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RESEARCH ARTICLE

Hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation *via* extracellular regulated kinases and phosphatidylinositol-3-kinase/protein kinase B pathways in HepG2 cells

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Hydroxytyrosol (HTy) is a natural polyphenol abundant in olive oil, which possesses multiple biological actions. Particularly, HTy has cytoprotective activity against oxidative-stress-induced cell damage, but the underlying mechanisms of action remain unclear. Here, we have investigated the molecular mechanism involved in the protection exerted by HTy on *tert*-butyl hydroperoxide-induced damage in human HepG2 liver cells. Treatment of HepG2 cells with HTy increased the expression and the activity of glutathione-related enzymes such as glutathione peroxidase, glutathione reductase and glutathione *S*-transferase. HTy also induced the nuclear transcription factor erythroid 2p45-related factor (Nrf2), a transcription factor implicated in the expression of several antioxidant/detoxificant enzymes. Moreover, two important signalling proteins involved in Nrf2 translocation, the protein kinase B and the extracellular regulated kinases, were also activated by HTy. Further studies with specific inhibitors confirmed that both molecular pathways are critical for the nuclear translocation of Nrf2, the increased enzyme expression and activity and the beneficial effect against oxidative stress induced by HTy. In conclusion, together with the inherent radical scavenging activity of HTy, our results provide an additional mechanism of action to prevent oxidative stress damage through the modulation of signalling pathways involved in antioxidant/detoxifying enzymes regulation.

Keywords:
Glutathione-related enzymes / Mediterranean diet / Oxidative stress / Phenolic compounds / Signalling pathways

1 Introduction

It is widely known that the Mediterranean diet, characterized by an abundance of fresh fruits and vegetables, is associated with the prevention of various chronic degenerative diseases such as cardiovascular diseases and cancer [1, 2]. At present, there is an increasing interest in extra-virgin olive oil, the major fat source of Mediterranean diet, for its antioxidant properties and its positive effects on oxidative stress associated processes [3]. Similar to fruits and vegetables, olive oil is rich in phenolic compounds with proven antioxidant activity, thus potentially contributing to

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Abbreviations: AKT/PKB, protein kinase B; **ARE,** antioxidant-response element; **DCFH,** dichlorofluorescin; **ERKs,** extracellular regulated kinase; **FBS,** fetal bovine serum; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **GPx,** glutathione peroxidase; **GR,** glutathione reductase; **GST,** glutathione *S*-transferase; **HTy,** hydroxytyrosol; **JNK,** c-jun amino-terminal kinase; **Keap-1,** Kelch-like ECH associated protein-1; **MAPK,** mitogen-activated protein kinase; **NADPH,** Nicotinamide adenine dinucleotide phosphate; **Nrf2,** nuclear transcription factor erythroid 2p45-related factor; **p-AKT,** antiphospho-AKT; **p-EKT,** antiphospho-EKT; **PI3K,** phosphatidylinositol-3-kinase; **p-JNK,** antiphospho-JNK; **ROS,** reactive oxygen species; **t-BOOH,** *tert*-butyl hydroperoxide

the beneficial effects attributed to the Mediterranean diet [4]. In particular, hydroxytyrosol (HTy) is considered one of the most abundant and representative olive oil phenols and its protective effect on cytotoxicity has been thoroughly investigated [5]. However, the precise molecular mechanisms responsible for this protection are still not fully understood.

Biological actions of phenolic compounds have been commonly related to their free radical scavenging activities, but current evidences strongly support that natural biophenols may also offer an indirect protection by increasing the endogenous defence systems [6]. A number of *in vitro* [7, 8] and *in vivo* [9, 10] studies have recently shown a positive effect of different olive phenols on diverse enzymes with antioxidant activities. These cytoprotective enzymes act as critically important regulators in cell protection from oxidative stress and chemical-induced damage by controlling the intracellular redox status. Therefore, their activation by phenolic compounds is considered an effective strategy to protect against oxidative stress and largely accounts for their chemoprotective activities [11].

Regulation of antioxidant and detoxifying enzymes is linked to the nuclear transcription factor erythroid 2p45-related factor (Nrf2). Nrf2 is normally repressed in the cytosol by specific binding to cytoskeleton-associated cytosolic Kelch-like ECH-associated protein 1 (Keap1). The presence of a stimulus leads to the disruption of this complex releasing Nrf2 for translocation to the nucleus where it binds to the electrophile responsive element or antioxidant responsive element (ARE) to regulate genes expression [12]. A number of dietary phytochemicals modulate Nrf2 pathway inducing antioxidant and detoxifying enzymes and contributing to the protection of cells against carcinogens and oxidative stress [13]. The mechanism by which these natural compounds liberate Nrf2 from the Keap1–Nrf2 complex remains to be established. However, accumulating data suggest that certain natural compounds possess the ability to differentially activate a number of cellular kinases, including mitogen-activated protein kinases (MAPKs), protein kinase C and phosphatidylinositol-3-kinase (PI3K), which have been speculated to phosphorylate Nrf2 [14]. Nrf2 phosphorylation stimulates the dissociation of Nrf2 from its repressor Keap1, thereby facilitating nuclear translocation of Nrf2 and gene transcription [12].

Considering the potential role of Nrf2 in protecting cells against oxidative stress, there has been much interest in the natural dietary compounds that could activate this pathway. Accordingly, there is limited information about the effects of biophenols in Nrf2 activation and on whether this signalling cascade mediates their cellular beneficial effects. Therefore, in the present study, we investigated the mode of action of the phenol HTy to protect against oxidative stress induced by the potent oxidant *tert*-butyl hydroperoxide (*t*-BOOH) in HepG2 cells, a useful model for evaluating the cytoprotective effect of natural antioxidants [15–17]. Previous studies have shown that HTy is absorbed and metabolized by human HepG2 cells in culture [18] where it exerted beneficial effects against oxidative stress [19]. Here, we found that HTy attenuated *t*-BOOH-induced injury by increasing the expression and the activity of three glutathione related enzymes, namely glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione *S*-transferase (GST) *via* a mechanism that involves Nrf2 translocation and activation of PI3K/protein kinase B (AKT) and extracellular regulated kinase (ERK) pathways.

