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ORIGINAL CONTRIBUTION

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Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells

Protection against oxidative stress induced by tert-butylhydroperoxide

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Abstract *Background* Scientific evidence suggests that olive oil's beneficial effects are related to the high level of antioxidants, including phenolic compounds such as hydroxytyrosol. In vivo studies have shown that olive oil HTy is bioavailable and its biological activities, similar to those reported for other natural antioxidants such as quercetin, include prevention of LDL oxidation. Previous studies from our laboratory have shown that HTy and other phenolics in olive oil are absorbed and metabolized by cultured human hepatoma HepG2 cells where glucuronidated and methylated conjugates were the main derivatives formed, resembling the metabolic profile of olive oil phenols observed in human plasma and urine. *Aim of the study* The effect of olive oil phenol (HTy) on cell viability and redox status of cultured HepG2 cells, and the protective effect of HTy against an oxidative stress induced by tert-butylhydroperoxide (t-BOOH) were investigated. *Methods* Lactate dehydrogenase activity as marker for cell integrity, concentration of reduced glutathione (GSH), generation of reactive oxygen species (ROS) and activity of the antioxidant enzyme glutathione peroxidase (GPx) as markers of redox status and determination of malondialdehyde (MDA) as marker of lipid peroxidation were measured. *Results* No changes in cell integrity or intrinsic antioxidant status resulted from a direct treatment with 10–40 μM HTy. Pre-treatment of HepG2 with 10–40 μM HTy for 2 or 20 h completely prevented cell damage as well as the decrease of reduced glutathione and increase of malondialdehyde evoked by t-BOOH in HepG2 cells. Reactive oxygen species generation and the significant increase of glutathione peroxidase activity induced by t-BOOH were greatly reduced when cells were pretreated with HTy. *Conclusion* The results clearly show that treatment of HepG2 cells with the olive oil phenolic HTy may positively affect their antioxidant defense system, favoring cell integrity and resistance to cope with a stressful situation.

Key words bioactive compounds – dietary antioxidants – olive oil phenolics – liver cell culture – antioxidant defenses – biomarkers for oxidative stress

EJN 633

Introduction

Lines of evidence suggest that olive oil's beneficial effects are related to its elevated oleic acid content and the high level of antioxidants in the nonsaponifiable fraction, including phenolic compounds absent in seed oils [1, 2]. The main phenolic compounds in virgin olive oil are secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) (HTy) and of 2-(4-hydroxyphenyl)ethanol or tyrosol (Ty), and 2-(3,4-dihydroxyphenyl)ethyl acetate (hydroxytyrosyl acetate) (HTyAc). Minor amounts of HTy, Ty, 2-(4-hydroxyphenyl)ethyl acetate (tyrosyl acetate), vanillin and phenolic derivatives of benzoic and cinnamic acids, together with some flavonoids and lignans, constitute the antioxidant fraction of olive oil [3, 4].

Several human [5–8] and animal [9–11] studies have shown that the main olive oil phenols HTy and Ty are bioavailable. Once absorbed in the intestinal tract, olive oil phenols are targeted to the liver where they both exert their biological effects and become metabolized. The phenolic fraction of virgin olive oil has proved to have antioxidant activity in vitro, scavenging peroxyl [12], other free radicals [13], and reactive nitrogen species [14], or breaking peroxidative chain reactions and preventing metal ion catalyzed production of reactive oxygen species [15, 16]. Also, the inhibitory action of olive oil constituents on LDL oxidation shown in vitro [17, 18] and in vivo [19, 20] would contribute to the protective effect of olive oil against cardiovascular disease.

Previous studies from our laboratory have shown that the main olive oil phenols, HTy and HTyAc, are absorbed and metabolized by human hepatoma HepG2 cells in culture [21]. Glucuronidated and methylated conjugates were the main derivatives formed, resembling the metabolic profile of olive oil phenols observed in human plasma and urine. The aim of the present study was to assess whether HTy directly affects cell integrity and steady-state values of cellular redox status (experiment A), and to investigate the potential protective effect of HTy against an oxidative stress induced by tert-butyl hydroperoxide (t-BOOH) in HepG2 cells in culture (experiment B). The overall results suggest a protective effect of HTy on HepG2 against an oxidative stress induced by t-BOOH.

