JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Alkyl Hydroxytyrosyl Ethers Show Protective Effects against Oxidative Stress in HepG2 Cells

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ABSTRACT: Alkyl hydroxytyrosyl ethers (methyl, ethyl, propyl, and butyl ethers) have been synthesized from hydroxytyrosol (HTy) in response to the increasing food industry demand of new lipophilic antioxidants. Having confirmed that these compounds reach portal blood partially unconjugated and thus are effectively absorbed, their potential antioxidant activity was evaluated in the human hepatocarcinoma cell line (HepG2). The effects of 0.5−10 μM alkyl hydroxytyrosyl ethers on HepG2 cell integrity and redox status were assessed as well as the protective effect against oxidative stress induced by *tert*-butylhydroperoxide (t-BOOH). Cell viability (Crystal violet) and cell proliferation (BrdU assay) were measured as markers of cell integrity, concentration of reduced glutathione (GSH), generation of reactive oxygen species (ROS), and activity of antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR) as markers of redox status and determination of malondialdehyde (MDA) as a marker of lipid peroxidation. Direct treatment of HepG2 with alkyl hydroxytyrosyl ethers induced slight changes in cellular intrinsic antioxidants status, reducing ROS generation and inducing changes in GPx and GR activities. Pretreatment of HepG2 cells with alkyl hydroxytyrosyl ethers counteracted cell damage induced by t-BOOH, partially after 2 h and completely after 20 h, by increasing GSH and decreasing ROS generation, MDA levels, and antioxidant enzyme (GPx and GR) activity. According to these results the alkyl hydroxytyrosyl ethers show clear protective effects against oxidative stress, related to their lipophilic nature, that are similar to or even higher than those of their precursor, HTy.

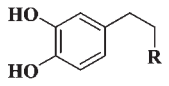
KEYWORDS: alkyl hydroxytyrosyl ethers, hydroxytyrosol, HepG2 cells, antioxidant defense capacity, oxidative stress biomarkers

INTRODUCTION

The phenolic content of virgin olive oil, the main source of fat in the Mediterranean diet, induces beneficial health effects due in part to its lipid oxidative damage protective effects,[1,2] which are mostly related to its antioxidant and free radical scavenger capacity. Hydroxytyrosol (HTy, **1**) is present in virgin olive as secoiridoid derivative, as acetate ester, and in free form and represents the major phenol fraction.[3] Several studies have demonstrated its cardiovascular disease preventive activity, showing protective effects against low-density lipoprotein oxidation,[4−6] averting oxidative stress induced by endothelial dysfunction,[7] inhibiting platelet aggregation,[8] and presenting anti-inflammatory activity.[9] In addition, HTy (**1**) induces a wide range of antitumor effects in human colon carcinoma cells, including suppression of cell proliferation and induction of apoptosis.[10,11] HTy (**1**) has also shown protective effects against induced oxidative stress by scavenging several free radical species in different cell lines, such as Caco-2 cells,[12,13] melanoma cells,[14] human hepatoma HepG2 cells,[15] renal tubular epithelial cells,[16] and red blood cells.[17] Moreover, it also offers indirect protection by increasing the endogenous defense system by inducing antioxidant enzymes.[18]

There is increasing interest within the food industry to develop new lipophilic antioxidants that may be used as functional ingredients in lipid food matrices. Nowadays, the only natural, lipophilic antioxidants available are tocopherols (e.g., vitamin E) and β-carotene. Synthetic, lipophilic, antioxidant compounds, which are currently used, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG) have not shown to be completely safe, and their usage in food may soon be restricted or banned. This is a challenge for food scientists as most phytochemicals with antioxidant activity are hydrophilic. With this purpose, series of hydrophobic derivatives (alkyl hydroxytyrosyl ethers, **2**−**5**, Figure 1) of HTy (**1**), a natural hydrophilic antioxidant, have been synthesized.[19] These derivatives have attracted much interest as they present antioxidant capacity similar to or even higher than that of their precursor (**1**).[20] Furthermore, the synthetic compounds are stable when digested in vitro,[21] rapidly absorbed, and partially metabolized by Caco-2/TC7 cells in a dose-dependent manner due to their lipophilic nature.[22] It is noteworthy that from 40 to 85% of the compounds transferred across the enterocyte monolayers are not metabolized and are expected to reach portal blood unmodified and, subsequently, the liver. These compounds have been shown to be well absorbed in human hepatoma cells.[23] Consequently, alkyl hydroxytyrosyl ethers (**2**−**5**) might play an important role in preventing oxidative stress, which has been associated with hepatic diseases.[18,24] In addition, the liver is particularly susceptible

Received: January 18, 2011
Revised: April 19, 2011
Accepted: April 19, 2011
Published: April 19, 2011



R	Name	Symbol
OH	Hydroxytyrosol (**HTy**)	1
OCH_3	Hydroxytyrosyl methyl ether	2
OC_2H_5	Hydroxytyrosyl ethyl ether	3
OC_3H_7	Hydroxytyrosyl propyl ether	4
OC_4H_9	Hydroxytyrosyl butyl ether	5

Figure 1. Chemical structures of hydroxytyrosol (**1**) and hydroxytyrosyl ethers (**2**−**5**).

to drugs and xenobiotics as blood is transferred straight to this organ after intestinal absorption through the portal vein. Taking into account these points, the aim of the present study was to assess whether alkyl hydroxytyrosyl ethers (**2**−**5**) at physiological concentrations (0.5−10 μM) directly affect HepG2 cell integrity and steady-state values of redox status and to further evaluate their capacity to protect human hepatoma HepG2 cells against oxidative cell damage induced by *tert*-butyl hydroperoxide (t-BOOH). With this purpose, cell viability (Crystal Violet) and cell proliferation (BrdU) were measured as markers of cell integrity; concentration of reduced glutathione (GSH), generation of reactive oxygen species (ROS), and activity of glutathione peroxidase (GPx) and glutathione reductase (GR) as redox status markers; and malondialdehyde (MDA) as a marker of lipid peroxidation.

MATERIALS AND METHODS

Materials. DMEM F-12 culture media and fetal bovine serum (FBS) were from Biowhitaker Europe (Lonza, Madrid, Spain). Gentamicin, penicillin, treptomycin, *tert*-butyl hydroperoxide (t-BOOH), glutathione reductase (GR), reduced and oxidized glutathione, nicotine adenine dinucleotide phosphate reduced form (NADPH), dimethyl sulfoxide (DMSO), *o*-phthaldehyde (OPT), dichlorofluorescin (DCFH), 2,4-dinitrophenylhydrazone (DNPH), EDTA, and mercaptoethanol were purchased from Sigma Chemicals (Madrid, Spain). Bradford reagent was from BioRad (Madrid, Spain). Crystal Violet indicator and dodecyl sulfate sodium salt (SDS) were also acquired from Sigma Chemicals. Cell proliferation ELISA 5-bromo-2′-deoxyuridine (BrdU) (colorimetric) assay kit was from Roche Diagnostics (Roche Molecular Biochemicals, Barcelona, Spain). Alkyl hydroxytyrosyl ethers (**2**−**5**) were chemically synthesized from hydroxytyrosol (HTy, **1**)[19] and purified by column chromatography, yielding >98% pure compounds All reagents were of analytical or chromatographic grade.

Cell Culture. Human HepG2 cells were grown in culture dishes (100 mm diameter) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C and maintained in DMEM−F12 supplemented with 2.5% fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin, and streptomycin,.

Hydroxytyrosyl Derivatives Treatments. Stock solutions of synthetic compounds (**2**−**5**) were dissolved in 10% of DMSO in deionized water and diluted with DMEM-F12 free serum to prepare test solutions (0.1% DMSO final concentration in cell culture media).

First, the effect of different concentrations (0.5, 1, 5, 10, 20, 50, 100, 200, and 500 μM) of the new lipophilic compounds on the HepG2 cell viability and proliferation after treatment for 24 h was evaluated using Crystal Violet and BrdU assays, respectively.