2 Materials and methods

2.1 Materials and chemicals

Gentamicin, penicillin G, streptomycin, LY294002 [2-(4-morpholinyl)-8-phenyl-1(4 H)-benzopyran-4- one hydrochloride], PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one], t-BOOH and 3, 4-dihydroxyphenylacetic acid were purchased from Sigma Chemicals (Madrid, Spain). Anti-AKT and antiphospho-AKT (p-AKT), anti-ERK1/2 and antiphospho-ERK1/2 (p-ERKs), anti-*c-jun* amino-terminal kinase (JNK)1/2 and antiphospho-JNK1/2 (p-JNKs), anti-phospho-p38 MAPK and anti-β-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-GPx, anti-GR, anti-GST, anti-p38α MAPK, anti-Nrf2, anti-poly (ADP-ribose) polymerase and anti-growth factor receptor-bound protein 2, were purchased from Santa Cruz (Quimigen, Madrid, Spain). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (Madrid, Spain). RNA isolation kit was obtained from Qiagen (Izasa, Madrid, Spain), primers for RT-PCR were from Isogen (Barcelona, Spain), dNTPs and reverse transcriptase were from Promega (Madrid, Spain) and Taq polymerase was obtained from Roche (Barcelona, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and fetal bovine serum (FBS) from Biowhittaker Europe (Innogenetics, Madrid, Spain).

2.2 Cell culture and HTy treatment

Human HepG2 cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37°C. They were grown in DMEM F-12 medium, supplemented with 2.5% FBS and 50 mg/L of gentamicin, penicillin and streptomycin. Plates were changed to FBS-free medium the day before the assay.

HTy was obtained by chemical synthesis from 3, 4-dihydroxyphenylacetic acid by reduction with $LiAlH_4$ [20]. The different concentrations of HTy (0.5, 1, 5 and 10 μM) were dissolved in serum-free culture medium and added to the cell plates for 20 h. In the experiments with the pharmacological inhibitors, cells were preincubated with 50 μM PD98059 (specific inhibitor of MAPK-ERK kinases) or with 10 μM LY294002 (inhibitor of PI3K) for 1 h prior to 6 or 20 h of HTy treatment. Both compounds were dissolved in

DMSO. To evaluate the protective effect of HTy, after HTy treatment the medium was discarded and fresh medium containing 100 μM of *t*-BOOH was added at different times.

2.3 Cell viability

Cell viability was determined by using the crystal violet assay [21]. HepG2 cells were seeded at low density (10^4 cells *per* well) in 96-well plates, grown for 20 h with the different treatments and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry and 1% SDS added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

2.4 Determination of GPx and GR activities

Cells were collected in PBS and centrifuged at low speed (300 *g*) for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at 3000 *g* for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity is based on the oxidation of GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance of Nicotinamide adenine dinucleotide phosphate (NADPH) by GR [22]. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [23]. Protein was measured by the Bradford reagent.

2.5 Determination of GST activity

GST activity was determined by using a commercial GST fluorimetric activity assay kit (Biovision Research Products, CA, USA). Cells were collected in PBS and centrifuged at low speed (300 *g*) for 5 min to pellet cells. Cell pellets were resuspended in sample buffer, sonicated and centrifuged at 3000 *g* for 15 min and enzyme activity were measured in the supernatants. The assay utilizes monochlorobimane as an artificial substrate and glutathione to determine total GST activity. Fluorescence was measured using a fluorescent microplate reader at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Bio-Tek). Protein was determined by the Bradford reagent.

2.6 RNA extraction and RT-PCR

The levels of antioxidant enzyme gene expression were quantified by semi-quantitative RT–PCR. Cellular RNA was extracted by a Qiagen RNA isolation kit (RNeasy Mini Kit) as described in the manufacturer's manual. Two micrograms of total RNA were submitted to reverse transcriptase and the cDNA products were amplified by PCR using the following couples of primers: 5′-CCTCAAGTACGTCCGACCTG-3′ and 5′-TAGGAGTTGCCAGACTGCTG-3′ for amplification of GPx; 5′-CAGTGGGACTCACGGAAGAT-3′ and 5′-TTCACTGCAACAGCAAAACC-3′ for GR; 5′-TCCGCTGCAAATACATCTCC-3′ and 5′-TGTTTCCCGTTGCCATTGAT-3′ for GST and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The samples were incubated in a Thermo Cycler (PCR Express, Thermo Hybaid, Ashford, UK) using the following parameters: 94°C for 1 min, 58°C for 1 min and 72°C for 2 min (using 31 cycles) followed by a 10-min extension at 72°C for GPx amplification; 92°C for 1 min, 52°C for 1 min and 72°C for 1 min (40 cycles) followed by a 10-min extension at 72°C for GR and 92°C for 1 min, 55°C for 1 min and 72°C for 1 min (35 cycles) followed by a 10-min extension at 72°C for GST and GAPDH amplification. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. The gel was photographed under ultraviolet transillumination and the bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA, USA). Band intensity was normalized to values for GAPDH that was used as an internal control.

2.7 Preparation of total cell lysates

To detect GPx, GR, GST, AKT, p-AKT, ERK1/2, p-ERKs, p38 MAPK, p-p38 MAPK, JNK1/2, p-JNKs and total Nrf2, cells were lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM 1,4-DTT, 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin and 1 mM PMSF. The supernatants were collected, assayed for protein concentration using the Bradford reagent, aliquoted and stored at −80°C until use.

