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Stabilization of membrane bound ATPases and lipid peroxidation by carotenoids from *Chlorococcum humicola* in Benzo(a)pyrene induced toxicity

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ABSTRACT

Objective: To identify the alteration of the membrane potential and the effect of carotenoid extracts from *Chlorococcum humicola* (*C. humicola*) on membrane bound ATPases and lipid peroxidation. **Methods:** The total carotenoids were extracted from *C. humicola*. Four groups of Swiss albino mice were treated as control, Benzo(a)pyrene [B(a)P], total carotenoids, B(a)P + total carotenoids respectively for a period of 60 days. Membrane lipid peroxidation and ATPases (Total ATPases, Ca^{2+}- ATPases, Mg^{2+} - ATPases, $Na^{+}K^{+}$ - ATPase) were determined in lung, liver and erythrocyte samples. **Results:** The activity of total ATPase was found to be significantly increased in the B(a)P treated liver and lung tissue. Erythrocyte membrane also showed higher ATPase activity which was significantly reverted on total carotenoid treatment. **Conclusions:** It can be concluded that the changes in membrane potential favour the functional deterioration of physiological system. The overall findings demonstrates that the animals post treated with carotenoid extract from *C. humicola* may maintains the alterations in membrane bound ATPase and lipid peroxidation in tissues against the carcinogenic chemical and hence aid in establishing the membrane potential action. Therefore *C. humicola* can be further extended to exploits its possible application for various health benefits as neutraceuticals and food additives.

1. Introduction

Carotenoids are the substances with very special and remarkable properties that no other groups of substances possess and these form the basis of their many varied functions in all kinds of living cells. Traditionally often thought of as plant pigments, carotenoids have a much wider distribution and occur extensively in animals and microorganisms. The natural functions and actions of carotenoids obviously are determined by the physical and chemical properties arising from their molecular structures. First of all their overall molecular geometry (size, shape, and presence of functional groups) is vital for ensuring their fitness into cellular and sub cellular structures (correct location and orientation) allowing them to function efficiently. Secondly, their conjugated double bond system determines the photochemical and chemical properties underlying their physiological functions. In addition, their specific interactions with other molecules in their immediate vicinity are crucial for their appropriate functioning. Carotenoids are known as biologically important micronutrients with many functions. Of all known carotenoids, about 50 display provitamin A activity. Carotenoids are also precursors of retinoids. More than 600 naturally occurring carotenoids have been identified and β-carotene is one of them[1].

Carotenoid supplementation has been further used for prevention and treatment of diseases with oxidative stress, such as cancer, UV-mediated skin diseases, neurodegenerative diseases, and cystic fibrosis. The majority of epidemiological studies have consistently shown that increased consumption of food rich in β-carotene is associated with a reduced risk of lung and other types of cancer[2]. For the studies of oxidative damage pathophysiology, erythrocyte membranes are often used (as a model system) because of their simplicity and availability. The form of oxygen radicals using oxidative stress may promote the oxidation of polyunsaturated fatty acids that are present in high concentrations within cell membranes. Lipid peroxidation causes polymerisation of the membrane components, their cross linking and/or fragmentation. This damage to the membrane leads to the alterations in the membrane fluidity and cell deformability[3].

In the search for feasible new sources, algae and microalgae have been suggested as possible raw materials

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for carotenoids. Numerous health benefits have been associated with their use. Algae and microalgae are potentially a great source of natural compounds that could be used as ingredients for preparing functional foods. Different compounds with antibacterial, antiviral and antifungal activity can be found in these types of organisms, along with compounds with antioxidant activity[4-6]. Therefore, the main target of the present study is to assess the effect of carotenoids from algae on membrane stability against the Benzo(a)pyrene [B(a)P] induced alterations.

2. Materials and methods

2.1. Algal source

Fresh water, unicellular, nonmotile green algae *Chlorococcum humicola* (*C. humicola*) was obtained from the culture collected from the Department of Plant Biotechnology and Algal Biotechnology, Vivekantha College, Chennai, India.

2.2. Culture conditions

Algal culturing was carried out with 100 mL Bold's basal medium[7] supplemented with sterile compressed air and kept under fluorescent light (20 μmol/m^2/s) with 16 h light period and at (25 ± 2) ℃ temperature.