Material and methods

Cell culture

Human hepatocarcinoma HepG2 cell line was grown in a humidified incubator containing 5% CO_2 and 95% air at 37°C. They were grown in DMEM F-12 medium from Biowhitaker (Innogenetics, Madrid, Spain), supplemented with 2.5% Biowhitaker foetal bovine serum (FBS) and 50 mg/l of each of the following antibiotics: gentamicin, penicillin, and streptomycin (all from Sigma, Madrid, Spain). Assays were carried out in FBS-free medium since serum factors might interfere in the running of the assays and affect the results [22].

Hydroxytyrosol treatment

The different concentrations of HTy, obtained by chemical synthesis from 3, 4-dihydroxyphenylacetic acid (Sigma Co.) by reduction with LiAlH4 [23] and dissolved in serum-free culture medium, were added to the cell plates for 4 or 24 h (experiment A), or for 2 or 20 h and then submitted to 200 μM t-BOOH for three hours (experiment B). Then, cell culture medium was collected (LDH assay), or eliminated (GSH, MDA, and GPx assays), and the cells washed with PBS, collected by scraping, and treated as described below for each assay. In the ROS assay of experiment A cells cultured in 24-wells multiwell plates were treated with HTy, the dichlorofluorescin (DCFH) probe was added for 30 min, then they were washed twice before being treated with the noted conditions for 90 min. In the ROS assay for experiment B, cell plates were treated with HTy for 2 or 20 h and the DCFH probe added for 30 min, plates were washed twice with PBS and new HTy-free medium containing 200 μM t-BOOH was added to all cultures except controls for the 90 min of the assay.

Evaluation of lactate dehydrogenase leakage, reduced glutathione, and malondialdehyde

Cells were plated in 60-mm diameter plates at a concentration of 1.5×10^6 per plate and the day after cells were treated as described in the section of hydroxytyrosol treatment and LDH leakage to the culture medium was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content [22, 24–27]. The content of reduced glutathione was quantitated by the fluorometric assay of Hissin and Hilf [28]. The method takes advantage of the reaction of reduced glutathione with o-phthalaldehyde (OPT) at pH 8.0. Fluorescence was measured at an emision wavelength of 460 nm and an excitation wavelength of 340 nm [22, 24, 25]. Cellular malondialdehyde (MDA) was analyzed by high-performance liquid chromatography (HPLC) as its 2,4-dinitrophenylhydrazone (DNPH) derivative [29]. Values are expressed as nmol of MDA/mg protein; protein was measured by the Bradford method [30].

Determination of reactive oxygen species

Cellular reactive oxygen species were quantified by the dichlorofluorescin (DCFH) assay using microplate reader [31]. After being oxidized by intracellular oxidants, DCFH will become dichorofluorescein (DCF), and emit fluorescence. By quantifying fluorescence over a period of 90 min, a fair estimation of the overall oxygen species generated under the different conditions was obtained [22, 24, 25].

Determination of glutathione peroxidase activity

For the assay of the GPx activity, cells were collected in phosphate-buffered saline (PBS) and centrifuged at low speed for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris, 5 mM EDTA, and 0.5 mM mercaptoethanol, sonicated, and centrifuged at 3,000 × g for 15 min. The enzyme activity was measured in the supernatants [22, 24, 25]. The determination of gluthathione peroxidase (GPx) activity is based on the oxidation of reduced glutathione by GPx, using tert-butyl hydroperoxide as a substrate, coupled to the disappearance of NADPH by GR [32]. Protein was measured by the method of Bradford [30].

Statistics

Statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. In the case of ROS assays, although one-way ANOVA was used for statistical analysis, to simplify the figures only comparisons of any sample data to control or t-BOOH have been depicted. The level of significance was $P < 0.05$. A SPSS version 12.0 program has been used.

