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Cytoprotective Effect of Hydroxytyrosyl Alkyl Ether Derivatives after Oral Administration to Rats in a Model of Glucose–Oxygen Deprivation in Brain Slices

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ABSTRACT: This study was designed to determine whether the oral administration of hydroxytyrosol (HT) alkyl ether derivatives has a neuroprotective effect in rats. The animals were treated for 7 days with HT or ethyl, butyl, hexyl, octyl, and dodecyl HT ether. A method of in vitro hypoxia–reoxygenation in brain slices was used. Hexyl, octyl, and dodecyl HT derivatives reduced brain cell death (LDH efflux). Lipid peroxidation and nitrite concentrations were inhibited most by hexyl, octyl, and dodecyl derivatives. Concentrations of 3-nitrotyrosine were reduced by HT butyl, hexyl, octyl, and dodecyl ether derivatives. Interleukin-1β was significantly reduced in brain slices from rats treated with all HT ether derivatives. LDH efflux showed a linear correlation with brain concentrations of lipid peroxides, nitrites plus nitrates, and interleukin 1β. The reduction in oxidative and nitrosative stress and decreased production of pro-inflammatory interleukins may be the basis for the observed neuroprotective effects.

KEYWORDS: *hydroxytyrosol alkyl ethers, neuroprotection, oxidative stress, nitrosative stress, interleukins*

INTRODUCTION

Because of their wide-ranging biological activities, phenolic compounds are the main components responsible for the cardiovascular benefits of virgin olive oil.[1–3] Hydroxytyrosol (3,4-dihydroxyphenylethanol) (HT) is an *o*-diphenolic compound present in virgin olive oil either as secoiridoid derivatives[4] or as an acetate ester.[5] Several studies have shown that olive oil can prevent cardiovascular disease through different mechanisms including a reduction in low-density lipoprotein oxidation[6] and inhibition of platelet aggregation.[7,8] Olive oil also has anti-inflammatory activities[9] and protective effects against induced oxidative stress.[10]

One of the most important consequences of cardiovascular events is cerebrovascular stroke. Virgin olive oil was shown to have a neuroprotective effect against this type of stroke in healthy rats.[11] Moreover, this protective effect has also been described for pure HT, HT acetate,[12] and HT extract prepared from olive mill wastewater.[13] Virgin olive oil intake supplies not only HT but also other antioxidant compounds that could exert a synergistic effect with HT itself. However, the studies with HT have been conducted for the possible future development of a compound from a natural source with potential use in the prevention of cardiovascular disease in humans. This requires increasing the lipophilicity of HT to try to increase the amount of product to interact with its molecular target in the cells. These conditions have led to efforts to synthesize HT derivatives with a better hydrophile/lipophile balance to improve their pharmacodynamic profile and increase their bioavailability. A series of lipophilic derivatives (alkyl HT ethers) of HT have recently been synthesized[14] and found to be stable when digested in vitro, rapidly absorbed, and partially metabolized by Caco-2/TC7 cells.[15] From 40 to 85% of the compound transferred across the enterocyte monolayer is not metabolized and would be expected to reach the portal blood, and subsequently the liver, unmodified. These compounds are absorbed well in human hepatoma cells.[16]

In a previous study we demonstrated the neuroprotective effect of some HT alkyl ether derivatives after incubation in vitro in rat brain slices.[17] The aim of this study was to determine whether this effect occurs after the oral administration of these compounds to rats.

MATERIALS AND METHODS

Chemicals. Lactate dehydrogenase (LDH) reagent kits were obtained from Biosystem SA (Barcelona, Spain). Interleukin 1β, interleukin 10, and 3-nitrotyrosine enzyme immunoassay kits were from GE Healthcare (Barcelona, Spain). Nitrite/nitrate enzyme immunoassay kits were obtained from Cayman Chemical (Ann Arbor, MI, USA). 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.[14] (Figure 1) and supplied by the Departament of Organic and Pharmaceutic Chemistry, School of Pharmacy, University of Seville (Spain).