2.3. Carotenoid extraction

Algal sample (1 g) was extracted with ethanol until all the pigments were removed, and then filtered through a sintered glass filter (porosity 3; pore size 20-30 μm). An equal volume of diethyl ether was added to the combined ethanol extracts, followed by the addition of water droplets until two layers were formed. The ethereal epiphase, containing all the pigments, were washed free from ethanol with water, and the solvent was removed. The residue was then saponified with equal volume of 10% methanolic KOH and kept in overnight in the room temperature at dark, after which the carotenoid solution was washed with water to remove the alkali (pH= 7.0) and dried over Na_2SO_4. The unsaponifiable residue was dissolved in a little ether and then in 10 mL of petroleum ether (b.p. 40-60 ℃). This extraction was used for further analysis[8].

2.4. Total carotenoid estimation

The total caroteoids were estimated spectrophotometrically at 450 nm[9].

2.5. Animals experiments

2.5.1. Animals

Swiss albino mice weighing 20-25 g used for the animal experiments were purchased from Mohamed Sathak AJ College of Pharmacy, Chennai, India. Animals were grouped and housed in polyacrylic cages (six animals per cage) and fed on standard pellet and given water *ad libitum*. Animals were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All experiments were performed in accordance with the guidelines for research with experimental animals, and animal ethical clearance was obtained from the institutional ethical committee (Reg. No.: 991/C/06/CPCSEA).

2.5.2. Assessment of the oral ED_{50} for total carotenoids

Preliminary investigation was carried out to calculate the median effective dose (ED_{50}) for the carotenoids to albino mice. Six groups of mice, each of six individuals (n = 6) were used for each the specified dose. Doses were prepared with different concentration in corn oil. Oral dosing was done by a special syringe that has a needle equipped with a ball tip. Mortality counts of animals were recorded after fifteen days of treatment. The ED_{50} values were calculated according to the statistical method[10].

2.5.3. Animal treatment

Group I (controls) were treated with corn oil given orally by gavages (0.1 mL). Group II were treated with B(a)P dissolved in corn oil (0.1 mL) and given by gavages in sixteen doses (1 mg per dose) twice per week for 8 weeks. Group III was orally administered with total carotenoid (TC) dissolved in corn oil for a period of 60 days. The mice belonging to the Group IV [B(a)P + TC] were treated the same way as mice in the second group. Food intake and body weight were monitored throughout the experimental period. At the end of treatment, animals were killed by cervical dislocation after deep anaesthesia with diethyl ether and the tissue samples of lung and liver were immediately dissected out, trimmed of excess fat and weighed. Blood samples were also collected.

2.6. Tissue preparation

The tissue was homogenized in 10 volumes of ice-cold (0-4 ℃) medium containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), pH 7.4 and 300 mM sucrose, using an ice-chilled glass homogenizing vessel at 900 rpm (4-5 strokes). Then, the homogenate was centrifuged at 1 000 × g for 10 min to remove nuclei and debris[11].

2.7. Preparation of erythrocyte ghost membrane

Hb (Haemoglobin B)- free erythrocyte membrane preparation was prepared according to the method of Arduini *et al*[12]. The washed erythrocytes were subjected to hypotonic lysis in 40 volumes of 5 mM sodium phosphate buffer (pH = 8.0) and centrifuged at 6000 × g for 20 minutes at 4 ℃ in a refrigerated centrifuge. The supernatant was discarded and pellet was washed at least five times in the same buffer until a colorless pellet was obtained. The erythrocyte ghosts were suspended in the same buffer and stored at -20 ℃ for future use.

2.8. Lipid peroxidation

The estimation of lipid peroxidation was done by the spectrophotometric method[13]. 0.1 mL of the tissue homogenate was incubated in the medium containing 150 mM KCl (0.1 mL) at 37 ℃ for one hour and at the end added 1 mL of 20% TCA. After thorough mixing 2 mL of 0.67% TBA was added and placed in the boiling water bath for 15 min, cooled. The absorbance of the clear supernatant was measured against reference blank at 535 nm.

