Oxidative stress in human lymphocytes treated with fatty acid mixture: Role of carotenoid astaxanthin

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1. Introduction

Fatty acids (FA) have been shown to alter leukocyte function, and depending on concentration and type, they can modulate both inflammatory and immune responses. These metabolites are important components of the diet and act as both intracellular and extracellular mediators, positively or negatively regulating physiological and pathological conditions (Pompeia et al., 2000). Polyunsaturated fatty acids (PUFAs) of the omega-3 family have overall suppressive effects on lymphocyte function by modulating cell-membrane fluidity and composition of lipid rafts, inhibiting lymphocyte proliferation, antibody and cytokine production, adhesion molecule expression, natural killer cell activity and triggering cell death (Costabile et al., 2005; Fan et al., 2003; Labri et al., 2005; Stulnig et al., 2000). The omega-6 PUFAs have both inhibitory and stimulatory effects on lymphocyte function. In addition to lymphocytes, FA has also been found to modulate phagocytosis of macrophages and neutrophils, reactive oxygen species production, cytokine production and leukocyte migration, also interfering with antigen presentation by macrophages (Calder et al., 1990; Endres et al., 1993; Meydani et al., 1991). The importance of FA has been corroborated by many clinical trials in which patients present enhancement or impairment of immune function depending on which FA is provided in supplementation. Several mechanisms have been proposed to explain fatty acid modulation of immune response, such as changes in membrane fluidity and signal transduction pathways, regulation of gene transcription, protein acylation, and calcium release (Pompeia et al., 2000).

Cell and plasma levels of FA are significantly increased under fasting conditions, hypoxia, obesity, strenuous exercise and type 1 and 2 diabetes. In these situations, we also observed a significant immune suppression (Bazan, 1970; Delarue et al., 2004; Gardiner et al., 1981; Itani et al., 2002; Otton et al., 2004). Indeed, diabetic individuals present a high occurrence of infections associated with complications such as heart disease, atherosclerosis, cataract formation, peripheral nerve damage, retinopathy, and others which contribute to decrease quality of life of the patients (Valko et al., 2007).

In our previous study (Otton et al., 2004) we showed that blood peripheral lymphocytes obtained from poorly controlled diabetic patients presented increased DNA fragmentation as compared with cells obtained from healthy patients. Lymphocytes from alloxan-induced diabetic rats also showed increased DNA fragmentation when compared with cells from controls. Comconitantly, there was also high occurrence of chromatin condensation and blebbing formation. These observations strongly support the proposition that uncontrolled diabetes leads to impaired immune function...
due to higher number of lymphocyte death. More recently our group showed that lymphocytes from healthy human subjects as well as leukemia cell lines (Raji and Jurkat cells) after treatment with a fatty acid mixture that mimics the proportion and concentration found in plasma from diabetic patients, raises the proportion of cells in apoptosis (Otton and Curi, 2005), by a mechanism involving the release of cytochrome c from mitochondria, activation of caspases, increase in the production of NO and superoxide, and induction of calcium release (Otton et al., 2007).

The production of free radicals is increased in diabetic patients, generating an oxidative stress condition as showed by many authors. According to these authors, many different pathways may contribute to increased oxidative stress in diabetes, including increased plasma levels of FA (Newsholme et al., 2007). The increase in fatty acid levels may alter reactive oxygen species (ROS) production via activation of NADPH-oxidase, by induction of mitochondrial uncoupling, by inducing calcium mobilization as well as the activation of the transcription factor NF-κb via Toll like receptor 4 (TLR-4) signaling (Atti et al., 2004; Baynes, 1991; Catherwood et al., 2002; Green et al., 2004; Inoguchi et al., 2000; Otton et al., 2007; Rolo and Palmeira, 2006; Sano et al., 1998). Based on these effects, many authors have suggested the use of antioxidants in the treatment of diabetic complications, especially those involving excessive production of free radicals.

