the experimental groups, especially by PC (82%) and SePC (85%). Cardiac production of superoxide anion significantly decreased by ~46–76% in the four experimental groups and especially in SePC group (76%). The expression of p22phox subunit of NAD(P)H oxidase decreased by 34% after consumption of SePC. The results indicate that chronic consumption of Se-rich spirulina phycocyanin powerfully prevents the development of atherosclerosis. The underlying mechanism is related mainly to inhibiting pro-oxidant factors and at a lesser extent improving the serum lipid profile.

KEYWORDS: Atherosclerosis; NADPH oxidase; selenium; spirulina; phycocyanin; hamsters

INTRODUCTION

Selenium has received considerable attention as an essential micronutrient for animals and humans. It functions in the active site of a large number of selenium-dependent enzymes such as glutathione peroxidase and is associated with anticancer and other physiological functions (*1, 2*). The lack of Se in food may cause different diseases such as cardiovascular disease (*3*), cancer (*4, 5*), rheumatoid arthritis (*6*), cataract (*7*), and anemia (*8*). The major forms of selenium occurring in foodstuffs are the organic, protein-associated forms, selenomethionine (SeMet, plant and animal sources) and selenocysteine (SeCys, animal sources). Selenate is also present in some foodstuffs (*9*), and in selenium-deficient areas inorganic selenium salts (selenite and selenate) are added to the food (*10*). Elsewhere, it is generally believed that organic Se compounds are better and safer than inorganic Se as dietary supplements; therefore, in response to the need for Se to support human health, selenium-enriched foods have been developed and commercialized (*11*). In the search for an economical source of organic nutritional forms of Se, we attempted to increase the normally low Se content of spirulina (*Spirulina platensis*) by growing it in Se-enriched medium (*12, 13*) and suggested it represents a promising source

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for commercial large-scale production of organic Se, as compared with other plants (*13*). Spirulina is a blue-green microalga belonging to the cyanobacteria family, commercially available for human consumption and used as a functional food for humans because of its high concentration of protein and other nutritional elements. Phycocyanin (PC) and allophycocyanin are blue water-soluble photosynthetic pigments derived from cyanobacteria (blue-green algae) such as spirulina. They have been used as nutrients for both human and animal consumption, as natural dyes for food and cosmetics, and as pharmaceuticals (*14*). In a recent work, we suggested that in Se-rich Spirulina organic Se was mainly located in phycocyanin (*13*); more recently, this was corroborated by a work of Huang et al. (*15*).

Previous reports have shown that a protean extract of *Spirulina platensis* is a potent free-radical scavenger (hydroxyl and peroxyl radicals) and inhibits microsomal lipid peroxidation; it has also been observed that an increase in phycocyanin content was related to an increase in the antioxidant activity in different fractions, and therefore phycobiliprotein phycocyanin is the component mainly responsible for the antioxidant activity (*16*). Phycocyanin has also been described as a strong antioxidant (*17,18*) and anti-inflammatory (*19*) natural compound. It is similar in chemical structure to bilirubin and acts as a powerful scavenger of reactive oxygen species (ROS) in various in vitro and in vivo experimental models (*20*).

It is now well recognized that an increased formation of oxygen radicals and other oxygen derivatives frequently accompanies tissue damage. Today, there is an explosive interest in the use of antioxidant nutritional supplements. Epidemiological evidence suggests that intake of some vitamins, minerals, and other food constituents may help to protect against heart disease, cancer, and the aging process and that antioxidants may have a protective effect, in either preventing these diseases or lessening the severity of the diseases upon their onset. Many of their activities are mediated by ROS, which are generated during the oxidative burst (*21, 22*). Hence, it is expected here that chronic diseases, particularly atherosclerosis, may be prevented by spirulina and phycocyanin and their Se-enriched forms.