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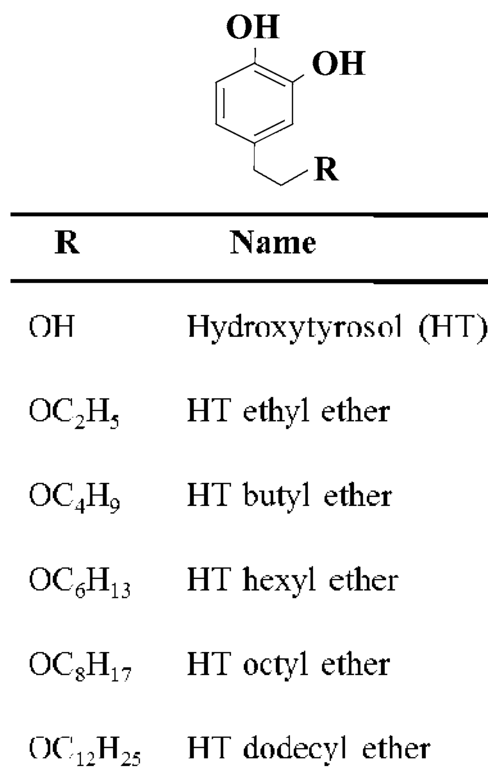

R	Name
OH	Hydroxytyrosol (HT)
OC_2H_5	HT ethyl ether
OC_4H_9	HT butyl ether
OC_6H_{13}	HT hexyl ether
OC_8H_{17}	HT octyl ether
$OC_{12}H_{25}$	HT dodecyl ether

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

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 86-23, revised 1985) were followed, as was the Spanish Law on the Protection of Animals when applicable. The study protocol was approved by the University of Malaga Ethics Committee for the Use of Animals.

A total of seven groups of animals ($N = 6$ animals per group) were used: a control group (treated with isotonic saline solution po), a group treated with HT (20 mg/kg/day po), and five groups treated with HT alkyl ethers (ethyl, butyl, hexyl, octyl, and dodecyl HT ether) at a dose of 20 mg/kg/day po. This dose was chosen according to the results obtained in a previous study with HT and HT acetate in the same experimental model.[12] All compounds were given once per day for 7 days via an endogastric cannula at 10:00 a.m. The last dose was given 1 h before the animals were killed.

At the end of the treatment period all rats were anesthetized with 40 mg/kg of sodium pentobarbital ip and then killed by decapitation. We used a previously described method of in vitro hypoxia−-reoxygenation in brain slices[12] with some modifications. Brains were isolated, the cerebellum and brain stem were discarded, and the remaining tissue was cut transversally into 0.1 mm slices with a vibrating microtome (Capdem Instruments, San Francisco, CA, USA). The slices were placed in buffer (composition in M: 0.1 NaCl, 5×10^{-4} KCl, 2.4×10^{-2} $NaHCO_3$, 5.5×10^{-4} KH_2PO_4, 5×10^{-6} $CaCl_2$, 2×10^{-3} $MgSO_4$, 9.8×10^{-3} 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 3×10^{-3} M, that of $MgSO_4$ was 1×10^{-6} M, and no glucose was included. This solution was perfused with a mixture of 95% N_2 and 5% O_2 for 60 min (hypoxia). Then the slices were placed in fresh buffer containing glucose, and the solution was perfused with a mixture of 95% oxygen and 5% CO_2 (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 within 7 days of freezing.

Analytical Techniques. *Lactate Dehydrogenase Assay.* Tissue damage was measured by examining LDH efflux to the incubation solution. Samples of this solution were obtained every 30 min, and enzyme activity was measured spectrophotometrically by observing oxidation of NADH (decrease in absorbance) in the presence of pyruvate with a manufactured kit (Roche Applied Science, Barcelona, Spain).

Lipid Peroxidation. To quantify lipid peroxidation in cell membrane-enriched fractions of the tissue samples, we measured thiobarbituric acid reactive substances (TBARS). Briefly, the tissue was diluted (1:10 wt/vol) in a buffer consisting of 0.1 M NaCl, 5×10^{-4} M KCl, 3.1×10^{-3} M $CaCl_2$, 1×10^{-3} M $MgSO_4$, 4.9×10^{-3} M glucose, 2.4×10^{-2} M Na_2CO_3, 5.5×10^{-4} M PO_4H_2K, and 0.32 M sucrose. The sample was homogenized and centrifuged at 10000*g* for 15 min at 4 °C, and the supernatant was collected and centrifuged again at 12000*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 of dilution buffer per tube (noninduced lipid peroxidation) or 100 μL of ferrous sulfate (induced lipid peroxidation). The tubes were shaken and incubated at 37 °C for 45 min, and 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 and then centrifuged at 2000*g* for 15 min at 4 °C. Absorbance of the resulting supernatant was determined spectrophotometrically at 532 nm (Perkin-Elmer C-532001 spectrophotometer, Waltham, MA, USA). Blank samples were prepared in an identical manner except that they were incubated at 4 °C to avoid TBARS production. The results were expressed as micromoles of TBARS per milligram of protein; the latter was determined accordig to the method of Bradford.[18]