Afterward, two different experiments were carried out. *In experiment A*, cells were exposed to different concentrations of alkyl hydroxytyrosyl ethers (**2**−**5**) (0.5, 1, 5, and 10 μM), dissolved in serum-free culture medium for 2 or 20 h, to evaluate the direct effect of these phenols. *In experiment B*, cells were exposed to different concentrations of alkyl hydroxytyrosyl ethers (**2**−**5**) (0.5, 1, 5, and 10 μM) dissolved in serum-free culture medium for 2 or 20 h and then submitted to 400 μM t-BOOH for 3 h to determine the chemoprotective effects of the synthetic compounds (**2**−**5**). All cells were washed with PBS and treated as described below for each assay. In both experiments, cell viability, GSH, MDA, ROS generation, and GPx and GR activities were evaluated. Control cells were treated with 0.1% DMSO in free serum culture medium to maintain the same ratio of DMSO in comparison with the treated cells.

Cell Viability. Cell viability was determined using the Crystal Violet assay.[25] HepG2 cells were seeded at low density (10^4 cells/well) in 96-well plates and exposed to the tested compounds. After the incubation period, cells were washed with PBS, and Crystal Violet (0.2% in ethanol) was added to each well for 20 min. Then, plates were rinsed with tap water three times and drained upside down on paper towels. Afterward, 100 μL of 1% sodium dodecyl sulfate (SDS) was added to release the dye and estimate cell viability. The absorbance of each well at 570 nm was measured using a microplate reader (Bio-Tek, Winooski, VT). Results are expressed as percentage of cell viability referred to the absorbance measurement obtained with untreated cells.

Cell Proliferation (BrdU). Cell proliferation was evaluated using a colorimetric immunoassay (ELISA) based on the measurement of BrdU incorporation into genomic DNA during its synthesis in proliferating cells. Briefly, HepG2 cells were seeded (10^4 cells/well) in 96-well plates and exposed to the indicated concentrations of the tested compounds for 24 h. Afterward, cells were labeled 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 absorbance at 620 nm using a scanning multiwell spectrophotometer (Bio-Tek). Results are expressed as the percentage of cell growth referred to untreated cells.

GSH Evaluation. HepG2 cells were seeded in 60 mm diameter plates at a concentration of 1.5×10^6/plate. The intracellular concentration of reduced glutathione was estimated by the fluorometric assay previously described.[15,26] Briefly, GSH reacts with *o*-phthalaldehyde (OPT) at pH 8.0, and fluorescence is measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm using a microplate reader (Bio-Tek). Results are expressed as percentage of GSH referred to GSH values obtained with untreated cells.

MDA Determination. HepG2 cells were seeded in 100 mm diameter plates at a concentration of 4.5×10^6/plate. Cellular MDA was analyzed by high-performance liquid chromatography (HPLC) as its 2,4-dinitrophenylhydrazone (DNPH) derivative.[27] Values are expressed as the coefficient nanomoles of MDA per milligram of protein. Protein concentration was measured using the Bradford method.[28]

ROS Determination. Cellular ROS were quantified by the DCFH assay using microplate reader (Bio-Tek).[15,26] After being oxidized with intracellular oxidants, DCFH will turn into dichlorofluorescein (DCF) and emit fluorescence. Results are expressed as fluorescence units.

GPx and GR Determination. The determination of the GPx activity is based on the oxidation of reduced glutathione by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR, which was monitored by following absorbance at 340 nm.[29] Alternatively, GR activity was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADPH utilized in the reduction of oxidized glutathione.[30] In both determinations, HepG2 cells were seeded in 100 mm diameter plates at a concentration of 4.5×10^6/plate. Results are expressed as milli- or microunits per milligram of protein for GPx or GR, respectively. Protein concentration was measured using the Bradford method.[28]

Statistical Analysis. Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene. Multiple comparisons were carried out using one-way ANOVA followed by Bonferroni tests when variances were homogeneous or by Tamhane test when

Table 1. Effects of Hydroxytyrosyl Methyl (2), Ethyl (3), Propyl (4), and Butyl (5) Ethers on Cell Viability and Cell Proliferation[a]

compd (μM)	% cell viability	% cell proliferation	compd (μM)	% cell viability	% cell proliferation
2			**4**		
0	100.0 ± 12.3 ab	100.0 ± 8.9 a	0	100.0 ± 16.5 ab	100.0 ± 10.1 a
0.5	98.9 ± 11.0 ab	97.9 ± 13.8 a	0.5	109.2 ± 5.2 a	94.7 ± 12.7 a
1	85.8 ± 5.5 ab	96.2 ± 9.2 a	1	94.1 ± 9.0 ab	80.6 ± 11.8 a
5	84.0 ± 6.4 b	97.3 ± 9.8 a	5	86.7 ± 11.3 b	84.5 ± 11.4 a
10	86.1 ± 6.2 ab	99.0 ± 6.7 a	10	84.8 ± 11.2 b	78.4 ± 2.8 a
20	94.1 ± 5.0 ab	99.2 ± 6.3 ab	20	81.7 ± 7.8 b	86.2 ± 4.0 a
50	101.6 ± 5.1 ab	99.2 ± 3.7 a	50	80.5 ± 8.0 b	88.4 ± 7.2 a
100	88.3 ± 11.1 ab	105.7 ± 4.2 a	100	58.0 ± 10.9 c	75.3 ± 17.1 a
200	86.2 ± 11.6 ab	33.9 ± 4.7 b	200	35.4 ± 16.8 cd	25.7 ± 4.0 b
500	64.3 ± 6.9 c	20.0 ± 5.1 b	500	24.5 ± 5.1 d	24.0 ± 2.3 b
3			**5**		
0	100.0 ± 7.4 a	100.0 ± 13.8 a	0	100.0 ± 8.0 a	100.0 ± 7.9 a
0.5	91.4 ± 11.8 a	91.4 ± 13.3 a	0.5	100.3 ± 17.2 a	89.5 ± 10.5 a
1	94.4 ± 6.6 a	89.2 ± 11.6 a	1	100.9 ± 9.3 a	79.8 ± 6.1 a
5	100.8 ± 8.3 a	97.7 ± 8.2 a	5	98.1 ± 7.4 a	78.7 ± 11.6 ab
10	91.5 ± 10.4 a	86.0 ± 14.5 a	10	85.7 ± 12.0 ab	78.0 ± 5.5 a
20	90.8 ± 16.9 a	91.0 ± 6.7 a	20	87.6 ± 18.9 ab	82.5 ± 8.1 a
50	88.3 ± 11.2 a	97.5 ± 4.0 a	50	65.3 ± 10.7 bc	70.9 ± 2.0 b
100	86.2 ± 11.3 a	96.8 ± 10.0 a	100	48.5 ± 6.2 cd	35.0 ± 8.5 c
200	46.1 ± 12.2 b	21.0 ± 4.7 b	200	35.1 ± 5.1 d	14.4 ± 1.8 d
500	31.7 ± 5.1 b	23.8 ± 1.6 b	500	24.2 ± 1.2 e	17.3 ± 4.4 d

[a] HepG2 cells were treated with the noted concentration of hydroxytyrosyl ethers during 24 h. Results are expressed as percent of cell viability and proliferation compared with controls, and values are the mean ± SD of eight determinations ($p < 0.05$).

variances were not. The level of significance was established at $p < 0.05$. The statistical package SPSS (version 17.0) was used.