Results

Experiment A: plain treatment of HepG2 with HTy

Cytotoxicity

Lactate dehydrogenase (LDH) leakage was used as an indicator of cell damage. A range of doses between 0.1 and 100 μM was tested for periods of 4 and 24 h for their potential deleterious effect on HepG2 cells, but only results of the four selected concentrations (10–40 μM) are presented (see Discussion). Figure 1a shows that no increase in LDH leakage to the culture medium was observed in any condition suggesting

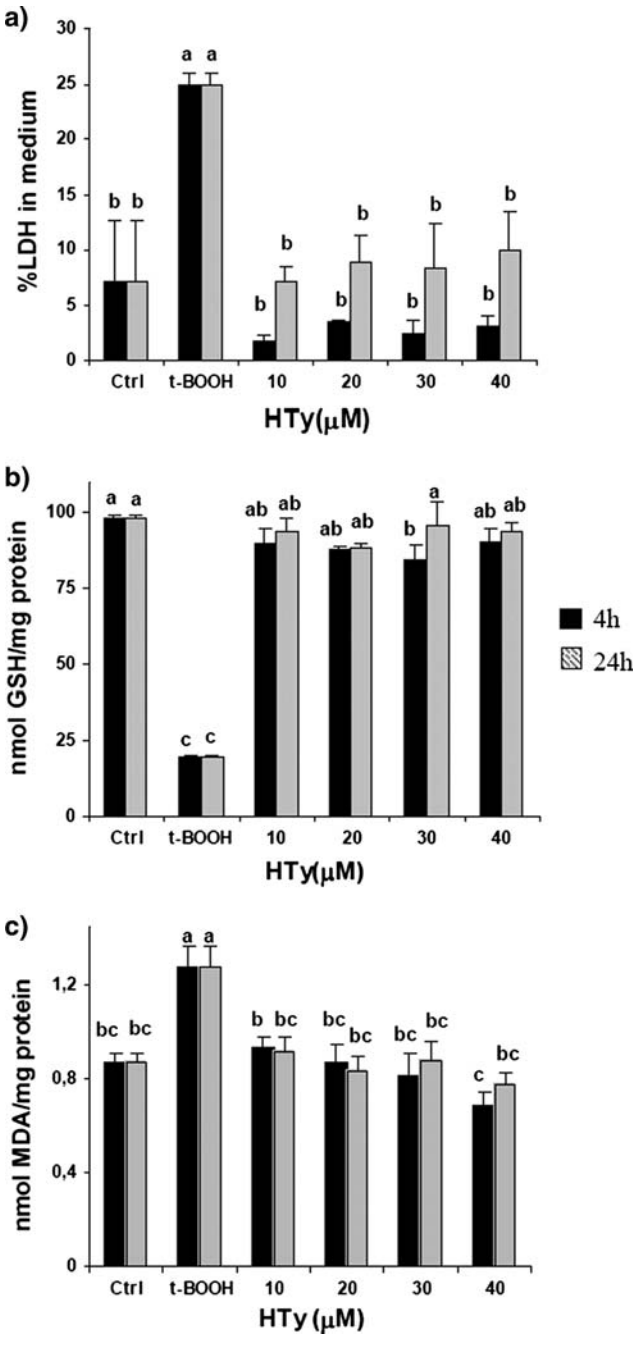

Fig. 1 *Experiment A.* Effect of HTy on cell viability and intracellular concentration of reduced glutathione and malondialdehyde. Cells were treated for 4 or 24 h; cells noted as t-BOOH were treated with 200 μM t-BOOH for 3 h. (**a**) Lactate dehydrogenase (LDH) leakage is expressed as percent of total LDH activity appearing in the culture medium. Values are means ± SD of 6–8 data. (**b**) Results of the fluorescent analysis for reduced glutathione (GSH), are means of 4–5 different samples per condition. (**c**) Analysis of malondialdehyde (MDA) was made by HPLC in cytoplasmatic contents. Values are means ± SD, $n = 4$. Different letters indicate statistically significant differences ($P < 0.05$) among different groups

that no significant damage was evoked in cell structure by the presence of HTy in concentrations up to

