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ORIGINAL CONTRIBUTION

Astaxanthin addition improves human neutrophils function: in vitro study

Rita C. Macedo · Anaysa P. Bolin · Douglas P. Marin · Rosemari Otton

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Abstract
Purpose The aim of the present study was to evaluate the in vitro effect of carotenoid astaxanthin (ASTA) on the phagocytic and microbicidal capacities, cytokine release, and reactive oxygen species production in human neutrophils.
Methods The following parameters were evaluated: cytotoxic effect of ASTA on human neutrophils viability, phagocytic and microbicidal capacities of neutrophils by using *Candida albicans* assay, intracellular calcium mobilization (Fura 2-AM fluorescent probe), superoxide anion (lucigenin and DHE probes), hydrogen peroxide (H_2O_2, phenol red), and nitric oxide (NO·) (Griess reagent) production, activities of antioxidant enzymes (total/Mn-SOD, CAT, GPx, and GR), oxidative damages in biomolecules (TBARS assay and carbonyl groups), and cytokine (IL-6 and TNF-alpha) release.
Results Astaxanthin significantly improves neutrophil phagocytic and microbicidal capacity, and increases the intracellular calcium concentration and NO· production. Both functional parameters were accompanied by a decrease in superoxide anion and hydrogen peroxide and IL-6 and TNF-α production. Oxidative damages in lipids and proteins were significantly decreased after ASTA-treatment.
Conclusions Taken together our results are supportive to a beneficial effect of astaxanthin-treatment on human neutrophils function as demonstrated by increased phagocytic and fungicide capacity as well as by the reduced superoxide anion and hydrogen peroxide production, however, without affecting neutrophils capacity to kill *C. albicans*. This process appears to be mediated by calcium released from intracellular storages as well as nitric oxide production.

Keywords Antioxidant · Astaxanthin · Carotenoids · Leukocytes · Neutrophil · Oxidative stress

R. C. Macedo · A. P. Bolin · D. P. Marin · R. Otton (✉)
Postgraduate Program, Health Science, CBS, Cruzeiro do Sul University, Avenida Regente Feijó, 1295. Tatuapé, São Paulo, SP CEP 03342-000, Brazil
e-mail: rosemari.otton@cruzeirodosul.edu.br

R. Otton
Postgraduate Program, Human Movement Sciences Institute of Physical Activity and Sport Sciences, ICAFE, Cruzeiro do Sul University, São Paulo, SP 01506-000, Brazil

Introduction

Carotenoids are phytochemicals considered beneficial in the prevention of a variety of major diseases [13, 29]. Evidence has suggested that the action of carotenoids on immunity and diseases may be mediated, at least in part, by their ability to quench and/or blench reactive oxygen species (ROS). Astaxanthin (ASTA), 3,3′-dihydroxy-β,β-carotene-4,4′-dione, a carotenoid without vitamin A activity [13, 24], has potential clinical applications due to its higher antioxidant activity than β-carotene and α-tocopherol [13, 26, 43]. In addition, it has many highly potent pharmacological effects, including antioxidant, anti-tumor, anti-cancer, anti-diabetic, and anti-inflammation activities [7, 21, 26, 37]. The potent antioxidant activity of ASTA has been observed to modulate biologic functions ranging from lipid peroxidation to tissue protection against light damage [8, 37]. The presence of the hydroxyl (OH) and keto (C=O) moieties on each ionone ring explains some of its unique features, namely, a higher antioxidant activity, which allows the molecule to protect both the inner and outer membrane surface [2, 13, 28, 37].

Astaxanthin has evident actions in the lymphocyte function as previously shown both by in vivo and by in vitro studies. Jyonouchi et al. [22–25] are systemically investigating the effects of ASTA on lymphocyte proliferation, interleukins and antibody production, and surface immunoglobulin expression. However, there is a lack of information in the literature concerning the effects of ASTA on human neutrophils function.

Polymorphonuclear phagocytes (PMNs), referred to as neutrophils, are short-lived (half-life of $\sim$12 h in circulation), phagocytic leukocytes that circulate the blood in large numbers. Neutrophils have been traditionally regarded as an important first line of defence against immunogenic material [44]. Their main function is in antibacterial or antifungal immune responses, where they comprise a major component of host defence [44]. Neutrophils phagocytosis and production of toxic substances work together to effectively eradicate infectious pathogens. An important mechanism to killing bacteria occurs via the production of oxygen-derived free radicals driven by a 'respiratory burst': a coordinated series of metabolic events that take place when phagocytes, such as neutrophils are exposed to appropriate stimulants. While a robust neutrophilic response is adaptive in the setting of infection it poses potential harm to the host, since overly robust production of neutrophil-derived substances can have pathological consequences. Release of these powerful substances can also lead to destruction of normal cells and dissolution of connective tissue at the site of inflammation. In addition to their involvement in acute inflammation, periodic neutrophilic infiltration is also typical of certain chronic inflammatory conditions, such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, and asthma, and may contribute to the progression of these diseases.

Several laboratories have shown that antioxidants preserve an adequate function of immune cells against homeostatic disturbances caused by oxidative stress. Since the immune system is an indicator of health and a longevity predictor, the protection of this system afforded by dietary antioxidant supplementation may play an important role in order to achieve health. The aim of the present study was to evaluate the in vitro effect of carotenoid astaxanthin on the phagocytic and microbicidal capacities, cytokine release, and ROS production in human neutrophils.

Materials and methods

Reagents

Astaxanthin (ASTA) and most of the other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), excepting the RPMI-1640 culture medium, lucigenin, pluronic acid, and acetoxymethylester (Fura-2AM) which were from Invitrogen (CA, USA). Common reagents for buffers (e.g. PBS) and regular laboratory solutions were obtained from Labsynth (Diadema, SP, Brazil).

