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Brain uptake of hydroxytyrosol and its main circulating metabolites: Protective potential in neuronal cells

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ABSTRACT

Hydroxytyrosol (HT) and oleuropein derivatives are the main phenolic compounds in virgin olive oil (VOO). After VOO intake, HT is extensively metabolized being hydroxytyrosol-sulphate (HT-S) and hydroxytyrosol-acetate sulphate (HT-AC-S) the main circulating metabolites detected in human plasma. The brain uptake and accumulation of HT and its metabolites were observed after 21 days of rat diet supplementation (5 mg/kg rat/day) of HT in its native form or through oleuropein derivatives. To establish their neuroprotective potential HT-S and HT-AC-S were chemically synthetized and their protective effects against oxidative stress at physiological concentrations (10 μM) in neuroblastoma (SH-SY5Y) and dopaminergic (LUHMES) neuronal cells were observed. The sulpho-conjugated HT structures showed a lower protective effect than native HT. Results showed brain accumulation of HT and HT-S, suggesting their neuroprotective activity by the reduction of the oxidative stress at neuronal level.

1. Introduction

Neurodegenerative diseases (NDD) normally begin in the elderly age, caused by a significant brain accumulation of reactive oxygen species (ROS) from unknown causes. NDD produce mitochondrial dysfunction, neuroinflammation and in some pathologies protein aggregation, neuronal death, culminating in brain dysfunction.

Regular consumption of virgin olive oil (VOO), the main lipid source in the Mediterranean diet, is associated with cardiovascular protection (EFSA, 2009). In addition, human interventions support that VOO consumption reduces the NDD prevalence (Rodríguez-Morató et al., 2015), decreasing the stroke incidence (Samieri et al., 2011) and improves human cognitive functions such as: visual memory, memory task, verbal fluency (Berr et al., 2009; Martínez-Lapiscina et al., 2013), frontal and global cognition (Valls-Pedret et al., 2015).

Studies performed in rodents and cell models have associated hydroxytyrosol (HT), the main phenolic compound released from VOO consumption, with the brain protective effects. For instance, Peng et al. (2016) used APP/PS1 mice model to mimic Alzheimer disease and supplemented with 5 mg HT/kg mice/day during 6 months, observing a reduction in brain oxidative stress and neural toxicity. Moreover, Fu & Hu (2016) administrated 100 mg/kg rat/day of HT in a mice model of subarachnoid haemorrhage; After 6 weeks of treatment HT reduced the oxidative stress, decreased the blood brain barrier permeability and increased the expression of the endogenous antioxidant enzymes. In addition, *in vitro* experiments conducted in neuronal cell lines exposed to HT showed a cytoprotective effect in adrenal gland (PC12 cells) by decreasing the DNA damage induced by H_2O_2 (Hashimoto et al., 2004; Young, Wahle, & Boyle, 2008).

The accumulation of HT in the brain tissue could be achieved by two ways, in low concentration as product of dopamine (DA) oxidative metabolism (Goldstein et al., 2016; Schroder et al., 2009), and also, after the intake of dietary products, being olive products the main dietary source of HT (Rodríguez-Morató et al., 2015). It has been

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DA, Dopamine; DOPAC, Dihydroxyphenylacetic acid; DOPAL, 3,4-Dihydroxyphenylacetaldehyde; HVAc, Homovanillic acid; HVAlc, Homovanillic alcohol; HT, Hydroxytyrosol, 3,4-Dihydroxyphenylethanol; HT-S, Hydroxytyrosol Sulphate; HT-AC, Hydroxytyrosol acetate; HT- AC-S, Hydroxytyrosol acetate Sulphate; K_2CO_3, Potassium carbonate; LUHMES, Lund human mesencephalic cells; MedDiet, Mediterranean diet; NDD, Neurodegenerative diseases; OLE, Oleuropein; PD, Parkinson's disease; ROS, reactive oxygen species; *t*-BHP, *tert*-butyl hydroperoxide; VOO, Virgin Olive Oil

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described that HT and its phase II metabolites reach the brain after the acute intake of HT or its derivatives in animal models (D'Angelo et al., 2001; López de Las Hazas et al., 2015; Rubió, Serra, et al., 2012; Rubió, Motilva, Macià, Ramo, & Romero, 2012; Suárez et al., 2011). However, no data exist about the brain uptake of HT and its metabolites after the sustained intake of VOO phenolics.

Previously, we have detected hydroxytyrosol sulphate (HT-S) and hydroxytyrosol acetate sulphate (HT-AC-S), in human plasma after VOO consumption, as the main circulating metabolites of HT (Rubió et al., 2014). However, the biological activities of these phase II metabolites are still unknown at the brain cell level.

The hypothesis of the present work is that both circulating metabolites and VOO phenolic compounds could promote protective effects against neurodegeneration. We report, for the first time the presence of HT metabolites in rat brain after the sustained intake during 21 days of HT in its free form or through its naturally occurring esterified precursors, namely oleuropein (OLE) and its aglycone forms known as secoiridoids (SEC). HT biological metabolites were then obtained by chemical synthesis for assessing its neuroprotective potential. We demonstrated that HT metabolites, found in human plasma, exert a protective effect in different neuronal systems (SH-SY5Y neuroblastoma cell line and LUHMES dopaminergic neuronal cells) in response to an oxidative injury. Additionally, we reveal that neuronal cells are able to further metabolize these compounds.