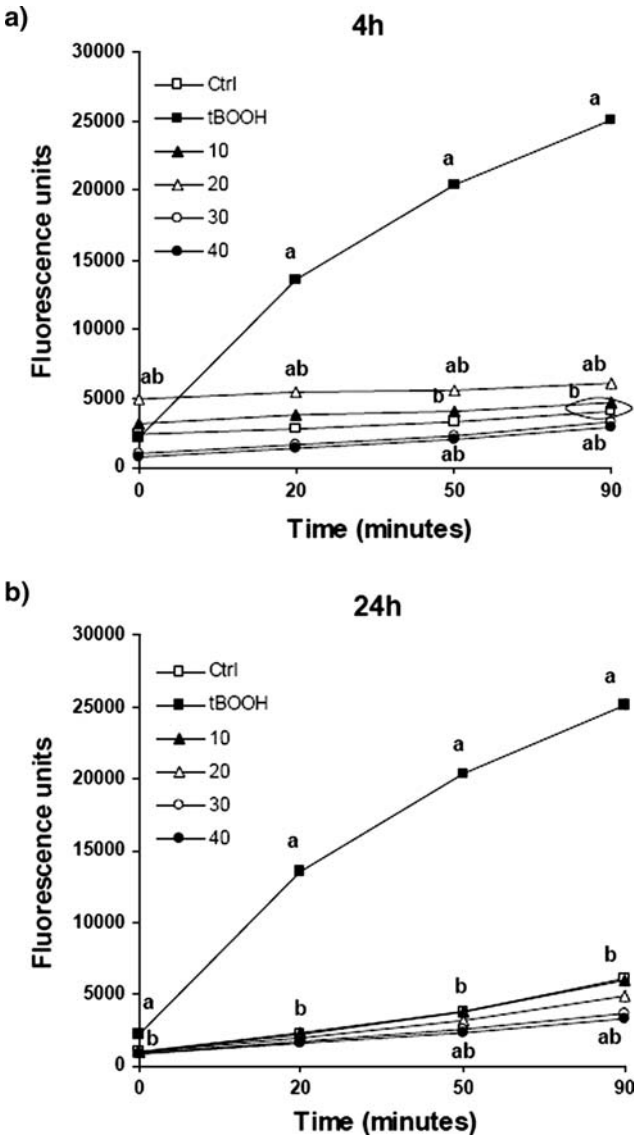

Fig. 2 *Experiment A*. Effect of HTy on intracellular reactive oxygen species (ROS) generation. Cells were treated with HTy for 4 (**A**) or 24 (**B**) h; cells noted as t-BOOH were treated with 200 μM t-BOOH. The results are expressed as fluorescence units. Letters a and b indicate statistically significant differences ($P < 0.05$) when that data or group of data are compared to their time-matched control (**a**) or t-BOOH (**b**). Values are means ± SD of 7–8 different samples per condition. SD values were not included due to intense bar overlapping

40 μM for as long as 24 h. Control values for both assays, 4 and 24 h, were pooled for the homogeneity of results. A batch of cultured cells was treated with 200 μM t-BOOH for 3 h as a positive control for cell damage and the results are shown within the same figure for comparison.

Reduced glutathione concentration

As an index of the intracellular non-enzymatic antioxidant defenses, the concentration of reduced glutathione was measured in HepG2 cells treated with increasing concentrations of HTy. As a positive control for a prooxidative status and GSH utilization, some cell plates were exposed to 200 μM t-BOOH for 3 h and the results of GSH are shown in the same figure for comparison (Fig. 1b). A significant decrease in GSH was observed in cells treated with t-BOOH whereas no changes were observed in HepG2 treated with 10 and 40 μM HTy for 4 or 24 h.

Malondialdehyde levels

As a biomarker for lipid peroxidation the cytoplasmic concentration of MDA was measured. As a positive control for increased lipid peroxidation a 3 h treatment of HepG2 with 200 μM t-BOOH evoked a significant increase of about 50% in the cellular concentration of MDA. No increase in MDA concentration was observed in cells treated with HTy for 4 or 24 h (Fig. 1c).