Subjects

The Ethical Committee of the Cruzeiro do Sul University approved the experimental procedure of this study. Around 30 healthy adult women and men (mean age 27.0 $\pm$ 9.0) were included in the present study. The subjects of the present study did not present any systemic or topical therapeutic regimen at least for the last 2 months. Subjects with a smoking history, alcohol habits, obesity, or any other systemic disease were excluded from the study (based on an anamnesis protocol).

Cell isolation and culture condition

Neutrophils were obtained through the collection of human peripheral blood by venipuncture procedure in vacuum/siliconized tubes containing 0.1 mM EDTA. Peripheral blood neutrophils were isolated under sterile conditions by using a density gradient present in the reagent Histopaque 1077 (Sigma-Aldrich) according to the manufacturer's instruction. Briefly, blood ($\sim$20 mL) was diluted (1:2) with phosphate-buffered saline PBS (133.8 mM NaCl, 2.7 mM KCl, 0.9 mM KH_2PO_4, 6.4 mM $Na_2H_2PO_2$, pH 7.4) and centrifuged for 30 min, 600*g* at room temperature. The plasma and intermediary layer were removed and both neutrophils and erythrocytes were collected from sediment. Erythrocytes were lysed with a hemolysis solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4) and subsequently centrifuged for 10 min (600*g* at 4 °C). This procedure was repeated twice for total red blood cell lyses. Thereafter, neutrophils were washed with PBS followed by centrifugation for 10 min (600*g* at 4 °C). After centrifugation, the neutrophils were counted in a Neubauer chamber using Trypan blue (1%). Neutrophils (1×10^6/mL) were cultured in 5 mL of RPMI-1640 medium supplemented with 10% foetal calf serum, 20 mM Hepes, 2 mM glutamine, and antibiotics (streptomycin 100 units/ml and penicillin 200 units/ml). The cells were treated with 5 μM of ASTA and cultured at 95% O_2 for 24 h at 37 °C. After this period, the cells were collected, centrifuged, and stored at −80 °C to assays of enzyme activities and oxidative damage in biomolecules.

Effect of ASTA and DMSO on cell viability

The toxicity assay of both ASTA and DMSO (vehicle for the lipophilic antioxidant) in human neutrophils was

performed using cell density of 1×10^6 cell/mL exposed to different ASTA concentrations (0, 0.025, 0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 30, and 40 μM) and DMSO percentages (0, 0.5, 1, 2, 3, 4, 5, 8, and 10%) for 24 h in the culture medium. Immediately after being isolated at the end of the incubation period, 5×10^5 cells were used to test their membrane integrity. This assay was carried out in a FACScalibur flow cytometer (Becton–Dickinson, Mountain View, CA) using propidium iodide (PI) (50 μg/mL) dissolved in PBS pH 7.4. PI is a highly water-soluble fluorescent compound that cannot pass through intact membranes and is generally excluded from viable cells. When cells lose membrane integrity it passes through the membrane and binds to DNA. Therefore, an increase in fluorescence to propidium iodide indicates a decrease in the proportion of viable cells. Fluorescence of PI was determined in FL2 channel (orange-red fluorescence 585/542 nm).

Intracellular Ca^{2+} concentration

Changes in cytosolic Ca^{2+} levels were monitored by fluorescence using the calcium-sensitive probe Fura 2-AM as previously described [36]. The loading period for 5 μM Fura 2-AM was 1 h at 37 °C in 1×10^6 cells/well in Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.49 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.6 mM D-glucose, and 5 mM acid HEPES), pH 7.4. Then, cells were washed and intracellular $[Ca^{2+}]_i$ was monitored for 15 min and fluorescence emission at 510 nm (excitation wavelengths alternating between 340 and 380 nm) of Fura 2-AM was measured in a microplate reader (Tecan, Salzburg, Austria). After a short resting period of monitoring (4 min), cells were stimulated with fMLP (formyl-Methionyl-Leucyl-Phenylalanine 1 μM) or opsonized zymosan particles (5×10^6 particles/well) to induce intracellular calcium release. Transformation of the fluorescent signal to $[Ca^{2+}]_i$ was performed by calibration with ionomycin (100 μM, maximum concentration) followed by EGTA addition (60 μM, minimum concentration) according to the Grynkiewicz equation, using the K_{diss} of 224 nM [18]. Negative controls were performed in cell-free systems with Fura-2 AM + fMLP or Fura-2 AM + ASTA; and Fura-2 AM + ASTA + fMLP (data not shown).

Phagocytic activity

Neutrophils (2×10^6 cell/mL) were incubated for 120 min at 37 °C in 1 mL RPMI 1640 medium with *Candida albicans* and 5 μM of ASTA. The particles (2×10^6 cell/mL) were opsonized by incubation in the presence of control serum for 60 min at 37 °C. The cells were incubated with particles and counted after cytocentrifugation. The score of phagocytosis was expressed by the number of cells that had one, two, three, four, or more *Candida* cells phagocyted [42].

Microbicidal activity

Yeast cells of *C. albicans* (kindly offered by Dr Sandra Sampaio from Butantan Institute-SP, Brazil) were cultured on Sabouraud agar medium and transferred onto fresh agar 24 h prior to the killing assay. *Candida* cells were harvested with saline phosphate buffer (PBS) and counted in a Neubauer chamber. Under these conditions, a suitable number of *Candida* cells can be obtained without germ tubes and hyphae. Their viability was checked by the Trypan blue exclusion technique. *Candida* cells were opsonized with 2.5% normal human serum for 60 min prior to each assay and 5 μM of ASTA-treated neutrophils were incubated for 2 h at 37 °C. After the incubation period, the homogenate was centrifuged and stained with Rosenfeld stain. The score of microbicidal capacity was expressed by the number of cells that had one, two, three, four, or more *Candida* cells killed [42].