RESULTS AND DISCUSSION

A series of alkyl hydroxytyrosyl ethers (**2**−**5**) were synthesized from HTy (**1**) to provide new lipophilic antioxidant ingredients as an alternative to the compounds currently used for fat food preservation. These new synthetic compounds (**2**−**5**) present similar or even higher antioxidant activity than HTy (**1**) [20] and are efficiently absorbed across the intestinal epithelial cell monolayers[22] and thus have the potential to reach the bloodstream and, subsequently, the liver, the main organ where xenobiotics and drugs are metabolized. It is well-known that HTy (**1**) is involved in the prevention of pathologies of which onset and progression have been related to ROS-mediated tissue injury as previously demonstrated by our group.[15] HepG2 cells are a suitable and well-characterized model of the human liver, which has been widely used in biochemical and nutritional studies.[15,18,26] The present study focuses on the effects of four hydroxytyrosyl derivatives of increasing lipophilic nature, hydroxytyrosyl methyl (**2**), ethyl (**3**), propyl (**4**), and butyl (**5**) ethers, on cell integrity and steady-state values of cellular redox status. It also evaluates the protective effects of these compounds against oxidative stress induced by t-BOOH in HepG2 cells. First, a cytotoxicity evaluation of the compounds was carried out to assess any possible deleterious effects on normal HepG2 cell growth.

Cytotoxicity Evaluation of Alkyl Hydroxytyrosyl Ethers (2−5) on HepG2 Cells. The effects of 0.5−500 μM alkyl hydroxytyrosyl ethers (**2**−**5**) on HepG2 cell viability analyzed using the Crystal Violet assay is shown in Table 1. There were no differences in cell viability after 24 h of exposure of up to 200, 100, 50, and 20 μM hydroxytyrosyl methyl (**2**), ethyl (**3**), propyl (**4**), and butyl (**5**) ethers, respectively. Interestingly, 24 h of incubation with hydroxytyrosyl methyl (**2**), ethyl (**3**), propyl (**4**), and butyl (**5**) ethers reduced HepG2 cell viability 13.8, 53.9, 64.6, and 64.9%, respectively, at 200 μM and 35.7, 68.3, 75.5, and 75.8%, respectively, at 500 μM. These results are in agreement with those previously described by our group after 24 h of incubation in Caco-2 cells.[22]

With regard to the antiproliferative effects of these alkyl hydroxytyrosyl derivatives (**2**−**5**), a significant arrest of HepG2 cell growth was observed when cells were treated for 24 h with 200 μM hydroxytyrosyl methyl (**2**), ethyl (**3**), and propyl (**4**) ethers, respectively, whereas hydroxytyrosyl butyl ether (**5**) highly inhibited cell proliferation from 50 μM compared to untreated cells (Table 1).

Direct Effect of Alkyl Hydroxytyrosyl Ethers (2−5) on HepG2 Cells. To evaluate the potential biological activity of the synthetic compounds, cell toxicity and cellular redox status were determined in cells directly treated for short (2 h) and long (18 h) periods with 0.5 to 10 μM alkyl hydroxytyrosyl ethers (**2**−**5**).

Cell Viability. When the previously indicated results (Table 1) were taken into account, no cytotoxic effects were observed after the exposure of cells to 0.5, 1, 5, and 10 μM of each of the alkyl hydroxytyrosyl ethers (**2**−**5**). The concentrations selected for this study correspond to human physiological levels that have been detected in plasma after the consumption of different types of phenolic-containing foods [31,32] including olive oil.[33,34]

GSH Concentration. GSH is the main nonenzymatic antioxidant defense within the cell. In hepatocytes, it is involved as a substrate in GPx-catalyzed detoxification of organic peroxides,

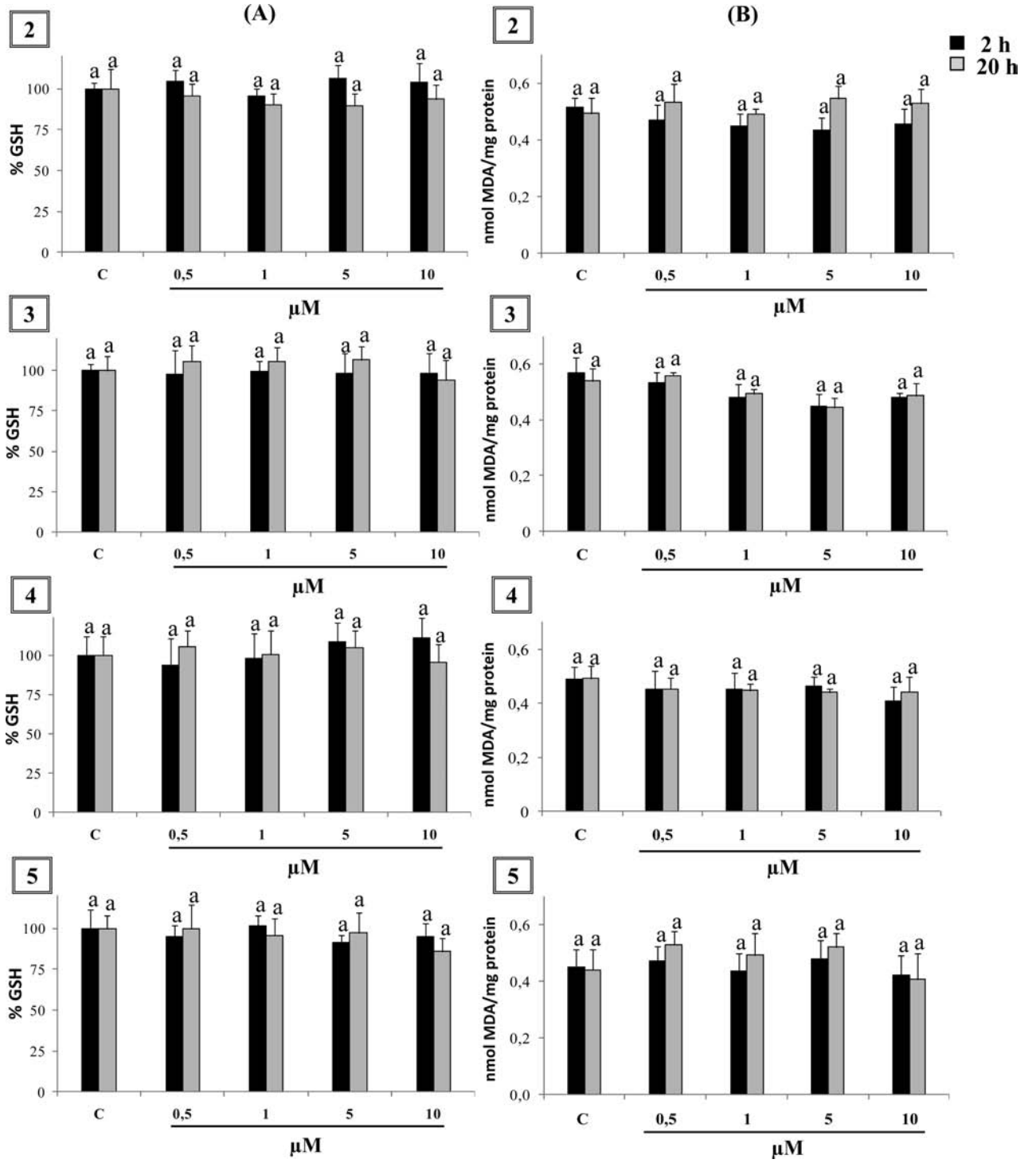


Figure 2. (A) Direct effects of hydroxytyrosyl ethers (**2**−**5**) on intracellular concentration of reduced glutathione and (B) cytoplasmatic concentration of malondialdehyde. HepG2 cells were treated with 0.5−10 μM of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), of hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. GSH values are expressed as percent of GSH compared with untreated cells. MDA levels are expressed as nanomoles of MDA per milligram of protein. Different letters denote statistically significant differences ($n = 4$) ($p < 0.05$).

reacting with free radicals and repairing free radical induced damage through electron-transfer reactions.[35] Figure 2A shows the results corresponding to the treatment of HepG2 cells with 0.5−10 μM alkyl hydroxytyrosyl ethers (**2**−**5**). No statistically significant differences in GSH concentration compared with control cells were found.

