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Comparative antioxidant effects of lycopene, apo-10′-lycopenoic acid and apo-14′-lycopenoic acid in human macrophages exposed to H_2O_2 and cigarette smoke extract

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ABSTRACT

Much of the beneficial effects of tomato lycopene in the prevention of chronic diseases has been attributed to its antioxidant properties, which could be mediated by its metabolites and/or oxidation products. However, the biological functions of these lycopene derivatives remain still unknown. In the present study, we evaluated and compared the antioxidant efficacy of the lycopene eccentric cleavage products apo-10′-lycopenoic acid and apo-14′-lycopenoic acid in counteracting the oxidative effects of H_2O_2 and cigarette smoke extract (CSE) in THP-1 macrophages. Both apo-10′-lycopenoic acid and apo-14′-lycopenoic acid were able to inhibit spontaneous and H_2O_2-induced ROS production in a dose-dependent manner. Such an effect was accompanied by an inhibition of MAPK phosphorylation, by NF-κB inactivation, and by inhibition of hsp-70 and hsp-90 expressions. Both apo-lycopenoic acids also decreased CSE-induced ROS production, 8-OHdG formation and reduced the increase in NOX-4 and COX-2 expressions caused by CSE. However, in both the models of oxidative stress, apo-14′-lycopenoic acid was much more potent as an antioxidant than apo-10′-lycopenoic acid, showing antioxidant properties similar to lycopene. These data strongly suggest that apo-lycopenoic acids, and particularly apo-14′-lycopenoic acid, may mediate some of the antioxidant functions of lycopene in cells.

1. Introduction

Lycopene, a major carotenoid from tomatoes and tomato products and a predominant carotenoid in human plasma and tissue, has been shown to be beneficial to human health against cardiovascular diseases and cancer (Rao and Rao, 2007). Although several mechanisms have been implicated in its beneficial effects, including the ability to act as an antioxidant agent (Palozza et al., 2011), to increase cell-cell communication (Zhang et al., 1991) to inhibit mutagenesis and inflammation (Palozza et al., 2010a) to inhibit tumor cell proliferation (Nahum et al., 2006) to enhance apoptosis (Liu et al., 2006; Palozza et al., 2010b) and to improve immune responses (Chew and Park, 2004), the mechanism(s) by which this carotenoid might exert its biological activities and thereby modulate disease processes is still unknown.

Recent research has focused on the metabolic fate of lycopene and the subsequent metabolites created (Carail and Caris-Veyrat, 2006; Khachik et al., 2002). Several reports suggest that the biological activities of lycopene may be mediated, at least in part, by lycopene metabolites and/or its oxidation products (Nagao, 2004; Mein and Wang, 2008; Linnewiel et al., 2009). Lycopene metabolites can possess either more or less activity than lycopene or can have independent functions.

Several products of lycopene have been formed by *in vitro* chemical oxidation (Kim et al., 2001; Caris-Veyrat et al., 2003; dos Anjos Ferreira et al., 2004; Rodriguez and Rodriguez-Amaya, 2009). Some of them have been reported to inhibit cancer cell proliferation (Kim et al., 2001; Aust et al., 2003; Zhang et al., 2003; Kotake-Nara et al., 2002), to induce cell apoptosis (Zhang et al., 2003; Kotake-Nara et al., 2002; Ben-Dor et al., 2001; Ford et al., 2011) and to enhance gap junction communication (Aust

Abbreviations: BHT, butylated hydroxytoluene; CMO2, carotenoid 9′10′-monooxygenase 2; Cox-2, cyclooxygenase-2; CSE, cigarette smoke extract; DCF, dichlorodihydrofluorescein diacetate; ERK1/2, extracellular signal-regulated kinase 1/2; HO-1, heme oxygenase-1; 8-OHdG, 8-hydroxy deoxoguanosine; JNK, Jun N-terminal kinase (JNK); LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinases; NOX, NAD(P)H oxidase-4; NF-κB, nuclear factor-kappaB; Nrf2, nuclear factor E(2)-related factor 2; ROS, reactive oxygen species; tBHQ, *tert*butylhydroquinone; THF, tetrahydrofuran.