Measurement of intra- and extracellular superoxide anion production

Dihydroethidium assay

Dihydroethidium (DHE) is a florescence probe and was used to measure the intracellular $O_2^{\cdot-}$ production. Once inside the cell, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by $O_2^{\cdot-}$ and/or H_2O_2 (in the presence of peroxidase). Neutrophils (5×10^5/well) were incubated with 5 μM DHE for 15 min at room temperature in the dark. At the beginning of the assay, cells were stimulated with PMA (20 ng/well) and ASTA (5 μM) in Tyrode's buffer and the fluorescence was analyzed in a microplate reader (Tecan, Salzburg, Austria) (396 nm wavelength excitation and 590 nm wavelength emission).

Lucigenin assay

The method utilized to measure the extracellular superoxide anion ($O_2^{\cdot-}$) content mainly produced through NADPH-oxidase activation was lucigenin. After being excited by superoxide anion, lucigenin releases energy in the form of light and the chemiluminescence produced can be monitored by a luminometer. Lucigenin (5 μM) was added to cells incubated (2.5×10^6/mL) in the absence and presence of ASTA (5 μM) in Tyrode's buffer. The experiments were carried out in the presence and absence of PMA (20 ng/well). The chemiluminescence response was monitored for 60 min in a luminometer (Tecan, Salzburg,

Austria). The results are expressed as chemiluminescence relative units.

Nitric oxide production

Nitric oxide production was performed according to Ding et al. [11] through nitrite determination. Nitric oxide is rapidly converted to nitrite in aqueous solutions and, therefore, the total nitrite can be used as an indicator of nitric oxide concentration. The spectrophotometric analysis of the total nitrite content was performed by using Griess reagent (1% sulfanilic acid, 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride) in supernatants. Neutrophils (5×10^5/well) were cultured with astaxanthin (5 μM) and LPS (10 μg/well) for 4 h. Then, 100 μL of Griess was added and the absorbance was measured in 550 nm. Nitrite concentration was determined using sodium nitrite as a standard.

Hydrogen peroxide production

Hydrogen peroxide (H_2O_2) production was measured according to Pick and Mizel [39], based on horseradish peroxidases, which catalyze the phenol red oxidation by H_2O_2. Neutrophils (5×10^5/well) were incubated in Dulbelco's PBS (136.9 mM NaCl, 2.68 mM KCl, 0.9 mM $CaCl_2$, 0.49 mM, $MgCl_2{\cdot}6H_2O$ 7.58 mM NaH_2PO_4, 1.47 mM KH_2PO_4, 5.55 mM D-Glucose pH 7.4), mixed with 0.28 mM phenol red and horseradish peroxidase (1,000 units/mg) at 37 °C for 1 h. The production of H_2O_2 was measured in the absence and presence of phorbol myristate acetate (PMA; 20 ng/well) a promoter of respiratory burst in leukocytes. The reaction was terminated by alkalinization (addition of 10 μL of NaOH 1 M solution) and absorbance at 620 nm was measured to evaluate H_2O_2 concentration (compared to a standard curve).

Preparation of homogenates for measurement of antioxidant enzymes and oxidative lesions

After the culture period, neutrophils (5×10^6/mL) were harvested and the cells were ruptured by ultrasonication in a Vibra Cell apparatus (Connecticut, USA) using 500 μL of the assay-specific extraction solution/buffer, then centrifuged for 10 min, 10,000*g* at 4 °C. The supernatant was used for further analysis.

Assay of superoxide dismutase activity (SOD)

The activity of superoxide dismutase (SOD) was measured according to Ewing and Janero [12], with adjustments. The complete reaction buffer for total SOD included 50 mM sodium phosphate buffer, pH 7.4, 0.1 mM EDTA, 50 μM nitrobluetetrazolium (NBT), 78 μM NADH, and 3.3 μM phenazine methosulphate (PMS) used as an $O_2^{\cdot-}$ generator. Analysis of Mn/SOD activity was performed similarly as for total/SOD but under strong inhibition (~88–100%) of the cytosolic CuZn/SOD isoenzyme with 3 μM KCN. The kinetic absorbance variation at 560 nm was continuously monitored for over 2 min in order to evaluate $O_2^{\cdot-}$-dependent reduction of NBT. Control system lacking PMS evidenced negligible change in absorbance at 560 nm.

Assay of catalase activity

The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm ($\varepsilon_{240} = 0.0394 \pm 0.0002$ L mM^{-1} cm^{-1}). One catalase unit is defined as the enzyme concentration required for the decomposition of 1 μmol of H_2O_2 per min at 25 °C. All assay solutions were prepared at room temperature as described by Aebi [1]. The complete reaction system for catalase consisted of 0.1 mM phosphate buffer, pH 7.4, and 10 mM H_2O_2. The reaction started by addition of 10 mM H_2O_2, and the absorbance was monitored over 2 min at 240 nm.

Assay of glutathione peroxidase activity (GPx)

The activity of GPx was measured according to the method of Mannervik [30]. The enzyme activity was determined by using 2.5 U/mL glutathione reductase (GR), 10 mM reduced glutathione (GSH), 250 μM sodium azide (as a catalase inhibitor), and 1.2 mM NADPH in the presence of 4.8 mM *tert*-butyl hydroperoxide used as the substrate in a cell homogenate. The oxidation of NADPH was monitored at 340 nm for 2 min in 0.2 M phosphate buffer (pH 7.4) in a Ultrospec 3000 spectrophotometer (Pharmacia Biotech).

Assay of glutathione reductase activity

The activity of glutathione reductase (GR) was measured using the same methodology described by Mannervik [30]. Alternatively, GR activity was determined by using 3.6 mM NADPH and 10 mM oxidized glutathione (GSSG). Again, the NADPH oxidation was monitored in 0.2 M phosphate buffer, pH 7.4, at 340 nm (for 2 min).