2.8 Preparation of nuclear and cytosolic extracts

To evaluate the cytosolic and nuclear Nrf2 content, cells were resuspended at 4°C in 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF (buffer A). Cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were centrifuged at 10 000 g for 2 min and the supernatant containing the cytosolic fraction was collected and stored at −80°C. The pellet was resuspended in cold buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation at 13 000 g for 10 min at 4°C, and the supernatant containing nuclear protein extract was stored at −80°C. Proteins were measured using the Bradford reagent.

2.9 Western blotting analysis

Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain) or peroxide-conjugated antimouse IgG (Sigma, Madrid, Spain) for GPx, GR and GST antibodies. Blots were developed with the ECL system (GE Healthcare). Anti-growth factor receptor-bound protein 2 and anti-poly (ADP-ribose) polymerase antibodies were used as markers for the cytosolic and nuclear extracts, respectively. Normalization of Western blot was ensured by β-actin and bands were quantified by laser scanning densitometry (Molecular Dynamics).

2.10 Determination of reactive oxygen species generation

Cellular reactive oxygen species (ROS) were quantified by the dichlorofluorescin (DCFH) assay. For the assay, cells were plated in 24-well at a rate of 2×10^5 cells *per* well. After the different treatments, 5 μM DCFH was added to the wells for 30 min at 37°C. Then, cells were washed twice with PBS and 0.5 mL of serum-free medium with or without 100 μM of *t*-BOOH were added *per* well. After being oxidized by intracellular oxidants, DCFH becomes dichlorofluorescein and emits fluorescence. ROS generation was evaluated at 5, 15, 30 60 and 120 min in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek).

2.11 Statistics

Prior to analysis, it was verified that all the variables analyzed follow a normal distribution using the Kolmogorov–Smirnov test. Then, 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 the Tamhane test when variances were not homogeneous (only in ROS assay). The level of significance was $p<0.05$. A SPSS version 15.0 program has been used.

3 Results

3.1 Protective effect of HTy in HepG2 cells

HepG2 cells were treated with increasing concentrations of HTy (0.5, 1, 5 and 10 μM) during 20 h and cell viability was measured by the crystal violet assay. Figure 1A shows that none of the tested concentrations of HTy induced cell injury in HepG2 cells. To determine whether treatment with HTy has a cytoprotective effect against an oxidative challenge, the effect of the phenol on *t*-BOOH-induced damage was investigated. HepG2 cells treated for 20 h with different concentrations of HTy were further exposed to 100 μM *t*-BOOH for 6 h and cell death was evaluated. As shown in Fig. 1B, 6 h of treatment with *t*-BOOH evoked a significant decrease in cell viability (about 40%) in control cells. However, the treatment with different concentrations of HTy significantly suppressed the deleterious effect induced by the stressor.

3.2 Enhancement of GPx, GR and GST enzymatic activities by HTy

Since induction of antioxidant cellular defences is considered one of the crucial mechanisms to protect cells against oxidative injuries, we evaluated whether HTy could increase the activity of different antioxidant/detoxifying enzymes. To this end, HepG2 cells were incubated with the indicated

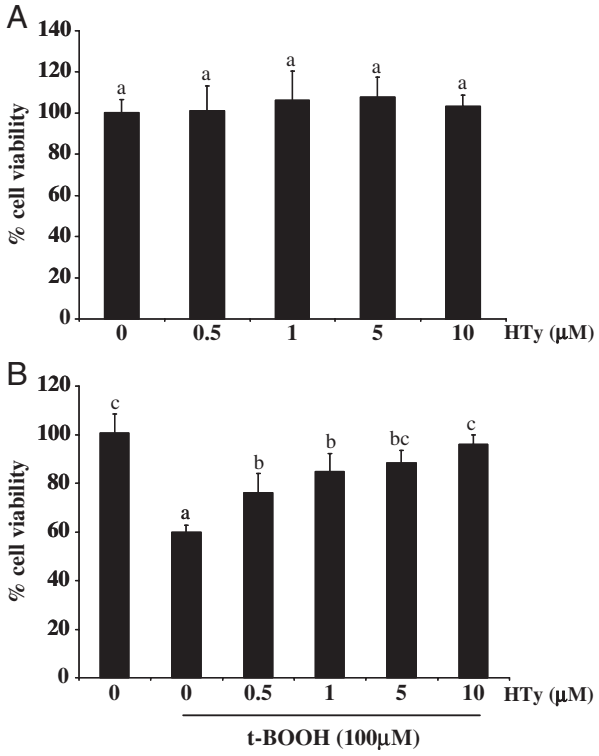

Figure 1. Protective effect of HTy against *t*-BOOH induced cell death. HepG2 cells were treated with the noted concentrations of HTy for 20 h. Cell viability was expressed as relative percentage of control cells staining (A). Control and HTy-treated cells were further exposed to 100 μM of *t*-BOOH during 6 h and cell viability were evaluated (B). Data represent means ± SD of 10–12 samples *per* condition. Different letters denote statistically significant differences, $p<0.05$.

concentrations of HTy for 20 h, and the activity of GPx, GR and GST were measured. As shown in Fig. 2, treatment with 0.5–10 μM of HTy significantly increased the activity of GR and GST enzymes while only the highest doses (5–10 μM) of HTy were able to increase GPx activity. Since 10 μM HTy evoked the maximal activity of the three glutathione-related enzymes in HepG2 cells, all subsequent experiments with HTy were performed using this concentration.

3.3 HTy induces GPx, GR and GST gene and protein expression

The effect of HTy on glutathione-related enzymes gene and protein expression was further examined. HepG2 cells were incubated with 10 μM HTy for 20 h and levels of mRNA and protein expression were analyzed by semiquantitative RT-PCR analysis and Western blot, respectively. As shown in Fig. 3A, HTy significantly increases the mRNA levels of GPx, GR and GST enzymes in HepG2 cells. Western blot analyses using anti-GPx, -GR and -GST antibodies confirmed that the increase in antioxidants enzymes mRNA levels by HTy was accompanied by a significantly increase in protein expression (Fig. 3B).

