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Lipophilic hydroxytyrosol esters significantly improve the oxidative state of human red blood cells

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ABSTRACT

Hydroxytyrosol (HT), a strongly hydrophilic phenol, shows a broad spectrum of biological properties due to its effectiveness as an antioxidant. A set of HT esters with varying acyl chain lengths and lipophilicity were tested to determine their effect on the oxidative state of human erythrocytes. All the esters tested, with the exception of HT stearate, showed a better activity than HT against haemolysis and lipid peroxidation. Maximum antioxidant efficiency was observed when the acyl chain of the HT derivatives was ten or twelve carbon atoms long. This is probably due to a better intercalation of molecules in the nonpolar internal bilayer of the erythrocyte membrane. A simple description of the system in which HT esters interact with the extracellular and membrane compartment of erythrocytes was then proposed. These encouraging results have prompted further investigations on the modulation of the hydrophobicity of HT, a promising tool for developing new potent antioxidants.

1. Introduction

Hydroxytyrosol (HT) is a well known natural antioxidant derived from the enzymatic (Briante et al., 2000) or chemical (Capozzi, Piperno, & Uccella, 2000) hydrolysis of oleuropein, one of the major phenolic compounds present in all parts of the olive tree. HT can also be effectively recovered from olive oil wastewater (Allouche, Fki, & Sayadi, 2004).

Several *in vitro* and *in vivo* studies have highlighted the biological importance of HT, mainly stemming from its capacity to protect cells against oxidative stress (Manna, Galletti, Cucciolla, Montedoro, & Zappia, 1999; Zhu et al., 2010) by scavenging reactive oxygen species (ROS), which may cause oxidation and damage cellular macromolecules. Clear epidemiological and biochemical evidence indicates that this biophenol reduces the risk of coronary heart disease and atherosclerosis (Efentakis et al., 2015; Vilaplana-Pérez, Auñón,

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García-Flores, & Gil-Izquierdo, 2014). Furthermore, HT has shown antimicrobial (Granados-Principal, Quiles, Ramirez-Tortosa, Sanches-Rovira, & Ramirez-Tortosa, 2010), anti-inflammatory (Tutino, Caruso, Messa, Perri, & Notarnicola, 2012), hypotensive (Khayyal et al., 2002) and hypoglycaemic activities (Gonzalez et al., 1992), as well as inhibition of platelet aggregation (Rubio-Senent, de Roos, Duthie, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2014) or induction of apoptosis in HL-60 cells (Sepporta, López-García, Fabiani, Maya, & Fernández-Bolaños, 2013).

The mechanism of action (MoA) of HT is currently associated with the catechol moiety, which prevents damage to the red blood cell (RBC) membrane by donating a hydrogen atom to alkylperoxy radicals (ROO•) formed in the initial step of lipid peroxidation (Visioli, Bellomo, & Galli, 1998).

In light of this considerable body of evidence, the European Food Safety Authority (EFSA) has recently issued a positive opinion regarding the capacity of HT and other olive oil phenols to protect low-density lipoproteins (LDL) against oxidation (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2011). In this context, the biological activities of HT and its affordable recovery from olive oil wastewater (Fernández-Bolaños et al., 2005) have been the focus of attention of many research groups, focusing on this biophenol as a potential functional food ingredient (Fki, Allouche, & Sayadi, 2005).

Accordingly, hydroxytyrosol-enriched sunflower oil was investigated by Vàzquez-Velasco et al. (2011) as functional food to improve certain cardiovascular disease biomarker values. In other studies, HT has been used as an additive with good results in tomato juice (Larrosa, Espín, & Tomás-Barberán, 2003), fish products (Pazos, Alonso, Sánchez, & Medina, 2008) as well as in beverage preparations (Zbakh & El Abbassi, 2012). However, the highly polar nature of HT reduces its solubility in lipophilic preparations, and thus chemically modified HT derivatives with increased lipophilicity were synthesized and tested for their antioxidant activity.