Parameters of oxidative lesions

The extension of lipid peroxidation in neutrophils was performed by measuring the concentration of thiobarbituric acid-reactive substances in cell homogenates as described previously (TBARS assay) [14]. Butylated hydroxytoluene was added to stop progressing oxidation reactions in 200 μL samples. To detect the colored adducts, 500 μL of each

sample was incubated at 100 °C for 15 min with 280 μL of 0.1 M phosphate buffer (pH 7.4), plus 500 μL of 0.25% thiobarbituric acid in 0.25 M HCl and 1% Triton X-100. After reaching room temperature, absorbance of the solutions was measured at 535 nm (blanks lack thiobarbituric acid) using malondialdehyde (MDA) as a standard [31].

Thiol and carbonyl groups were evaluated as parameters of amino acid oxidation in total protein fractions, which were isolated from crude homogenate by precipitation with 20% trichloroacetic acid solution in ice. After washing once with 0.3 M $HClO_4$, 5 mM EDTA and 0.06% 2.2′-bipyridine solution, and twice with the mixture 1:1 ethyl acetate:ethanol (v/v), the protein precipitate for thiol assay was dried at room temperature to remove traces of any organic solvent. The pellet was subsequently dissolved in 500 μL of 6 M guanidine. HCl and reduced thiol groups were detected by the formation of colored adducts upon reaction with 4 mM 5.5′-dithiobis (2-nitrobenzoic acid) solution (DTNB). A control treatment with 10 mM *N*-ethylmaleimide solution—a specific thiol-blocking compound—was introduced to discount non-specific DTNB bonds formed with other organic groups present in the samples. The absorbance of DTNB-treated samples at 412 nm was calculated using GSH as a standard [5].

The same procedure was employed in protein isolation for carbonyl analysis, although pre-treatment with 10% streptomycin was included to remove nucleic acids from homogenates. The protein carbonyl groups were identified by the hydrazones formed with 10 mM dinitrophenylhydrazine (DNPH) in 0.25 M HCl, whereas the controls lacked DNPH. Absorbance at 380 nm was measured, and the carbonyl group concentration was estimated based on $\varepsilon = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$ [33].

ELISA assay

Neutrophils (1×10^6 cells/ml) suspended in RPMI 1640 and supplemented with 0.3 g/l glutamine, 2.32 g/l Hepes, 2 g/l sodium bicarbonate, 100 μg/ml streptomycin, 100 UI/ml penicillin, and 10% low endotoxin fetal serum were cultured at 37 °C and 5% CO_2, with and without ASTA 5 μM and LPS (10 μg). After 18 h of culture, the supernatants were collected and frozen at −80 °C until the IL-6 and TNF-α were determined by ELISA (Quantikine, R&D System, Minneapolis, MN, USA) following manufactures' instructions.

Protein determination

The total protein content of lymphocytes was measured by the method of [6], using BSA as standard.

Statistical analysis

The results are expressed as mean ± SEM, and (n) is the number of experiments (at least 3). ANOVA was employed to detect differences among groups followed by the Tukey's post-test. $^*p < 0.05$.

Results

Cytotoxic effect of ASTA and DMSO on human neutrophils

Our study was started by performing an experiment to evaluate the cytotoxicity of ASTA and DMSO on viability of human neutrophils. At this point, it was important to find an ASTA and DMSO concentration that were not toxic for human cells. For this purpose, human neutrophils (1×10^6/mL) were treated for 24 h with increasing concentrations of ASTA or percentages of DMSO in the culture medium. The viability scores from flow cytometry analysis are presented in Fig. 1. DMSO showed a significant cytotoxic effect in neutrophils cultivated during 24 h at concentrations over 2% in the culture medium (data not shown). Based on these results, all experiments were carried out using up to 1% of DMSO. The cytotoxicity of ASTA was demonstrated by the reduction in the viability of neutrophils due to the increased concentration of that carotenoid in the culture medium. Neutrophils viability was decreased by 15, 70, 88, and 92% with ASTA at 10, 20, 30, and 40 μM, respectively. Based on these results, all experiments of the present study were carried out with 5 μM of ASTA.

Intracellular calcium concentration $[Ca^{2+}]_i$

Alterations on intracellular calcium concentration due to acute treatment of cells with 5 μM of ASTA were

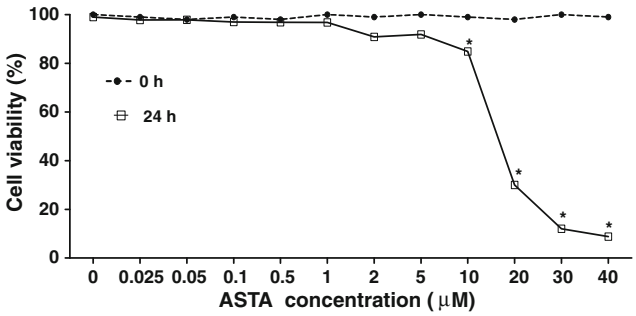

Fig. 1 Cytotoxicity of ASTA in neutrophils cultured for 24 h. The cells (1×10^6/mL) were cultured in different ASTA (0, 0.025, 0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 30, and 40 μM) concentrations. The results are presented as mean ± SEM ($n = 5$). $^*p < 0.05$ as compared to control group (0 μM ASTA)

performed by using Fura 2-AM fluorescent probe. The kinetic of intracellular calcium release was monitored during 15 min and has shown that ASTA-treatment increased by 48% the intracellular calcium concentration as compared with the control group (area under curve (AUC) control 3,841 ± 95 vs. ASTA 5,701 ± 162, analysis; $p < 0.05$). Similar increase (~30%) was observed when cells were treated with ASTA, and fMLP (a calcium agonist), and particles of opsonized zymosan as compared with stimulated-control group (Fig. 2a).