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et al., 2003; Stahl et al., 2000) in biological systems. Among the more interesting lycopene metabolites formed by oxidative cleavage of the hydrocarbon chain there are the apo-lycopenoids. These compounds have been found *in vitro* and *in vivo*, in animals fed lycopene-enriched diets. It has been shown that lycopene can be converted into apo-10′-lycopenal by CMO2[1] both *in vitro* and *in vivo* (Kiefer et al., 2001; Hu et al., 2006). Interestingly, apo-lycopenoids can be detected in the lungs of ferrets (Hu et al., 2006) and in the liver of rats (Gajic et al., 2006) after lycopene treatment. Recently, several apo-lycopenals have been isolated in fruits, vegetables, and human plasma (Kopec et al., 2010).

However, although it has been reported that apo-10′-lycopenoic acid acted as chemopreventive agents in cancer (Lian et al., 2007) in hepatic steatosis (Chung et al., 2012; Gouranton et al., 2011) the physiological roles of apo-lycopenoids remain unknown. In particular, there is little evidence to support an antioxidant role of lycopene metabolites. It has been recently provided evidence of a possible indirect antioxidant effect of apo-10′-lycopenoic acid in BEAS-2B cells (Lian and Wang, 2008). The treatment with this compound increased total intracellular glutathione levels and suppressed both endogenous reactive oxygen species (ROS) generation and H_2O_2-induced oxidative damage. Moreover, apo-10'-lycopenoic acid has been reported to induce Nrf2-mediated expression of phase II detoxifying/antioxidant enzymes, including HO-1, NAD(P)H:quinone oxidoreductase 1, glutathione *S*-transferases, and glutamate-cysteine ligases (Lian and Wang, 2008). Recently, several apo-lycopenoids have been also shown to act as *in vitro* antioxidants, being able to inhibit lipid peroxidation of linoleic acid induced by metmyoglobin, although at different extent (Goupy et al., 2012).

In this study, we evaluated and compared, for the first time in a cell model, the antioxidant potency of lycopene, apo-10′-lycopenoic acid and apo-14′-lycopenoic acid (Fig. 1) in inhibiting oxidative stress and modulating redox-sensitive molecular pathways induced by two different sources of free radicals: H_2O_2 and cigarette smoke extract (CSE).

2. Materials and methods

2.1. Cell culture

THP-1 (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI Dutch Modified (Sigma, Milan, Italy) without antibiotics and supplemented with 10% fetal calf serum, non essential aminoacids, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were maintained in log phase by seeding twice a week at density of 3×10^8 cells/L at 37 °C under 5% CO_2/air atmosphere. Purified lycopene (kindly supported by Conesa, Barcelona), apo-10′-lycopenoic acid and apo-14′-lycopenoic acid previously synthesized in our team (Reynaud et al., 2011) were delivered to the cells using THF as a solvent. To avoid the formation of peroxides, the solvent used contained 0.025% BHT. The stock solutions of lycopene and the two apo-lycopenoids in THF were prepared immediately before each experiment. From the stock solutions, aliquots of the compounds were rapidly added to the culture medium to give the final concentrations indicated. The amount of THF added to the cells was not greater than 0.5% (v/v). Control cultures received an amount of THF equal to that present in lycopene- and apo-lycopenoid-treated ones. No differences were found between cells treated with THF and untreated cells in terms of cell number, viability and ROS production. After the addition of the compounds, the medium was not further replaced throughout the experiments. Experiments were routinely carried out on triplicate cultures and three independent experiments were performed on different batches. At the times indicated, cells were harvested and quadruplicate haemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

[1] CMO2 = carotenoid 9′10′-monooxygenase 2; ROS = reactive oxygen species; Nrf2 = nuclear factor E(2)-related factor 2; HO-1 = heme oxygenase-1; CSE = cigarette smoke extract; BHT = butylated hydroxytoluene; THF = tetrahydrofuran; DCF = dichlorodihydrofluorescein diacetate; ERK1/2 = extracellular signal-regulated kinase 1/2; JNK = jun N-terminal kinase (JNK) ; NOX = NAD(P)H oxidase-4; Cox-2 = cyclooxygenase-2; NF-kB = nuclear factor-kappaB; 8-OhdG = 8-hydroxy deoxoguanosine; MAPK = mitogen-activated protein kinases; LDH = lactate dehydrogenase; tBHQ = *tert*butylhydroquinone.