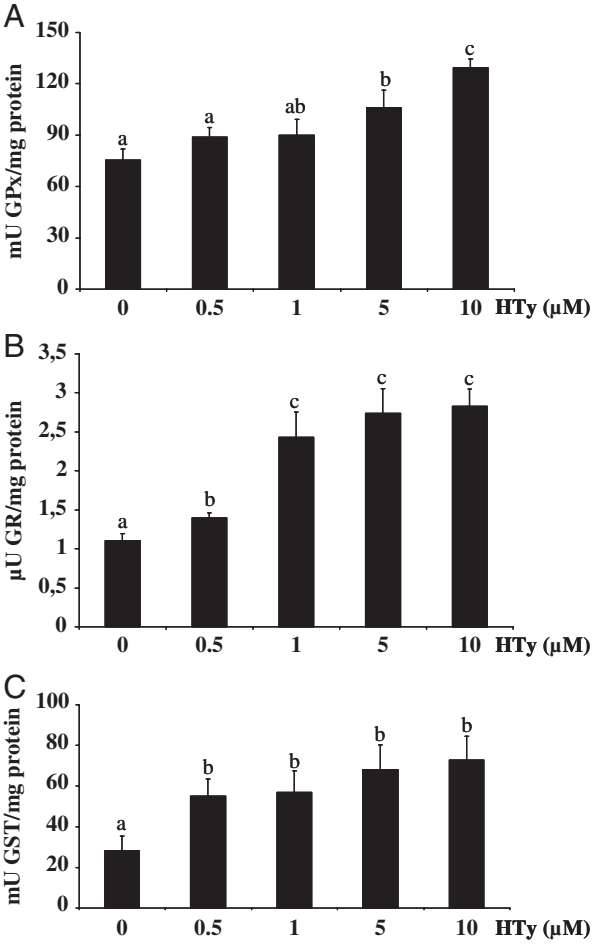

Figure 2. Induction of GPx, GR and GST activity by HTy. HepG2 cells were treated with 0.5-10 μM of HTy for 20 h. GPx (A), GR (B) and GST (C) activities were measured as described in Section 2. Values are means of six to eight different samples *per* condition. Means without a common letter differ, $p < 0.05$.

3.4 HTy induces the nuclear translocation of Nfr2

Next, we investigated the effect of HTy in the expression and the nuclear translocation of the transcription factor Nrf2. Accordingly, HepG2 cells were treated with 10 μM of HTy for 3, 6 and 20 h, and Nrf2 proteins in total lysates and in the cell nuclear or cytosolic compartment were measured by Western blot. As shown in Fig. 4, treatment of cells with HTy increased the protein levels of Nrf2 in the nucleus at 3 h, peaked at 6 h and continued elevated up to 20 h of treatment. The increase of Nrf2 in the nuclear fraction was accompanied by a parallel decrease of the protein in the cytosolic compartment whereas Nrf2 in total cell lysates remained unchanged at any time.

3.5 HTy increases the phosphorylation of AKT and ERKs in HepG2 cells

To further elucidate the upstream signalling pathways involved in the activation of Nrf2, we examined the phosphorylation states of AKT and MAPKs subfamilies, JNKs, ERKs and p38 MAPK. HepG2 cells were exposed to 10 μM HTy during 1, 2 or 3 h and then immunoblots were performed using phospho- and non-phospho-antibodies against p-38 MAPK, JNKs, ERKs, and AKT. HTy-treated cells showed a significant increase in the levels of phosphorylation of AKT and ERKs protein that was evident after 1 h of treatment with HTy and remained enhanced up for 3 h. Conversely, there was no difference in the phosphorylated levels of p38 MAPK and JNKs or in the total levels of AKT, ERKs, p-38 MAPK or JNKs (Fig. 5).

3.6 Role of PI3K/AKT and ERK pathways in Nrf2 nuclear translocation

To address the involvement of PI3K/AKT and ERK pathways in the HTy-induced nuclear translocation of Nrf2, we examined the effects of LY294002 and PD98059, specific inhibitors for the PI3K and ERKs, respectively, on this process. HepG2 cells were pre-treated with PI3K or ERKs inhibitors during 1 h and then treated with 10 μM HTy for 6 h. Figure 6 shows that the inhibition of the two kinases decreased the nuclear levels of Nrf2 in control cells and significantly reduced the nuclear translocation of Nrf2 induced by HTy. The observed increase of cytosolic Nrf2 in