Phagocytic and microbicidal capacities

Considerable increase in both phagocytic (30%) and fungicide (28%) capacities were observed when neutrophils were treated with 5 μM of ASTA (Fig. 2b, c), in comparison to the control group.

Intra- and extracellular superoxide anion production

Regarding DHE and lucigenin protocols, appropriate controls were carried out to exclude possible quenching effects of ASTA in those fluorescence/chemiluminescence assays. In cell-depleted systems, ASTA did not directly affect the ROS-detected fluorescences (data not shown). However, 5 μM of ASTA caused a drastic reduction by 100% on extracellular superoxide anion production as showed by lucigenin assay (Fig. 3a). A significant reduction on intracellular superoxide anion production was observed after ASTA-treatment; however, this reduction was slight (25%) as compared with the control group (Fig. 3b).

Hydrogen peroxide and nitric oxide production

To evaluate the production of hydrogen peroxide in human neutrophils, cells were treated with ASTA (5 μM) in presence and absence of PMA (20 ng/well) for 1 h. ASTA significantly reduces by 20% the hydrogen peroxide production, when compared to the control PMA-stimulated group (Fig. 4a). The LPS-triggered NO· formation showed a significant increase by 109% in ASTA-treated group as compared with the control group (Fig. 4b).

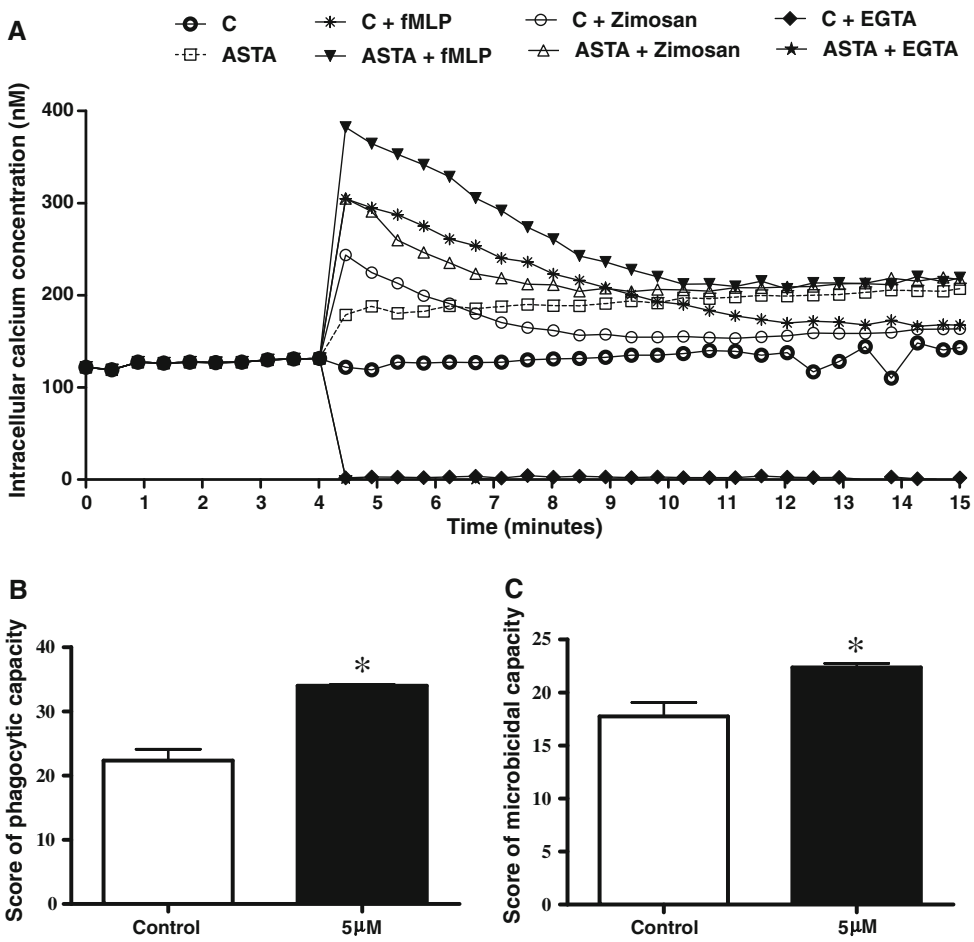

Fig. 2 **a** Changes of intracellular calcium content $[Ca^{2+}]_i$ (nM) in human neutrophils induced by ASTA-treatment. Cells (1×10^6/well) were previously loaded with Fura 2-AM (5 μM) during 1 h and then incubated with the ASTA at 5 μM for 15 min. After a resting calcium release, cells were treated with fMLP (1 μM) or zymosan particles (5×10^6 particles/well). EGTA (100 μM) was used as a calcium chelator. The results are presented as mean ± SEM. Area under curve (AUC) analysis. **b** Scores of phagocytic capacity and **c** microbicidal activity of human neutrophils. Both assays were carried out using *Candida albicans* and neutrophils were treated with 5 μM of ASTA for 2 h. Results are presented as mean ± SEM of 3 experiments carried out in triplicate. *$p < 0.001$ as compared with the control group. *C* control group