Glutathione Levels. Glutathione concentration was measured spectrofluorometrically according to the technique described by Hissin and Hill.[19] Brain tissue was homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid at a proportion of 1:20 and then centrifuged at 13000*g* 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 and 440 nm emission wavelength. To determine the proportions of oxidized and reduced glutathione, the supernatant from each sample was incubated with 4 vinylpyridine and then continued as for as total glutathione.

Nitrite + Nitrate Concentration. 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 Co.) based on the Griess reaction, after the nitrates were converted to nitrites with nitrate reductase. Levels of nitrite and nitrate were determined spectrophotometrically at 540 nm and compared with a standard curve obtained with sodium nitrite.

3-Nitrotyrosine Determination. Brain tissue was homogenized (1:10 w/v) 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.

Interleukin Determination. The tissue was homogenized in a volume of 1 mL of a buffer (4 °C, pH 7.2) containing 50 mM Tris, 1 mM EDTA, 6 mM $MgCl_2$, 1 mM phenylmethanesufonyl fluoride, 5 μg/mL leupeptin, 1 mg/mL antipain, 1 mg/mL aprotinin, and 1 mg/mL soybean trypsin inhibitor. After mixing, the samples were transferred to silicone tubes and then sonicated (VC-50T Vibracell,

Sonics Materials Inc., Newtown, CT, USA) for 5−10 s and centrifuged at 14000*g* for 10 min at 4 °C. Then the supernatant was collected and processed according to the instructions for the commercial kits for interleukin 1*β* and interleukin 10 determinations.

Statistical Analysis. The data in the text, tables, and figures represent the mean ± standard error of the mean of six animals per group. The results were analyzed statistically with v. 19.0 of the SPSS personal computer program (Statistical Program for Social Sciences, licensed by the Central Computer Services at the University of Malaga). The significance of the differences between drugs with respect to the control group was determined with Student's *t* test for independent samples. We used analysis of variance to determine the differences between groups for different alkyl ethers derivatives. To determine the possible relationship between mechanisms of action and the neuroprotective capacity of the compounds tested here, we calculated individual Pearson correlation coefficients. In all cases, statistical significance was assumed at a value of $p < 0.05$.

RESULTS

After the reoxygenation period, all variables quantified here in brain slices changed significantly with respect to control samples that were subjected to oxygenation throughout the experimental period (Table 1).

Table 1. Mean Values of the Variables Quantified in Rat Brain Slices Subjected to Hypoxia−Reoxygenation at the End of Reoxygenation, Compared to Control Slices[a]

	oxygenation ($N = 6$)	hypoxia−reoxygenation ($N = 6$)
LDH (arbitrary units)	0.11 ± 0.02	3.00 ± 0.31*
TBARS (nmol/mg protein)	1.20 ± 0.11	3.18 ± 0.36*
GSH (μmol/g tissue)	9.91 ± 0.95	7.63 ± 0.27*
%GSSG with respect to GSH +GSSG	19.65 ± 2.04	25.60 ± 1.04*
NO^-_2 + NO^-_3 (μmol/0.1 g tissue)	10.10 ± 1.20	22.10 ± 1.50*
3-nitrotyrosine (nmol/0.1 g tissue)	0.04 ± 0.01	2.12 ± 0.58*
IL-1*β* (pg/0.1 g tissue)	5.80 ± 0.90	19.40 ± 0.95*
IL-10 (pg/0.1 g tissue)	11.02 ± 0.96	8.41 ± 0.53*

[a]LDH, lactate dehydrogenase; TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; GSSG, oxidized glutathione; NO^-_2 + NO^-_3, nitrites + nitrates; IL-1*β*, interleukin 1*β*; IL-10, interleukin 10; *, $p < 0.05$ with respect to oxygenation.