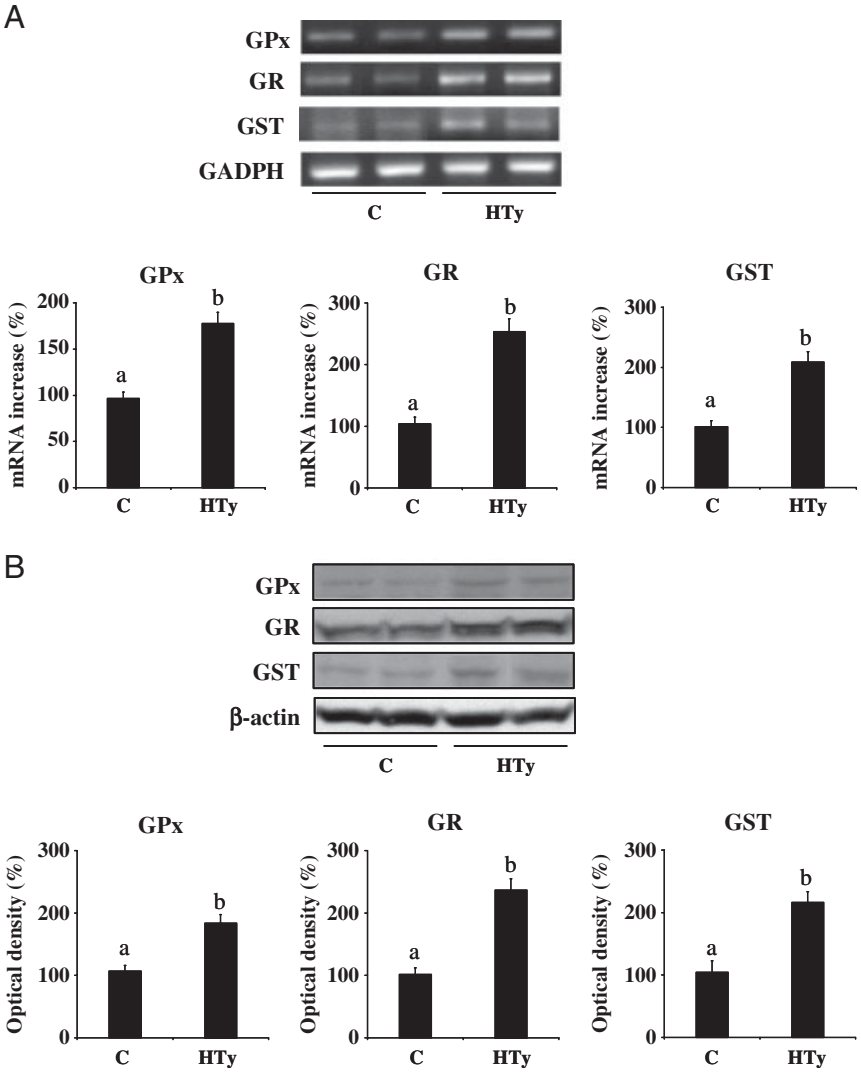

Figure 3. Induction of GPx, GR and GST mRNA and protein expression by HTy. HepG2 cells were treated with 10 μM of HTy for 20 h. Representative RT-PCR of two different experiments and percentage values of mRNA levels of GPx, GR and GST relative to control condition (means ± SD) (A). Representative Western blot of two different experiments and percentage values of GPx, GR and GST relative to the control conditions (means ± SD) (B). Means without a common letter differ, $p < 0.05$.

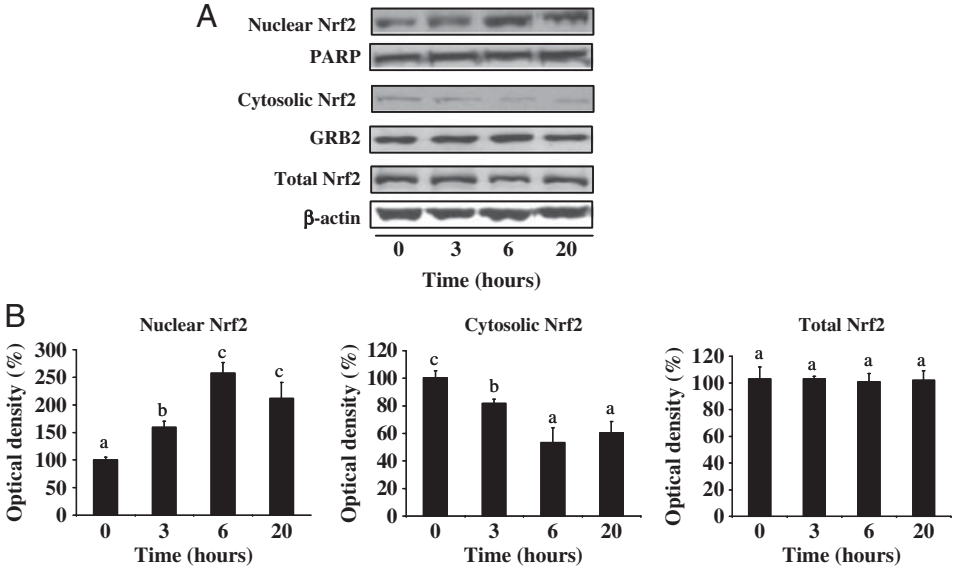

Figure 4. Effects of HTy on nuclear, cytosolic and total Nrf2 levels. HepG2 cells were incubated with 10 μM of HTy for 3, 6 and 20 h and subjected to Western blot analysis. Representative bands of four to six experiments (A). Percentage values of nuclear, cytosolic and total Nrf2 relative to the control condition (means ± SD) (B). Means without a common letter differ, $p < 0.05$.

