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Hydroxytyrosyl alkyl ether derivatives inhibit platelet activation after oral administration to rats

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ABSTRACT

The low lipophilicity of hydroxytyrosol (HT) has motivated efforts to synthesize homologous series with better lipid solubility, such as the ethers, which are more lipophilic than HT. Because HT inhibits platelet aggregation, the aim of the study was to assess the possible anti-platelet effect of five HT ether derivatives (ethyl, butyl, hexyl, octyl and dodecyl) after oral administration to rats. Whole blood collagen-induced platelet aggregation and calcium-induced thromboxane B_2 (TxB_2), aortic 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and nitrites + nitrates, plasma concentration of lipid peroxides (TBARS) and red blood cell content of reduced glutathione (GSH) were measured. The administration of 20 mg/kg/day inhibited platelet aggregation, TxB_2 and TBARS in a non-linear manner related to the length of the carbon chain, with a cut-off effect in the hexyl derivative. Aortic nitrite and red blood cell GSH production were also increased. The aortic production of 6-keto-$PGF_{1\alpha}$ was unaltered except in the group treated with the dodecyl derivative. The administration of 50 mg/kg/day showed a similar pharmacodynamic profile but without the non-linear effect. In conclusion, HT ethers, especially the hexyl derivative, are a potential alternative to hydroxytyrosol, and their effect merits additional research to determine their role in the prophylaxis of vascular disease.

1. Introduction

There is solid evidence of the effect of the Mediterranean diet in preventing cardiovascular disease (de Lorgeril and Salen, 2011; Martínez-González et al., 2012; van de Laar et al., 2012). The important contribution of virgin olive oil as the main source of fat in the diet has also been documented, and olive oil is known to influence the cardioprotective effects of the Mediterranean diet (Buckland et al., 2012; Urpi-Sarda et al., 2012). Among the components of virgin olive oil, phenolic compounds play an important role in most of the recognized effects of this edible oil (Delgado-Lista et al., 2011; Lou-Bonafonte et al., 2012). Hydroxytyrosol (HT) and hydroxytyrosol acetate (HTa), in particular, exert several effects on pathways of vascular damage such as the oxidation of LDL cholesterol (Covas et al., 2006), vascular oxidative stress (Zrelli et al., 2011), inflammatory pathways (González-Correa et al., 2009; Richard et al., 2011) and platelet aggregation (Dell'Agli et al., 2008; González-Correa et al., 2008, 2009; Petroni et al., 1995), among others.

The low lipophilicity of HT has motivated attempts to synthesize homologous series with increased lipid solubility. Among these derivatives are the esters and ethers, which are more lipophilic than HT (Madrona et al., 2009; Mateos et al., 2008; Trujillo et al., 2006). Previous studies showed that HTa (present in virgin olive oil) exerts an anti-platelet effect both *in vitro* (González-Correa et al., 2009) and after administration to experimental animals (González-Correa et al., 2008). Hydroxytyrosol ethers are more chemically stable compounds (Pereira-Caro et al., 2012) with greater lipid solubility than HT (Pereira-Caro et al., 2010a, 2010b). Moreover, HT ethers have a cytoprotective (Guerrero et al., 2012; Muñoz-Marín et al., 2012; Pereira-Caro et al., 2011) and inhibitory effect on platelet aggregation *in vitro*, which was greater than the inhibitory effect of HT (Reyes et al., 2013). These laboratory findings raised the possibility that these effects also occur *in vivo* after oral administration to experimental animals.

The aim of this study was to determine the potential anti-platelet effect of some etherified derivatives of HT after oral

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administration to healthy rats. Our secondary objectives were to evaluate the possible influence of these compounds on vascular endogenous anti-platelet pathways (prostacyclin and nitric oxide), and to characterize the relationship between the length of the hydrocarbon chain of each compound and its anti-platelet effect.

2. Materials and methods

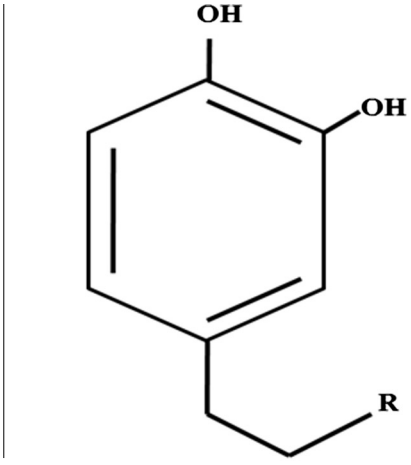
2.1. Materials

Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ enzyme immunoassay kits were from GE Healthcare (Barcelona, Spain). The nitrite/nitrate ELISA kit and HT were obtained from Cayman Chemical (Ann Arbor, MI, USA). Collagen was obtained from Menarini Diagnóstica (Barcelona, Spain). All other reagents were from Sigma Chemical Corp. (St. Louis, MO, USA). Alkyl HT ethers were chemically synthesized from HT according to the methods of Madrona et al. (2009) (Fig. 1).

2.2. Study design

Male Wistar rats weighing 200–250 g were used. All animals were housed at the University of Malaga Centro de Experimentación Animal under standard temperature and light/dark conditions, with access to food and water ad libitum. The rats were used in accordance with current Spanish legislation for animal care, use and housing (RD 223/1998, based on European Directive 86/609/CEE). The recommendations in Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) were followed, as was the Spanish Law on the Protection of Animals where applicable.