Only two studies have suggested that *Spirulina platensis* concentrate imparts a hypocholesterolemic effect in rats (*23*) and humans (*24*), and very recently, Nagaoka et al. (*25*) provided the first direct evidence that phycocyanin is a novel hypocholesterolemic protein derived from *Spirulina platensis* that can powerfully influence rat serum cholesterol concentrations. Atherosclerosis may be characterized according to three theories (oxidative, inflammatory, and hypercholestrolemic); since selenium takes part to the antioxidant defense and PC has been shown to possess antioxidant, anti-inflammatory, and hypocholesterolemic properties, we hypothesized that PC and SePC might prevent this pathology. To do so, an aortic wall response to a high-cholesterol/low antioxidant diet was triggered in Syrian hamsters to induce fatty streak formation and atherosclerosis emergence; we then evaluated the possible preventive effect of the administration of spirulina and phycocyanin, prepared from low-selenium or Se-rich spirulina.

MATERIALS AND METHODS

Spirulina Selenium Fortification and Phycocyanin Preparation. Aquamer S.A. (Mèze, France) and algae (*Spirulina platensis*) were grown in a 130 L photobioreactor under continuous lighting on Zarouk's medium at 22 °C and pH 10.5 in the presence of SeO_2. This medium contained $NaHCO_3$, 16.8 g/L; K_2HPO_4, 0.5 g/L; $NaNO_3$, 2.5 g/L; K_2SO_4, 1.0 g/L; NaCl, 1.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $CaCl_2$, 0.04 g/L; $FeSO_4 \cdot 7H_2O$, 0.01 g/L; EDTA, 0.08 g/L; H_3BO_3, 2.86 mg/L; $MnCl_2 \cdot 4H_2O$, 1.81 mg/L; $ZnSO_4 \cdot 7H_2O$, 220 mg/L; $CuSO_4 \cdot 5H_2O$, 79 mg/L; MoO_3, 15 mg/L; and Na_2MoO_4, 21 mg/L and was supplied with light aeration (30 L/min) and the addition of 0.03% CO_2. At the end of the growth, the biomass was recovered and filtered through a 20 mm membrane, thoroughly washed with distilled water, frozen, and lyophilized. Phycocyanin was prepared by suspending spirulina in distilled water (50 g/L). Then, the suspension was submitted to two freezing/thawing cycles to induce cell breaking and centrifuged for 20 min at 8000*g*; the supernatant was submitted to three successive frontal filtrations (membrane porosities of 11.2, 1.2, and 0.2 μm). An ultrafiltrate was obtained by passing the filtrate, at 4 °C under nitrogen pressure, through an Amicon (Millipore S.A., Saint-Quentin-en-Yvelines, France) stirred ultrafiltration cell fitted with a 30000 *M*r exclusion membrane. The ultrafiltrate was discarded, and retentate (phycocyanin) (*13*) was frozen in liquid nitrogen and lyophilized.

Se Analysis. Samples were wet-ashed overnight with concentrated nitric acid and hydrogen peroxide in borosilicate test tubes placed in a hot block (Bioblock Scientific, Illkirch, Paris, France) at 110 °C. Selenium concentration in spirulina and phycocyanin was determinated by inductively coupled plasma mass spectrometry (ICP-MS) using a Varian Vista spectrometer (Varian, Les Ulis, France).

Animals. Male golden Syrian hamsters ($n = 40$; Janvier, Le Genest-St-Isle, France) weighing 95–105 g were randomly divided into five groups of eight with approximately equal mean group body weights. Hamsters were housed in groups of eight per plastic cage in a temperature-controlled room (23 ± 1 °C) subjected to a 12 h light: dark cycle (lights on at 0700 h) with free access to both food and water. Hamsters were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines (*26*).