Carotenoids act as antioxidants by quenching singlet oxygen and free radicals (Palozza and Krinsky, 1992; Tsuchiya et al., 1992). These compounds are colored pigments widely distributed in vegetables, fruits and seafood and are implicated in the prevention of degenerative diseases including coronary heart disease and cancer (Gerster, 1993; Morris et al., 1994). The xanthophyll carotenoid astaxanthin (3,3′-dihydroxy-β,β′-rotenone-4,4′-dione; ASTA), a reddish-colored C-40 compound, is a powerful broad-ranging antioxidant that occurs naturally in a wide variety of living organisms, such as microalgae, fungi, complex plants, and crustaceans (Hussein et al., 2006). It is a quencher of ROS and reactive nitrogen species (RNS) single- and 2-electron oxidants as well as a chain-breaking scavenger of free radicals. ASTA, unlike other carotenoids, contains two additional oxygenated groups on each ring structure, resulting in enhanced antioxidant properties (Guerin et al., 2003). It has been reported that ASTA has a high antioxidant activity: 10 times higher than other carotenoids such as lutein, canthaxantin, and β-carotene and 100 times higher than α-tocopherol (Goto et al., 2001; Naguib, 2000). This potent antioxidant activity has been observed to modulate biological functions ranging from lipid peroxidation to tissue protection against light damage (McNulty et al., 2007; Santonovo et al., 2006).

At the same time, ASTA displays interesting anti-inflammatory effects by preserving redox-sensitive (and essential) structures of human lymphocytes, although the applied dose apparently hinders lymphocyte proliferation (Bolin et al., 2010). As fatty acids are potent inducers of oxidative stress and as reported by many authors that ASTA has an important and prominent antioxidant activity, we propose to evaluate the oxidative stress caused by a mixture of fatty acids previously used by our group, and the possible ASTA protective role of oxidative stress induced by the FA mixture.

2. Materials and methods

2.1. Reagents

Astaxanthin (ASTA) and most of other chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), excepting the RPMI–1640 culture medium, pluronic acid, Vybrant MTT Cell Proliferation kit and acetoxymethylester (Pura-2 AM) which were from Life Technologies (California, 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 Universidade Cruzeiro do Sul (protocol number 030/07) approved the experimental procedure of this study. Around 30 healthy adult women and men (mean age 27.0 ± 9.0) were included in the present study. All subjects did not present systemic or topical therapeutic regimen at least for the last 2 months. Subjects with a smoking history, alcohol habits, obesity or any other systemic diseases were excluded of the study (based on an anamnesis protocol).

2.3. Cell isolation and culture condition

Lymphocytes were obtained through the collection of human peripheral blood by venipuncture procedure in vacuum/siliconized tubes containing 0.1 mM EDTA. Peripheral blood lymphocytes were isolated under sterile conditions by using a density gradient present in the reagent Histopaque 1077 (Sigma–Aldrich) according to the manufacturer’s instructions. After centrifugation, lymphocytes were counted in a neubauer chamber using Trypan blue (1%). Lymphocytes (1 × 10⁸/ml) were cultured in 5 ml of RPMI 1640 supplemented as described above. The cells were treated with 0.3 mM of the fatty acid mixture added or not of 2 μM of ASTA solubilized in DMSO and cultured at 5% CO₂ for up to 24 h at 37 °C. After this period, the cells were collected, centrifuged and stored at −80 °C. To perform the assays of enzymes activities and oxidative damages in biomolecules, cells were defrosted and immediately used. For acute effects of FA on cell ROS production and intracellular calcium mobilization, after isolation lymphocytes were suspended in Tyrode’s solution (137 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl₂, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5.6 mM d-glucose, and 5 mM acid HEPES, pH 7.4) and freshly used.

The fatty acid mixture used in the present study was previously described (Otton and Curi, 2005). Briefly, the proportion of fatty acids was as follows: 1.74% lauric (C12:0), 5.2% myristic (C14:0), 31% palmitic (C16:0), 1.1% palmitoleic (C16:1), 41% stearic (C18:0), 4.6% oleic (C18:1), 9.6% linoleic (C18:2), 1.3% linolenic (C18:3), 3.2% arachidonic (C20:4), 0.45% eicosapentaenoic (C20:5), and 1.8% docosahexaenoic (C20:6) acids. In this study, the 0.3 mM FA concentration used is frequently found in plasma from diabetic patients (Bajaj et al., 2002; Woerle et al., 2002). The percentage of ethanol used to prepare the FA mixture, was always lower than 0.05% of the total volume of culture medium. This concentration of ethanol has shown not to be toxic for the cells (Siddiqui et al., 2001). All experiments were performed with cells left untreated (control) or treated with ethanol (vehicle). Bovine serum albumin (BSA) was added at 0.2% as an extracellular fatty acid chelator. There was no difference between untreated and ethanol-treated cells in all cases.

