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Impact of the carotenoid astaxanthin on phagocytic capacity and ROS/RNS production of human neutrophils treated with free fatty acids and high glucose

Beatriz Alves Guerra, Rosemari Otton *

Postgraduate Program — Health Sciences — CBS, Cruzeiro do Sul University, Sao Paulo, SP, 03342000, Brazil

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A B S T R A C T

Background: The purpose of the present study was to evaluate the effect of carotenoid astaxanthin (ASTA) on human neutrophils treated with high glucose and free fatty acids (FFA) on the phagocytic capacity and reactive oxygen/nitrogen species production.

Methods: The following parameters were evaluated: phagocytic capacity of neutrophils by using zymosan particles, intracellular and extracellular superoxide anion (lucigenin and DHE probes), hydrogen peroxide (H_2O_2 — phenol red), nitric oxide (Griess reagent) production, and maximal activity of G6PDH.

Results: There was a decreased phagocytic capacity of human neutrophils treated with high glucose (30 mM) or FFA (0.1 mM) and a partial restoring of the phagocytic capacity after ASTA-treatment was observed. ROS and RNS production was increased in neutrophils due to both high glucose and FFA. This increase in ROS/RNS production was also partially prevented by ASTA treatment. Both glucose and FFA increased the G6PDH activity. We show that ASTA provides a modest improvement of cellular functions after cells have been treated with high glucose and FFA.

Conclusions: In summary, this study showed that both high glucose and a mixture of FFA are potent inducers of ROS/RNS production on neutrophils as observed by higher levels of superoxide anion, hydrogen peroxide and NO• production. Also, these metabolites decrease the phagocytic capacity of neutrophils and increase the G6PDH activity. Overall, ASTA-treatment was able to reduce partially ROS/RNS production by reducing the availability of NADPH and recover phagocytic capacity of neutrophils.

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1. Introduction

Diabetes is a special condition where both glucose and free fatty acids (FFA) are increased in plasma. This fact occurs by a reduced insulin action on target tissues or a failure of insulin secretion by pancreatic β-cells. Chronic exposure to high glucose and FFA can cause damage in different cell types by a variety of mechanisms including 'glucolipotoxicity'. Because an elevated level of glucose is one of the metabolic hallmarks of diabetes, much attention has been given to the sugar's oxidative chemistry. One widely studied mechanism involves autoxidation of glucose itself, which generates reactive oxygen species (ROS) such as hydroxyl radical and also cross-links proteins [1,2]. Other potential mechanisms include glucose and FFA-stimulated mitochondrial oxidative phosphorylation, production of reactive intermediates by phagocytic white blood cells at sites of inflammation (e.g. atherosclerotic tissue), and peroxidation of polyunsaturated fatty acids, all of which have been implicated in the generation of reactive intermediates in vitro [3]. In fact, it has been reported that glucose and FFA initiate the formation of ROS in muscles, adipocytes, pancreatic β-cells and other cells [4–6].

Patients with diabetes are affected by frequent episodes of acidosis caused by increasing metabolism of fatty acids. This pathological condition is correlated with the high incidence of vascular disease, for which there a strong correlation with tissue damage mediated by oxidative stress [7–9]. However, there is considerable controversy regarding the nature, magnitude, and mechanisms of oxidative stress in the diabetic state. Overproduction of ROS or a failure in intracellular defenses against ROS has been held as one major contributor to long-term diabetic complications [6,10–14].

Peripheral blood cells such as phagocytes (macrophages and neutrophils) are well known to be activated in diabetes and thus produce large amounts of ROS. The major pathway through which neutrophils generate oxidants begins with their membrane-bound NADPH-oxidase, which produces superoxide with subsequent hydrogen peroxide production [15,16]. The peroxide can then be used by another phagocyte enzyme, myeloperoxidase. The connection between this pathway and diabetes may be that, at least in the artery wall of diabetic animals, hyperglycemia can activate protein kinase C [17,18], which leads to phagocyte activation, secretion of myeloperoxidase, and oxidant generation. These changes might enhance the production of superoxide and hydrogen peroxide, which myeloperoxidase

* Corresponding author at: Av. Regente Feijó, 1295, 03342000, São Paulo, SP, Brazil. Tel./fax: +55 11 26726200.
E-mail address: rosemari.otton@cruzeirodosul.edu.br (R. Otton).