Diets and Feeding Procedures. Hamsters were fed for 12 weeks a semipurified atherogenic diet consisting of 200 g/kg casein and 3 g/kg L-methionine, 393 g/kg corn starch, 154 g/kg sucrose, 50 g/kg cellulose, 150 g/kg lard, and 0.5 g/kg cholesterol. Vitamin and mineral mixes (respectively 10 and 35 g/kg) were formulated according to AIN-93 guidelines (*27*) and supplied by Scientific Animal Food & Engineering (SAFE, Augy, France); mixes did not contain selenium, vitamin C, and vitamin E. The hamsters of each group additionally received daily by gavage either tap water (group 1; control), crude spirulina (group 2; 2.66 mg day^{-1} 100 g BW^{-1}), Se-rich spirulina (group 3; 2.66 mg day^{-1} 100 g BW^{-1}), crude PC (group 4; 3.63 mg day^{-1} 100 g BW^{-1}), or Se-rich PC (group 4; 3.63 mg day^{-1} 100 g BW^{-1}). Spirulina and phycocyanin were given in water (7.14 mL kg BW^{-1}). Se-rich spirulina and Se-rich PC supplied 0.4 μg Se 100 g BW^{-1}.

Analytical Procedures. At the end of the 12 week experimental period, hamsters were deprived of food for 18 h and were anesthetized with an IP injection of pentobarbital (60 mg/mL at a dosage of 60 mg/kg body weight). Blood was drawn by cardiac puncture with heparin-moistened syringes, and plasma was prepared by centrifugation at 2000*g* for 10 min at 4 °C and then stored at −80 °C until analysis. Plasma total cholesterol (TC) and HDL cholesterol (HDL-C) were assayed with a quantitative colorimetric technique according to the kit supplier's instructions (respectively 401 and 352-4, Sigma Chemicals, Saint Quentin Fallavier, France). Plasma very low- and low-density lipoprotein cholesterol (nonHDL-C) was precipitated with phosphotungstate reagent (*28*) and HDL-C was measured in the supernatant. Plasma nonHDL-C was calculated from the difference between TC and HDL-C.

The antioxidant capacity of plasma was measured as Trolox equivalent; that is a quantitative value for general antioxidant levels in biological samples (*29*) which was assayed in plasma with a quantitative colorimetric technique according to the kit supplier's instructions (Kit NX2332; Randox, Mauguio, France). The assay is based on the incubation of a peroxidase and H_2O_2 with 2,2′-azinodi-(3-ethylbenzthiazoline sulfonate) (ABTS) to produce the radical cation $ABTS^{\bullet+}$. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants (albumin, uric acid, ascorbic acid, α-tocopherol, glutathione, beta-carotene, etc.) in the sample suppressed $ABTS^{\bullet+}$ color production to a degree proportional to their concentration.

Table 1. Effects of Ingestion of Phycocyanin (PC), Se-Rich Phycocyanin (SePC), Spirulina (SP), and Se-Rich Spirulina (SeSP) on Body Weight, Food Intake, and Organs Weight of Hamsters Fed an Atherogenic Diet[a]

group	controls	PC	SePC	SP	SeSP
initial body weight, g	103.7 ± 4.9 a	101.5 ± 5.8 a	100.1 ± 6.1 a	99.9 ± 4.7 a	101.4 ± 7.0 a
final body weight, g	134.4 ± 6.9 a	131.6 ± 6.4 a	132.4 ± 9.2 a	129.7 ± 7.6 a	132.2 ± 5.0 a
food intake, g/day	5.02 ± 0.43 a	5.15 ± 0.50 a	5.05 ± 0.49 a	5.18 ± 0.44 a	5.19 ± 0.42 a
liver weight (% BW)	7.02 ± 0.88 a	6.71 ± 0.47 a	7.34 ± 1.26 a	6.72 ± 0.69 a	6.78 ± 1.33 a
kidneys weight (% BW)	1.10 ± 0.27 a	1.01 ± 0.12 a	1.10 ± 0.22 a	1.04 ± 0.13 a	1.00 ± 0.14 a
spleen weight (% BW)	0.13 ± 0.02 a	0.13 ± 0.04 a	0.14 ± 0.05 a	0.12 ± 0.03 a	0.13 ± 0.02 a

[a] Values are means ± SEM, $n = 8$. Data were analyzed by one-way ANOVA followed by the Least Significant Difference test. For each dietary treatment, means in a column with different letters differ, $P < 0.05$.

