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Role of the catechol group in the antioxidant and neuroprotective effects of virgin olive oil components in rat brain

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Abstract

The aim of the present study was to determine the role of the catechol group in the antioxidant and neuroprotective effects of minor components of virgin olive oil in rat brain tissue. Hydroxytyrosol ethyl ether (HT, 2 OH), tyrosol ethyl ether (Ty, 1 OH) and 3,4-di-*ortho*-methylidene-hydroxytyrosol ethyl ether (MET, no OH) were compared. Oxidative stress was induced with ferrous salts (lipid peroxidation induction), diethylmaleate (depletion of glutathione) and hypoxia-reoxygenation in brain slices. Lipid peroxidation was inhibited in direct proportion to the number of OH groups: HT>Ty>MET. Exposure to HT led to partial recovery of the glutathione system after chemical inhibition or hypoxia-reoxygenation. All three compounds inhibited cell death in hypoxia-reoxygenation experiments (HT≥Ty>MET). Peroxynitrite formation (3-nitrotyrosine) and inflammatory mediators (prostaglandin E_2 and interleukin 1ß) were inhibited by all three compounds. In conclusion, the presence of OH groups in the molecule of these phenolic compounds from virgin olive oil is a determinant factor in their antioxidant effect in brain tissue, but this antioxidant effect is not the only explanation for their neuroprotective effect.

Keywords: Virgin olive oil; Hydroxytyrosol; Neuroprotection; Oxidative stress; Nitric oxide; Interleukins

1. Introduction

The cardioprotective characteristic of the Mediterranean diet is of potential relevance not only in diseases of the heart but also in all those related to the cardiovascular system as well as the nervous system and digestive tract [1]. The Mediterranean diet also contributes to the control of risk factors recognized as incidents in the further development of these diseases [2]. Although other elements or associations between them (particularly nutritional balance) are likely to be involved, the presence of olive oil as the main source of fat in the Mediterranean diet has become a focus of research interest [3]. This has led to studies to determine which olive oil components are mainly responsible for this effect. Research to date has found that the so-called minor components, mainly tyrosol and hydroxytyrosol, seem to exert the beneficial effects of olive oil in cardiovascular phenomena [4].

Related studies were designed to investigate the possible mechanism of action that would explain the cardiovascular effects of these compounds. Their antioxidant potential has been linked directly to their biological actions [5]. In this connection, the antioxidant effect of olive oil phenolic compounds was shown to depend on several factors such as chemical composition, lipid solubility, the ability to scavenge free radicals, the number of atoms that donate hydrogen and free radical-associated chain reactions. There is now clear evidence that the presence of a catechol group with hydroxyl groups (OH) in the 3,4-*ortho* position is involved in the antioxidant activity of these compounds [6].

However, some studies in experimental models have raised questions about the involvement of phenolic compounds, especially with regard to the role of antioxidant mechanisms in biological effects, in light of a hypothetical relationship between chemical structure and activity [7]. Moreover, some minor components of virgin olive oil with weak antioxidant effects, such as tyrosol, have shown a cytoprotective effect [8–10].

The aim of the present study was to determine whether the presence of the catechol group (1,2-dihydroxybenzene) in a series of compounds related to phenolic compounds in olive oil are responsible for their higher antioxidant effect in a biological environment, specifically in brain tissue. As secondary objectives, we aimed to elucidate the possible relationship between the antioxidant and neuroprotective effects of these compounds (possible prevention of cell death and inhibition of biochemical pathways that induce cell injury) and to assess the possible involvement of biochemical mechanisms that supplement the antioxidant effect in the neuroprotective effect of these compounds. For that reason, two phenolic

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compounds present in virgin olive oil have been used (hydroxytyrosol with 2 OH and tyrosol with 1 OH). In order to support the results, a compound (no present in virgin olive oil) with the same chemical structure but without OH was used as negative control (3,4-di-ortho-methylidene-hydroxytyrosol).