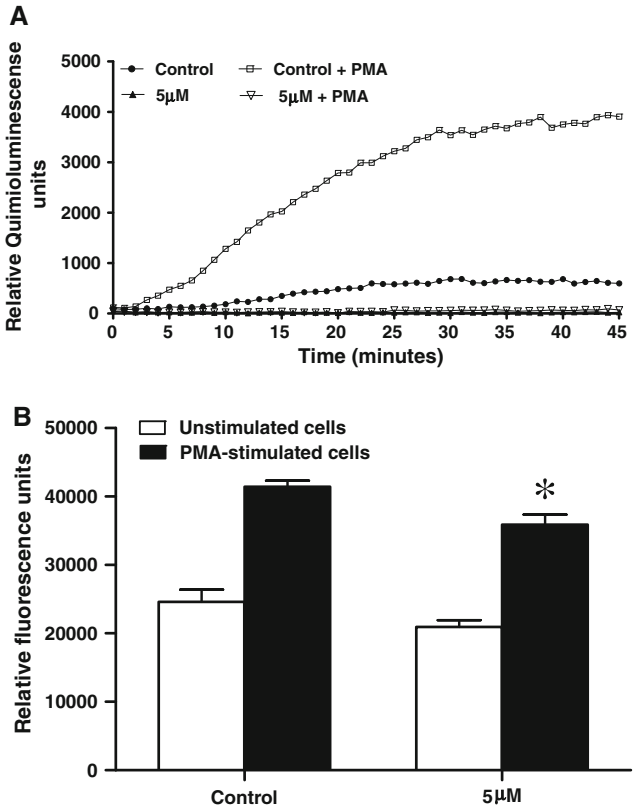

Fig. 3 Superoxide anion production in human neutrophils (5×10^5/well) freshly treated with ASTA (5 μM) and PMA (20 ng/well). **a** Extracellular superoxide anion production was measured using lucigenin (5 μM) assay. **b** Dihydroethidium (DHE 5 μM) was used to measure intracellular superoxide anion production. The values are presented as mean ± SEM of 10 determinations. $*p < 0.05$ compared to control-stimulated group

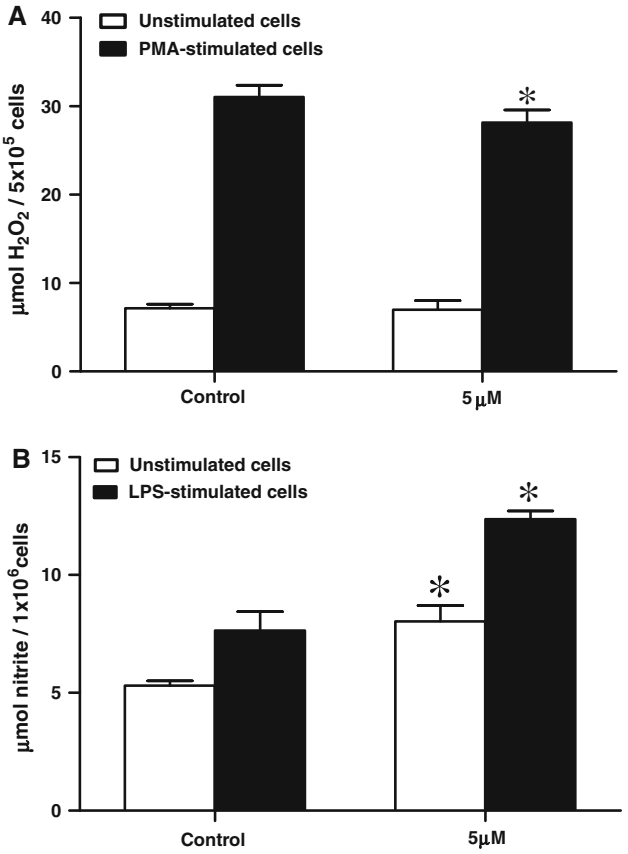

Fig. 4 Measurement of hydrogen peroxide (**a**) and nitric oxide (**b**) production in human neutrophils treated with 5 μM of ASTA. Cells (5×10^5/well) were stimulated with PMA (20 ng/well **a**) or LPS (10 μg/well **b**) and then were evaluated by using the phenol red or Griess reagent, respectively. The results are presented as mean ± SEM ($n = 12$) in at least four experiments $*p < 0.05$ compared as control-stimulated group

Oxidative damage in biomolecules

In vitro ASTA-treatment promoted a significant decrease in biomolecule damages by 35% in MDA content as demonstrated by T-BARS assay and carbonyls groups (60%) as compared with the control group (Fig. 5a, b).

Antioxidant enzyme activities

Total SOD, MnSOD, and catalase antioxidant enzyme activities evaluated in the present study were not changed in neutrophils after treatment of cells with 5 μM of ASTA. However, GPx was increased by 3.9-fold and GR was decreased by 40% as compared to the control group (Table 1).

Cytokine release

LPS-stimulated neutrophils significantly altered the pro-inflammatory cytokines production. After 18 h of culture with ASTA and LPS, the release of IL-6 and TNF-α was significantly reduced by 45 and 60%, respectively in comparison to the control LPS-stimulated group (Fig. 5c, d).

Discussion

PMNs are multifunctional cells that play a role in both innate immune responses as well as adaptive immune responses. Although circulating PMN are short-lived, once they infiltrate into tissues in response to injury or foreign invaders, their life span is prolonged, and they can be activated to perform multiple functions associated with innate and adaptive immune responses [15]. In this condition, neutrophils utilize an extraordinary array of microbicidal mechanisms to destroy and remove infectious agents, and these mechanisms can be generally classified as being oxygen-dependent and oxygen-independent. Among the oxygen-dependent mechanisms, NADPH-oxidase