The liver was perfused with ice-cold 0.1 mol/L potassium phosphate buffer (pH 7.4) to remove residual blood, rapidly excised, rinsed in ice-cold saline, blotted dry, weighed, sectioned for analysis, and stored in liquid nitrogen. Liver was homogenized in 5 vol of the same buffer (pH 7.4), and the homogenate was spun at 13000*g* for 15 min at 4 °C. The supernatant was stored at −80 °C for subsequent assay of glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) activity. GSHPx activity was measured on an automat Pentra 400 (HORIBA ABX, Montpellier, France) by the method of Randox (Randox Laboratories LTD, Crumlin, UK) using a commercial kit (no. RS505). The assay is based on the oxidation of reduced glutathione by GSHPx, coupled to the disappearance of NADPH at 340 nm (*30*). Superoxide dismutase activity was measured according to McKersie et al. (*31*). Briefly, supernatant (20 μL) was applied to a 13% polyacrylamide gel with a 4% stacking gel. One lane contained 0.5 unit of bovine Cu/Zn-SOD as an internal standard. The gel was stained with nitroblue tetrazolium and riboflavin at 4 °C and then developped on a light box for 20 min. The areas of SOD activity were clear against a blue background. An image of the gel was captured using a video camera, digitized, and then analyzed using Gel analyst device (Clara Vision, Orsay, France). Specific activity was expressed as units per milligram of protein. Protein content of samples was determined by a commercial protein assay (Sigma, Saint Quentin Fallavier, France) according to Smith et al. (*32*) and using bovine serum albumin as standard.

Aortic Tissue Processing. Following blood collection and liver removal, the intact aorta was first perfused with phosphate buffered saline containing 1 mmol/L $CaCl_2$ and 15 mmol/L glucose for 5 min and then with 0.1 mmol/L sodium cacodylate buffer pH 7.4 containing 2.5 mmol/L $CaCl_2$, 2.5% paraformaldehyde, and 1.5% glutaraldehyde for the fixation of the vasculature. The aorta was carefully dissected between sigmoid valves and 3–4 cm after the aortic arch and thoroughly cleaned of loose adventitial tissue; the aortic arch was cut free, open longitudinally along the outside of the arch, pin cork, immersed in fresh fixative solution, and stored at 4 °C until staining. The aortic arches were then first rinsed for 48 h in 0.1 mol/L sodium cacodylate buffer pH 7.4 containing 30 mmol/L $CaCl_2$ and 250 mmol/L sucrose. The arches were then rinsed in distilled water, stained for 40 s in Harris hematoxylin, and rinsed in distilled water and then quickly in 70% isopropyl alcohol; finally, they were stained in Oil red O for 30 min according to Nunnari et al. (*33*) and rinsed in 70% isopropyl alcohol and back to distilled water. Each aortic arch was then directly displayed on a glass slide, endothelium side up, covered with Aquamount mounting medium and coverslips and observed en face by light microscopy. All segments were photographed using a video digitizer. A computerized image analysis system (ImageJ, Scion Corp., Frederick, MD) attached to a compound light microscope was used to measure the total Oil Red O stained area of each aortic arch. The area covered by foam cells (aortic fatty streak lesion area or AFSA) was expressed as a percentage of the total area surveyed.

Determination of Superoxide Anion Production. Briefly, the left ventricle (150 mg) (*34*) was placed in Krebs buffer containing 250 μM of lucigenin, and the intensity of luminescence was recorded on a luminometer (Perkin-Elmer Wallac, Victor, Turku, Finland). Results were expressed as counts/mg of protein.

Immunoblotting of NADPH Oxidase Component. Proteins were extracted as previously described (*34*) from frozen left ventricle of hamsters. Samples were homogenized using an ultra turrax T25 basic (Irka-Werke) in an ice-cold extraction buffer containing 120 mM NaCl, 25 mM KCl, 2 mM $CaCl_2$, 15 mM Tris-Cl pH 7.5, 0.5% Triton, 1 mM PMSF, 0.1 mM DTT, 10 μM leupeptin, and 1 μM pepstatin. Protein concentrations in sample were determined by Bio-Rad Dc protein assay using BSA as a standard. Proteins (50 μg) were separated with 12% SDS-PAGE and then transferred to a nitrocellulose membrane (45 min, 100 V). Membranes were then incubated for 2 h with primary antibody against p22 phox (1/100[e], Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer. After six washes (6 °C, 5 min) in TBS/Tween under gentle agitation, blots were incubated for 45 min with horseradish peroxidase-labeled antibody (1/30000[e]). After further washes, blots were reavealed by enhanced chemiluminescence detection reagents (ECL, Amersham) and areas (mm^2) measured using the BIO-Profil 1D software (Fisher Bioblock).