Malondialdehyde Levels. An important step in the degradation of cell membranes is the reaction of ROS with the double bonds of polyunsaturated fatty acids (PUFAs), yielding lipid hydroperoxides, which break down to yield a large variety of aldehydes.[36] Elevated levels of MDA have been described in different diseases related to free radical damage, and thus MDA

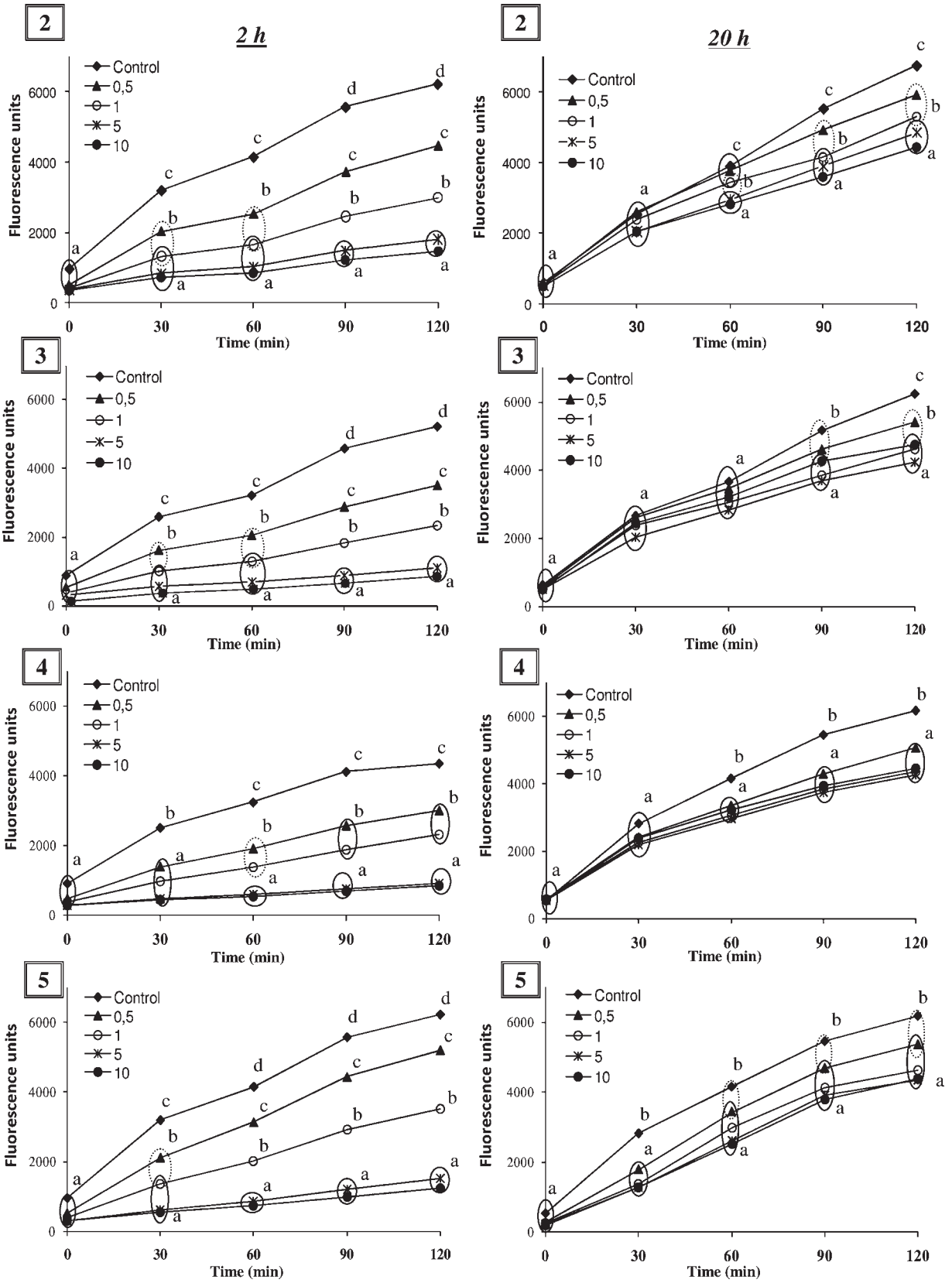


Figure 3. Direct effects of hydroxytyrosyl ethers (**2**−**5**) on intracellular ROS production. HepG2 cells were treated with different concentrations of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. Results are expressed as fluorescence units. Values are means ($n = 7-8$). SD values were not included due to intense bar overlapping. Means without a common letter differ statistically ($p < 0.05$). In the ROS assay, only data within each time point were compared.

dx.doi.org/10.1021/jf2002415 |*J. Agric. Food Chem.* 2011, 59, 5964–5976

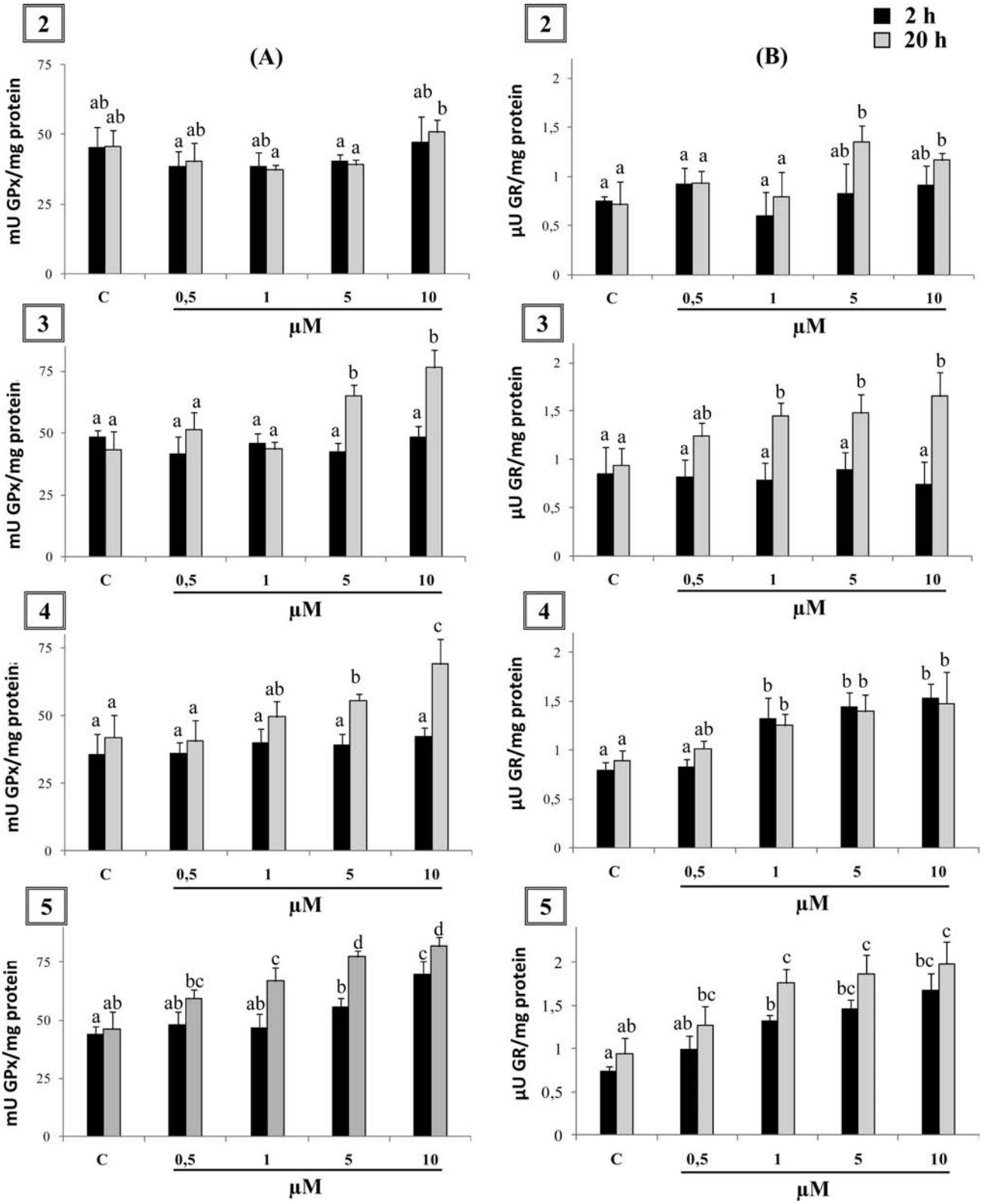


Figure 4. Direct effects of hydroxytyrosyl ethers (**2**−**5**) on the activity of glutathione peroxidase (A) and glutathione reductase (B). HepG2 cells were treated with different concentrations of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. Values are the mean ± SD of four determinations. Different letters indicate statistically significant differences ($p < 0.05$).

concentration has been widely used as an index of lipoperoxidation in biological and medical sciences.[37] In the present study MDA levels in human hepatoma cells treated with increasing concentrations of alkyl hydroxytyrosyl ethers (**2**−**5**) did not

change compared with untreated cells (Figure 2B) at any of the incubation periods.