The administration of HT alkyl ethers to rats reduced brain cell death in all experimental periods of hypoxia−reoxygenation. After the reoxygenation period, LDH efflux was significantly reduced (in comparison to brain slices from nontreated rats) only by HT (26.60%), HT hexyl (46.13%), HT octyl (45.33%), and HT dodecyl ether derivatives (66.00%) (Figure 2).

With regard to oxidative stress variables, lipid peroxidation was inhibited after the reoxygenation period in brain slices from rats treated with all compounds (Table 2). The hexyl, octyl, and dodecyl derivatives had the greatest inhibitory effect on TBARS concentration (74.52, 79.24, and 84.90%, respectively, compared to the control group; $p < 0.05$ with respect to HT and HT ethyl and butyl ether derivatives).

None of the HT alkyl ether derivatives led to significant changes in either reduced glutathione concentrations (GSH) or percentage oxidized glutathione (Table 2).

With regard to nitrosative stress, brain slice production of nitrites plus nitrates, as an index of nitric oxide accumulation after reoxygenation, was significantly lower in samples from

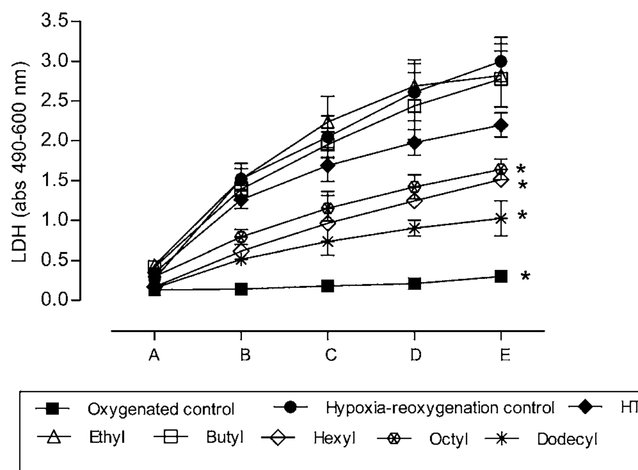

Figure 2. Lactate dehydrogenase efflux measured in different periods in the experimental hypoxia−reoxygenation model: (A) equilibration period; (B) hypoxia; (C) 1 h of reoxygenation; (D) 2 h of reoxygenation; (E) 3 h of reoxygenation. *, $p < 0.05$ with respect to hypoxia−reoxygenation control samples.

animals treated with HT hexyl (23.42% inhibition), octyl (26.77% inhibition), and dodecyl ether derivatives (31.08% inhibition) (Table 2). Concentrations of 3-nitrotyrosine, an indirect index of peroxynitrite formation, were reduced after the reoxygenation period in brain slices from rats treated with HT butyl (46.22%), hexyl (50.00%), octyl (40.56%), and dodecyl ether derivatives (67.45%) (Table 2).

Interleukin-1*β* concentrations after reoxygenation in brain slices from rats treated with all HT ether derivatives were significantly lower than in the control group. The percentages of inhibition were 20.16% with HT, 16.85% with ethyl, 31.19% with butyl, 45.90% with hexyl, 39.81% with octyl, and 48.42% with the dodecyl HT derivative (Table 2). None of HT ether derivatives modified the concentration of interleukin 10 after reoxygenation (Table 2).

There were significant linear correlations between LDH efflux after reoxygenation and brain slice concentrations of TBARS, nitrites plus nitrates, and interleukin 1*β* (Figure 3). None of the correlations for other variables were statistically significant.

DISCUSSION

Our results show that the oral administration of long-carbon-chain HT alkyl ethers (6, 8, and 12 carbon atoms) has a neuroprotective effect, which decreases sensitivity to tissue injury after hypoxia−reoxygenation in a rat brain slice model.

The neuroprotective effect of HT and some ester derivatives has been previously demonstrated in models of chemically induced neurotoxicity with oxidizing agents,[13] as well as models of hypoxia−reoxygenation such as that used in this study.[8] In this connection, the administration of virgin olive oil rich in HT also exerted a neuroprotective effect in this experimental model, as well as in healthy animals and in a model of experimental diabetes.[11,20]

The neuroprotective effect of these compounds has been directly related to their antioxidant effect in different tissues, as occurs with other polyphenols derived from various types of food.[21] However, other possible mechanisms may complement this main effect, such as inhibition of the inflammatory process or an effect on the nitric oxide pathway.[22]