Reactive oxygen species (ROS) generation

A significant increase in ROS generation was observed over time in the cell wells treated with 200 μM t-BOOH (Fig. 2). Cells treated for 4 or 24 h with HTy generated ROS levels that were in the range of those of control unstressed cells, much lower than those of t-BOOH-treated cells. Due to inter-assay variation of fluorescent emission, only results obtained within the same DCF assay should be compared. Leakage of probe was not observed in cells throughout the assay, as determined in our laboratory in previous tests during method set-up [24]. Thus, any potential contribution of extracellularly oxidized DCF to the final fluorescence can be ruled out.

Experiment B: pretreatment of HepG2 with HTy before exposure to t-BOOH

Cytotoxicity

In our experimental conditions a 3-h treatment with 200 μM t-BOOH evoked a great increase in LDH activity in the cell culture medium indicating cell damage in HepG2 (Fig. 3a). Pretreatment for 2 or 20 h of HepG2 cultures with 10–40 μM HTy completely prevented cell damage induced by t-BOOH.

Reduced glutathione concentration

200 μM t-BOOH evoked a dramatic decrease of cytoplasmic GSH, which was partly or completely overcome by a pretreatment for 2 or 20 h with any of the above doses of HTy (Fig. 3b). A recovery of above 50% of the control values was observed with all but