Statistical Analyses. Data are shown as the means ± SEM, $n = 8$ measurements/group. Data were subjected to logarithmic transformation where necessary to achieve homogeneity of variances. Statistical analysis of the data were carried out using the Stat View IV software (Abacus Concepts, Berkeley, CA) by one-way ANOVA followed by Fisher's Protected Least Significant Difference test. Differences were considered significant at $P < 0.05$.

RESULTS

Se-rich spirulina contained 150 μg Se g^{-1} and issuing phycocyanin 110 μg Se g^{-1}. Crude spirulina contained 5 μg Se g^{-1}, and selenium was not detected in issuing phycocyanin. Nutritional parameters are summarized in **Table 1**. No significant difference appeared in food intake, final body weight, and organ weight (liver, kidneys, and spleen) between the five groups. Plasma lipid concentration and plasma antioxidant capacity are shown in **Table 2**. Only Se-rich phycocyanin (SePC) and PC significantly reduced plasma total cholesterol by 10% ($p = 0.009$) and 7.5% ($p = 0.039$), respectively. Non-HDL cholesterol decreased by 34% ($p = 0.0132$) after feeding SePC in comparison with the control group, and there was no difference between the four experimental groups (PC, SePC, SP, SeSP). HDL-cholesterol was never modified. Consequently, atherogenic index calculated as total cholesterol/HDL-cholesterol was lowered by 12% ($p = 0.032$) in hamsters receiving SePC. Elsewhere, SePC significantly prevented the 42% decrease in plasma antioxidant capacity (PAC) induced by the atherogenic diet ($p = 0.0128$); this effect is closely related to the PAC measuered on hamsters fed a nonatherogenic diet for 12 weeks (laboratory chow) exhibiting a PAC at 1.23 ± 0.02 mmol/L (value not shown in **Table 2**) vs 1.25 ± 0.09 mmol/L in SePC fed hamsters. At a lesser extent, PC, SP, and SeSP were also efficient in preventing PAC decrease (34%, 13%, and 9%, respectively). The highest GSHPx and SOD activity in liver was found in the control group (41.07 and 6.72 U/mg protein, respectively) compared to all other groups (**Table 2**). There was no difference between PC, SePC, SP, and SeSP groups where average activity was 5.24 U/mg protein for GSHPx and 2.95 U/mg protein for SOD. Average aortic fatty streak accumulation (AFSA), measured as the percentage of Oil Red O staining

Table 2. Effects of Ingestion Phycocyanin (PC), Se-Rich Phycocyanin (SePC), Spirulina (SP), and Se-Rich Spirulina (SeSP) on Plasma Lipid Concentrations and Antioxidant Capacity (PAC) and on Liver Antioxidant Enzymes Activity (GSHPx and SOD) in Hamsters Fed an Atherogenic Diet[a]