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Table 2. Mean Values of Each Variable at the End of the Reoxygenation Period[a]

	control	HT	ethyl	butyl	hexyl	octyl	dodecyl
TBARS (nmol/mg protein)	3.18 ± 0.36	2.29 ± 0.13*	2.48 ± 0.16*	2.49 ± 0.19*	0.81 ± 0.11*†	0.66 ± 0.05*†	0.48 ± 0.03*†
GSH (μmol/g tissue)	7.63 ± 0.27	7.77 ± 0.56	7.21 ± 0.21	6.99 ± 0.19	7.12 ± 0.62	7.09 ± 0.53	7.16 ± 0.51
%GSSG with respect to GSH + GSSG	25.60 ± 1.04	29.25 ± 2.96	26.57 ± 0.53	24.12 ± 1.86	25.00 ± 1.86	25.56 ± 1.47	24.67 ± 1.74
$NO^-_2 + NO^-_3$ (μmol/0.1 g tissue)	22.71 ± 1.91	20.73 ± 2.66	21.31 ± 1.41	22.93 ± 1.62	17.39 ± 1.83*	16.63 ± 1.59*†	15.65 ± 12.02*†
3-NTy (nmol/0.1 g tissue)	2.12 ± 0.58	2.01 ± 0.44	2.15 ± 0.54	1.14 ± 0.23*††	1.06 ± 0.09*†	1.26 ± 0.12*†	0.69 ± 0.08*†
IL-1β (pg/0.1 g tissue)	19.04 ± 0.95	15.20 ± 1.30*	15.83 ± 0.88*	13.10 ± 0.84*	10.93 ± 0.64*†	11.46 ± 0.44*†	9.82 ± 0.50*†
IL-10 (pg/0.1 g tissue)	8.41 ± 0.53	8.49 ± 1.32	8.37 ± 0.90	9.26 ± 1.22	9.54 ± 1.13	9.66 ± 0.71†	9.65 ± 0.62†

[a] N = 6 samples per group. HT, hydroxytyrosol; TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; GSSG, oxidized glutathione; $NO^-_2 + NO^-_3$, nitrites + nitrates; 3-NTy, 3-nitrotyrosine; IL-1β, interleukin 1β; IL-10, interleukin 10; *, $p < 0.05$ with respect to the control samples; †, $p < 0.05$ with respect to HT, ethyl, butyl, and hexyl; ††, $p < 0.05$ with respect to HT, ethyl and dodecyl.

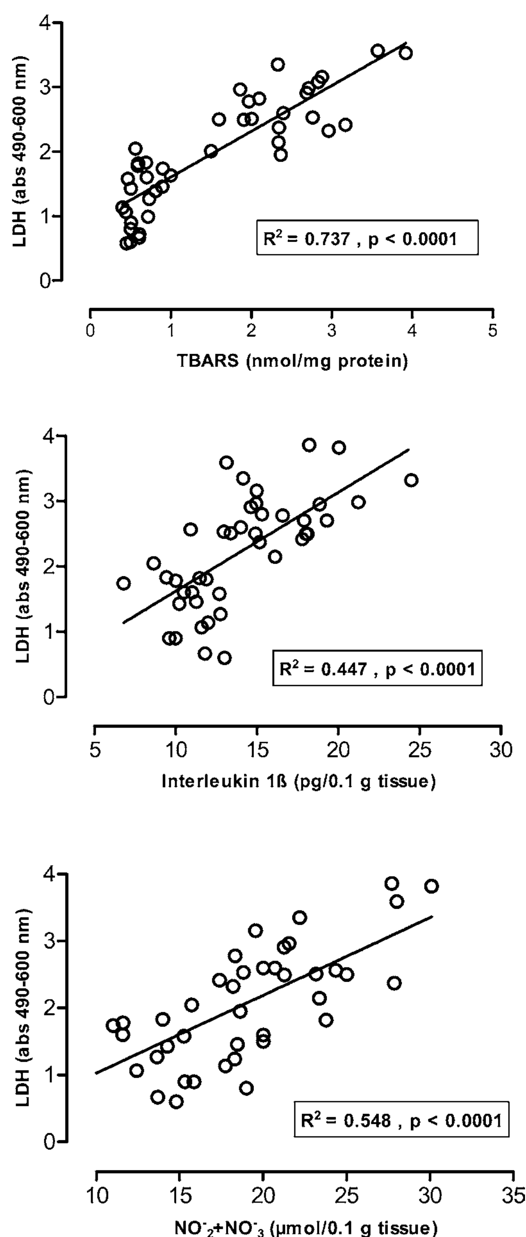

Figure 3. Linear correlations between lactate dehydrogenase efflux and thiobarbituric acid reactive substances (TBARS) (top), interleukin 1β (middle), and nitrites + nitrates ($NO^-_2 + NO^-_3$) (bottom) after reoxygenation.