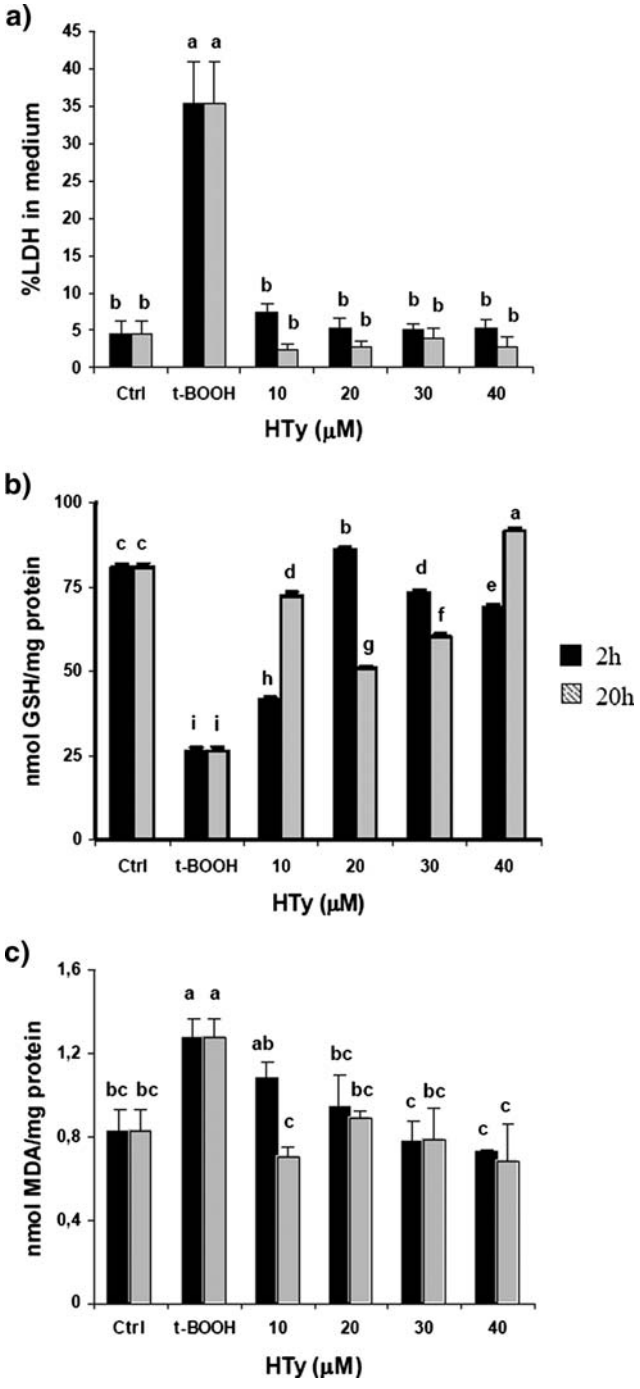

Fig. 3 *Experiment B*. Protective effect of HTy against t-BOOH-induced oxidative stress; cell viability, intracellular GSH, and malondialdehyde. Cells were treated with HTy for 2 or 20 h, washed twice and 200 μM t-BOOH was added to all the cultures except controls for 3 h. (**a**) Results of lactate dehydrogenase (LDH) leakage are expressed as percent of total LDH activity in the culture medium. Values are means ± SD of 6–8 data. (**b**) Results of the fluorescent analysis for reduced glutathione (GSH) are means of 4–5 different samples per condition. (**c**) Malondialdehyde (MDA) in cytoplasmatic contents are means ± SD, $n = 4$. Different letters indicate statistically significant differences ($P < 0.05$) among different groups

one condition, 10 μM HTy for 2 h, the lightest treatment, whereas 20, 30, and 40 μM for 2 h and 10 and 40 μM for 20 h recovered values to more than 80% of those of controls.

Malondialdehyde levels

Pretreatment for 20 h with all four doses of HTy prevented the MDA increase induced by 200 μM t-BOOH, indicating a reduced level of lipid peroxidation in response to t-BOOH in cells that had been previously in the presence of doses of HTy (Fig. 3c). A similar response of MDA was observed after a pretreatment for 2 h with 20, 30, and 40 μM HTy and only the lowest concentration of HTy (10 μM) for the shorter treatment (2 h) was unable to prevent the increase of MDA induced by t-BOOH.

Reactive oxygen species (ROS) generation

Pretreatment for 2 h of HepG2 cultures with 10–40 μM HTy decreased ROS production as compared to t-BOOH stressed cells but ROS levels at 90 min were still above those of control unstressed cells (Fig. 4a). When HepG2 cells were pretreated for 20 h, ROS production in the presence of t-BOOH was dependent on the HTy concentration, i.e., the higher the HTy dose the closer the ROS value to those of unstressed cells; in fact, control values were achieved when cells were pretreated for 20 h with 40 μM HTy.

Activity of glutathione peroxidase

The presence of 200 μM t-BOOH in the culture medium for 3 h induced a significant increase in the enzyme activity of GPx (Fig. 5). When cells were pretreated for 2 or 20 h with HTy, the t-BOOH-induced increase in enzyme activity of GPx was mostly suppressed.

Discussion

There is considerable interest in the cytoprotective effects of natural antioxidants against oxidative stress and the different defense mechanisms involved. This study demonstrates that the main phenolic in olive oil, hydroxytyrosol, does not modify the cellular antioxidant status, yet it has the ability to protect the cell against an oxidative insult by modulating ROS generation, reduced glutathione concentration, MDA production, and GPx activity.

Biological activities of HTy are similar to those reported for other natural antioxidants such as quercetin and include prevention of LDL oxidation [2], platelet aggregation [33], and inhibition of 5 and