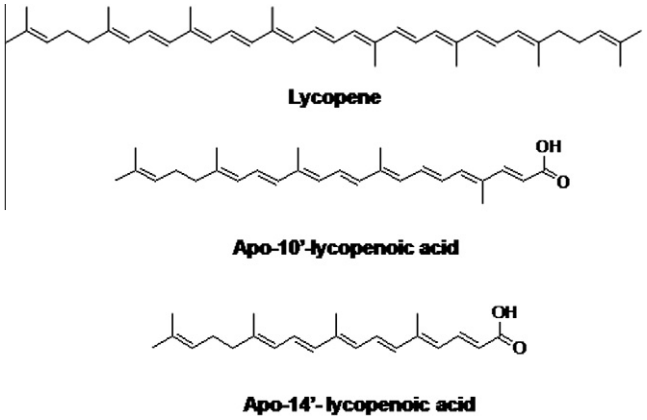

Fig. 1. Chemical structures of lycopene, apo-10′-lycopenoic acid and apo-14′-lycopenoic acid.

2.2. Preparation of aqueous CSE

Research grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). The total particulate matter (TPM) content of 1R3F was 17.1 mg/cigarette, tar (15 mg/cigarette) and nicotine (1.16 mg/cigarette). Prior to each smoke exposure, CSE was freshly prepared within an hour by bubbling smoke from one cigarette at a rate of one cigarette per minute as described previously, using a modification of the method described by Carp and Janoff (1978). This undiluted material was regarded as 100% CSE. A 10% CSE was prepared in a culture medium supplemented with 1% FBS. The pH of the CSE was adjusted to 7.4 and was sterile filtered through a 0.45-μm filter (25-mm Acrodisc; Pall, Ann Arbor, MI). The CSE preparation was standardized by monitoring the absorbance at 320 nm (optical density of 0.74 ± 0.05). The spectral variations observed between different CSE preparations at 320-nm wavelength were found to be less than 10%. Control medium was prepared by bubbling air through culture medium supplemented with 1% FBS, adjusting pH to 7.4, and sterile filtered as described for 10% CSE. The final concentration of CSE was 0.5%. At this concentration, cell viability was always greater than 95% as measured by trypan blue exclusion.

2.3. Measurement of ROS

Cells were harvested to evaluate reactive oxygen species (ROS) production using the di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR) as described (Palozza et al., 2002). Before the addition of the fluorescent probes, 2×10^6 cells were washed to eliminate the amount of lycopene not cell-associated. Fluorescent units were measured in each well after 30 min incubation with DCF (10 μM) by use of a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore Corp., Bedford, MA). Lycopene and the apo-lycopenoic acids did not alter the basal fluorescence of DCF.

2.4. Western blot analysis of p38 and p-p38, ERK1/2, pERK1/2, JNK, p-JNK, p-IKK, p-IkB, IkB, hsp-70, hsp-90, NOX-4 and Cox-2 expression

Cells (10×10^6) were harvested, washed once with ice-cold PBS, and gently lysed for 30 min in ice-cold lysis buffer (1 mM MgCl2, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na4P2O7, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na3VO4, 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4 °C (10,000*g*) to obtain the supernatants, which were used for Western blot analysis. The anti-p-p38 (D-8, Cat. No. SC-7973), anti-p38 (clone C-20, Cat. No. SC-535), anti p-ERK1/2 (E-4, Cat. No. SC-7383), anti-ERK1/2 (clone K-23, Cat. No. SC-94), anti-p-JNK (G-7, Cat. No. SC-6254), anti-JNK (clone C-17, Cat. No. SC-474) and the anti-Cox-2 (clone C-20, Cat. No. 1745) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-hsp70 (clone K-20, catalog No. sc-1060), anti-hsp90α (clone C-20, Cat. No. sc-8262), anti-NOX-4 (clone N-15, Cat. No. sc-21860) and anti-IKKα (clone M280, Cat. No. SC-7182)-polyclonal antibodies were also purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-IkBα and the anti-p-IkBα (Ser32/36) monoclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The blots were washed and exposed to horseradish peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

2.5. Electrophoretic mobility-shift assay

Frozen cell pellets were processed to obtain nuclear extracts. The pellet was treated as indicated in (Lee et al., 2006). Binding reactions containing 5 μg nuclear extracts, 10 mmol/L Tris–HCl (pH 7.6), 5% glycerol, 1 mmol/L EDTA, 1 mmol/L DTT,