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converts into more potent cytotoxic oxidants, such as hypochlorous acid. Superoxide anion can react with nitric oxide (NO·) and produces peroxinitrite, a powerful oxidant agent. Free fatty acids that commonly are overabundant in diabetes can also activate phagocytes in vitro [19,20].

Uncontrolled ROS production can cause oxidative damage to a variety of biologic macromolecules such as nucleic acids (DNA, RNA), lipids, proteins and carbohydrates [21]. However, there are intracellular antioxidant defense mechanisms which involve both enzymatic and non-enzymatic strategies. Among the non-enzymatic compounds we can find carotenoids that have antioxidant properties and a therapeutic role in various diseases [22].

The ketocarotenoid astaxanthin (ASTA), 3,3′-dihydroxy-β,β-carotene-4,4′-dione is ubiquitous in nature, especially in the marine environment [23]. It is a red pigment common in many marine animals, such as salmonids, shrimps, lobsters and crayfish, contributing to the pinkish-red color of their flesh [24]. ASTA may act as a strong antioxidant by donating the electrons and reacting with free radicals to convert them into a more stable product and terminate free radical chain reaction in a wide variety of living organisms [25,26]. In recent years, a major number of studies on ASTA have demonstrated its 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 [27–30]. In addition, several other biologic activities of ASTA, including anti-cancer, anti-inflammatory, antidiabetic, immunomodulatory activities and a neuroprotective effect also have been reported [25,31]. Antioxidants, including a number of carotenoids, have been hypothesized to inhibit oxidative stress and play a protective role against chronic diseases such as diabetes. The purpose of this study was to evaluate the effects of ASTA in the phagocytic capacity and ROS/RNS production induced by FFA and high glucose in human neutrophils.

2. Materials and methods

2.1. 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 and lucigenin, which came from Invitrogen (CA, USA). Common reagents for buffers (e.g. PBS) and regular laboratory solutions were obtained from Labsynth (Diadema, SP, Brazil).

2.2. Subjects

The Ethical Committee of the Cruzeiro do Sul Univerity approved the experimental procedure of this study. Around 30 healthy adult women and men (mean age 28.0 ± 9.0) were included in the present study. The subjects recruited did not present any systemic or topical therapeutic regimen, a smoking history, alcohol habits, obesity or any other systemic diseases at least for the last 2 months (based on an anamnesis protocol).

2.3. 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 (~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, 300 *g* at room temperature. The plasma and the intermediary layer were removed and both neutrophils and erythrocytes were collected from the 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 1 mL of RPMI-1640 medium supplemented with 10% fetal bovine serum, 20 mM Hepes, 2 mM glutamine, and antibiotics (streptomycin 100 units/mL and penicillin 200 units/mL).

The cells were treated with 0.1 mM of a mixture of free fatty acid prepared as described in our previous study [32,33] and with high glucose (30 mM) added or not of 2 μM of ASTA solubilized in DMSO. Then, after treatment we had the following experimental groups: control (without treatment), high glucose, high glucose + ASTA, FFA and FFA + ASTA. The cells were cultured at 5% CO_2 for up to 24 h at 37 °C. After this period, the cells were collected, centrifuged and stored at −80 °C to assay enzyme activity. For acute effects of FFA and high glucose on cell ROS production, after isolation, neutrophils were resuspended 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) and freshly used.