2.9. Determination of ATPases

Adenosine triphosphatase (ATP phosphohydrolase; EC 3.6.1.3) activity was measured as the release of inorganic phosphate from ATP using the procedure of Evans with slight modifications[14]. The assay mixture contained (in 1

mL) Tris/HCl buffer (pH = 8.0)– 100 mM; ATP– 5 mM, $CaCl_2$– 2 mM, $MgCl_2$– 2 mM, NaCl– 60 mM, KCl–20 mM and enzyme protein. The tubes were incubated at 37 ℃. After 30 min, the enzyme was inactivated by adding 1 mL of chilled 10% (w/v) trichloroacetic acid (TCA) and the tubes were kept in ice for 15 min. Precipitated proteins were removed by centrifugation. A control was run simultaneously, in which enzyme was added after TCA at the end of the incubation period. Inorganic phosphate was measured according to the procedure of Fiske and Subbarow[15]. Protein was estimated by the method of Lowry *et al*[16].

2.10. Statistical analysis

All data were analyzed with SPSS 12 student software. Hypothesis testing methods included two way analysis of variance (ANOVA) followed by least significant difference (LSD) test. The values are expressed as Mean ± SEM. *P*–values of less than 0.05 were considered to indicate statistically significant.

3. Results

3.1. Assessment of the oral ED_{50} for total carotenoids

Results showed that, the oral ED_{50} value for total carotenoids was found to be 8 mg/kg body weight.

3.2. Lipid peroxidation

The lipid peroxidation, measured as malondialdehyde (MDA) in tissue and erythrocyte ghost of mice was shown in Figure 1. Tissue and erythrocyte MDA levels were significantly increased ($P<0.001$) following B(a)P administration. These adverse changes were significantly reverted to normal level on carotenoid administration when compared to its vehicle control and to some extent in combination with B(a)P treated mice.

3.3. Total ATPases

A significant increase ($P<0.001$) of total ATPase activity was observed in the lung, liver and erythrocyte samples on B(a)P administration. This increase was significantly reduced by carotenoid treatment depicted in Table 1.

3.4. Ca^{2+}/ Mg^{2+}/ Na^+ K^+ ATPases

Effect of carotenoid on Ca^{2+}/ Mg^{2+}/ Na^+ K^+ ATPases in the tissues and in erythrocytes are presented in Table 1 and it was found to be significantly increased in B(a)P treatment and the activity was decreased on carotenoid treatment alone and to some extent in combination treatment (Table 1).

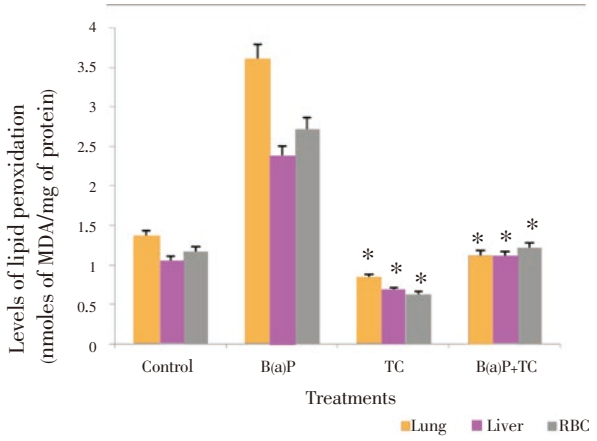

Figure 1. Levels of lipid peroxidation in various membranes on carotenoid treatment alone and in combination with B(a)P.
*$P<0.001$ compared with B(a)P. Values are Mean±SEM of six animals.

Table 1
Levels of membrane bound ATPases of lung, liver and RBC (Mean ± SEM).