2. Material and methods

2.1. Reagents

Hydroxytyrosol (HT) and hydroxytyrosol acetate (HT-AC) were purchased from Seprox Biotech (Madrid, Spain), oleuropein (glycosylated form) by Extrasynthese (Genay, France) and homovanillic alcohol (HVAlc) from Carbosynth (Berkishire, UK). Ortophosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Methanol and acetonitrile (HPLC-grade) were acquired from Scharlab (Barcelona, Spain). Milli-Q water was obtained from a Milli-Q water purification system (Millipore Corp., Medford, MA, USA).

Reagents and solvents were purified and dried according to Armarego and Chai (2003). All reactions were carried out under an inert atmosphere (argon) except when the solvents were not dried. The reactions progress was monitored by TLC on aluminium-backed Silica gel (Merck 60 F254. Darmstadt, Germany). Medium pressure preparative column chromatography: Silica Gel Merck 60H. ^{1}H NMR spectra were obtained at 400 MHz in D_2O with chemical shift values (δ) in ppm, and ^{13}C NMR spectra were obtained at 100.61 MHz in the same deuterated solvents. Assignments were supported by 2D correlation NMR studies.

2.2. Animals and experimental procedure

Thirty-two male and female Wistar rats from Charles River Laboratories (Barcelona, Spain) weighing between 300 and 350 g were divided into four groups of 8 rats in each group (4 males, 4 females). Each group was supplemented during 21 days with 5 mg/kg rat/day of HT or HT derivatives. Group A: Control (standard feed); Group B: standard feed supplemented with HT; Group C: standard feed supplemented with secoiridoid extract (SEC), and Group D: standard feed supplemented with oleuropein (OLE). The animals were housed two per cage at a temperature (21 ± 1 °C) and humidity-controlled (55 ± 10%) room with a 12 h light/dark cycle. Food and water were available *ad libitum*. The animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the Animal Ethics Committee of Universitat de Lleida (CEEA 10-06/14, 31st July 2014).

The elaboration of the secoiridoid extract and the preparation of the supplemented diets followed the method previously described (López de las Hazas et al., 2016). Commercial feed pellets (Harlan Laboratories, Madison, WI, USA) were crushed and reconstituted with Milli-Q water containing the equivalent of 5 mg of OLE, SEC or HT/kg rat weight, respectively based on the daily feed consumption of each rat. Prepared pellets were then freeze-dried and stored in a freezer until their use.

After 21 days, rats were anesthetized with isoflurane (IsoFlo, Veterinarian Esteve, Bologna, Italy) and then sacrificed by exsanguination, after that rats were perfused with NaCl (0.9%) during at least 8 min to remove all the blood brain irrigation. Then, brain samples were collected and frozen immediately with liquid nitrogen. Then, the brain samples were lyophilized, and the phenolic content was determined following the method previously described (Serra et al., 2012). Sixty mg of freeze-dried brain sample was mixed with 50 μL of ascorbic acid (1%), 100 μL of phosphoric acid (4%), and 50 μL of catechol (20 ppm as Internal Standard). The samples were treated four times with 400 μL of water/methanol/phosphoric acid 4% (94:4.5:1.5, v/v/v). In each extraction, the sample was sonicated for 30 s maintaining the sample in ice and then centrifuged. Aliquots of 350 μL of supernatant were mixing with 350 μL of phosphoric acid (4%) and centrifugated during 10 min at 8784*g*. Micro-SPE cartridge was conditioned sequentially by using 250 μL of methanol and acidified Milli-Q water. The sample (medium or cellular lysate) was loaded into each preconditioned cartridge, eluted with 100 μL of methanol and directly analysed by AcQuity UPLC™ coupled to PDA detector and a triple quadrupole detector (TQD)™ mass spectrometer (Waters, Milford USA).

2.3. Chromatographic analysis

The chromatographic method employed was previously described (Catalán et al., 2015). The analytical column was a High Strength Silica (HSS) T3 column (100 × 2.1 mm, 1.8 μm). The injection volume was 2.5 μL and the column was kept at 30 °C, the flow rate was 0.3 mL/min using 0.2% acetic acid as solvent A and methanol as solvent B. The elution started at 3% of eluent B and was linearly increased to 15% of eluent B. After 6 min the flow rate of B was linearly increased to 70% during 8 min and further increased to 100% of B for 3 min. Finally, it was returned to the initial conditions in 1 min, and the re-equilibration time was 2 min. Ionization was done by electrospray (ESI) in the negative mode and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters were capillary voltage of 3 KV, source temperature of 150 °C, and desolvation temperature of 400 °C with a flow rate of 800 L/h. Nitrogen (99% purity, N_2.LC-MS nitrogen generator, Claind, Como, Italy) and argon (≥ 99.99% purity, Alphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. Two transitions were acquired for HT and its metabolites, one for quantification and a second for confirmation purposes. The software used was MassLynx 4.1. HT and HT sulphate metabolites were quantified with their own calibration curves.

2.4. Synthesis of sulphate metabolites

2.4.1. General sulphation procedure

HT-AC was used as starting material to obtain HT-S and HT-AC-S metabolites. The sulphation procedure was performed as previously described (Pimpão, Ventura, Ferreira, Williamson, & Santos, 2015). HT-AC (500 mg, 2.5 mmoles) and sulphur trioxide–pyridine (one equivalent) were dissolved in 10 mL of anhydrous pyridine and kept at 65 °C with constant stirring for 24 h. The reaction was quenched by the addition of water. Solvents were removed *in vacuo* and the residue was dissolved in water. The unreacted starting materials were separated by washing with ethyl acetate and the product was purified on a Dowex 50W-X8 ion-exchange resin (Sigma-Aldrich, Sintra, Portugal) loaded with Na^+. The final residue was dried *in vacuo* to give the mixture of