2.4. Phagocytic capacity

Neutrophils (1×10^6 cell/mL) were incubated for 30 min at 37 °C in 1 mL RPMI 1640 medium with opsonized zymosan particles and treated with or without 2 μM of ASTA, 0.1 mM of FFA and 30 mM of glucose. The particles (5×10^6/mL) were opsonized by incubation in the presence of control serum for 30 min at 37 °C. The cells were incubated with particles and counted after cytocentrifugation. The score of phagocytosis was expressed after the counting of one hundred cells. The number of particles phagocyted was multiplied by one, two and three, according to a criterion established to score [34].

2.5. Dihydroethidium assay

Dihydroethidium (DHE) is a fluorescence probe used to measure the intracellular superoxide anion production. Once inside the cell, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by superoxide 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, the cells were stimulated with phorbol myristate acetate (PMA — 20 ng/well), a promoter of respiratory burst in phagocytes and treated with or without 2 μM of ASTA, 0.1 mM of FFA and 30 mM of glucose in Tyrode's buffer for 30 min. The fluorescence was analyzed in a microplate reader (Tecan, Salzburg, Austria) (396 nm wavelength excitation and 590 nm wavelength emission). Results are expressed as relative fluorescence units (RFU).

2.6. Lucigenin assay

The lucigenin chemiluminescent probe was utilized to measure the extracellular superoxide anion content mainly produced through NADPH-oxidase activation. Lucigenin releases energy in the form of light after excitation by superoxide anion. The chemiluminescence produced was monitored by a luminometer for 20 min (Tecan, Salzburg, Austria). Lucigenin (5 μM) was added to cells (5×10^5/well) treated with or without 2 μM of ASTA, 0.1 mM of FFA and 30 mM of glucose in Tyrode's buffer. The experiments were carried out in the presence and absence of PMA (20 ng/well). The results are expressed as chemiluminescence relative units. The statistical analysis was performed by AUC calculation (area under the curve).

2.7. Hydrogen peroxide production

Hydrogen peroxide (H_2O_2) production was measured according to Pick and Mizel [35], based on horseradish peroxidases, which catalyze the phenol red oxidation by H_2O_2. Neutrophils (5×10^5/well) were incubated with or without 2 μM of ASTA, 0.1 mM of FFA and 30 mM of glucose in Tyrode's buffer, mixed with 0.28 mM phenol red and horseradish peroxidase (1000 units/mg) at 37 °C for 1 h. The production of H_2O_2 was measured in the absence and presence of PMA (20 ng/well). 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).

2.8. Nitric oxide production

NO• production was performed according to Ding et al. [36] through nitrite determination. Nitric oxide is rapidly converted into 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 the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride) in supernatants. Neutrophils (5×10^5/well) were cultured with or without 2 μM of ASTA, 0.1 mM of FFA and 30 mM of glucose and LPS-stimulated (10 ng/well) for 4 h in RPMI-1640 medium supplemented with 10% fetal bovine serum. Then, the same volume of Griess was added to cells and the absorbance was measured in 550 nm. The nitrite concentration was determined using sodium nitrite as a standard (0–60 μM).

2.9. Assay the glucose-6-phosphate dehydrogenase (G6PDH) activity

Glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.l.49, is a key regulatory enzyme of the oxidative segment of the pentose-phosphate pathway and produces reducing equivalents in the form of NADPH to meet some cellular needs for reductive biosynthesis and as a contribution to the maintenance of the cellular redox state [37]. The neutrophils (5×10^6/well) after a culture of 24 h were disrupted in the pH 8.0 extraction buffer consisting of 50 mM Tris–HCl and 1 mM EDTA. The activity was assayed as described in [38] and 0.05% (v/v) Triton-X-100 was added to the assay system to complete the extraction of the enzyme. The following 334 μL assay medium at pH 7.6 composed of 8.6 mM Tris–HCl, 6.9 mM $MgCl_2$, 0.4 mM NADP, 1.2 mM glucose-6-phosphate. The spectrophotometric measurements were made in a microplate reader (Tecan, Salzburg, Austria). Enzyme activity is expressed as nmol/min/mg of protein.