Some alkyl ether derivatives of HT have been shown to exert a cytoprotective effect in vitro in cultured HepG2 cells subjected to cytotoxicity with *tert*-butyl hydroperoxide (t-BOOH).[23] This effect was directly related to the antioxidant potency of these compounds and was even greater than the effect of HT itself. In this regard, our results appear to be consistent with these effects, although our study provides evidence of this effect ex vivo after oral administration to experimental animals. In addition, an important difference in the experimental method used in our study is that tissue damage was not induced by a chemical agent but all biochemical alterations that occur in the process of hypoxia−reoxygenation-induced cell damage in rat brain slices.[24]

With regard to the role of the antioxidant effect of these compounds in their neuroprotective action, in our experimental model we found an increase in cellular lipid peroxidation and a decrease in the glutathione system. Oral administration of HT ethers reduced the accumulation of lipid peroxide after hypoxia−reoxygenation, and the effect of hexyl, octyl, and dodecyl derivatives was greater than the other derivatives and even greater than the effect of HT per se. However, none of the compounds tested here modified the glutathione system. These results agree with those reported previously in rat brain tissues treated with HT and HT acetate: these compounds inhibited lipid peroxidation and modified the glutathione system slightly.[8] However, experiments of tissue damage induced by chemical agents (t-BOOH) indicated that HT ether derivatives prevented the decrease in glutathione concentration after chemical damage.[23,25,26] The in vitro design of these experiments as well as the type of cell used may be the main explanations for the differences in the results. However, the effect of HT ether derivatives on lipid peroxidation showed a direct relationship with its neuroprotective action, as demonstrated by the linear correlation between the levels of TBARS and LDH in the experimental groups (Figure 3).

Despite the importance of oxidative stress in the mechanisms of neuronal damage in hypoxia−reoxygenation, other pathways of damage can increase the effect of excess free radicals. Some of these pathways are the excessive production of nitric oxide, the formation of peroxynitrites (nitrosative stress), and the increased release of inflammatory cytokines.[27,28] The oral administration of HT alkyl ether derivatives modified these pathways. Some studies have shown that HT inhibits the expression of interleukins[7,27] and inducible nitric oxide synthase[7,28] in activated leukocytes. Moreover, HT limited the neuronal damage produced by incubation with nitric oxide donors such as sodium nitroprusside.[26] Our study shows that

the oral administration of various HT alkyl ether derivatives reduces the production of neurotoxic mediators in our model of hypoxia–reoxygenation in rat brain slices. Furthermore, the importance of these mechanisms is further supported by the linear correlation between LDH efflux and brain concentrations of nitrite and IL-1β. These effects may act in concert with the inhibition of lipid peroxidation caused by HT alkyl ether derivatives. However, octyl and dodecyl ether derivatives may exhibit the effect through IL-10, which is a well-known anti-inflammatory cytokine.

The greater effect observed with long carbon chain HT alkyl ethers suggests that their higher lipophilicity may facilitate penetration into the brain tissue after oral administration. This statement is based on previous studies showing that the hydrocarbon chain length of alkyl HT ether derivatives is directly related with higher lipophilicity.[15] With respect to the pass across the blood–brain barrier, there are no in vivo studies on the kinetic parameters of these compounds; however, it has been demonstrated that hydroxytyrosol is found in rat brain tissue after a single oral administration of a phenolic extract from olive cake,[29] but this aspect requires further kinetics studies to verify the effect.

These results do not exclude the participation of these compounds in other neuroprotective pathways, including protection of mitochondrial respiratory function and stimulation of other endogenous antioxidant pathways (superoxide dismutase, Nrf2, etc.). Moreover, according to Son et al.[30] it is valuable a possible hormetic effect, defined as a biphasic dose–response with a low-dose beneficial effect and a high-dose nonbeneficial effect. These authors postulate the activation of adaptive cellular stress response pathways in the central nervous system with low doses of these compounds; these adaptive mechanisms include antioxidant and cytoprotective factors. However, confirmation of the real role of these potential specific effects requires further studies.