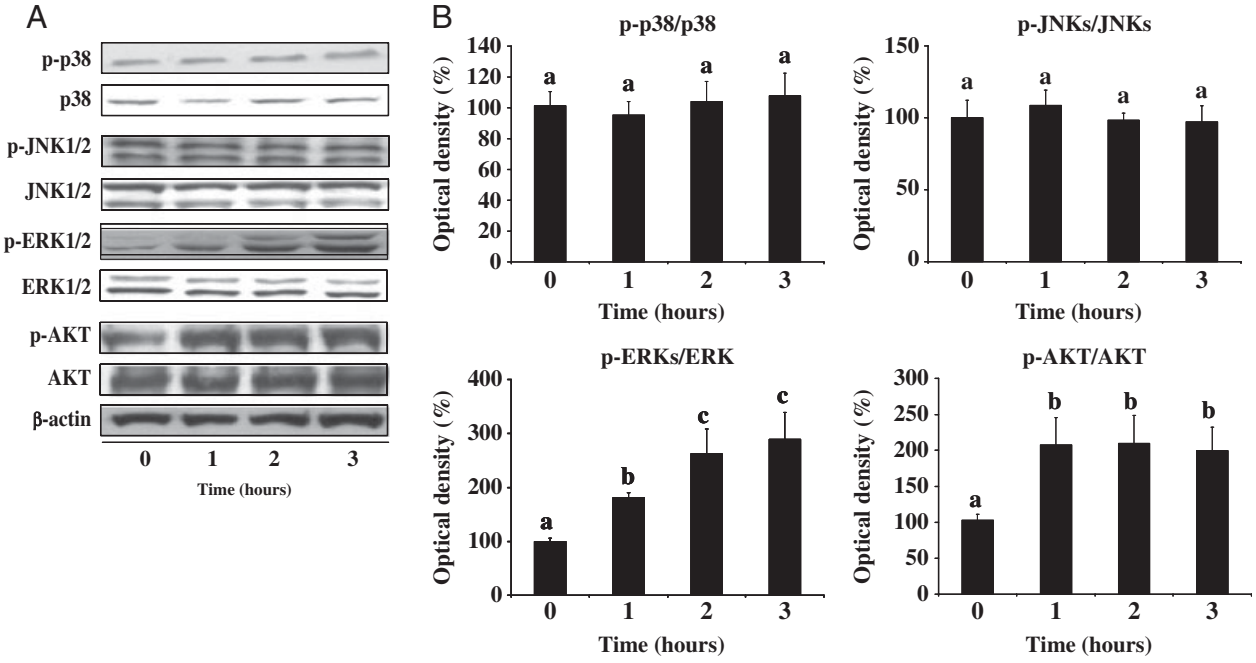

Figure 5. Effect of HTy on total and phosphorylated levels of p-38, JNKs, ERKs and AKT. HepG2 cells were treated with 10 μM of HTy during 1, 2 or 3 h and subjected to Western blot analysis. Bands are representative of four to six experiments (A). Percentage values of the p-p38/p-38, p-JNK/JNK, p-ERK/ERK and p-AKT/AKT ratio relative to the control condition (means ± SD) (B). Means without a common letter differ, $p < 0.05$.

the presence of PI3K or ERKs inhibitors also suggested the involvement of both pathways in its HTy-induced translocation.

3.7 Involvement of PI3K/AKT and ERKs pathways in HTy-induced antioxidant/detoxificant enzymes

Next, we examined whether the inhibition of PI3K/AKT or ERKs would also abolish the increase in the expression and the enzymatic activities provoked by HTy. HepG2 cells were pre-treated with LY and PD for 1 h and then treated with HTy for 20 h. Treatment with the PI3K/AKT and ERKs inhibitors completely suppressed both the increased mRNA expression (Fig. 7A) and the increased protein expression (Fig. 7B) of GPx, GR and GST induced by HTy. Similarly, inhibition of PI3K/AKT by LY completely blocked the HTy-increased GPx and GST activities and almost totally prevented the increase in GR activity. Similarly, the presence of ERKs inhibitor abolished the effect of HTy in GPx activity and significantly reduced the increase in GR and GST activities (Fig. 7C).

3.8 Role of PI3K/AKT and ERK pathways in cytoprotection by HTy

Finally, we investigated whether the activation of AKT and ERKs could be involved in the cytoprotective effect of HTy from t-BOOH induced injury. Since t-BOOH-induced injure results from increased ROS generation, we examined the effect of HTy and the specific inhibitors (LY and PD) in ROS levels and cell damage induced by t-BOOH. HepG2 cells were pre-treated with LY or PD during 1 h and then treated with or without HTy for 20 h. After that, the HTy-treated cells and the controls were exposed to 100 μM t-BOOH, and ROS generation at different times (5–120 min) and cell death at 6 h were evaluated. LY or PD alone did not have any effect on ROS generation or cell viability (data not shown).

As presented in Table 1, pre-treatment of cells with 10 μM HTy for 20 h significantly reduced the generation of oxidative radicals caused by t-BOOH. However, this reduction was completely blocked by the specific inhibitors of PI3K and ERKs. Similarly, inhibition of these pathways significantly blocked the cytoprotective effect of HTy against cell death induced by the pro-oxidant (Fig. 8).

4 Discussion

Olive oil, a relevant component of the Mediterranean diet, is considered an important source of dietary antioxidants. Its phenolic compound, HTy, has been shown to be highly efficient in protecting cells against oxidative stress-induced damage [19]. The positive effect of HTy has been long related to its proficient action as radical scavenger [5]. In this study we demonstrate that HTy modulates important signalling proteins involved in the induction of cytoprotective enzymes,

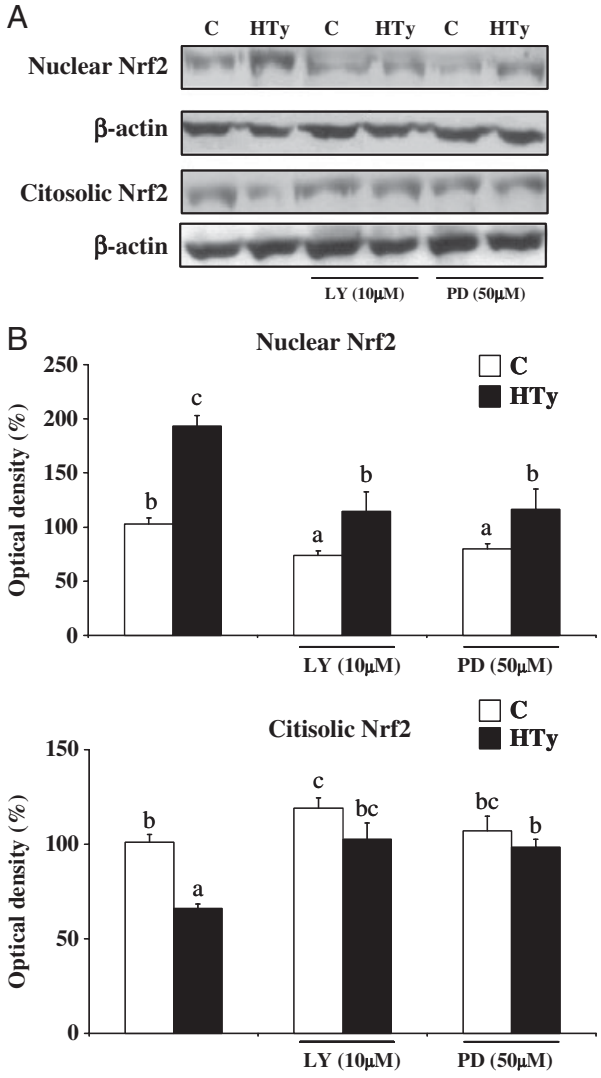

Figure 6. Effects of HTy and selective inhibitors LY294002 (LY) and PD98059 (PD) on nuclear, cytosolic and total Nrf2 levels. HepG2 cells pre-treated with 10 μM LY or 50 μM PD for 1 h and treated with or without 10 μM HTy for 6 h were subjected to Western blot analysis. Bands are representative of four experiments (A). Percentage values of nuclear, cytosolic and total Nrf2 relative to the control condition (means ± SD) (B). Means without a common letter differ, $p < 0.05$.

thereby fortifying the inherent cellular defence capacity as an additional mechanism of cell protection.