2.4. Determination of lymphocyte proliferation capacity

The proliferation response of lymphocytes was determined using the Vybrant MTT Cell proliferation (Life Technologies) according to the manufacturer’s instructions. Briefly, the MTT assay involves the conversion of the water soluble compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the insoluble formazan. The formazan is then solubilized, and the concentration determined by optical density at 570 nm. The cells (5 × 10⁵ cell/well) were treated for 48 h with 0.3 mM of the fatty acid mixture added or not of 2 μM of ASTA and stimulated with concavalin A (Con A) (20 μg/mL) or lipopolysaccharide (LPS) (100 μg LPS/mL) to stimulate T and B cell proliferation, respec-
2.6.1 Phenol red assay

Changes in cytosolic Ca²⁺ levels were monitored by fluorescence using the calcium-sensitive probe Fura 2-AM (Otton et al., 2010). Briefly, cells (1 x 10⁶/300 µL) were acutely treated with 0.3 mM of the FA mixture added or not by 2 µM of ASTA. The loading period for 5 µM Fura 2-AM was 1 h at 37 °C in 1 x 10⁶ cells/well in Tyrode’s solution. Afterwards, cells were washed and intracellular [Ca²⁺] was monitored for 20 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). Transformation of the fluorescent signal to [Ca²⁺] 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 Kₐ of 224 nM (Grynkiewicz et al., 1985).

2.6.2 Dihydroethidium assay

Dihydroethidium (DHE) is a fluorescence probe and was used to measure the intracellular superoxide anion production. Once inside the cell, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by superoxide with minor collaboration of other ROS. Lymphocytes (5 x 10⁵/well) were incubated with 5 µM DHE for 15 min at room temperature in the dark. At the beginning of the assay control cells were stimulated with PMA (20 ng/well) and 0.3 mM of the FA mixture added or not by 2 µM of ASTA. Cells were incubated in the dark at room temperature for additional 30 min. DAPI (diphenyle iodonium 10 µM), an inhibitor of NADPH oxidase (Chen et al., 2007), was used to investigate if superoxide anion production occurred through NADPH-oxidase activation. Sodium azide (SA – 400 µM) was used as a mitochondrial inhibitor. Afterwards, fluorescence was analyzed in a microplate reader (Tecan, Salzburg, Austria) (wavelengths of excitation and emission were 396 and 590 nm, respectively).

2.6.3 DCFH-DA assay

The probe DCFH-DA was primarily used as an indicator of the production of H₂O₂ (Keston and Brandt, 1965) but is also described as being oxidized by other ROS such as HO·, ROO·, NO and peroxynitrite (Crow, 1997; Wang and Joseph, 1999). The cells (5 x 10⁵/well) were preloaded with DCFH-DA (5 µM) by incubation in culture medium for 30 min. DCFH-DA is cleaved intracellularly by non specific esterase and turns into high fluorescent 2,7-dichlorofluorescein (DCF) upon oxidation by ROS. After the loading period, cells were treated with FA with or without ASTA at 2 µM and cultured for 18 h. The experiments were conducted in the presence and absence of PMA (20 ng/well). After the culture period, cells were centrifuged and resuspended in 300 µL of Tyrode’s buffer and the fluorescence was monitored in spectrofluorimeter Tecan (Salzburg, Austria) with excitation at 485 nm and emission at 530 nm. The results of this experiment were expressed as relative units of fluorescence.