In conclusion, the oral administration of HT alkyl ether derivatives to rats led to a reduction in the sensitivity of brain tissue to hypoxia–reoxygenation. The reduction in oxidative and nitrosative stress and decreased production of pro-inflammatory interleukins may be the basis for the neuro-protective effect of these derivatives.

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Notes

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■ REFERENCES

(1) Bendini, A.; Cerretani, L.; Carrasco-Pancorbo, A.; Gomez-Caravaca, A. M. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules* **2007**, *12*, 1679–1719.

(2) Trípoli, E.; Giammanco, M.; Tabacchi, G.; Di Majo, D.; Giammanco, S.; La Guardia, M. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutr. Res. Rev.* **2005**, *18*, 98–112.

(3) Tuck, K. L.; Hayball, P. J. Major phenolic compounds in olive oil: metabolism and health effects. *J. Nutr. Biochem.* **2002**, *13*, 636–644.

(4) Montedoro, G.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A. Simple and hydrolyzable compounds in virgen olive oil. Spectroscopic characterizations of the secoiridoid derivatives. *J. Agric. Food Chem.* **1993**, *41*, 2228–2234.

(5) Brenes, M.; Garcia, A.; Garcia, P.; Rios, J. J.; Garrido, A. Phenolic compounds in Spanish olive oils. *J. Agric. Food Chem.* **1999**, *47*, 3535–3540.

(6) Gimeno, E.; de la Torre-Carbot, K.; Lamuela-Raventós, R. M.; Castellote, A. I.; Fitó, M.; de la Torre, R.; Covas, M. I.; López-Sabater, M. C. Changes in the phenolic content of low density lipoprotein after olive oil consumption in men. A randomized crossover controlled trial. *Br. J. Nutr.* **2007**, *98*, 1243–1250.

(7) González-Correa, J. A.; López-Villodres, J. A.; Asensi, R.; Espartero, J. L.; Rodríguez-Gutiérez, G.; De La Cruz, J. P. Virgin olive oil polyphenol hydroxytyrosol acetate inhibits in vitro platelet aggregation in human whole blood: comparison withhydroxytyrosol and acetylsalicylic acid. *Br. J. Nutr.* **2009**, *101*, 1157–1164.

(8) González-Correa, J. A.; Navas, M. D.; Lopez-Villodres, J. A.; Trujillo, M.; Espartero, J. L.; De La Cruz, J. P. Neuroprotective effect of hydroxytyrosol and hydroxytyrosol acetate in rat brain slices subjected to hypoxia-reoxygenation. *Neurosci. Lett.* **2008**, *446*, 143–146.

(9) Bitler, C. M.; Viale, T. M.; Damaj, B.; Crea, R. Hydrolyzed olive vegetation water in mice has anti-inflammatory activity. *J. Nutr.* **2005**, *135*, 1475–1479.

(10) Paiva-Martins, F.; Fernandes, J.; Santos, V.; Silva, L.; Borges, F.; Rocha, S.; Belo, L.; Santos-Silva, A. Powerful protective role of 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde against erythrocyte oxidative-induced hemolysis. *J. Agric. Food Chem.* **2010**, *58*, 135–140.

(11) González-Correa, J. A.; Muñoz-Marín, J.; Arrebola, M. M.; Guerrero, A.; Narbona, F.; López-Villodres, J. A.; De La Cruz, J. P. Dietary virgin olive oil reduces oxidative stress and cellular damage in rat brain slices subjected to hypoxia-reoxygenation. *Lipids* **2007**, *42*, 921–929.

(12) González-Correa, J. A.; Navas, M. D.; Muñoz-Marín, J.; Trujillo, M.; Fernández-Bolaños, J.; De La Cruz, J. P. Effects of hydroxytyrosol and hydroxytyrosol acetate administration to rats on platelet function compared to acetylsalicylic acid. *J. Agric. Food Chem.* **2008**, *56*, 7872–7876.

(13) Schaffer, S.; Podstawa, M.; Visioli, F.; Bogani, P.; Müller, W. E.; Eckert, G. P. Hydroxytyrosol-rich olive mill wastewater protects brain cells in vitro and ex vivo. *J. Agric. Food Chem.* **2007**, *55*, 5043–5049.