group	controls	PC	SePC	SP	SeSP
TC[b] (mmol/L)	7.54 ± 0.31 a	7.02 ± 0.16 b	6.79 ± 0.28 b	7.22 ± 0.28 ab	7.09 ± 0.18 ab
HDLC[c] (mmol/L)	4.51 ± 0.44 a	4.25 ± 0.33 a	4.58 ± 0.43 a	4.59 ± 0.28 a	4.39 ± 0.30 a
non-HDLC (mmol/L)	3.04 ± 0.42 a	2.78 ± 0.29 ab	2.21 ± 0.39 b	2.53 ± 0.21 ab	2.70 ± 0.33 ab
atherogenic index[d]	1.68 ± 0.29 a	1.66 ± 0.15 ab	1.48 ± 0.11 b	1.58 ± 0.06 ab	1.61 ± 0.11 ab
PAC (mmol/L)	0.88 ± 0.06 a	1.18 ± 0.09 bc	1.25 ± 0.09 b	0.99 ± 0.08 c	1.05 ± 0.07 c
GSHPx[e] (U/mg protein)	41.07 ± 1.23 a	4.67 ± 0.74 b	5.72 ± 1.27 b	5.00 ± 0.62 b	5.59 ± 1.41 b
SOD[f] (U/mg protein)	6.72 ± 0.52 a	3.17 ± 0.38 b	3.00 ± 0.32 b	2.29 ± 0.26 b	3.36 ± 0.90 b

[a] Values are means ± SEM, $n = 8$. Data were analyzed by one-way ANOVA followed by the Least Significant Difference test. For each dietary treatment, means in a column with different letters differ, $P < 0.05$. [b] TC = total cholesterol. [c] HDLC = high-density lipoprotein cholesterol. [d] Total cholesterol/HDL cholesterol. [e] Glutathione peroxidase. [f] Superoxide dismutase.

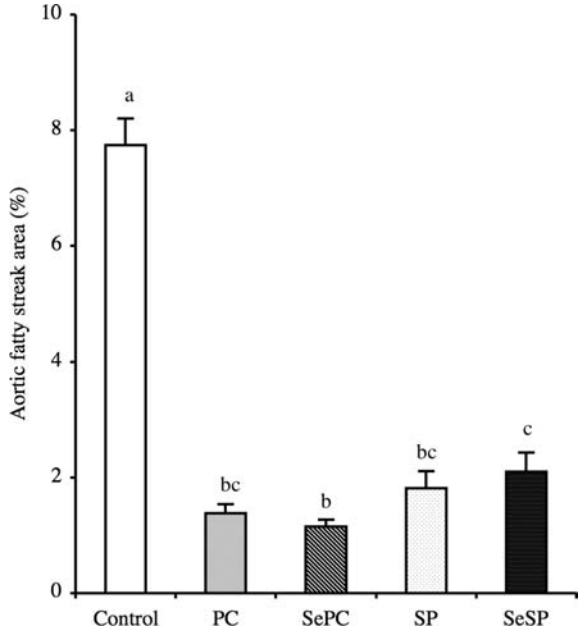

Figure 1. Effects of daily force-feeding water (control) or phycocyanin (PC), Se-rich phycocyanin (SePC), spirulina (SP), or Se-rich spirulina (SeSP) on aortic fatty streak area in hamsters fed an atherogenic diet for 12 weeks. Values are means ± SEM ($n = 8$). Bars with different index letters differ, $P < 0.05$.

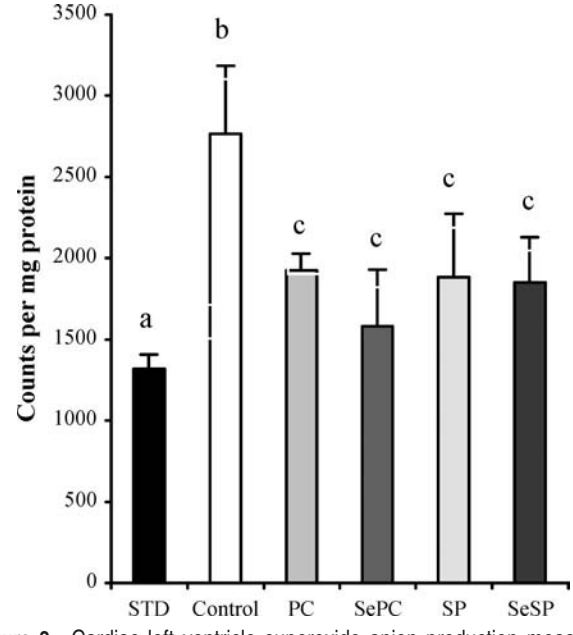

Figure 2. Cardiac left ventricle superoxide anion production measured by chemiluminescence probe (lucigenin 250 μM) in hamsters fed a standard diet (STD), in controls fed an atherogenic diet for 12 weeks, and in hamsters receiving an atherogenic diet plus phycocyanin (PC), Se-rich phycocyanin (SePC), spirulina (SP), or Se-rich spirulina (SeSP). Values are expressed as mean ± SEM of triplicate wells ($n = 8$). For each dietary treatment, bars with different index letters differ, $P < 0.05$.