2.7 Nitric oxide production

Nitric oxide production was performed according to Ding et al. (1988) through nitrite (NO₂⁻) determination. Nitric oxide (NO) is rapidly converted into NO₂ in aqueous solutions and, therefore, the total NO₂ concentration can be used as a stoichiometric indicator of NO production in culture. Lymphocytes (5 x 10⁵/well) were cultured with 0.3 mM of the FA mixture with or without 2 µM of ASTA and LPS (10 µg/well) for 4 h. EGTA (ethylene glycol tetaacetic acid, 500 µM) was used as a calcium quencher and therefore to discard NO production by constitutive calcium-dependent NOS. N-Acetyl-Cysteine (NAC – 500 µM) was added as an antioxidant to discard ROS induction on iNOS activation. The absorbance was measured in 550 nm to estimate NO₂ concentrations based on a standard NaNO₂ solution.

2.8 Preparation of homogenates

For the enzymatic activities, oxidative lesions in biomolecules and glutathione content cells were pelleted (5 x 10⁶) after 24 h culture and mixed with 0.6 mL of the assay-specific extraction buffer and ruptured by ultrasonication in a Vibra Cell apparatus (Connecticut, USA), then centrifuged for 10 min, 10,000g at 4 °C. The supernatant was used for further analysis.

2.9 Lymphocyte antioxidant enzyme activities

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were determined in lymphocytes using a microplate reader (Tecan, Salzburg, Austria). CAT activity was measured as described by Aebi (1984) based on the direct decomposition of hydrogen peroxide (H₂O₂). SOD activity was measured using the method described by Ewing and Janero (1995), which involves the reduction of O₂·⁻ radicals by nitroblue tetrazolium (NBT) following a linear first order kinetic during 3 min. Glutathione peroxidase (Mannervik, 1985) and glutathione reductase (Carlberg and Mannervik, 1985; Rahman et al., 2006) were measured based on the oxidation of β-NADPH in the presence of tert-butyldihydroperoxide used as substrate.

2.10 GSH/GSSG ratio

Lymphocytes (5 x 10⁵) were used for determination of glutathione status, using the method described by Rahman et al. (2006). Both total GSH and GSSG were analyzed using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to combine with reduced glutathione (GSH) to form 5-thio-2-nitrobenzoic acid (TNB). The GSH/GSSG concentrations were calculated from a standard curve prepared with pure GSH/GSSG standards and were expressed as µM of GSH and GSSG.

2.6.8 Oxidative damages

The lipid peroxidation in lymphocytes was performed by measuring the concentration of thiobarbituric acid-reactive substances in cell homogenates as described previously by Fraga and colleagues (Fraga et al., 1988). The assay evaluated the formation of a colored adduct after the stoichiometric reaction between thiobarbituric acid (TBA) and several lipid derived aldehydes, including malondialdehyde (MDA). The absorbance at 535 nm was measured after the mixture reached room temperature and the TBARS content was estimated by a standard curve of 10 µM 1,1,3,3-tetraethoxypropane.
Thiol and carbonyl groups were evaluated as biomarkers of amino acid oxidation in total protein fractions, which were isolated from crude homogenate of cells (5 × 10⁶) by precipitation with 20% trichloroacetic solution in ice. Reduced thiol groups were detected by the formation of colored adducts after reaction with 4 mM 5,5′-dithio-bis (2-nitrobenzoic acid) solution (DTNB). The absorbance of DTNB-treated samples at 412 nm was calculated using GSH as a standard (Biteau et al., 2003; Murphy and Kehrer, 1989). The same procedure was used to estimate protein carbonyls. The protein carbonyls were identified by the hydrazones formed with 10 mM dinitrophenylhydrazine (DNPH) in 0.25 M HCl. Absorbance of the peak detected within the range of 340–380 nm was measured, and the carbonyl group concentration was calculated based on the molar coefficient of ε = 2.2 × 10⁴ M⁻¹ cm⁻¹ (Murphy and Kehrer, 1989).

2.11. Protein determination

The total protein content of lymphocytes was measured by the method of Bradford (Bradford, 1976), using BSA as standard.

2.12. Statistical analyses

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

3. Results

3.1. Lymphocyte proliferation

The functional activity of lymphocytes was assayed by their capacity to proliferate in response to a specific stimulation. Fig. 1 shows the MTT assay results after stimulation with Con A (α T lymphocytes mitogen) or LPS (α B lymphocytes mitogen) for 48 h. FA at 0.3 mM increased both basal (without stimulation) and LPS-stimulated proliferative capacity of human lymphocytes by 38% and 30%, respectively as compared with non stimulated control group. The addition of astaxanthin to cells treated with FA caused a decrease in the proliferation of lymphocytes in basal, Con A and LPS-stimulated conditions by 43%, 26% and 30%, respectively as compared with 0.3 mM of FA mixture.