2.10. Protein measurement

The measurement of the specific enzyme activity of G6PDH was entirely related to protein concentrations, which were estimated by the Bradford method [39] using bovine serum albumin as a standard.

2.11. Statistical analysis

All data points are the mean values with standard errors of at least three independent experiments. The data were analyzed by one-way ANOVA followed by the Tukey's post-test. The software employed for statistical analysis was GraphPad Prism (version 4; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Phagocytic capacity

The phagocytic capacity was measured by the incorporation of opsonized zymosan particles (Fig. 1). There was a significant decrease in the phagocytic capacity of neutrophils after the cells were treated with FFA and glucose of 46% and 39% respectively, when compared with the control group. ASTA treatment promoted a significant increase in phagocytosis of neutrophils, as compared with the FFA and glucose group, by 51% and 45%, respectively. The addition of ASTA on the FFA group improved the phagocytic capacity of neutrophils by 47% when compared with the FFA group. The same effect was observed in the glu + ASTA group, which presented an improvement of 22% as compared with the glucose group.

3.2. Superoxide anion production

There was an increase in intracellular superoxide anion production as assayed by DHE after the treatment of the cells with glucose and FFA to levels of control-stimulated group, indicating that both metabolites can induce neutrophil ROS production (Fig. 2A). When compared with the basal control group, glucose and FFA promoted an increase of 98% and 97%, respectively. In cells treated with ASTA, a decrease in superoxide anion production of about 43% was observed as compared with the control group. The addition of ASTA was not effective in reducing the superoxide anion production induced by FFA and glucose. On the contrary, it promoted an increase of 51% in superoxide production when compared to the FFA group.

The extracellular superoxide anion production was measured by using lucigenin probe (Fig. 2B). There was a direct induction of NADPH-oxidase by FFA which promoted a significant increase in $O_2{\bullet}^-$ production in cells. The treatment of FFA with ASTA was unable to reduce superoxide anion production.

3.3. Hydrogen peroxide production

To promote hydrogen peroxide production, human control neutrophils were activated with PMA (20 ng/well). The cells were then treated with or without 2 μM of ASTA and 30 mM of glucose or 0.1 mM of FFA for 1 h. Hydrogen peroxide production was increased by 70%, 60%, 100% and 65% in ASTA, glucose, FFA, and FFA + ASTA respectively when compared with the control-unstimulated group. A decrease of 34% in hydrogen peroxide production was observed in the glu + ASTA

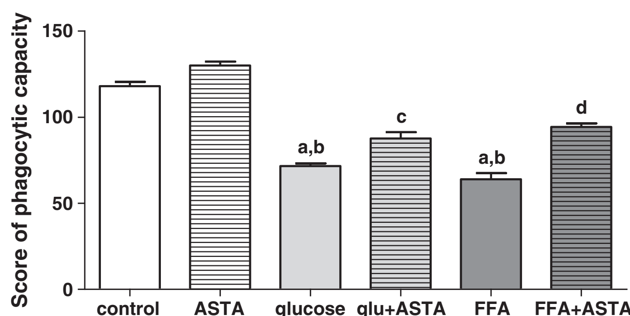

Fig. 1. The phagocytic capacity was measured by incorporation of opsonized zymosan particles in human neutrophils (1×10^6/mL) treated with or without 2 μM of ASTA, 30 mM glucose and 0.1 mM FFA. Results are expressed as mean ± SEM of at least 4 determinations. (a) Significantly different compared with the control group ($p<0.0001$). (b) Significantly different compared with the respective group without ASTA ($p<0.0001$). (c) Significantly different compared with the glucose group ($p<0.01$). (d) Significantly different compared with the FFA group ($p<0.001$).

