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Hydroxytyrosyl ethyl ether exhibits stronger intestinal anticarcinogenic potency and effects on transcript profiles compared to hydroxytyrosol

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ABSTRACT

The anticarcinogenic activity of hydroxytyrosyl ethyl ether (HTy-Et) compared to its precursor hydroxytyrosol (HTy) has been studied in human Caco-2 colon adenocarcinoma cells. 451 and 977 genes were differentially expressed in Caco-2 cells exposed to HTy or HTy-Et for 24 h, respectively, compared with untreated cells ($P < 0.005$; FDR = 0), using Affymetrix microarrays. Results showed that both HTy and HTy-Et inhibited cell proliferation and arrested the cell cycle by up-regulating p21 and CCNG2 and down-regulating CCNB1 protein expression. HTy and HTy-Et also altered the transcription of specific genes involved in apoptosis, as suggested by the up-regulation of BNIP3, BNIP3L, PDCD4 and ATF3 and the activation of caspase-3. Moreover, these polyphenols up-regulated xenobiotic metabolizing enzymes UGT1A10 and CYP1A1, enhancing carcinogen detoxification. In conclusion, these results highlight that HTy and its derivative HTy-Et modulate molecular mechanisms involved in colon cancer, with HTy-Et being more effective than HTy.

1. Introduction

Olive oil has been identified as a major component of the Mediterranean diet that contributes to reduction of the risk of cardiovascular diseases, ageing processes and certain kinds of cancer, mainly breast, colorectal and prostate cancer (López-Miranda et al., 2010; Pauwels, 2011). Several reports have described the beneficial effects of virgin olive oil polyphenols, including reducing *in vivo* lipid oxidative damage (Covas et al., 2006), and improving endothelial dysfunction (Ruano et al., 2005), prothrombotic profiles (Ruano et al., 2007), atherosclerosis progression (Konstantinidou et al., 2010) and inflammatory status (Castañer et al., 2012) in healthy volunteers, as well as in patients with stable cardiovascular diseases or hypercholesterolemia. In this sense, the recent claim on the effectiveness of ingesting olive oil polyphenols (5 mg/d) on protecting LDL from oxidation (EFSA, 2011) is noteworthy. Among olive oil polyphenols, it is important to highlight hydroxytyrosol (HTy, Fig. 1), which is one of the main phenol components in virgin olive oil, largely present as secoiridoid derivatives or as acetate and free forms (Mateos et al., 2001). HTy has shown potent antioxidant, anti-atherogenic, anti-thrombotic and anti-inflammatory properties (Visioli & Bernardini, 2011). More recently, the role of HTy as a chemopreventive anticarcinogenic compound has received major attention. It has been shown that HTy suppresses induced DNA damage in human blood mononuclear cells and HL60 cells (Fabiani et al., 2008a) as well as in HepG2 cells (Li et al., 2012), suggesting that this compound may inhibit cancer by preventing the mutagenic activity caused by oxidative stress. In addition, studies in cell cultures (Bouallagui, Han, Isoda, & Sayadi, 2011; Fabiani et al., 2012; Han, Talorete, Yamada, & Isoda, 2009; Rafehi et al., 2012) and in animal models (Granados-Principal et al., 2011) demonstrate that HTy inhibits cell proliferation, induces apoptosis and also has gene regulatory activities acting as a potential anti-cancer agent. Its ability to reduce colon cancer development, by inhibiting initiation, promotion and metastasis of colon carcinogenesis processes has raised particular interest (Gill et al., 2005; Hashim et al., 2008).

Most phytochemicals with antioxidant activity are hydrophilic. This fact raises the challenge for scientists to provide the industry with fat soluble antioxidants that may be incorporated into lipid food matrices. Hydroxytyrosyl ethers (Madrona et al., 2009) are a promising group of lipophilic compounds obtained from HTy by chemical synthesis, which maintain the *ortho*-diphenolic moiety of HTy intact. In particular, hydroxytyrosyl ethyl ether (HTy-Et) shows higher antioxidant activity (Pereira-Caro et al., 2009), and is more efficiently absorbed across the intestinal epithelial

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Hydroxytyrosol HTy | Hydroxytyrosyl ethyl ether HTy-Et

Fig. 1. Chemical structure of hydroxytyrosol (HTy) and hydroxytyrosyl ethyl ether (HTy-Et).

monolayers than HTy (Pereira-Caro et al., 2010). Furthermore, HTy-Et has the ability to protect human HepG2 cells against oxidative damage induced by t-BOOH, preventing radical formation and modulating antioxidant defences (Pereira-Caro et al., 2011).

Transcriptional profiling using DNA microarrays is a powerful technology to determine changes in gene expression that accompany specific cellular responses induced by food components at a molecular level and to identify genes with potential significance in relation to chemoprevention. The overall aim of the present study was to provide new insights into the chemopreventive effects of HTy compared with its lipophilic derivative, HTy-Et, at physiological concentrations by evaluating changes in gene expression in human adenocarcinoma cells (Caco-2 cells) using transcriptome analysis. In addition, quantitative RT-PCR was used to confirm the microarray results. Transcriptional changes were validated by analysis of cell proliferation using BrdU assay, cell cycle using flow cytometry and apoptosis by means of caspase-3 activity assays.