3.2. Intracellular calcium concentration [Ca²⁺]i

Intracellular Ca²⁺ mobilization was significantly enhanced by the mixture of FA in human lymphocytes (about 31-fold) when compared to the control group (Fig. 2). The increase in Ca²⁺ levels was sustained during 20 min of kinetic monitoring. Treatment with ASTA was unable to prevent the calcium increase induced by FA. BSA (0.2%) addition was able to partially decrease calcium mobilization probably by chelating free FA.

3.3. ROS and RNS production

To measure intracellular superoxide anion, hydrogen peroxide and nitric oxide production, cells were acutely treated with the FA mixture with or without ASTA as indicated in the material and methods section. As shown in Fig. 3A, the treatment of human lymphocytes with the FA mixture increased the intracellular superoxide anion levels by 135% as compared with the PMA-control group and as assessed by using DHE probe. The addition of ASTA to FA-treated cells promoted a reduction of 20% in superoxide production. Treatment of PMA-control cells with DPI, a NADPH-oxidase inhibitor, totally inhibited superoxide anion production, whereas sodium azide (SA) partially inhibited superoxide anion production. DPI addition in cells treated with fatty acid mixture partially decreased (20%) the superoxide anion production (Fig. 3A).

A similar pattern was observed when DCH-DA probe was used as a general ROS probe (Fig. 3B). An increase of threefold in total ROS production was observed in lymphocytes treated with the FA mixture as compared with PMA-control group. ASTA-treatment decreased the ROS production induced by FA in 20%. Addition of BSA, used as a FA chelating agent, reduced the ROS production in about 32%.

We observed an increase of up to twofold in the production of hydrogen peroxide induced by the FA mixture as compared with control without stimulation. ASTA-treatment partially reduced (23%) the H₂O₂ production observed in FA group as compared with PMA-control group (Fig. 3C).

There was an increase of 97% in NO production after treatment with 0.3 mM of FA as compared with control group without LPS. Treatment of cells with ASTA in the FA group did not prevent the increase caused by the presence of FA. ASTA per se, raises nitric oxide production by 99% as compared with control group without LPS (Fig. 3D). N-acetyl-L-cysteine (NAC) and BSA partially reduced the NO production induced by the FA mixture.

3.4. Antioxidant enzyme activities and oxidative damage

To determine whether the increased levels of ROS induced by the FA mixture can modulate antioxidant status of cells, we evaluated the antioxidant enzyme activities after 24 h of treatment (Table 1). The FA mixture decreased the activity of CAT by 23% and increased the total-SOD activity by 27% as compared to the control

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![Fig. 1](image-url) Proliferation response of human lymphocytes to Con A and LPS-stimulation. Cells (5 × 10⁶/well) were cultured for 48 h with 0.3 mM of the fatty acid mixture (FA) added or not by 2 μM of ASTA. The results are presented as mean ± SEM (four different experiments). *p < 0.05 compared to control group. **p < 0.05 as compared with the cells treated with the fatty acid mixture 0.3 mM.
group. The FA group with ASTA restored total-SOD activity to those of the control group, whereas CAT activity decreased by 71% and GR activity increased by 80% as compared with the control group. Among all front line antioxidant enzymes tested, total-SOD was increased by 52% and GR activity was decreased by 28% due to ASTA treatment.

Oxidative damages in biomolecules were also modulated by the FA mixture. TBARS levels were dramatically increased by treatment of cells with a FA mixture (210%) and ASTA treatment partially restored TBARS levels (112%) as compared with the control cells. Free protein SH-group was decreased in 69% in cells treated with FA. After treatment with 2 μM of ASTA a partial restoring of 41% was observed in thiol content groups. Carbonyl groups were not modulated by the treatment of cells with FA and ASTA (data not shown).

