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

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ABSTRACT

Fatty acids (FA) have been shown to alter leukocyte function, and depending on concentration and type, they can modulate both inflammatory and immune responses. Astaxanthin (ASTA) is a carotenoid that shows notable antioxidant properties. In the present study we propose to evaluate the oxidative stress in human lymphocytes induced by a FA mixture and the possible protective role of ASTA. The present study showed that the FA mixture at 0.3 mM 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, in TBARS levels and a reduction of catalase activity and content of GSH and free thiol groups. The FA mixture also promoted an increase in intracellular Ca^{2+} mobilization and in the proliferative capacity of B-lymphocytes. The addition of ASTA (2 μM) partially decreased the ROS production and TBARS levels and increased the levels of free thiol groups. ASTA decreased the proliferative capacity of cells treated with FA but not by reducing intracellular calcium concentration. Based on these results we can conclude that ASTA can partially prevent oxidative stress in human lymphocytes induced by a fatty acid mixture, probably by blenching/quenching free radical production.

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 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; Larbi 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. Concomitantly, 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

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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 (Atli 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′-rotene-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; Santocono 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 (Fura-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 (1×10^6/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% CO_2 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 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.

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 (5×10^5 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-