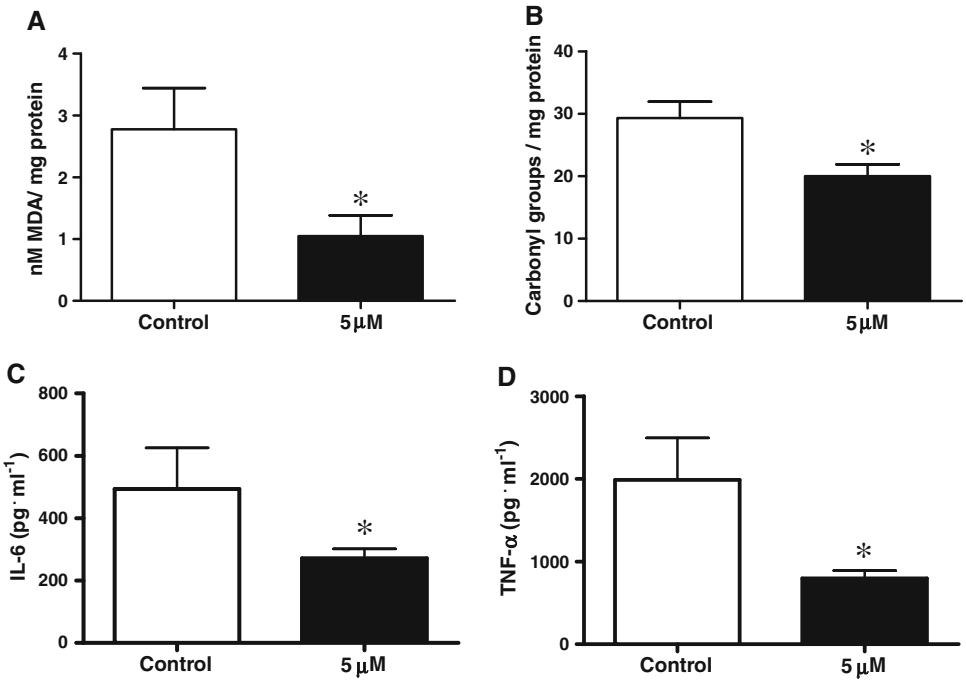

Fig. 5 Oxidative damages in biomolecules were performed to evaluate the presence of lipid peroxidation (**a**), and carbonyl groups (**b**) in human neutrophils. Cells (1×10^6/ml) were cultured with 5 µM of ASTA for 24 h. Lipopolysaccharide (LPS)-stimulated release of interleukin (IL)-6 (**c**) and tumor necrosis factor (TNF)-α (**d**) from neutrophils (1×10^6 cells/ml). Cytokines were measured in 18-h culture supernatants with 5 µM of ASTA by enzyme-linked immunosorbent assay (ELISA). The results are expressed as mean ± SEM of 11 evaluations. *$p < 0.05$ compared to control group

Table 1 Activities of the antioxidant enzymes

	Control	ASTA (5 µM)
Total/SOD (U/mg protein)	14.05 (0.71)	12.76 (0.46)
Mn/SOD (U/mg protein)	10.46 (0.52)	13.16 (0.75)
Catalase (µmol/min/mg protein)	2.3 (0.26)	2.38 (0.40)
GPx (mU/mg protein)	132.5 (10,27)	519.7 (66.16)*
GR (mU/mg protein)	87.93 (14.07)	52.39 (7.43)*

Total superoxide dismutase (total/SOD; U/mg protein), Mn-dependent superoxide dismutase (Mn/SOD; U/mg protein), catalase (CAT; µmol H_2O_2 min^{-1} L^{-1} mg $protein^{-1}$), glutathione peroxidase (GPX; mU/mg protein), and glutathione reductase (GR; mU/mg protein) in human neutrophils treated with 5 µM ASTA for 24 h

Results are expressed as mean (SEM) of 5 experiments and 11 evaluations

* $p < 0.05$ compared to control group

activation plays a major and essential role. NADPH-oxidase activation results in the generation of superoxide anion, however, subsequent biochemical events can convert this radical into much more potent microbicidal oxidant species.

In this study, we have demonstrated that astaxanthin, a natural carotenoid present in aquatic animals, significantly improves neutrophil phagocytic and microbicidal capacity. Both functional parameters were accompanied by a decrease in superoxide anion and hydrogen peroxide production. This is a conflicting result at first, since ASTA while promoting a significant improvement in phagocytic and microbicide capacity, promotes a significant reduction in the production of microbicide molecules responsible for exterminate foreign microorganisms. However, this result confirms our initial idea that ASTA can, in fact, reduce the deleterious effects caused by ROS in lipids and proteins of neutrophils and in tissues underlying lesion by quenching the exacerbated production of oxidant species (due to the potent antioxidant properties of ASTA) without causing impairment of the neutrophil ability to phagocytose and kill microorganisms.

Lipid peroxidation is probably the most extensively investigated process induced by free radicals. The abundant presence of membrane phospholipids at sites, where radicals in general and, more specifically, ROS are formed to render them easily accessible endogenous targets were rapidly affected by free radicals. Especially the group of polyunsaturated fatty acids (PUFAs) is highly susceptible to reactions with free radicals. In our study, lipid peroxidation evaluated by TBARS assay as well as the presence of carbonyl groups, assessed as an index of oxidative damages were significantly decreased after ASTA treatment. These data are in agreement with antioxidant properties of ASTA showing a protective role on biomolecule damages induced by ROS. Although the TBARS assay is heavily criticized in the literature, we assume that ASTA reduces oxidative damages in lipids and this protection may be related with the better response of neutrophils phagocytosis after treatment with ASTA.

As previously shown, increased production of ROS, which are known to exhibit direct tissue damaging properties, may contribute to a number of diseases [3, 10, 35]. In fact, there are recent concerns that many human diseases are associated to an increased oxidative stress resulting

either from the altered production of free radicals or from the altered antioxidant content or activity. Undeniable support for the involvement of free radicals in disease development comes from epidemiological studies showing that an enhanced antioxidant status is associated with reduced risk of several diseases. Free radicals can be neutralized by endogenous and exogenous antioxidants, such as vitamin E, vitamin C, and thioctic (lipoic) acid. More recently, the carotenoid astaxanthin was shown to reduce oxidative stress in several conditions [20, 34, 37].