3.5. Glutathione redox status

A significant reduction in the content of GSH and GSSG of 73% and 35%, respectively was observed in lymphocytes treated with 0.3 mM of the mixture compared to the control group. This reduction was not prevented by ASTA addition (Fig 4).

4. Discussion

It has been postulated that FA may influence cells of the immune system, including lymphocytes by modifying cell–membrane composition (Fan et al., 2004; Li et al., 2006), altering intracellular signaling pathways (Gorjao et al., 2007; Lee et al., 2004; Madani et al., 2001; Mizota et al., 2009), proliferation capacity, interleukins release (Nunes et al., 2008; Sacerdote et al., 2005; Verlengia et al., 2004), ROS production (Cury-Boaventura and Curi, 2005; Stentz and Kitabchi, 2006; Otton et al., 2007), gene expression (Verlengia et al., 2004) and calcium mobilization (Otton et al., 2007).

Overall, the mixture of FA used in the present study caused a marked increase in the production of superoxide anion, hydrogen peroxide and nitric oxide, which was accompanied by an increase in total-SOD activity and in levels of TBARS as well as a reduction of catalase, levels of free thiol groups and GSH content. These parameters could be understood as an increase in oxidative stress. The FA mixture also promoted an increase in intracellular Ca²⁺ mobilization and in the proliferative capacity of B-lymphocytes. Treatment of cells with the antioxidant ASTA partially decreased the oxidative stress imposed by the FA mixture.

Ca²⁺ signaling is essential for diverse biological processes. Ca²⁺ ions are especially suited as intracellular second messengers because of the strong homeostatic mechanisms that maintain intracellular free Ca²⁺ concentrations ([Ca²⁺]ᵢ) in resting cells at 100 nM or less. In the face of extracellular Ca²⁺ concentrations ([Ca²⁺]ₒ) that are four orders of magnitude higher (1–2 mM), Cytoplasmic Ca²⁺ concentrations are maintained at low levels primarily through the action of plasma membrane Ca²⁺-ATPases (PMCA) that pump Ca²⁺ out of the cell across the plasma membrane. Additionally, the sarco-endoplasmic reticulum Ca²⁺-ATPases (SERCA) pumps Ca²⁺ into the lumen of the endoplasmic reticulum (ER). In the longer term (hours), sustained Ca²⁺ entry is critical for essentially all responses initiated through T cell, B cell, and Fc receptors, including proliferation and cytokine production by T cells, cytokine production by mast cells and natural killer (NK) cells, differentiation of B cells into plasma cells, and the differentiation of naive T cells into Th1, Th2, and Th17 effectors subtypes (Hogan et al., 2010). As showed in our work, intracellular calcium concentration was exceptionally enhanced and sustained during 20 min of monitoring in cells treated with FA mixture (Fig 2) and addition of ASTA to FA-treated cells was unable to restore calcium to basal levels. At the same time, proliferative capacity of lymphocytes was increased by the presence of FA mixture, and ASTA addition restored proliferative capacity of lymphocytes to control values (Fig 1). Based on this data we are able to suggest that proliferative response of lymphocytes, which is a well-known calcium-dependent process is not the only mechanism involved in this process since ASTA decreased proliferative capacity of cells treated with FA but did not reduce intracellular calcium concentration. It has been shown that ASTA is a potent inhibitor of tyrosine kinases, inhibiting the MAPK pathway, decreasing the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK and MEK pathway, down regulating the NF-κB activation and ERK1/2 and pMSK-1 pathway (Lee et al., 2003; Kim et al., 2010). Whether ASTA is reducing lymphocyte proliferation by inhibiting the phosphorylation of key proteins implicated in the process of lymphocyte proliferation remains to be elucidated.