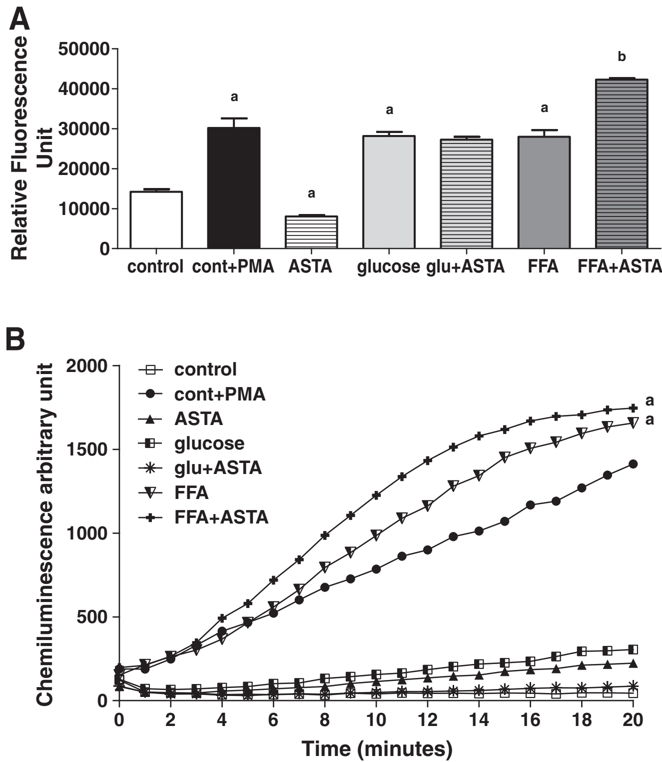

Fig. 2. (A) Intracellular (DHE) and (B) extracellular (lucigenin) superoxide anion production in human neutrophils. Cells (5×10^5/well) were treated with or without 2 μM of ASTA and activated with PMA (20 ng/well) or 30 mM glucose or 0.1 mM FFA for 30 min. Results are expressed as mean ± SEM of at least 5 determinations. (a) Significantly different compared with the cont + PMA group ($p<0.001$). (b) Significantly different compared with the ASTA group ($p<0.001$).

group as compared to glucose group and of 37% in FFA + ASTA when compared to the FFA group (Fig. 3).

3.4. Glucose-6-phosphate dehydrogenase activity

The maximum G6PDH activity was assessed by the reduction of the co-factor NADP+ to NADPH in human neutrophils. G6PDH activity was increased 97% and 107% in glucose and FFA groups respectively when compared to the control group. The addition of ASTA in glucose and FFA groups promoted a decrease of 39% and 32% as compared with glucose and FFA respectively (Fig. 4).

3.5. Nitric oxide production

The NO• production was evaluated in cells treated with glucose (Fig. 5A) and FFA (Fig. 5B) in both basal and LPS-stimulated conditions. An increase of 77%, 190% and 146% was observed in NO• production in ASTA, glucose, and glu + ASTA groups respectively when compared with the control-unstimulated group. After LPS-stimulation, the glucose and glu + ASTA groups were increased by 74% and 50% respectively as compared with the control-stimulated group. The treatment of cells with FFA did not promote an increase in NO• production as observed after the treatment of cells with high glucose. However, ASTA promoted an increase of ~30% at both basal and LPS-stimulation conditions as compared with the respective control group.

4. Discussion

In the present study we observed a decreased phagocytic capacity of human neutrophils (Fig. 1) when they were treated with high glucose (30 mM) or FFA (0.1 mM) mimicking a diabetic condition in vitro. A partial restoring of the phagocytic capacity was observed after ASTA-treatment. Overall, ROS and RNS production was increased in neutrophils due to both high glucose and FFA. This increase in ROS/RNS production was also partially prevented by ASTA treatment. Both glucose and FFA increased the G6PDH activity (Fig. 4), probably by increasing the flux of these metabolites throughout oxidative pathways which result in increased NADPH co-factor content. While ASTA has a potent antioxidant effect as shown by other authors, in this study we show that ASTA has a modest improvement of cellular functions after cells has been treated with high glucose and FFA. This effect could be in part due to high stress imposed to cells by the presence of high glucose and FFA. ASTA was unable to totally abrogate the huge oxidative stress induced by these metabolites.