relative to the total area surveyed (**Figure 1**), was significantly decreased in hamsters given SePC (85%, $p = 0.006$) and PC (82%, $p = 0.009$) in comparison with controls. Selenium-rich spirulina and native spirulina led to a less important AFSA decrease (73% and 76%, respectively). In the left cardiac ventricule, the superoxide anion production (**Figure 2**) was significantly reduced by PC (54%, $p = 0.004$), SP (56%, $p = 0.003$), and SeSP (46%, $p = 0.011$) in comparison with controls and SePC (76%, $p = 0.0003$) led to the strongest reduction, although this production was significantly higher than in hamsters fed a standard diet (1318 ± 90 counts per mg protein). The expression of p22phox (**Figure 3**) was significantly decreased by 22% ($p = 0.023$), 34% ($p = 0.001$), 25% ($p = 0.012$), and 33% ($p = 0.002$) respectively in hamsters receiving PC, SePC, SP, and SeSP, and there was no significant difference between the treatements. However, the lowest expression (SePC) was 33% higher than that exhibited by hamsters fed a standard diet for 12 weeks (**Figure 3**).

DISCUSSION

In this study we have demonstrated for the first time the cardiovascular and oxidative stress protective effect of spirulina and phycocyanin supplementation in hypercholesterolemic golden Syrian hamsters. Phycocyanin, either native or Se-rich, clearly showed a cholesterol-lowering effect. This confirmed the findings of Nagoaka et al. (*25*) that phycocyanin and also spirulina produce such effects on rats, but these authors used ~8 times higher dietary doses. We also showed that SePC decreases plasma non-HDL cholesterol concentration. Additionally, spirulina reduced plasma cholesterol but in a nonsignificant way. These effects involve the inhibition of both jejunal cholesterol absorption and ileal bile acid reabsorption (*25*).

The oxidative hypothesis of atherosclerosis allows to explain the initiation mechanism of the pathology according to an accumulation of oxidized LDL in monocyte-derived macrophages that leads to the development of foam cells (*35*). Oxidative stress results from an imbalance between ROS generation and antioxidant defence mechanisms, increasing superoxide production particularly when using a cholesterol-rich antioxidant-deficient diet that modifies hamsters antioxidant status. Elsewhere, the development of atherosclerosis is associated with an increased cardiac ROS production (*36*), i.e., here a superoxide

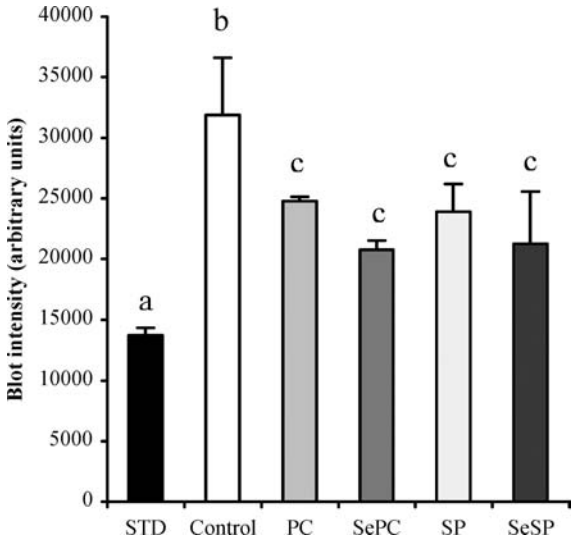

Figure 3. Expression of cardiac p22phox subunit of NAD(P)H oxidase in hamsters fed a standard diet (STD), in controls fed an atherogenic diet for 12 weeks, and in hamsters receiving an atherogenic diet plus phycocyanin (PC), Se-rich phycocyanin (SePC), spirulina (SP), or Se-rich spirulina (SeSP). Values are expressed as mean ± SEM ($n = 8$). For each dietary treatment, bars with different index letters differ, $P < 0.05$.