The endogenous antioxidant gluthatione and its related enzymes GR, GPx and GST play a key role in defence mechanisms against reactive free radicals and other oxidant species. GST and GPx are involved in eliminating peroxides providing an important cellular defence mechanism against oxidative damage [24, 25], whereas GR is responsible for the regeneration of oxidized glutathione [26]. Consequently, enhancement of glutathione-related enzymes seems to prepare cells to prevent ROS generation in the presence of stressors and thus to suppress oxidative-stress induced damage [27]. Consistent with this, our results revealed that long-term pre-treatment of HepG2 cells with HTy increased the activity of these protective enzymes and effectively inhibited the cytotoxicity exerted by the potent prooxidant t-BOOH. Furthermore, the enhancement in GPx, GR and GST enzyme activities was correlated with a parallel augment in mRNA and protein levels, suggesting that HTy can modulate GSH-related enzymes gene expression, as previously described with other phenolic compounds [6, 28]. Therefore, we propose that the increased endogenous defence capacity evoked by HTy could be a critical determinant to protect against oxidative stress-induced injury.

The mode of action of phenolic compounds to induce the endogenous antioxidant defence is the subject of extensive investigations. At present, the transcriptional factor Nrf2 is considered a key protein that up-regulates the activity and expression of numerous antioxidant/detoxifying enzymes through interaction with the ARE [12]. Many antioxidants derived from dietary and medicinal plants have been shown to induce gene expression by enhancing Nrf2 expression and/or its nuclear translocation [13]. Similarly, phenolic compounds such as curcumin [29], quercetin [30] or chlorogenic acid [31] have been reported to induce cytoprotective enzymes through stimulation of Nrf2 transactivation [14]. Accordingly, in the present study, we found that HTy clearly induces the nuclear translocation of Nrf2, a necessary event to exert its regulatory effects. Moreover, this observation was consistent with the increased GPx, GR and GST gene expression, suggesting that the nuclear translocation of Nrf2 might contribute to the induction of ARE-mediated glutathione-related enzymes gene expression after HTy treatment. Supporting these, it has been previously described that HTy is able to increase the mRNA expression of a cystine transporter, involved in the synthesis of GSH, *via* Nrf2 translocation in human skin cells [32]. Our results also agree with those of Liu *et al.* [33] showing that HTy pretreatment significantly prevent the decrease of Nrf2 induced by acrolein in retinal pigment epithelial cells.

One of the major contributing mechanisms of Nrf2 activation by phenolic compounds is the phosphorylation of Nrf2 at specific serine and/or tyrosine residues *via* activation of upstream signalling pathways such as MAPKs or AKT [13]. Many dietary phenols have shown to exert modulatory actions in cells by interacting with a wide variety of molecular targets central to the cell signalling machinery [34, 35]. Therefore, to identify upstream regulatory mechanisms involved in the nuclear translocation of Nrf2 induced by HTy, the effect of HTy on the phosphorylated levels of AKT and MAPKs, including ERKs, JNKs and p38 MAPK were also examined in this study. We found that HTy induced the phosphorylation of AKT and ERKs, whereas JNK and p38 MAPK were not activated by the phenol. Furthermore, the use of selective inhibitors of ERKs and PI3K prevents the nuclear translocation of Nrf2 induced by HTy, indicating that the activation of these pathways is intimately involved in the process. Similarly, the increased expression and activity of GPx, GR and GST was suppressed in the presence of any of the inhibitors, pointing