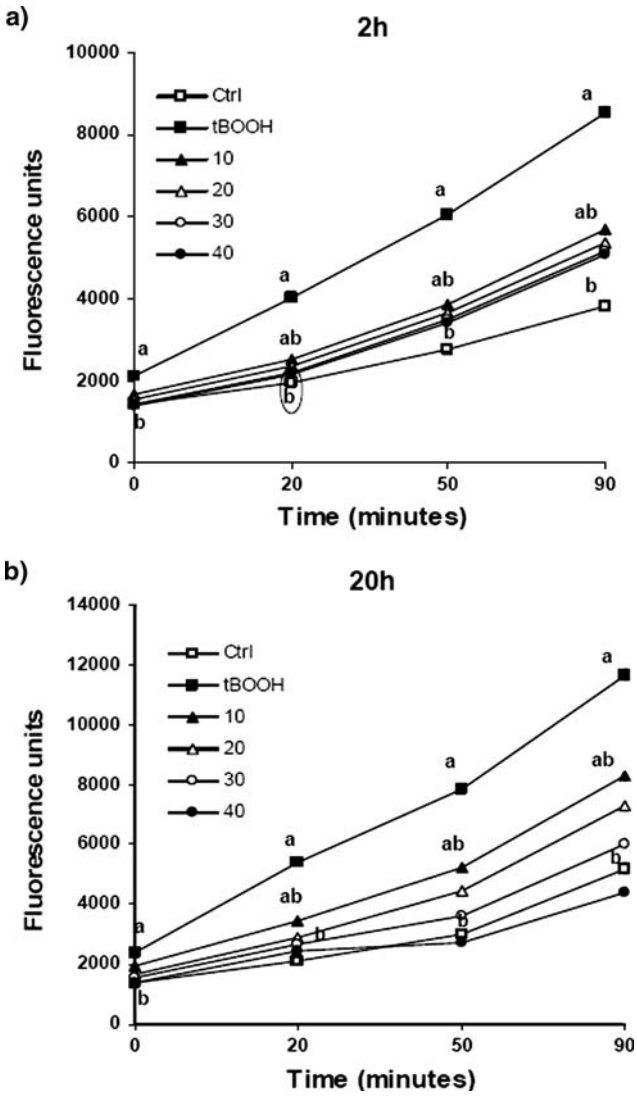

Fig. 4 *Experiment B*. Protective effect of HTy against t-BOOH-induced intracellular ROS generation. Cells were treated with HTy for 2 (**a**) or 20 (**b**) h, washed twice, and 200 μM t-BOOH was added to all cells except controls. Results are expressed as fluorescence units. Letters a and b indicate statistically significant differences ($P < 0.05$) when the data or group of data are compared to their time-mate control (**a**) or t-BOOH (**b**). Values are means ± SD of 7–8 different samples per condition. SD values were not included due to intense bar overlapping

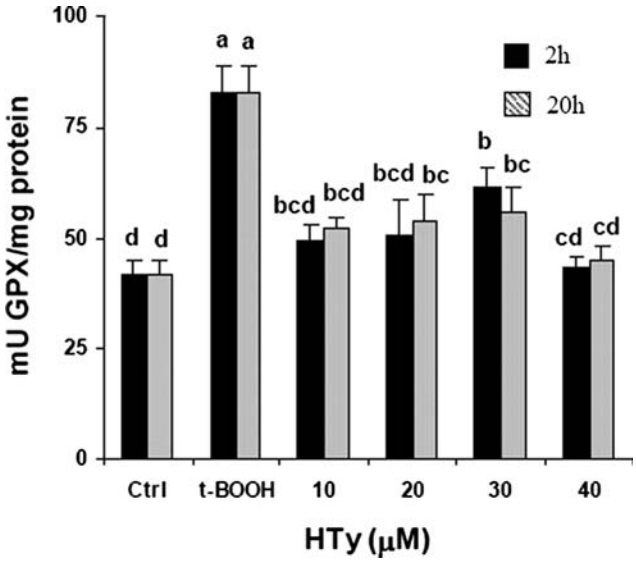

Fig. 5 *Experiment B*. Protective effect of HTy against t-BOOH-induced changes in the activity of GPx. Cells were treated with the noted concentrations of HTy for 2 or 20 h, washed, and 200 μM t-BOOH was added for 3 h to all cultures except controls. Different letters indicate statistically significant differences ($P < 0.05$) among different groups. Values are means ± SD of 4–5 different samples per condition

lipooxygenases [34]. Moreover, HTy has been reported to counteract cytotoxycity induced by ROS in some human cellular models such as Caco-2 [15] and erythrocytes [35]. To date, no research on the antioxidant effects of the main olive oil phenolics in cultured cells from liver origin has been reported. However, the liver is not only the main target for phenolic antioxidants once absorbed from the gastrointestinal tract but is the major place for phenolic metabolism. Therefore, studies dealing with the effect of antioxidant dietary phenolics at a physiological level (liver in live animals) and at a cellular level (cultured cells from liver origin) should be prioritized. Human hepatoma HepG2, a well-differentiated transformed cell line, is a reliable model, easy to culture, well characterized, and widely used for biochemical and nutritional studies where many antioxidants and conditions can be assayed with minor inter-assay variations [21, 22, 24, 25, 29, 36–39].