Parameters		Treatment			
		Control	B(a)P	TC	B(a)P+ TC
Total ATPases	Lung	0.210 ± 0.030	0.287 ± 0.050**	0.237 ± 0.030	0.255 ± 0.040*
	Liver	0.233 ± 0.010	0.296 ± 0.020***	0.238 ± 0.010	0.255 ± 0.020*
	RBC	0.168 ± 0.010	0.221 ± 0.020***	0.170 ± 0.010	0.181 ± 0.020*
Ca^{2+} ATPases	Lung	0.022 ± 0.020	0.045 ± 0.040**	0.022 ± 0.020	0.032 ± 0.030*
	Liver	0.027 ± 0.020	0.054 ± 0.090**	0.025 ± 0.010	0.034 ± 0.070*
	RBC	0.030 ± 0.020	0.053 ± 0.090**	0.028 ± 0.010	0.039 ± 0.070*
Mg^{2+} TPases	Lung	0.174 ± 0.010	0.192 ± 0.030**	0.173 ± 0.010	0.182 ± 0.020*
	Liver	0.192 ± 0.010	0.215 ± 0.020**	0.188 ± 0.010	0.191 ± 0.010*
	RBC	0.132 ± 0.010	0.166 ± 0.020**	0.132 ± 0.010	0.148 ± 0.010*
Na^+K^+ATPases	Lung	0.017 ± 0.010	0.030 ± 0.020***	0.014 ± 0.010	0.023 ± 0.020*
	Liver	0.021 ± 0.010	0.034 ± 0.030***	0.023 ± 0.020	0.027 ± 0.010**
	RBC	0.015 ± 0.010	0.030 ± 0.030***	0.016 ± 0.020	0.024 ± 0.010**

ATPases were expressed as μg of phosphorus min/ mg protein. As compared with control, statistical significance was observed in B(a)P treated group alone and in combination with carotenoid. No significant difference was found in carotenoid fed group with control. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

4. Discussion

There are clear links between human cancers and diet, dietary risk factors rank higher than tobacco usage and much higher than pollution or occupational hazards in their association with cancer deaths. In addition to avoidance of carcinogenic agents, regular intake of chemopreventive compounds is a promising approach for reducing cancer incidence. A number of substances naturally occurring in foodstuffs, particularly antioxidant compounds in plant products, have shown promise as potential chemopreventive agents[17]. Among these phytonutrients, the yellow, orange and red carotenoid pigments have recently sparked much interest. In epidemiological studies, vegetable and fruit

consumption has consistently been associated with reduced incidence of various cancers, and dietary carotenoid intake from these sources has similarly been correlated with reduced cancer risk[18]. However, several recent large-scale intervention trials failed to find the beneficial effect of long-term supplementation with β-carotene, the most abundant dietary carotenoid. Several naturally occurring carotenoids other than β-carotene have exhibited anticancer activity, and are being considered further as potential chemopreventive agents[19].

B(a)P the polycyclic aromatic hydrocarbon (PAH) is an environmental carcinogen which promotes the lipid peroxidation and Reactive oxygen species (ROS) production, through that it causes the cellular dysfunction[20]. The active metabolites of B(a)P are 3-OH-B(a)P, 6-quinone B(a)P, 9-OH-B(a)P, B(a)P-1, 6-quinone, trace amounts of B(a)P-4, 5-dihydrodiol, and B(a)P-7, 8-dihydrodiol are primarily produced in liver and then bio transformed into various organs and causes cellular toxicity[21,22].

In the present investigation it was proved that, an increase in lipid peroxidation indicates serious damage to cell membranes, inhibition of several enzymes and cellular dysfunction. A number of ROS are generated during normal aerobic metabolism, such as superoxide, hydrogen peroxide and the hydroxyl radical. In addition, singlet oxygen can be generated through photochemical events, and lipid peroxidation can lead to peroxyl radical formation[23,24]. These oxidants collectively contribute to aging and degenerative diseases such as cancer through oxidation of DNA, proteins and lipids[25].

It was well documented that during cancer development the ROS were generated and the increased levels of ROS can cause increased lipid peroxidation markers. In the present study, the elevated levels of MDA in tissues and erythrocyte membrane are consistent with earlier reports[26]. Treatment with carotenoids reduced the levels of MDA to near normal, which could be associated with its protective role. ROS contribute towards increased transendothelial and transepithelial permeability. The increase of transepithelial permeability allows toxins to permeate through the barrier, which leads to inflammation. ROS produced by the B(a)P induction may possibly cause the above events, leading to the B(a)P induced tissue damage. Carotenoids significantly reduced the membrane lipid peroxides and ROS production in toxicity induced animals. Antioxidant compounds can decrease those effects, and thus carcinogenesis, both by decreasing oxidative damage to DNA and by decreasing oxidant stimulated cell division[27].