In recent years, a number of studies on astaxanthin have demonstrated its in vitro and in vivo antioxidant effect, for example, the quenching effect on singlet oxygen, a strong scavenging effect on superoxide, hydrogen peroxide, and hydroxyl radicals and an inhibitory effect on lipid peroxidation [28]. However, neither of these studies have shown the effects of ASTA in neutrophil function nor the antioxidant effects of ASTA on neutrophils that is a cell with potential capacity to produce a great amount of free radical during their activation.

Neutrophils have long been known to release hydrogen peroxide, which in association with neutrophil myeloperoxidase and a halide ion constitutes a potent bactericidal system. The production of hydrogen peroxide in leukocytes, involves a membrane-associated NADPH-oxidase that removes electrons from NADPH to reduce O_2 into superoxide anion, which is rapidly dismuted to H_2O_2 [17, 35, 42]. H_2O_2 and superoxide are generated in inflamed tissues and are reported to contribute to the pathogenesis of inflammatory lung diseases including chronic obstructive pulmonary diseases, bronchial asthma, cystis fibrosis, and idiopathic pulmonary fibrosis [48]. It was also shown that hydrogen peroxide, which breaks down to produce hydroxyl radicals, can also activate NF-κB, a transcription factor involved in stimulating inflammatory responses. Excess production of these ROS is toxic, exerting cytostatic effects, causing membrane damage, and activating pathways of cell death (apoptosis and/or necrosis) [45]. Cytokines are particularly relevant to inflammatory processes, because of their ability to selectively recruit leukocytes into sites of injury. Given neutrophils are usually the first blood cells to infiltrate inflamed tissues, their ability to generate various inflammatory cytokines is likely to have a significant impact on the initiation and development of inflammatory response. In the present study, we found that ASTA significantly reduced the production of pro-inflammatory cytokines, such as TNF-α and IL-6 in LPS-stimulated neutrophils. Intracellular ROS seems to exert a central role in modulating the expression of pro-inflammatory mediators [16] through activation of NF-κB [32]. A significant correlation ($R^2 = 0.917$, $p < 0.028$) was observed when both TNF-α and lipoperoxidation were decreased in cells treated with ASTA. We believe that ASTA suppressed activity of NF-κB by scavenging the ROS, and decreased the expression of pro-inflammatory cytokines decreasing the level of lipoperoxidation. In this respect, further studies are required to establish the effect of ASTA on NF-κB activation in neutrophils.

Another important signaling molecule, nitric oxide (NO·) takes action as an immunomodulator molecule, which regulates various functions of lymphocytes, macrophages, and polymorphonuclear leukocytes (PMNs) [41]. The role of NO· is proposed to inhibit chemotaxis, endothelium adhesion and aggregation, NF-κB activation [38], and in the PMN-mediated tissue damage. Studies have demonstrated that NO· modulates ROS generation from PMNs [47]. In addition to ROS scavenging, NO· attenuated NADPH-oxidase activity and inhibited ROS generation. NO·-mediated modulation of ROS generation has also been demonstrated in the conditions associated with the increased accumulation of NO·, such as hypoxia-reoxygenation and lipopolysaccharide treatment. Tissue damage arises as a consequence of NO· interacting with free radicals or the transition metals and its ability to cause nitration or nitrosation of proteins. Clancy et al. [9] demonstrated that NO· inhibited neutrophil superoxide anion production via direct action on a membrane component of the NADPH oxidase. The effect of NO· must be exerted before the assembly of the activated complex and may involve a reaction with one or more thiol groups, which are important in the function of cytochrome b_{558}. This could result from interference with the conformation of the assembled oxidase necessary for electron transfer. Whenever NO· interacts in vivo with superoxide anions, the local formation of peroxynitrite $ONOO^-$ causes tissue damage of the endothelial cells at sites of inflammation. As presented herein, ASTA-treatment in vitro promoted an increase of NO· production after LPS-stimulation as well as a significant decrease in superoxide anion production, possibly by inhibiting NADPH-oxidase activation as verified in other studies.

Intracellular calcium homeostasis, regulated by the redox status of cellular thiols and cell calcium concentration, may play a critical role in the control of a wide variety of cellular functions, including gene transcription and expression [27]. Increased intracellular calcium mobilization was observed in neutrophils after acute treatment with ASTA. Intracellular calcium, in particular the cytosolic free ionized calcium concentration $[Ca^{2+}]_i$ is tightly regulated under physiological conditions. Stimulation of receptors, belonging to almost all the classes so far described, will result in changes in $[Ca^{2+}]_i$. Either Ca^{2+}-influx or Ca^{2+}-mobilization from intracellular stores, or indirectly by a number of other mechanisms [19, 46], might directly induce these changes. Following stimulation of the neutrophils with agonists that activate the nicotinamide

adenine dinucleotide phosphate (NADPH-oxidase), there is a marked increase in the concentration of cytosolic free Ca^{2+}. This Ca^{2+} signaling was thought to cause oxidase activation. Evidence shows that both internal and external Ca^{2+} are required for full activation of the respiratory burst, and external Ca^{2+} influx via capacitative Ca^{2+} entry, a process triggered by depletion of Ca^{2+} stores, contributes significantly to the generation of reactive oxygen metabolites [4, 40]. Our study shows that ASTA-treatment causes a significant increase in intracellular calcium mobilization both in a basal and in fMLP-stimulated condition (Fig. 2); however, this event was accompanied by a decrease in the NADPH-oxidase activation as indicated by decreased superoxide anion production (Fig. 3). The exact mechanism behind this observation remains to be elucidated.

Taken together our results are supportive of a beneficial effect of astaxanthin-treatment on human neutrophil function as demonstrated by increased phagocytic and microbicidal capacities as well as by the reduced superoxide anion and hydrogen peroxide production without affecting neutrophils capacity to kill *C. albicans*. This process appears to be mediated by calcium released from intracellular storages as well as nitric oxide production.

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