Although plant phenolics may have potent antioxidant effects in vitro and in vivo, both in cell culture and live animals, elevated doses of these dietary compounds can also be toxic and mutagenic in cell culture systems and excess consumption by mammals could cause adverse metabolic reactions [40]. The range of doses between 10 and 40 μM was finally selected after an extensive search in the literature showing that only concentrations of and above 10 μM HTy were effective in most conditions. In addition, preliminary experiments (data not shown) proved that concentrations of HTy below 50 μM can be safely used in order to test its potential protective effect against a condition of oxidative stress and concentrations below 10 μM showed no protection against the oxidative insult induced by t-BOOH. Therefore, in experiment A, cell toxicity and cellular redox status were determined in cells treated for short- and long-terms with different concentrations of HTy in the micromolar range. When human hepatoma HepG2 cells were pretreated with HTy for 2 or 20 h prior to being submitted to an oxidative stress, cell toxicity was completely prevented, indicating that the antioxidant-treated cells were totally protected against the oxidative insult.

Reduced glutathione (GSH) is the main non-enzymatic antioxidant defense within the cell, reducing different peroxides, hydroperoxides, and radicals (alkyl, alkoxyl, peroxyl, etc.) [41]. It is usually assumed that GSH depletion reflects intracellular oxidation whereas an increase in GSH concentration could be expected to prepare the cell against a potential oxidative insult [22, 24, 25, 39, 40, 42]. In our experimental conditions, treatment of HepG2 cells with 200 μM t-BOOH induced a marked decrease in the concentration of reduced glutathione which was completely prevented by pretreatment with all four doses of HTy for 2 or 20 h. Other reduced soluble thiols including cysteine, gamma-glutamyl cysteine and homocysteine, are able to react with OPT, and although their relevance as compared to the reducing effect of glutathione might be relatively low, some interference with the results should not be ruled out [22, 24, 25].

An important step in the degradation of cell membranes is the reaction of ROS with the double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. On breakdown of such hydroperoxides MDA, a three-carbon compound formed by scission of peroxidized PUFAs, is one of the main products of lipid peroxidation [43, 44]. Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences [45]. However, determination of MDA levels in cell culture conditions is less common in the literature [22, 24, 25, 29, 46]. The protection against lipid peroxidation by HTy in a cell culture, reported here for the first time, is in concert with previous studies that showed a similar protection by tea catechins [36–38], beta carotene, or lutein [36], and quercetin [25] in the same cell line, human hepatoma HepG2.

Direct evaluation of ROS by the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells [47]. A prooxidant such as t-BOOH can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus increasing fluorescence. The results of this study clearly show that HTy strongly reduces the generation of ROS induced by t-BOOH in cultured HepG2, thus preventing or delaying conditions which favor oxidative stress in the cell [22, 24, 25].

GPx catalyses GSH oxidation to GSSG at the expense of H_2O_2 or other peroxides [48], and therefore, is essential for the intracellular quenching of cell-damaging peroxide species. Enzyme defenses activate in order to face the increasing generation of reactive oxygen species such as peroxides induced by the potent prooxidant t-BOOH [22, 24, 25, 40, 48]. The significant increase in the activity of GPx observed after a 3 h treatment with 200 μM t-BOOH, clearly indicates a positive response of the cell defense system to face an oxidative insult [22, 24, 25]. Other natural antioxidants have been tested and significant changes in the enzyme activity of the antioxidant enzymes have been observed only at very high doses [49, 50]. In our experimental conditions, we show, for the first time, that treatment of human hepatoma cells with HTy concentrations in the micromolar range prevents the increase in the activity of GPx induced by oxidative stress either by direct action or via the decrease in ROS production or the increase in GSH. This could represent a major benefit for the cell when dealing with a stressful situation.

In summary, our results extend the protective effect reported for other dietary polyphenols to one of the most common of them in the Mediterranean diet, HTy. The presence of HTy may prepare the antioxidant defense system of the cell to succesfully face a condition of oxidative stress by molecular mechanisms that are currently being studied in our laboratory. Therefore, HTy may contribute to the protection afforded by fruit- and vegetable-rich diets against diseases, such as cardiovascular disease, for which excess production of ROS has been implicated as a causal or contributory factor.

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