Concerning NO: calcium also seems not to be involved in both ASTA and FA-induction of its production (Fig 3D) since the treatment of cells with EGTA (used as a calcium chelating agent) was not able to reduce NO levels, which demonstrates that NO production induced by FA and ASTA occurs by activation of iNOS gene expression, which is a calcium-independent isoenzyme. In fact, it has been demonstrated that the saturated FA are potent inducers.
Fig. 3. ROS and RNS production by using DHE (A), DCFH-DA (B), phenol red (C) and Griess reagent (D) in human lymphocytes treated with 0.3 mM of the fatty acid mixture added or not by 2 μM of ASTA, NAC (N-acetylcysteine), BSA (bovine serum albumin) and EGTA (ethylene glycol tetraacetic acid). Cells (5 × 10^5/well) were stimulated with PMA (20 ng/well) or LPS (10 μg/well) when indicated. The results are presented as mean ± SEM from at least four different experiments. (a) p < 0.05 compared to group control without stimulation; (b) p < 0.05 compared to stimulated-control group; (c) p < 0.05 as compared with the cells treated with the fatty acid mixture 0.3 mM.
of activation of the transcription factor NF-κB, through its connection with the Toll like receptor 4 (TLR4) (Lee et al., 2004). When the FA binds to the receptor TLR4, there is an immediate activation of intracellular pathway leading to NF-κB activation and increased gene transcription of iNOS with subsequent increase in NO production. In FA-treated cells with BSA, there was a total inhibition of NO production. Therefore, we can assume that the increase of NO production induced by FA could be due to activation of NF-κB and increased iNOS expression by direct activation of TLR4. It was recently shown by our group that ASTA also increases the production of NO in human lymphocytes and neutrophils (Bolin et al., 2010; Macedo et al., 2010). As previously shown, ASTA was able to reduce the arterial blood pressure mediated by increase of NO production (Hussein et al., 2005). However, ASTA reduced the activation the transcription factor NF-κB and decreased the IL-6 production in microglial cells (Kim et al., 2010). In the current study, ASTA led to an increase in NO production and association of ASTA and FA-treated cells was not able to restore the NO production (Fig 3D). Therefore, we can suggest the ROS production on NO induction, since a slight reduction on ROS production promoted by ASTA also promoted a small reduction in NO levels on FA + ASTA group. In fact, NAC treatment partially reduced the production of NO induced by FA, indicating a partial contribution of ROS in the NO production by FA. Contrasting results were obtained by Choi et al. (2008) which showed astaxanthin inhibiting the production of inflammatory mediators by blocking iNOS and COX-2 activation or by the suppression of iNOS and COX-2 degradation. Then, as in our FA mixture there is a great content of saturated FA and this FA can induce both the activation of TLR4 pathway which in turn activates nuclear transcription factor NFκB by different ways as previously described by other authors (Lee et al., 2004), we can assume there is the activation of TLR4-pathway, with a consequent induction of NFκB, followed by iNOS activation, which culminates in increased NO levels. ASTA was unable to abrogate the NO producing induced by the FA mixture.

Excessive levels of reactive oxygen species not only directly damage cells by oxidizing DNA, protein and lipids, but indirectly damage cells by activating a variety of stress-sensitive intracellular signaling pathways such as NF-κB, p38 MAPK, JNK/SAPK, hexosamine and others. Activation of these pathways results in the increased expression of numerous gene products that may cause cellular damage and play a major role in the etiology of the late complications of diabetes (Newsholme et al., 2007). In our study we showed that the FA mixture caused a large increase in superoxide anion and hydrogen peroxide production as showed by dihydroethidium, DCFH-DA and phenol red assays (Fig 3). This increase in ROS production was accompanied by an increase of damage in lipids and proteins (Table 1), whereas catalase activity and GHS content were decreased. In an attempt to reduce the ROS production induced by the mixture of FA we added ASTA which resulted in a partial reduction of 20% (on average) in ROS production. Many antioxidants are particularly known to provide protection from ROS-mediated cellular damage. This effect is considered to be a defense mechanism against the attack of ROS. In addition, antioxidants have been linked to regulatory functions in cell growth, survival, cytotoxicity, and transformation possibly involving redox regulation and chemical toxicity (Larcombe et al., 2010).