ROS Generation. The major cause of molecular injury leading to cell aging and age-related degenerative diseases such as cancer and coronary heart disease[38] is the accumulation of ROS in several cellular components. In fact, direct evaluation of ROS yields a very good indication of the oxidative damage in living cells.[39] In this study, ROS generation in cultured HepG2 cells was monitored over time after exposure to the tested concentrations of alkyl hydroxytyrosyl ethers (**2**−**5**). After 2 h of treatment, a significant dose-dependent decrease in ROS generation was observed over time (from 0 to 120 min) with all of the hydroxytyrosyl ethers tested (**2**−**5**) compared with control cells (Figure 3). However, a significant decrease in ROS levels was observed after 20 h of incubation with all of the studied phenols, not following a dose−response at any of the four concentrations assayed (Figure 3). It is noteworthy that a higher decrease in ROS production was observed when cells were incubated with phenols for 2 h rather than for 20 h. Considering that ROS generation takes place at a high rate, the protection effects of the hydroxytyrosyl ethers counterbalanced ROS generation after 20 h of incubation to a lower extent than after a shorter incubation time; moreover, the concentration of the polyphenol compounds is likely to be lower due to their slight degradation and cell metabolism over time. In general, all of the evaluated compounds presented similar activity not showing any difference associated with their different lipophilic nature. These results support that alkyl hydroxytyrosyl ethers (**2**−**5**) strongly decrease the steady-state generation of ROS in HepG2 cells in culture.

Antioxidant Enzyme (GPx and GR) Activity. The antioxidant enzyme system of cells plays a crucial role in the defense against oxidative stress. This system includes GR and GPx, the activities of which are determined as indices of the intracellular enzymatic antioxidant defense and, thus, are considered important antioxidant biomarkers.[40,41] GPx is involved in eliminating peroxides,[42,43] and GR is responsible for the regeneration of oxidized glutathione.[44] Therefore, their activities are essential for an effective recovery of the steady-state concentration of GSH. In the present study, GPx and GR were measured after the treatment of HepG2 with 0.5−10 μM alkyl hydroxytyrosyl ethers (**2**−**5**). As shown in Figure 4A, after treatment for 2 h, exclusively hydroxytyrosyl butyl ether (**5**) over 5 μM induced significant changes in GPx. Treatment of HepG2 for 20 h enhanced GPx activity at 10 μM for hydroxytyrosyl methyl ether (**2**), from 5 μM for hydroxytyrosyl ethyl (**3**) and propyl (**4**) ethers, and from 1 μM for the particular case of hydroxytyrosyl butyl ether (**5**), respectively. Therefore, a direct relationship between activity and lipophilic nature was observed. With regard to GR activity (Figure 4B), treatment with hydroxytyrosyl methyl (**2**) and ethyl (**3**) ethers during 2 h did not induce significant alterations at any of the phenolic concentrations tested. However, treatment with hydroxytyrosyl propyl (**4**) and butyl (**5**) ethers at 1 μM and higher concentrations significantly enhanced GR activity compared with control cells. After the longer incubation, all compounds were able to significantly increase GR activity, although at different doses according to their lipophilic nature. The reducing power balancing activity of GPx and GR after treatment with the hydroxytyrosyl ethers leads to a compensated concentration of GSH (Figure 2).

Up to this point, the results obtained indicate that alkyl hydroxytyrosyl ethers (**2**−**5**) may reduce the generation of ROS and induce changes in the GRx and GR activities, thus favoring conditions for human hepatic cells to face an oxidative stress challenge. The outcome is in agreement with that previously published with HTy in olive oil (**1**).[15,18,45] Similarly,

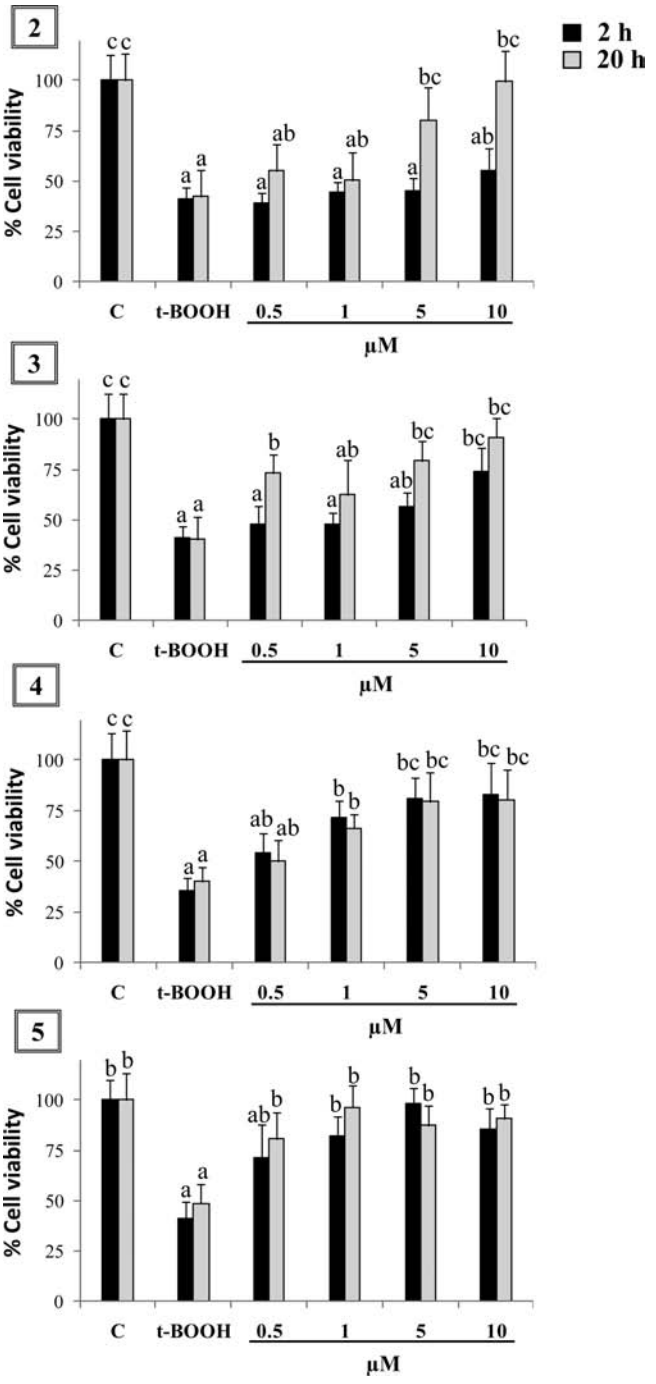


Figure 5. Protective effects of hydroxytyrosyl ethers (**2**−**5**) against oxidative stress on HepG2 cell viability. Cells were treated for 2 and 20 h with the noted concentrations of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**). After that, the cultures were washed and 400 μM t-BOOH was added to all cultures except controls for 3 h. Results are expressed as relative percentage compared with control cells. Values are the mean ± SD, $n = 8$. Different letters indicate statistically significant differences ($p < 0.05$).