Proposed mechanisms for the pathogenesis of diabetic complications include the formation of advanced glycosylation end products (AGEs), oxidative stress, carbonyl stress, increased protein kinase C activity, altered growth factor or cytokine activities, and mitochondrial dysfunction [3,40,41]. Two factors that strongly affect the risk of diabetic complications are disease duration and degree of glycemic control. These observations have given rise to the "glucose hypothesis" which suggests that glucose mediates many of the deleterious effects of diabetes [3]. However, strict glycemic control alone does not prevent diabetic complications, suggesting the involvement of additional factors. High levels of plasma FFA are also implicated in diabetic complications [6,42].

A variety of types of recurrent infections draws attention to diabetic patients, not because of its ease of acquired infections, but also

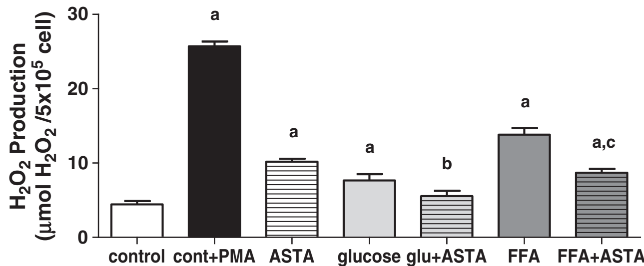

Fig. 3. Hydrogen peroxide production evaluated by phenol red oxidation. Human neutrophils (5×10^5/well) were treated with or without 2 μM of ASTA and activated with PMA (20 ng/well) or 30 mM glucose or 0.1 mM FFA for 60 min. Results are expressed as mean ± SEM of at least 5 determinations. (a) Significantly different compared with the unstimulated-control group ($p<0.001$). (b) Significantly different compared with the glucose group ($p<0.001$). (c) Significantly different compared with the FFA group ($p<0.001$).

Fig. 4. Maximum G6PDH activity assessed by the reduction of the cofactor NADP+ to NADPH in human neutrophils (1×10^6/mL) cultured for 24 h and treated with or without 2 μM of ASTA, 30 mM glucose and 0.1 mM FFA. Results are expressed as mean ± SEM of at least 5 determinations. (a) Significantly different compared with the control group ($p<0.001$). (b) Significantly different compared with the respective group without ASTA ($p<0.001$). (c) Significantly different compared with the glucose group ($p<0.001$). (d) Significantly different compared with the FA group ($p<0.001$).

due to the fact that they cannot deal well with invading pathogens. Even simple and relatively benign pathogens can cause severe infections in these patients [43]. Hyperglycemia and high levels of FFA were shown to decrease bactericidal activity, impair phagocytosis and decrease the release of lysosomal enzymes, and reduce production of reactive oxygen species by neutrophils of diabetic patients [44–46]. In the present study glucose and a mixture of fatty acids used at 0.1 mM caused a marked decrease in phagocytic capacity of human neutrophils similar to the one observed in the diabetic condition. ASTA addition significantly improves phagocytic capacity of neutrophils treated with glucose and FFA. A similar result was obtained by our group previously [30].