One mechanism to explain the increase in ROS production induced by FA could be by the interaction of polysaturated, saturated and monounsaturated FA, which are present in our FA mixture, with components of the respiratory chain, thereby inhibiting the electron transport chain, when electrons are directly delivered to Complex III, e.g. from succinate. FA strongly enhance complex III-associated superoxide anion generation (Schonfeld and Reiser, 2006; Schonfeld and Wojtczak, 2007). Also, an elevation of intracellular Ca²⁺ induced by increased Ca²⁺ influx through voltage-gated Ca²⁺ channels caused by the FA mixture can stimulate mitochondrial generation of ROS. Moreover, Ca²⁺ via protein kinase C (PKC) activation enhances NADPH oxidase-dependent generation of ROS, and thus induces oxidative stress (Kruman et al., 1998; Morgan et al., 2007; Yu et al., 2006). Interestingly, the high levels of ROS induced by FA were not totally inhibited by DPI (Fig 3A), whereas in PMA-control group there was a reduction on ROS production to basal levels. This phenomenon indicates that not only NADPH-oxidase is involved in ROS production of lymphocytes treated with FA. Furthermore, when SA was used as an electron transport chain inhibitor there was no reduction in ROS production induced by FA (Fig 3A).

In summary, our data suggest that FA induces oxidative stress through increased production of superoxide anion, hydrogen peroxide and NO production, decreasing enzymatic activity of catalase and GSH content and increasing intracellular calcium concentration, which can be involved in increasing B-lymphocyte proliferation. Moreover, the increase in ROS and NO production explains the increase in lipid peroxidation and damage to cell proteins. Our data also show that ASTA can decrease the exacerbated production of ROS induced by FA, but only partially. Based on these results we can conclude that ASTA can partially prevent oxidative damage to human lymphocytes after 24 h of treatment with 0.3 mM of the fatty acid mixture added or not by 2 μM of ASTA. (a) p < 0.05 compared to group control. (b) p < 0.05 as compared with the cells treated with the fatty acid mixture 0.3 mM.

Table 1 Activities of the antioxidant enzymes and oxidative damages in biomolecules.

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<tr>
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<th>Control</th>
<th>FA</th>
<th>FA + ASTA</th>
<th>ASTA</th>
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<td>Total/SOD</td>
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<td>12 ± 0.3</td>
<td>9 ± 0.8**</td>
<td>14 ± 0.9*</td>
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<td>1 ± 0.1**</td>
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<td>38 ± 1.5</td>
<td>25 ± 2.3**</td>
<td>20 ± 2.1**</td>
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<tr>
<td>GR</td>
<td>33 ± 0.9</td>
<td>47 ± 4.5</td>
<td>60 ± 5.2</td>
<td>24 ± 0.8*</td>
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<tr>
<td>TBARS</td>
<td>75 ± 3.6</td>
<td>233 ± 35.4</td>
<td>160 ± 12.9**</td>
<td>131 ± 1.3</td>
</tr>
<tr>
<td>Protein-SH</td>
<td>287 ± 19</td>
<td>89 ± 4.4</td>
<td>167 ± 18.6**</td>
<td>291 ± 19.4</td>
</tr>
</tbody>
</table>

* Total/SOD expressed in (unit mg protein⁻¹).
# Mn/SOD expressed in (unit mg protein⁻¹).
+= CAT expressed in (μmol H₂O₂ min⁻¹ L⁻¹ mg protein⁻¹).
** GPx expressed in (μmol mg protein⁻¹).
% GR expressed in (unit mg protein⁻¹).
! TBARS expressed in (nmol MDA mg protein⁻¹).
& Protein-SH expressed in (μmol-SH mg protein⁻¹).

p < 0.05 as compared with group control.

p < 0.05 as compared with the cells treated with the fatty acid mixture 0.3 mM.
stress in human lymphocytes induced by a fatty acid mixture, probably by blenching/quenching free radical production.

Disclosure statement

All authors of the present manuscript disclose no actual potential conflict of interest including any financial, or other relationships with people or organizations.

Acknowledgements

The authors are grateful to the technical assistance of Fineto J., C., Guerra, B.A., Marin, D.P. and Bolin, P.A. This research is supported by Fundação de Amparo a Pesquisa do Estado de São Paulo – FAPESP (2008/08886-6 and 2007/03334-6), Cruzeiro do Sul University and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Malgras, A., Huet, J.C., Mathieu, J., Kruse, C., Guerra, B.A., Marin, D.P. and Bolin, P.A. This research is supported through grants from the Brazilian Research Fund and the National Council for Scientific and Technological Development (CNPq).