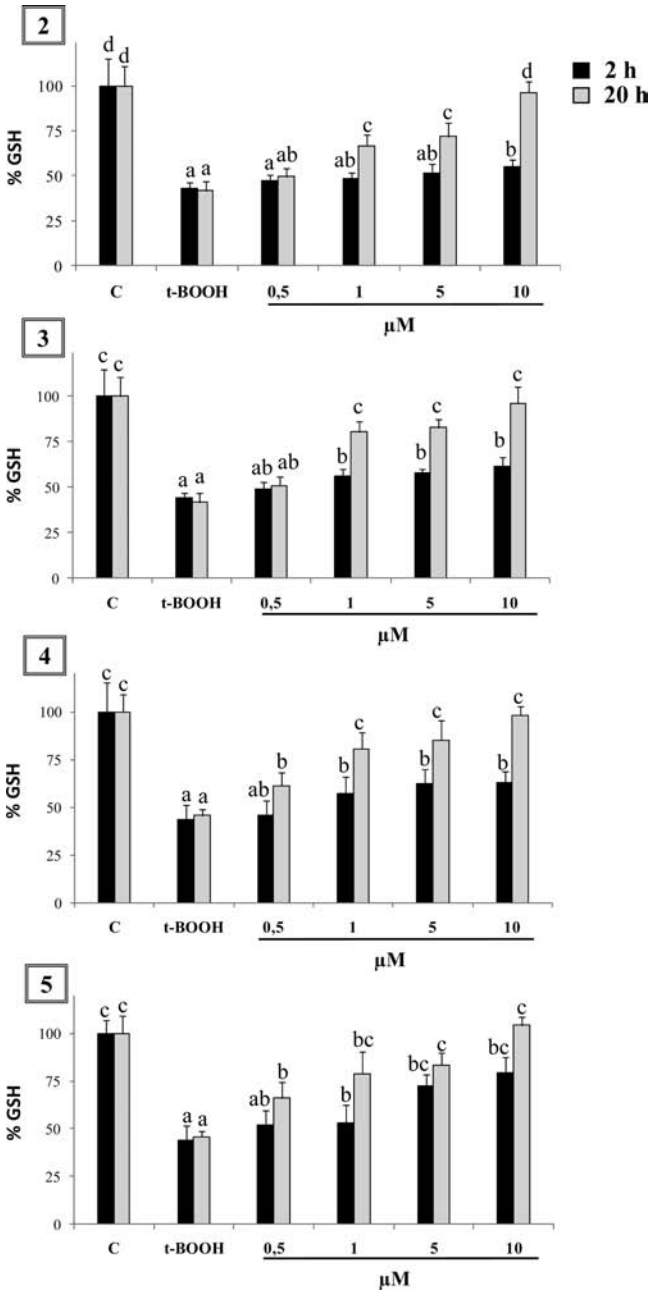


Figure 6. Protective effects of hydroxytyrosyl ethers (**2**−**5**) against oxidative stress on intracellular concentration of reduced glutathione. HepG2 cells were treated with 0.5−10 μM hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. After that, cells were washed and 400 μM t-BOOH was added to all cultures except controls for 3 h. GSH values are expressed as percent compared with untreated cells. Different letters denote statistically significant differences ($n = 4$) ($p < 0.05$).

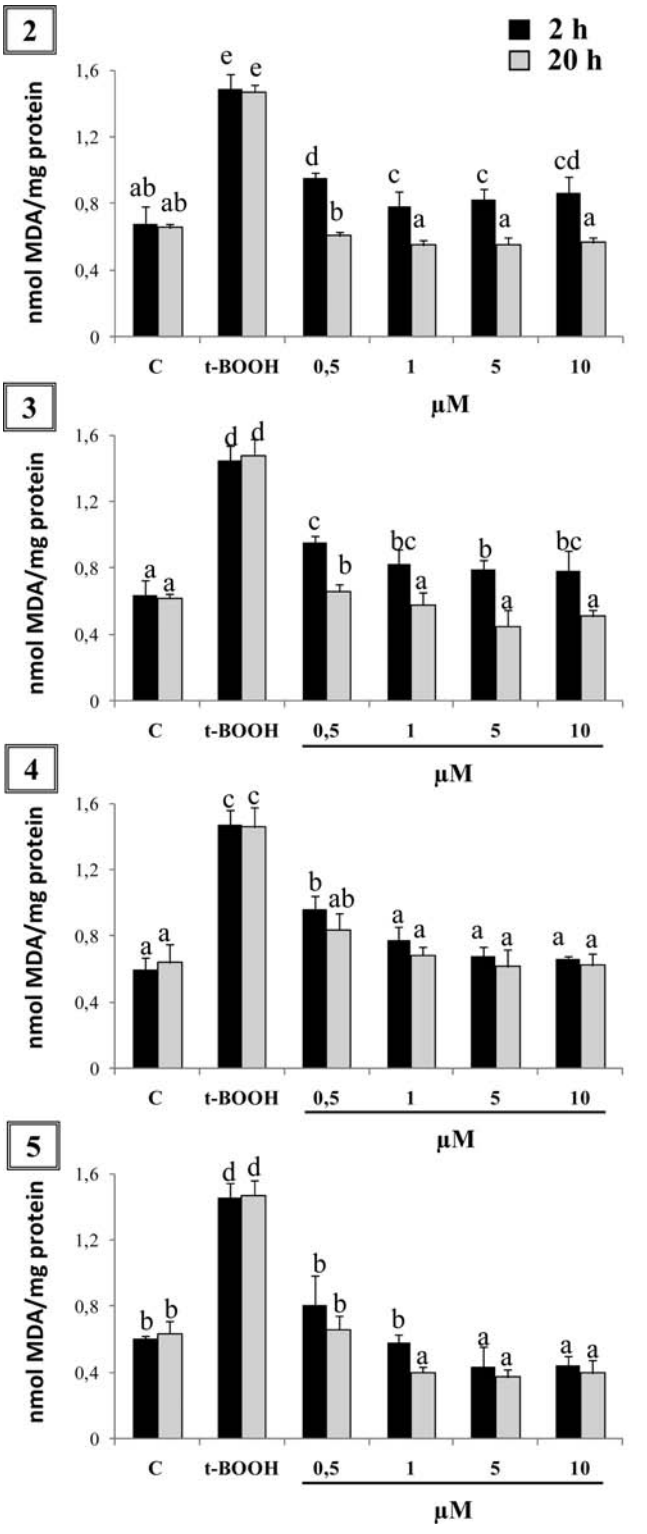


Figure 7. Protective effects of hydroxytyrosyl ethers (**2**−**5**) against oxidative stress on cytoplasmatic concentration of malondialdehyde. HepG2 cells were treated with the noted concentration of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. After that, cells were washed and 400 μM t-BOOH was added to all cultures except controls for 3 h. MDA levels measured by HPLC in cytoplasmatic content are expressed as nanomoles of MDA per milligram of protein as the mean ± SD ($n = 4$). Different letters denote statistically significant differences ($p < 0.05$).

flavanols, such as epicatechin-3-gallate and procyanidin B2, which are present in wine, tea, or cocoa among other foodstuffs, induce a significant increase in several antioxidant-related enzymes activities, accompanied by a decrease in ROS production not affecting GSH content in colon-derived Caco-2 cells.[46]