anion production induced by the atherogenic diet, which is closely dependent on the overexpression of NAD(P)H oxidase subunit p22-phox. As suggested by previous studies in rat model (*37*) and in humans (*38*), we suspected that the source of superoxide anion generation may be NADPH oxidase. In agreement with that, the expression of p22phox was significantly decreased by 34% in cardiac left ventricle from hamsters given SePC. On the other hand, high cardiac levels of superoxide play an important role in the pathogenesis of atherosclerosis and linked coronary arteries disease. In our model, the origin of cardiovascular alterations in cholesterol-fed hamster is accompanied by an increase of cardiac superoxide production and NAD(P)H oxidase expression. To our knowledge, this cardiovascular complication in the cholesterol-fed hamster and the implication of oxidative stress in the process of aortic fat deposition have never been reported. Determination of the activity of the liver antioxidant defense system as a marker of the putative protective role of dietary selenium from spirulina and phycocyanin failed to show any major differences in our experimental conditions. We only observed a significant decrease of the glutathione peroxidase activity in liver homogenates from PC, SePC, SP, and SeSP fed hamsters after 84 days in comparison with the control animals. The activity of the other main enzyme involved in the antioxidant defense mechanism, superoxide dismutase, was reduced according to an identical pattern. According to Breinholt (*39*), a decreased activity of antioxidant enzymes may be a consequence of the sparing effect of dietary antioxidants, reducing the requirement for enzymatic antioxidant function when elevated concentrations of exogenous antioxidants are present in the circulatory system, and the lack of difference in liver antioxidant enzymes activities between PC or SP and their respective selenated forms could imply more powerful preliminary mechanisms of protection allowing to save the intervention of these enzymes. We have shown that the antioxidant phycocyanin prevents the early development of atherosclerosis, characterized by the lipid deposition on the aorta and formation of foam cells. Huang et al. (*15*) have shown in vitro that, compared with phycocyanin without bound Se, the superoxide and hydrogen peroxide radical-scavenging activities of SePC were significantly higher and proved to be positively correlated with its Se content. Here, the same decrease of aortic fatty steak area by PC and SePC suggests that the ROS-scavenging effect by phycocyanin is an unlikely mechanism. In fact, it has been established that phycocyanin not only scavenges peroxyl, hydroxyl (*18*), and superoxide radicals (*40*) but also acts as a potent antioxidant and inhibits the lipid peroxidation mediated by reactive oxygen species (*41*). This could mean that the phycobiliprotein phycocyanin prevented the progression of atherosclerosis in aortic cross of cholesterol-fed hamsters throughout the improvement of antioxidant defense mechanisms in plasma lipoproteins. Thus, taken into account the hypercholestrolemic and oxidative hypothesis of atherosclerosis (*1, 7, 17, 35*), this study supports and agrees with previous studies which reported the beneficial in vitro and in vivo effects of phycocyanin on lipid profile and antioxidant status (*15–20, 23–25*). Moreover, our findings suggest that the modulation of NAD(P)H oxidase expression is implicated in SePC antioxidant capacity.

Here, improvement of PAC, decrease in superoxide anion production and reduction of NADPH expression (p22phox subunit) by SePC, and to a lesser extent by PC and spirulina, were observed, and AFSA development was prevented. All of these results suggest that they acted by mechanisms operating both inside and outside a hypolipemic effect, especially an antioxidant effect, although anti-inflammatory effects do not be ruled out.

Taking into account that spirulina are used as nutritional supplements in many countries, it is conceivable that phycocyanin, particularly Se-enriched PC, could be potentially used as a dietary supplement, becoming a helpful potential therapeutic agent in oxidative stress-induced diseases. As spirulina has been found to be a heath-improving agent by experts of the World Health Organization, SePC isolated from this cyanobacterium will have a new application in the health food market.

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