(14) Madrona, A.; Pereira-Caro, G.; Mateos, R.; Rodríguez, G.; Trujillo, M.; Fernández-Bolaños, J.; Espartero, J. L. Synthesis of hydroxytyrosyl alkyl ethers from olive oil waste waters. *Molecules* **2009**, *14*, 1762–1772.

(15) Pereira-Caro, G.; Mateos, R.; Saha, S.; Madrona, A.; Espartero, J. L.; Bravo, L.; Kroon, P. A. Transepithelial transport and metabolism of new lipophilic ether derivatives of hydroxytyrosol by enterocyte-like Caco-2/TC7 cells. *J. Agric. Food Chem.* **2010**, *58*, 11501–11509.

(16) Pereira-Caro, G.; Bravo, L.; Madrona, A.; Espartero, J. L.; Mateos, R. Uptake and metabolism of new synthetic lipophilic derivatives, hydroxytyrosyl ethers, by human hepatoma HepG2 cells. *J. Agric. Food Chem.* **2010**, *58*, 798–806.

(17) Abdel-Karim, M.; Muñoz-Marin, J.; Lopez-Villodres, J. A.; Guerrero, A.; Reyes, J. J.; Ruiz-Moreno, M. I.; Madrona, A.; Gonzalez-Correa, J. A.; De La Cruz, J. P. Neuroprotective effect of alkyl hydroxytyrosyl ethers in rat brain slices subjected to hypoxia-reoxygenation. *Basic Clin. Pharmacol. Toxicol.* **2011**, *109* (Suppl. 2), 50.

(18) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(19) Hissin, P. J.; Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **1976**, *74*, 214−226.
(20) De La Cruz, J. P.; Del Río, S.; Arrebola, M. M.; López-Villodres, J. A.; Jebrouni, N.; González-Correa, J. A. Effect of virgin olive oil plus acetylsalicylic acid on brain slices damage after hypoxia-reoxygenation in rats with type 1-like diabetes mellitus. *Neurosci. Lett.* **2010**, *471*, 89−93.
(21) Craggs, L.; Kalaria, R. N. Revisiting dietary antioxidants, neurodegeneration and dementia. *Neuroreport* **2011**, *22*, 1−3.
(22) Simonyi, A.; Wang, Q.; Miller, R. L.; Yusof, M.; Shelat, P. B.; Sun, A. Y.; Sun, G. Y. Polyphenols in cerebral ischemia: novel targets for neuroprotection. *Mol. Neurobiol.* **2005**, *31*, 135−147.
(23) Pereira-Caro, G.; Sarriá, B.; Madrona, A.; Espartero, J. L.; Goya, L.; Bravo, L.; Mateos, R. Alkyl hydroxytyrosyl ethers show protective effects against oxidative stress in HepG2 cells. *J. Agric. Food Chem.* **2011**, *59*, 5964−5976.
(24) Sahota, P.; Savitz, S. I. Investigational therapies for ischemic stroke: neuroprotection and neurorecovery. *Neurotherapeutics* **2001**, *8*, 434−451.
(25) Pereira-Caro, G.; Madrona, A.; Bravo, L.; Espartero, J. L.; Alcudia, F.; Cert, A.; Mateos, R. Antioxidant activity evaluation of alkyl hydroxytyrosyl ethers, a new class of hydroxytyrosol derivatives. *Food Chem.* **2009**, *115*, 86−91.
(26) Schaffer, S.; Müller, W. E.; Eckert, G. P. Cytoprotective effects of olive mill wastewater extract and its main constituent hydroxytyrosol in PC12 cells. *Pharmacol. Res.* **2010**, *62*, 322−327.
(27) Zhang, X.; Cao, J.; Zhong, L. Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. *Naunyn−Schmiedeberg's Arch. Pharmacol.* **2009**, *379*, 581−586.
(28) Zhang, X.; Jiang, L.; Geng, C.; Cao, J.; Zhong, L. The role of oxidative stress in deoxynivalenol-induced DNA damage in HepG2 cells. *Toxicon* **2009**, *54*, 513−518.
(29) Serra, A.; Rubió, L.; Borrás, X.; Macià, A.; Romeri, M.-P.; Motilva, M. J. Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. *Mol. Nutr.* **2011**, *55*, 1−11.
(30) Son, T. G.; Camandola, S.; Mattson, M. P. Hormetic dietary phytochemicals. *Neuromol. Med.* **2008**, *10*, 236−246.