Protective Effect of Alkyl Hydroxytyrosyl Ethers (2−5) on HepG2 Cells against Oxidative Damage Induced by t-BOOH. In this part of the study, the chemoprotective effect of alkyl

dx.doi.org/10.1021/jf2002415 |*J. Agric. Food Chem.* 2011, 59, 5964–5976

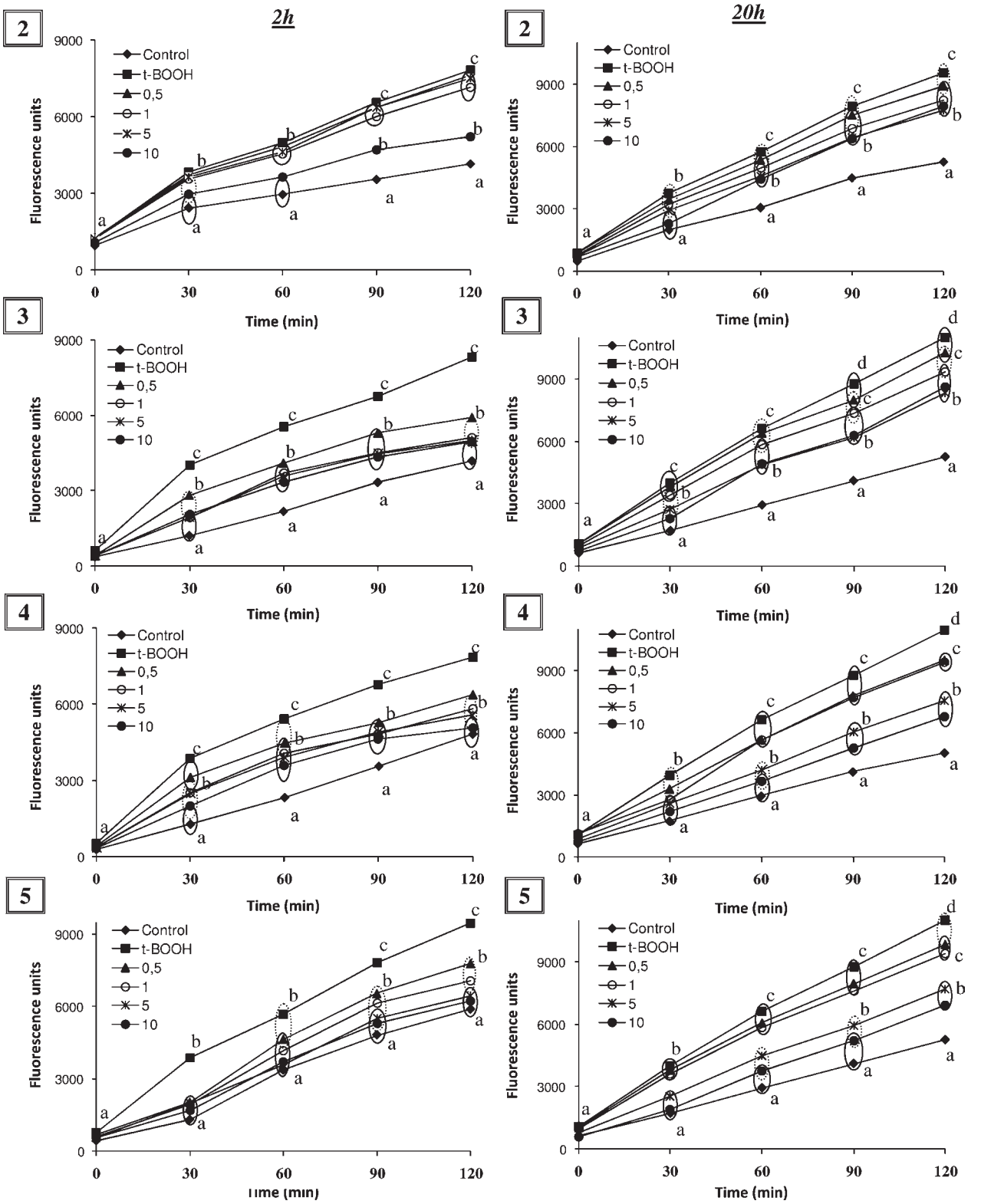


Figure 8. Protective effects of hydroxytyrosyl ethers (**2**−**5**) against t-BOOH-induced intracellular ROS generation. HepG2 cells were treated with different concentrations of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. After that, the cell culture were washed with PBS, and 400 μM t-BOOH was added to all cells except controls for 120 min. Results are expressed as fluorescence units (n = 7−8). SD values were not included due to intense bar overlapping. Means without a common letter differ statistically ($p < 0.05$). In the ROS assay, only data within each time point were compared.

hydroxytyrosyl ethers (**2**−**5**) before the exposure of HepG2 cells to oxidative stress induced by t-BOOH was evaluated. t-BOOH has been extensively used for studying the protective effects of natural compounds and plant extracts against oxidative damage.[26,47]

dx.doi.org/10.1021/jf2002415 |*J. Agric. Food Chem.* 2011, 59, 5964–5976

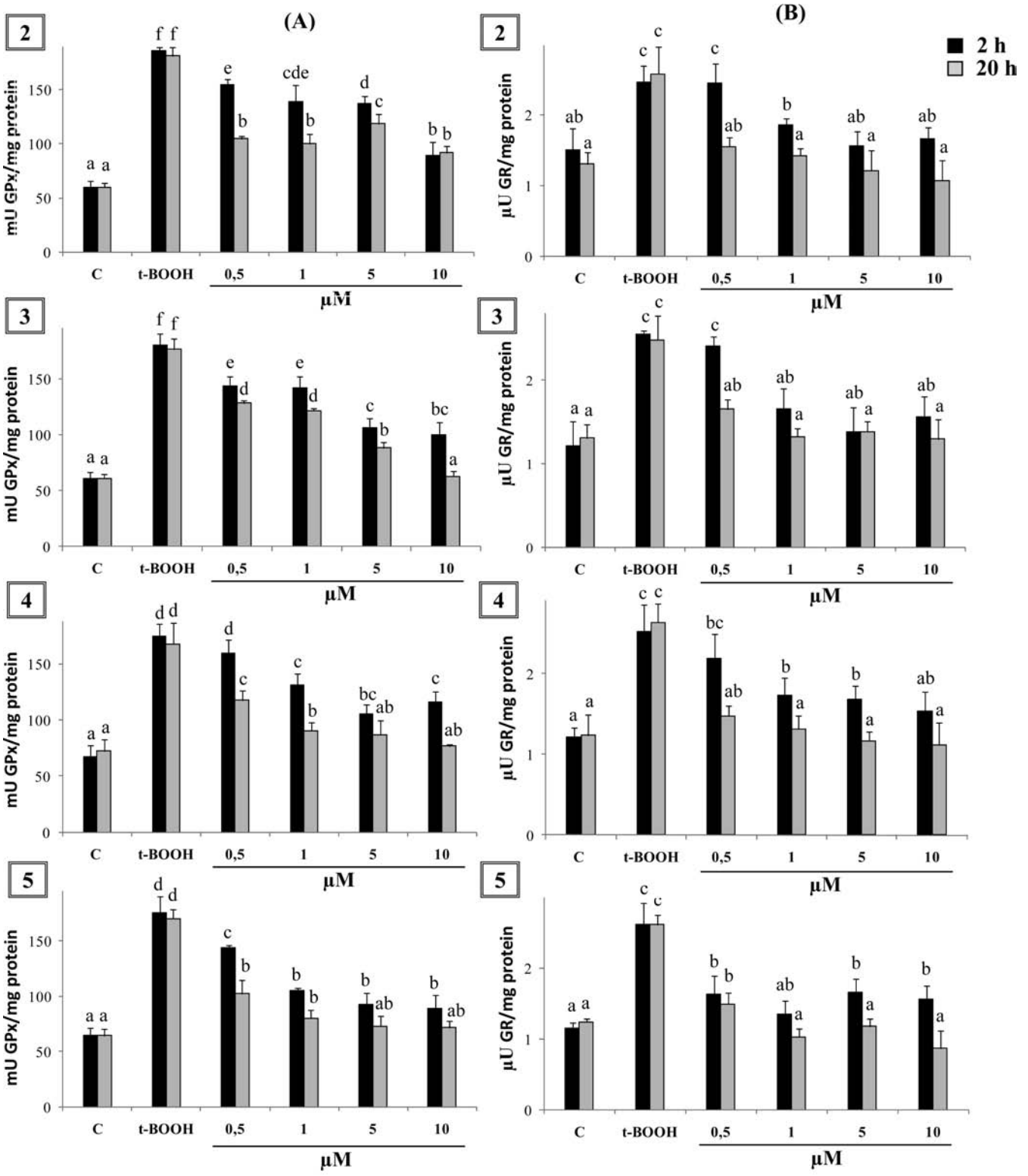


Figure 9. Protective effects of hydroxytyrosyl ethers (**2**−**5**) against oxidative stress on the activities of glutathione peroxidase (A) and glutathione reductase (B). HepG2 cells were treated with different concentrations of hydroxytyrosyl methyl ether (**2**), hydroxytyrosyl ethyl ether (**3**), hydroxytyrosyl propyl ether (**4**), or hydroxytyrosyl butyl ether (**5**) for 2 and 20 h. After that, the cell cultures were washed with PBS, and 400 μM t-BOOH was added to all cells except controls for 3 h. Values are the mean ± SD of four determination. Different letters indicate statistically significant differences ($p < 0.05$).