Among synthetic HT derivatives, the synthesis and evaluation of HT esters have been the object of many investigations in recent years because of their interesting antioxidant activity profile (Mateos et al., 2008; Medina, Lois, Alcántara, Lucas, & Morales, 2009; Trujillo et al., 2006). Trujillo et al. (2006) investigated the chemical antioxidant activity of HT esters of C2-C18 fatty acids in different matrices and in a brain homogenate as an *ex vivo* model in order to evaluate the capacity of HT esters to protect proteins and lipids against oxidation. HT esters, such as palmitate, oleate and linoleate, showed a greater capacity with respect to HT to prevent the generation of carbonyl groups in protein in lipid protein oxidation induced by cumene hydroperoxide.

Similar results were reported by Tofani, Balducci, Gasperi, Incerpi, and Gambacorta (2010) testing eleven HT esters of C2-C18 fatty acids using 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays in L6 cells.

Furthermore, we have reported the greater antioxidant capacity of HT laurate (C12) compared to HT against H2O2-induced apoptosis in U937 cells and C2C12 murine myoblasts (Burattini et al., 2013).

In order to gain additional information on the biological activities of HT derivatives as potential functional ingredients to improve the quality and nutritional properties of foods, we evaluated the antioxidant properties of a number of lipophilic HT esters in human erythrocytes. Erythrocytes were selected as a metabolically simplified model system on which we have long-standing expertise (Blasa, Candiracci, Accorsi, Piacentini, & Piatti, 2007).

Erythrocytes are particularly sensitive to oxidative damage due to the high polyunsaturated fatty acid content of their membranes and their high cellular concentrations of haemoglobin (Hb). Hence, they are well suited for the evaluation of the effects of oxidative stress (Ko, Hsiao, & Kuo, 1997). Another advantage of using erythrocytes as oxidizable targets stems from the fact that the results obtained reflect the effective radical-scavenging activity of the antioxidants since protein synthesis is not operative in these anucleate cells. This allowed us to rule out the possibility that the antioxidant activity of HT esters might be promoted by the presence of antioxidant enzymes.

Indeed, Manna et al. (1999) have reported that HT has a protective effect on H_2O_2-induced oxidative alterations in human erythrocytes. According to the authors, under the experimental condition (200 μM H_2O_2) used to induce a moderate haemolysis (~20%), a significant protective effect is observed when the cells are pretreated with 50 μM of HT, whereas a dose of 100 μM completely prevents oxidative damage. In addition, the same concentration of 50 μM HT exerts effective protection against lipid peroxidation, and no increase in TBARS is observable upon pre-incubation with 100 μM of HT.

More recently, similar results have been reported by Paiva-Martins et al. (2009). These authors studied and compared the capacity of four important olive oil polyphenolic compounds, oleuropein, hydroxytyrosol, and the oleuropein aglycones 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA), to protect RBCs from oxidative haemolysis induced by the physiological initiator H_2O_2. At 2% haematocrit and in the presence of H_2O_2 (7.5 mM), all the compounds were shown to protect RBCs from oxidative-induced haemolysis; in particular, HT yielded a 14% haemolysis inhibition.

Along the same lines, different research groups have evaluated the influence of lipophilicity modification on natural antioxidants to expand their application on different lipophilic environments as well as to increase the cellular absorption (Liu, Jin, & Zhang, 2014; Zhong & Shahidi, 2011). In particular, Zhong and Shahidi (2011) have evidenced that the esterification of the water-soluble epigallocatechin gallate (EGCG) with selected long-chain fatty acids improved its lipophilicity and exhibited greater antioxidant activity in scavenging the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical than EGCG itself.

In the present study, a series of HT derivatives (Fig. 1), esterified with different acyl groups (C2-C18), were evaluated for their capacity to inhibit oxidative stress in human RBCs induced by 2,2-azobis(2-amidinopropane hydrochloride) (AAPH) and *tert*-butyl hydroperoxide (t-BOOH). All the HT esters tested, with the exception of HT stearate, were found to be more effective antioxidants than HT and may be suitable to be used as lipophilic functional components in nutraceutical and pharmacological preparations.