The correlation between lipid peroxidation, fluidity and membrane function has been documented in several studies. Free radical induced change in membrane structure may have very specific effects on membrane proteins as documented by selective modification of different active sites in tissues. Although the plasma membrane is thought to be a critical site of free radical reaction, membranes of intracellular organelles could also be an important site of free radical attack. Recently, it is showed that free oxygen radicals alter net Ca^{2+} uptake in rabbit brain endoplasmic reticulum. Because the decrease in Ca^{2+} uptake preceded inhibition of Ca^{2+}-ATPase activity and it can be concluded that membrane lipid peroxidation occurs as an early consequence of free radical attack and leads to an increase in passive Ca^{2+} leak. In the present study the carotenoids maintains the membrane Ca^{2+} ATPaes against the potent carcinogenic enzyme inhibitor[28].

Mg^{2+}-ATPase is widely believed to be responsible for the control of membrane permeability. Since action of carcinogen in the erythrocytes involves distortion of the membrane, there is the tendency for impaired permeability to be increased and in an attempt by carotenoids to regulate the permeability. It is suggested that affects ion fluxes across the membranes in the brain, liver and kidney tissues which may lead to disruption of the cation balance with the attendant consequences in the affected organs. Moreover, the role of Mg^{2+}-ATPase is to maintain intracellular Mg^{2+}, changes of which can control rates of protein synthesis and cell growth[29].

The alteration in the activities of the liver Ca^{2+}, Mg^{2+} ATPase kidney cells that are reported along with Na^+ K^+-ATPases suggests an altered biosynthesis during infection. In the Na^+K^+-ATPase, the Na^+ and K^+ ions across cell membranes act as a link with metabolism during an epithelial transport of sugars and amino acids. Furthermore it serves to restore resting membrane potential in excitable tissues. The changes in the activity of the Na^+K^+-ATPase would also result in a disturbance of the transport across the membrane. This would probably produce the favourable condition for the existence of the carcinogen within the cell[30].

Hepatic Na^+/K^+-ATPase is known to be responsible for the Na^+ gradient and consequently for the bile acids/Na^+ secondary active transport across the plasma membrane[29]. Increased activity of Na^+/K^+-ATPase has been implicated in the development of complications and adaptive changes. However, in this study with mice, higher Na^+K^+-ATPase activity in the erythrocyte membrane of carcinogen treated mice was observed compared with the control. Carcinogenic effect results depolarization of the membrane at a higher rate than before, efflux of Na^+ ion will be less in the cancerous condition relative to the normal mice[31].

Antioxidants (*e.g.* β-carotene) can counteract the changes in ATPase activity and the increase in oxidative stress that are induced by carcinogenic chemicals. Many epidemiologic studies have associated high carotenoid intake with a decrease in the incidence of chronic disease. Multiple possibilities exist - certain carotenoids, can be converted to retinoids, can modulate the enzymatic activities of lipoxygenases, can have antioxidant properties which are seen with vitamin A, can activate the expression of genes which encode the message for production of a protein, which is an integral component of the gap junctions required for cell to cell communication. Such gene activation is not associated with antioxidant capacity and is independent of pro-vitamin A activity[32]. Carotenoids also serve as precursors for retinoids. Some carotenoids also appear to have effects on cell communication and proliferation in animals. Because animals cannot synthesize carotenoids *de novo*, they must obtain them from dietary sources[33].

The present study concludes that the free radical effects on fluidity of membranes are consistent with those reported for various kinds of membranes from different tissues and erythrocytes. Alteration in membrane fluidity as a result of lipid peroxidation can affect membrane properties since permeability and function of membrane-bound proteins are known to be intimately associated with the dynamic state of the membrane lipids. Carotenoids significantly reduced the membrane lipid peroxides, ROS production and improved the blood and cellular membrane potential by acting against the toxicity. Carotenoids, the most abundant dietary source from *C. humicola* can be considered further as potential bioactive compound.

Conflict of interest statement

We declare that we have no conflict of interest.

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