Cell Viability. When HepG2 cells were treated with 400 μM t-BOOH during 3 h, a significant decrease in cell viability (50−60%) was observed (Figure 5). The cell damage induced by t-BOOH was partially or almost totally prevented by pretreatment with alkyl hydroxytyrosyl ethers (**2**−**5**) for 2 or 20 h, respectively, in a dose-dependent and lipophilic nature dependent manner.

Reduced Glutathione Concentration. The treatment of HepG2 cells with 400 μM t-BOOH induced a remarkable decrease (~70%) in the concentration of GSH compared to untreated cells (Figure 6). GSH decrease was slightly prevented by the pretreatment for 2 h with 10 μM methyl derivative (**2**) and 1−10 μM for the rest of the alkyl hydroxytyrosyl derivatives (**3**−**5**), whereas pretreatment for 20 h showed a dose-dependent recovery of GSH levels, reaching control levels at 10 μM hydroxytyrosyl methyl ether (**2**) and from 1 μM onward with hydroxytyrosyl ethyl (**3**), propyl (**4**), and butyl (**5**) ethers, respectively. The different lipophilic natures of these compounds could explain the higher effect obtained with **3**−**5** compared with **2**, especially at the longer incubation time.

MDA Levels. The treatment of HepG2 cells with 400 μM t-BOOH induced a significant increase (2−2.5-fold) in MDA cellular concentrations, compared with control cells, indicating oxidative damage in cell lipids (Figure 7).

The oxidative damage was partially reverted with pretreatment for 2 h with 0.5−10 μM hydroxytyrosyl methyl (**2**) and ethyl (**3**) ethers and totally prevented with 1−10 μM hydroxytyrosyl propyl ether (**4**), Lower MDA levels than in control cells were obtained after pretreatment with 5−10 μM hydroxytyrosyl butyl ether (**5**). Complete inhibition of lipid peroxidation induced by t-BOOH was observed in cells pretreated for 20 h with all of the assayed polyphenols, reaching levels below that of control cells in the case of hydroxytyrosyl butyl (**5**) ether. This outcome points out that alkyl hydroxytyrosyl ethers (**2**−**5**) provide significant protection against the increased lipid peroxidation induced by t-BOOH in HepG2 cells, preserving the integrity of the biological membranes from detrimental oxidative processes caused by free radicals.

Reactive Oxygen Species Generation. HepG2 cells treated with t-BOOH showed a significant 2-fold increase in ROS generation after 2 h compared with nonstressed cells (Figure 8). However, when cells were pretreated with alkyl hydroxytyrosyl derivatives (**2**−**5**) for 2 and 20 h, ROS production was partially prevented, especially at higher doses. At both incubation times, the most lipophilic compounds (**4** and **5**) reduced more significantly the progressive formation and accumulation of ROS. These data suggest that high levels of ROS are more efficiently quenched in cells pretreated with alkyl hydroxytyrosyl ethers (**2**−**5**), resulting in reduced cell damage and lipid peroxidation.

Antioxidant Enzyme (GPx and GR) Activity. The treatment of HepG2 cells with 400 μM t-BOOH during 3 h, induced a significant increase, between 2.5- and 3-fold, in GPx and GR activities (Figure 9) indicating a positive response of the cell defense system to face the increasing generation of ROS induced by a pro-oxidant as t-BOOH.[48] When cells were pretreated for 2 and 20 h with 0.5−10 μM of alkyl hydroxytyrosyl ethers (**2**−**5**), the t-BOOH induced increase in GPx activity was partially counteracted in a dose, time and lipophilic nature dependent manner. Similar results were observed with GR activity. The rapid recovery of antioxidant enzyme activity basal values places the cell in favorable conditions to face further oxidative insults.

From the results described we may gather that the new synthetic compounds (**2**−**5**) show the potential to prevent cell damage induced by t-BOOH and maintain unaltered cellular redox status, partially and almost completely after 2 and 20 h of pretreatment, respectively. This outcome is in accordance with that obtained with their precursor HTy (**1**),[15,18,45] which shows protective ability in HepG2 cells, similar to the less lipophilic ethers (**2** and **3**) and slightly lower than that of more lipophilic compounds (**4** and **5**). Recently, Zhang et al.[49] described that HTy at concentrations between 12.5 and 50 μM attenuated GSH depletion in HepG2 cells treated with acrylamide as an oxidative agent. Moreover, HTy (**1**) shows the capacity to scavenge ROS in red blood cells under natural and chemically simulated oxidative stress conditions.[50]

It is important to note the relevant role of the lipophilic nature of the synthetic phenolic compounds on their antioxidant potential against induced cell damage; hence, hydroxytyrosyl methyl (**2**) and ethyl (**3**) ethers are less effective in protecting against cellular damage, whereas hydroxytyrosyl propyl (**4**) and butyl (**5**) ethers are more active, especially at higher doses (5−10 μM). This finding is in agreement with that reported for oleuropein aglycones, which, having higher log *P* (partition coefficient of a chemical compound provides a thermodynamic measure of its lipophilicity/hydrophilicity balance) values than HTy (**1**) shows higher protection against ROS-induced oxidative injury in human red blood cells (RCB) than HTy (**1**). This outcome may be attributed to the higher capacity to penetrate the membranes and reach the radicals formed.[17] In the same line, Lima et al.[24] evaluated the protective effects of two phenolic acids, caffeic and rosmarinic acids, which are esters of caffeic acid and 3,4-dihydroxyphenylactic acid, respectively, and luteolin (flavone), luteolin-7-glucoside (flavone glycoside), and quercetin (flavonol) against oxidative damage in HepG2 cells and pointed out that the hydrophobicity plays an important role in the hepatoprotective potential of the polyphenols. Likewise, Spencer et al.[51] suggested that the antioxidant biological activity of polyphenols strongly depends on the extent to which they interact and permeate cell membranes. Recently, Pavlica et al.[52] compared two urinary metabolites of quercetin, the lipophilic metabolite, 3,4-dihydroxytoluene, was more protective than the hydrophilic metabolite, 3,4-dihydroxyphenylacetic acid, against oxidative stress induced by peroxides in neuronal PC12 cells.

It may be concluded that (1) alkyl hydroxytyrosyl ethers (**2**−**5**) protect human HepG2 cells against the damage induced by t-BOOH, preventing radical formation and modulating antioxidant defenses. The latter involves complex mechanisms, including signaling processes that remain to be investigated. (2) The lipophilic nature of the alkyl hydroxytyrosyl ethers is relevant in the regulation of the antioxidant defense mechanism of phenolic compounds, so that hydroxytyrosyl methyl (**2**) and ethyl (**3**) ethers are less effective than hydroxytyrosyl propyl (**4**) and butyl (**5**) ethers, especially at higher doses. (3) Hydroxytyrosol ethers (**2**−**5**) could be used as bioactive ingredients in the production and development of functional fat foods having the added value of contributing to the protection against oxidative stress related diseases.

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Funding Sources

This work was supported by Projects AGL2007-64042, AGL2007-66373-C04/ALI, and CSD2007-00063 from the Consolider-Ingenio Program of the Spanish Ministry of Science and

Innovation (CICYT), a grant (RTA2007-000036-00-00) from the Research National Institute of Food, Agriculture and Technology (INIA), and a contract (110105090014) with CSIC-IFAPA . G.P.-C. is a predoctoral fellow of the National Research Institute of Food, Agriculture and Technology (INIA).

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