Long-chain FFA and some of their derivatives and metabolites can modify intracellular production of reactive oxygen species (ROS), in particular superoxide and H_2O_2. In mitochondria, FFA exerts a dual effect on ROS production. Because of slowing down the rate of electron flow through Complexes I and III of the respiratory chain due to an interaction within the complex subunit structure, and between Complexes III and IV due to the release of cytochrome c from the inner membrane, FFA increases the rate of ROS generation in the forward mode of electron transport [47]. FFA can also modulate mitochondrial ROS production by other mechanisms: (a) being protonophores, they abolish RET (reverse electron transport) dependent on the superoxide generation; (b) having a potential ability to interfere with various enzymatic processes, FFA could inactivate the regeneration of reduced glutathione (GSH) from its oxidized form (GSSG), thus impeding removal of H_2O_2 by glutathione peroxidase; (c) the amphiphilic nature of FFA favors their incorporation into the inner mitochondrial membrane with a consequent change of membrane fluidity [47]. FFA are also long known as an activators of the NADPH oxidase-dependent superoxide production of neutrophils [48–51]. This non-mitochondrial oxygen uptake is accompanied by the generation of superoxide and H_2O_2 [52]. In our study there was a huge increase in superoxide anion production in cells treated acutely with high glucose and FFA as assayed by DHE and lucigenin probes (Fig. 2). The addition of ASTA on the FFA group promoted a higher increase in superoxide production. Vascular cells increase their ROS generation through the activation of NADPH oxidase induced by high glucose levels and FFA [53]. The neutrophil respiratory burst seems to be related directly to glycemic control with an increase in protein kinase C (PKC) and NADPH oxidase activity [54–56]. Mechanistically, hyperglycemia results in increased phosphorylation of $p47^{phox}$, leading to an increase in the generation of superoxide anion [54–56]. Recent works have shown that $p47^{phox}$, a key protein in the assembly of NAPDH oxidase, prematurely translocates to the membrane and associates with $p22^{phox}$ in neutrophils from diabetic subjects [57]. In the present study the mechanism by which FFA and high glucose induce ROS production needs to be clarified.

Neutrophils are dependent on the production of reactive oxygen species to exert their role in destroying pathogens during phagocytosis. But these reactive oxygen species being produced in large amounts can damage the cell, mainly because their membranes have a large amount of polyunsaturated fatty acids, which are easily oxidized by these reactive species.

Superoxide is rapidly converted into hydrogen peroxide (H_2O_2), either spontaneously or enzymatically by superoxide dismutase. H_2O_2 is then reduced to water by catalase or converted into hypochlorous acid (HOCl) by myeloperoxidase [58]. H_2O_2 can also be transformed into hydroxyl radicals in the presence of transition metal ions [59]. In this study we observed that the hydrogen peroxide production in neutrophils was increased (Fig. 3) when cells were treated with glucose and FFA. Many authors have reported that astaxanthin has a greater affinity with hydrogen peroxide when compared to superoxide [27–29]. Recently, Macedo et al. [30] showed that astaxanthin significantly reduced the production of pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6 in lipopolysaccharide-stimulated neutrophils. The authors also showed that astaxanthin improved neutrophil phagocytic and microbicidal capacity and reduced superoxide anion and hydrogen peroxide production, which appeared to be mediated by calcium released from intracellular storages and nitric oxide production.

The interaction of glucose with proteins leading to the formation of Amadori products and then AGEs is an important source of free radicals in diabetes [40]. These AGEs, via their receptors (RAGE), inactivate enzymes and alter their structures and functions [60], promote free radical formation [41,61], and quench and block antiproliferative effects of nitric oxide [62,63]. By increasing intracellular oxidative stress, AGE activate the transcription factor NFκB (nuclear factor kappa B), thus promoting up-regulation of various NFκB controlled target genes [64,65]. NFκB enhances production of NO•, which is believed to be a mediator of islet beta cell damage [66]. We observed an increase of 190% in NO˙ production (Fig. 5A) in cells treated with high glucose. FFA was not a potent inducer of NO• production (Fig. 5B). Whether the NFκB pathway is activated in these cells remains to be elucidated. ASTA, as previously shown by our group [29,30,33], increased NO• production both in lymphocytes and neutrophils, which was associated with a beneficial anti-inflammatory role of ASTA. In the present study ASTA also promoted a significant increase in NO•. Hussein and coworkers [25] also found an increase in NO• production associated with a reduction of the arterial blood pressure in spontaneously hypertensive rats, suggesting an anti-hypertensive role of ASTA. Recently, Kim and colleagues [67] found that ASTA reduced the activation of the transcription factor NF-κB and decreased the IL-6 production in microglial cells. Since NFκB is the most important pathway in immune cells, responsible of inducing NO• production by inducing iNOS expression, we can speculate that ASTA can promote a NF-κB activation which leads to an increase in NO• production.