2. Materials and methods

2.1. Materials

Hydroxytyrosol (HTy) was recovered with 95% purity from olive oil wastewaters following a patented industrial system (Fernández-Bolaños et al., 2005), and further purified by column chromatography. Hydroxytyrosyl ethyl ether (HTy-Et) was prepared by chemical synthesis from HTy (Madrona et al., 2009), further purified by column chromatography to yield practically pure compounds (>98% purity). All reagents were purchased from Invitrogen (Paisley, UK), unless otherwise stated.

2.2. Cell culture

The human Caucasian colon adenocarcinoma cell line Caco-2 (TC7 clonal cells) was kindly provided by Dr. Monique Rousset (INSERM, Paris, France). Caco-2/TC7 cells were grown in 75 cm^2 flasks and maintained in Dulbecco modified Eagle's medium (DMEM) (without phenol red) supplemented with 20% foetal calf serum, 1% (v/v) non-essential amino acids, 2 mmol/L glutamine, 100 IU/mL penicillin and 100 μg/L streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. All cells used were between passages 50 and 60.

2.3. Preparation of test solutions

Standard stock solutions (50, 20 and 10 mM) of HTy and HTy-Et were prepared in 10% DMSO with deionised water. Next, 10 and 100 times diluted solutions were prepared from stock solutions by adding distilled water, yielding a range of concentrations (5000, 2000, 1000, 500, 200, 100, 50, 20 and 10 μM, 1% DMSO), which were ultimately diluted with serum-free DMEM to prepare test solutions (500, 200, 100, 50, 20, 10, 5, 2 and 1 μM, 0.1% of DMSO). Concentrations were checked by HPLC to confirm complete dissolution in aqueous DMEM media.

2.4. Cell proliferation assay (BrdU)

The effects of HTy and HTy-Et on Caco-2 cell proliferation was evaluated using a chemiluminescent immunoassay ELISA kit (Cat No. 11 669 915 001, Roche Applied Science, Germany) based on the measurement of BrdU incorporation into genomic DNA during its synthesis in proliferating cells. Briefly, Caco-2 cells were seeded (10^4 cells per well) in black 96-well plates with clear bottoms and exposed to different concentrations of HTy and HTy-Et (1, 2, 5, 10, 20, 50, 100, 200 and 500 μM) for 24 h in a humidified atmosphere (37 °C, 5% CO_2). Afterwards, cells were labelled by adding BrdU for 4 h and subsequently anti-BrdU antibodies. The immune complexes were detected by the subsequent substrate (tetramethylbenzidine) reaction and quantified by measuring the luminescence using a scanning multiwell spectrophotometer, by microplate reader (BIO-TEK Instruments, Inc., Winooski, VT) after shaking the plate thoroughly for 1 min. Each phenol was tested six times. Results are expressed as the percentage of cell growth referred to untreated cells.

2.5. Quantification of apoptosis using CaspACE™

Apoptosis induced by the treatment of different concentrations of HTy and HTy-Et in Caco-2 cells were evaluated by the measurement of caspase-3 protease activity by using a colorimetric CaspACE™ Assay System kit (G7220, Promega), which used a substrate labelled with the chromophore *p*-nitroaniline (pNA). Briefly, cells were grown in 6-well culture plates (10^4 cells per well) in a humidified atmosphere (37 °C, 5% CO_2). Cells were allowed to adhere to the plate surface for 36 h before being exposed to 2, 10 and 50 μM, of HTy and HTy-Et, respectively for 24 h. Each polyphenol concentration was tested three times. Afterwards, 2 μl of DEVD-pNA substrate (10 mM stock solution) and 20 μM of caspase inhibitor Z-VAD-FMK were added to each well at the same time. Cleavage of the substrate by caspase-3 produced yellow pNA, which was measured spectrophotometrically at 410 nm. Untreated cells and cells treated with 5 μM of camptothecin, which induces apoptosis in Caco-2 cells under these conditions, were used as negative and positive controls, respectively.

2.6. Cell cycle analysis using flow cytometry

Caco-2 cells were seeded in 25 cm^2 flask (10^6 cells) and were allowed to adhere to the flask surface for 36 h before being exposed to different concentrations of HTy and HTy-Et (2, 5, 10, 50, 100 and 200 μM, 0.1% DMSO final concentration) for 24 h. After phenol treatment, cells were washed twice with PBS and collected by trypsinization. Cells were suspended in DMEM supplement with 20% foetal calf serum (FBS), further centrifuged at 200*g* for 6 min at 4 °C and resuspended in 0.5 ml of DMEM supplement with 20% FBS. Subsequently, cells were fixed in 3.5 ml of ice-cold ethanol/PBS (70:30) for 2 h. Fixed cells were centrifuged at 200*g* for 5 min and the pellet was resuspended in 4 ml of PBS and centrifuged again at the same conditions in order to completely remove the ethanol solution. After removing the supernatant, cells were treated with 0.5 ml of 0.1% Triton X-100 in PBS containing DNAse free RNase A (10 mg/ml) and 1 mg/mL propidium iodide (PI, Invitrogen Molecular Probes) for 30 min at 4 °C in the dark, to stain nuclei cells. Samples were filtered through a 50 μm nylon mesh filter and analysed by acquiring 20,000 events using a FACSCalibur flow cytometer (Becton Dickinson, BD) equipped with Cell QuestPro software (BD) for data acquisition and with FlowJo software (Tree