2. Materials and methods

2.1. Materials

Lactate dehydrogenase (LDH) reagent kits were obtained from Biosystem SA (Barcelona, Spain). Hydroxytyrosol ethyl ether (HT) (2 OH in the chemical structure), tyrosol ethyl ether (Ty) (1 OH in the chemical structure) and 3,4-di-*ortho*-methylidene-hydroxytyrosol ethyl ether (MET) (no OH in the chemical structure) (Fig. 1) were chemically synthesized according to the methods of Madrona et al. [11].

2.2. Study design

The animals were 2-month-old adult male Wistar rats (body weight 200–250 g). Only male rats were used in order to avoid a possible influence of female sex hormones in brain metabolism. All rats were used in accordance with current Spanish legislation for animal care, use and housing (EDL 2013/80847. BOE-A-2013-6271). The recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 86-23, revised 1985) were followed, as well the Spanish Law on the Protection of Animals, where applicable. The study protocol was approved by the University of Malaga Ethics Committee for the Use of Animals.

Three typed of experiments were carried out: (1) measurement of oxidative stress parameters in oxygenated brain slices; (2) oxidative stress induction with 100 μM of ferrous salts (increase of lipid peroxidation), 10 mM diethylmaleate (DEM; depletion of glutathione) and after hypoxia-reoxygenation (see below); (3) nitrosative stress (nitrite and 3-nitrotyrosine concentrations) and inflammatory mediators (prostaglandin E_2 and interleukin 1ß) induction after hypoxia-reoxygenation.

2.3. Sample processing

Rats were anesthetized with pentobarbital sodium (40 mg/kg ip), then decapitated with a guillotine. The compounds were incubated in the experimental buffer (see below) from the beginning of the experiment.

2.4. In vitro model of rat brain hypoxia-reoxygenation

We used a previously described *in vitro* method of hypoxia-reoxygenation in brain slices [12]. The cortex and midbrain were cut coronally into 1-mm slices with a vibrating microtome (Capdem Instruments, San Francisco, CA, USA). Each slice was placed in 1 ml of buffer (composition in mmol/L: 100 NaCl, 0.05 KCl, 24 $NaHCO_3$, 0.55 KH_2PO_4, 0.005 $CaCl_2$, 2 $MgSO_4$, 9.8 glucose, pH 7.4) and perfused with a mixture of 95% O_2 and 5% CO_2. After 30 min to reach equilibrium, the slices were placed in fresh buffer of the same composition, except that the concentration of $CaCl_2$ was 3 mmol/L, that of $MgSO_4$ was 0.001 mmol/L, and no glucose was included. This solution was perfused with a mixture of 95% N_2 and 5% CO_2 for 20 min (hypoxia). Then the slices were placed in fresh buffer containing glucose, and the solution was perfused with a mixture of 95% O_2 and 5% CO_2 (reoxygenation).

Brain slices or incubation buffer were analyzed: (1) after 30 min of incubation and before N_2 perfusion, (2) after 20 min of perfusion with N_2, and (3) after 180 min of reoxygenation. For all studies, the tissues were quickly frozen in liquid nitrogen and stored at −80°C until the day of the experiment, which was done not more than 7 days after the sample was frozen.

2.5. Analytical techniques

All techniques were run in a single-blind manner; that is, the persons who did the assays were unaware of the origin and nature of the samples.

2.5.1. Lipid peroxidation

To quantify lipid peroxidation in cell membrane-enriched fractions of the tissue samples, we measured thiobarbituric acid reactive substances (TBARSs). Briefly, the tissue was diluted (1:10 wt/vol) in a buffer consisting of (composition in M): 0.1 NaCl, 5×10^{-4} KCl, 3.1×10^{-3} $CaCl_2$, 1×10^{-3} $MgSO_4$, 4.9×10^{-3} glucose, 2.4×10^{-2} Na_2CO_3, 5.5×10^{-4} PO_4H_2K and 0.32 sucrose. The sample was homogenized and centrifuged at 10,000*g* for 15 min at 4°C, and the supernatant was collected and centrifuged again at 12,000*g* for 20 min at 4°C. The resulting pellet was resuspended in the same buffer without sucrose at a proportion appropriate for the determination of lipid peroxide production.