Defense mechanisms against free radical-induced oxidative stress involve: (i) preventive mechanisms, (ii) repair mechanisms, (iii) physical defenses, and (iv) antioxidant defenses. Enzymatic antioxidant

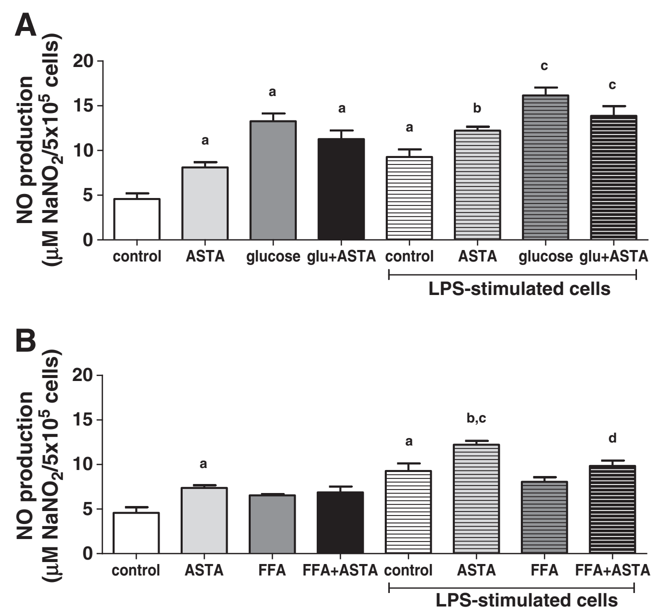

Fig. 5. Nitric oxide production evaluated by Griess reagent. Human neutrophils (5×10^5/well) were treated with or without 2 μM of ASTA and stimulated with LPS (10 ng/well) or 30 mM glucose (A) or 0.1 mM FA (B) for 4 h. Results are expressed as mean ± SEM of at least 5 determinations. (a) Significantly different compared with respective control group ($p<0.001$). (b) Significantly different compared with the ASTA group ($p<0.001$). (c) Significantly different compared with the control-stimulated group ($p<0.001$). (d) Significantly different compared with the ASTA-stimulated group ($p<0.001$).

defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health [68]. Some antioxidant enzyme systems need the cofactor NADPH, which is produced mainly by the pentose pathways through the enzyme glucose-6-phosphate dehydrogenase (G6PDH). This cofactor is extremely important for several cellular processes.

In this study we evaluated the maximum activity of G6PDH and our findings show that there was an increase in G6PDH activity in cells treated with glucose and FFA. Probably, the high flux of these metabolites throughout the metabolic pathways was responsible for increased G6PDH activity. In the pentose pathway, higher G6PDH activity can raise the level of NADPH, which is an important co-factor for antioxidants enzymes such as glutathione peroxidase and glutathione reductase. In addition, NADPH is an essential co-factor for NADPH-oxidase, an important pathway for ROS/RNS production. Treatment with ASTA was able to restore G6PDH activity to control levels, reducing the availability of NADPH for participation in ROS production. Perhaps, the antioxidant role of ASTA observed in this study is due to this specific action on pentose pathway.

In summary, this study showed that both high glucose and a mixture of FFA are potent inducers of ROS/RNS production on neutrophils as observed by higher levels of superoxide anion, hydrogen peroxide and NO• production. Also, these metabolites decrease the phagocytic capacity of neutrophils and increase G6PDH activity. Overall, ASTA-treatment was able to reduce partially the ROS/RNS production by reducing the availability of NADPH and to recover phagocytic capacity of neutrophils. However, it is important that further human studies be conducted to evaluate the effect of astaxanthin in relation to its bioavailability, bioconversion and bioefficacy to complement the findings in this study. Soon the use of antioxidants such as astaxanthin may be indicated as an adjunctive treatment to diabetes mellitus.

Conflict of interest statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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