A total of 13 groups of animals (N = 10 animals per group) were used. Rats in the control group were given isotonic saline solution p.o., and the treated groups received HT or its ethyl, butyl, hexyl, octyl or dodecyl alkyl ether derivative. Two doses were used in each group: 20 and 50 mg/kg/day p.o. These doses were chosen on the basis of results in a previous study with HT and HT acetate in the same experimental model (González-Correa et al., 2008). All compounds were given once per day for 7 days via an endogastric cannula at 10:00 h. The last dose was given 1 h before the animals were killed.



R	Name
OH	Hydroxytyrosol (HT)
OC_2H_5	HT ethyl ether
OC_4H_9	HT butyl ether
OC_6H_{13}	HT hexyl ether
OC_8H_{17}	HT octyl ether
$OC_{12}H_{25}$	HT dodecyl ether

Fig. 1. Chemical structures of hydroxytyrosol and hydroxytyrosyl alkyl ethers.

At the end of the treatment period all rats were anaesthetized with 40 mg/kg sodium pentobarbital i.p. and immobilized in a supine position. The abdominal cavity was opened, the intestinal packet was moved to the right side, and the abdominal aorta was dissected at the level of its bifurcation into the iliac arteries. Blood was obtained via puncture of the abdominal aorta with 3.8% sodium citrate at a proportion of 1:10 as the anti-coagulant. Part of the blood sample was centrifuged at 1500g for 25 min at 25 °C; the plasma was frozen at −80 °C until laboratory analysis for biochemical parameters.

The thoracic and abdominal portions of the aorta were carefully washed in isotonic saline solution and placed in containers with buffer consisting of NaCl (100 mM), KCl (4 mM), $NaHCO_3$ (25 mM), Na_2SO_4 (2.1 mM), sodium citrate (20 mM), glucose (2.7 mM) and Tris (50 mM) (pH 8.3).

2.3. Laboratory analyses

Samples of blood, plasma and aorta were used in the following analyses:

2.3.1. Platelet aggregation

We tested whole blood with 3.8% sodium citrate at a proportion of 1:10 by electric impedance with a Chrono-Log 540 aggregometer (Chrono-Log Corp., Haverton, PA, USA), using collagen (10 μg/mL) to induce platelet aggregation. After 10 min of aggregation at 37 °C we measured maximum aggregation as the maximum change in electric impedance after the addition of collagen to the sample.

2.3.2. Platelet thromboxane B_2 production

We incubated samples of whole blood with calcium A23187 ionophore (10 μM) for 30 min at 37 °C. Samples were then centrifuged at 10 000g for 3 min at 4 °C, and the supernatant was frozen at −80 °C until use to measure thromboxane B2 (stable metabolite of thromboxane A2) by enzyme immunoassay.

2.3.3. Vascular 6-keto-$PGF_{1\alpha}$ production

A segment of the thoracic aorta measuring approximately 50 mg was incubated in fresh buffer at 37 °C for 3 min, after which 6-keto-$PGF_{1\alpha}$ production was induced with 10 μM calcium ionophore A23187 for 30 min. After this period the arterial tissue was weighed and the supernatant was frozen at −80 °C until use to measure 6-keto-$PGF_{1\alpha}$ by enzyme immunoassay.

2.3.4. Vascular nitric oxide production

The concentration of nitrite + nitrate was quantified as an indirect indicator of nitric oxide (NO) production. A segment of the thoracic aorta measuring approximately 50 mg was incubated in fresh buffer at 37 °C for 3 min, after which NO production was induced with 10 μM calcium ionophore A23187 for 30 min. After this period the arterial tissue was weighed and the supernatant was frozen at −80 °C until use to measure nitrite + nitrate by enzyme immunoassay.

2.3.5. Plasma lipid peroxidation

Thiobarbituric acid reactive substances (TBARSs) were measured as an index of plasma lipid peroxide concentration. Samples of plasma were incubated with 500 μL 0.5% thiobarbituric acid in 20% trichloroacetic acid. The samples were shaken and incubated at 100 °C for 15 min, then centrifuged at 2000g for 15 min at 4 °C. Absorbance of the resulting supernatant was determined spectrophotometrically at 532 nm (FluoStar, 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.

2.3.6. Glutathione levels

Reduced glutathione (GSH) was measured in red blood cells after they were homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid, then centrifuged at 13,000g for 15 min at 4 °C to obtain the supernatant. Duplicate cuvettes were prepared for spectrofluorometry with sodium phosphate buffer, the supernatant for each sample, and o-phthaldehyde. Measurements were made at 350 nm excitation wavelength and 440 nm emission wavelength.

2.4. Statistical analysis

The data in the text, tables and figures are expressed as the mean ± standard error of 10 experiments. All statistical analyses (ANOVA followed by Bonferroni transformation) were done with the Statistical Program for Social Sciences v. 20.0 (SPSS Co., Chicago, IL, USA), and differences were considered significant when $P < 0.05$.

3. Results

The administration of HT ether derivatives reduced the maximum intensity of collagen-induced platelet aggregation (Fig. 2).