Lipid peroxides were determined by dividing the tissue into 850-μl aliquots and adding 100 μl dilution buffer per tube (noninduced lipid peroxidation) or 100 μl of 100 μM ferrous sulfate (induced lipid peroxidation). The tubes were shaken and incubated at 37°C for 45 min, then 500 μl of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added. The samples were shaken and incubated at 100°C for 15 min, then centrifuged at 2000*g* for 15 min at 4°C. Absorbance of the resulting supernatant was determined spectrophotometrically at 532 nm (FLUOstar-POLAEstar, BMG Labtechnologies, Offenburg, Germany). Blank samples were prepared in an identical manner, except that they were incubated at 4°C in order to avoid TBARS production. A standard curve was carried out using malonaldehyde-diethyl-acetal. The results were expressed as μmoles TBARS per mg protein.

2.5.2. Glutathione levels

Reduced glutathione (GSH) was measured spectrofluorometrically. Brain tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% (vol/vol) phosphoric acid at a proportion of 1:20 (wt/vol), then centrifuged at 13,000*g* for 15 min at 4°C to obtain the supernatant. Duplicate cuvettes were prepared for spectrofluorometry with sodium phosphate buffer (900 μl), the supernatant for each sample (50 μl), and *o*-phthaldehyde (50 μl). Measurements were made at 350-nm excitation and 440-nm emission wavelength. To determine the concentration of oxidized glutathione (GSSG), we incubated the supernatant from each sample with 4 vinylpyridine (200 μl), then proceeded as for as GSH.

2.5.3. LDH assay

Tissue damage was measured by measuring LDH. Enzyme activity was measured spectrophotometrically at 340 nm with a commercial kit (Cytotoxicity Detection Kit; Roche Applied Science, Barcelona, Spain) according to the manufacturer's instructions.

2.5.4. Nitrite+nitrate

As an indirect indicator of overall nitric oxide production, we determined nitrite+nitrate levels in the incubation buffer. One milliliter of buffer was filtered through Ultrafree MC microcentrifuge filters to remove high-molecular-weight substances released by cell lysis. The nitrite+nitrate level was measured with a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA) based on the Griess reaction, after the nitrates were converted to nitrites with nitrate reductase. The levels of nitrite+nitrate were measured spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite according to the manufacturer's instructions.

2.5.5. 3-Nitrotyrosine

Brain tissue was homogenized (1:10 wt/vol) in a buffer containing 100 mM KH_2PO_4/K_2HPO_4 and 0.1% digitonin (pH 7.4). Then the homogenate was centrifuged at 5000*g* for 10 min at 4°C. The amount of 3-nitrotyrosine in the supernatant was measured according to the manufacturer's instructions for the enzyme immunoassay kit.

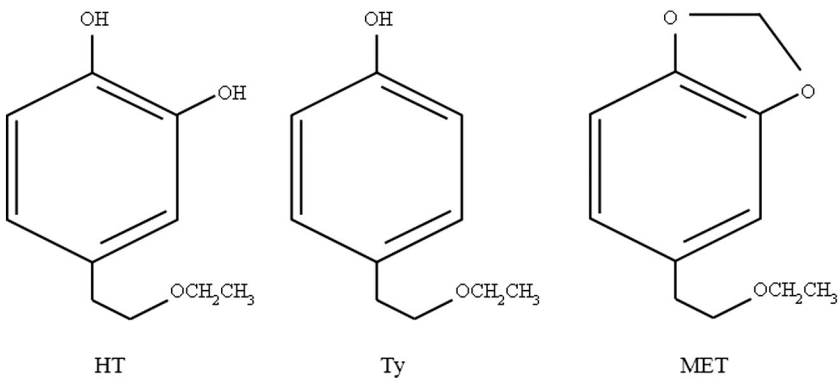

Fig. 1. Chemical structures of HT, Ty and MET.