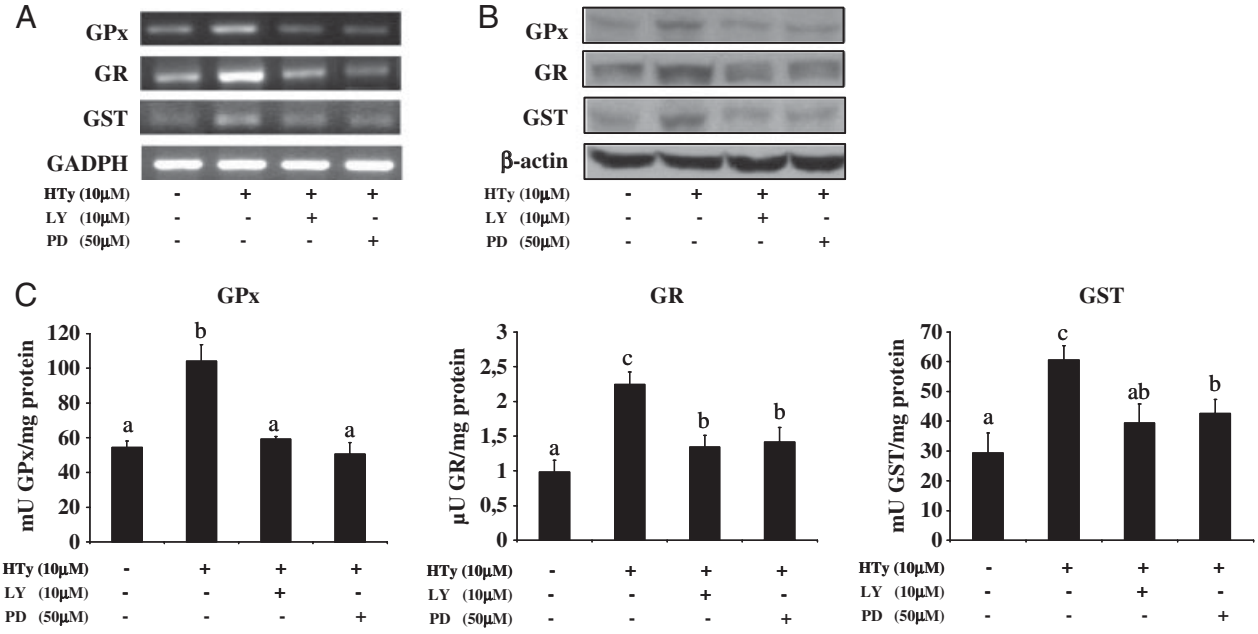

Figure 7. Effect of LY294002 (LY) and PD98059 (PD) on GPx, GR and GST mRNA, protein level and activity. HepG2 cells were pre-treated with 10 μM LY or 50 μM PD for 1 h and then treated with 10 μM HTy for 20 h. Representative RT-PCR of three different experiments (A). Representative Western blots of three different experiments (B). GPx, GR and GST activities were measured as described in Section 2. Values are means of six to eight different samples *per* condition (C). Means without a common letter differ, $p<0.05$.

Table 1. Effect of treatment with 10 μM HTy, 10 μM HTy plus 10 μM LY294002 (HTy+LY) and 10 μM HTy plus 50 μM PD98059 (HTy+PD) on intracellular ROS generation induced by t-BOOH (T)[a)]

	ROS (fluorescence units)				
	5 min	15 min	30 min	60 min	120 min
C	537±36[a]	1292±60[a]	3029±105[a]	5128±121[a]	6744±104[a]
C+T	1023±42[c]	2294±84[c]	5185±114[c]	8478±225[c]	14281±963[c]
HTy+T	716±37[b]	1615±75[b]	3749±119[b]	6564±154[b]	10906±718[b]
(HTy+LY)+T	660±75[b]	2276±195[c]	4908±104[c]	7901±236[c]	13865±695[c]
(HTy+PD)+T	526±21[a]	2185±65[c]	4884±145[c]	7856±265[c]	13776±947[c]

a) Data represent the means (±SD) of 10–12 different samples *per* condition. There was a significant increase in ROS generation through time within every condition. At any time period, the different conditions were analyzed using one-way ANOVA followed by the appropriate test. Means in a column with different letters differ, $p<0.05$.

out the close association between Nrf2 translocation and the induction of glutathione-related enzymes. The present study demonstrates for the first time that the phenol HTy induces the expression levels and the activity of antioxidant/detoxifying enzymes through a mechanism involving nuclear translocation of Nrf2 and the activation of PI3K/AKT and ERKs pathways. In a previous study, the phenol epigallocatechin gallate was also reported to activate Nrf2 *via* PI3-kinase/AKT and ERK1/2 in endothelial cells to induced heme oxygenase-1 expression [36]. Similarly, other natural compounds have been reported to increase the cellular antioxidant defence capacity in different cell types through the induction of cytoprotective enzymes *via* the PI3K/AKT and/or the MAPKs pathways [37].

Since induction of cytoprotective enzymes has been shown to be an important pathway to protect cells against oxidative damage [38], we further investigated the role that phosphorylation of ERKs and AKT played in cell protection. We have previously shown that *t*-BOOH-induced injury in HepG2 cells is mediated by increased ROS generation [27]. In this study, the treatment of cells with HTy prevented the increase in intracellular ROS and the cell death induced by *t*-BOOH. However, HTy-mediated cytoprotection against *t*-BOOH was significantly reduced in the presence of specific inhibitors of ERKs and PI3K. These observations strongly suggest that phosphorylation of ERKs and AKT and the subsequent activation of glutathione related enzymes are involved in the protective mechanism exerted by HTy against oxidative stress in HepG2 cells. Therefore, we can assume that HTy-treated cells are able to maintain cellular homeostasis during oxidative stress, at least in part, by their

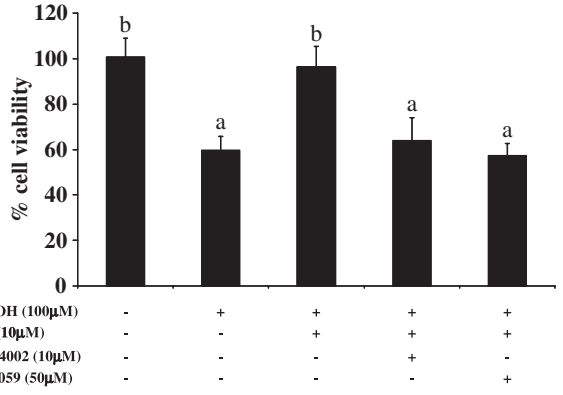

Figure 8. Effects of HTy and selective inhibitors LY294002 (LY) and PD98059 (PD) on cell death induced by *t*-BOOH. HepG2 cells incubated with 10 μM of HTy for 20 h in the presence or absence of 10 μM LY or 50 μM PD were further exposed to 100 μM *t*-BOOH during 6 h. Cell death was expressed as relative percentage of control cells staining. Data represent means ± SD of ten separate experiments. Different letters denote statistically significant differences, $p < 0.05$.

ability to induce glutathione related enzymes, which probably decrease the level of oxidative stress caused by ROS.

It is worth noting that olive oil phenols, particularly HTy, are well absorbed and metabolized *in vivo* [39]. A recent study has shown that a single ingestion of 40 mL of olive oil leads to a plasma HTy concentration around 20 μM. [40]. Consequently, data here reported showed clear protective effects of HTy at doses that could be reached *in vivo*. Moreover, it would be predictable that HTy has additive or synergistic effects *in vivo* with other phenolic compounds present in olive oil and/or with antioxidants that are abundant in the Mediterranean diet [41].

In summary, we have shown that HTy is able to enhance the cellular antioxidant defence capacity, thereby protecting cells from oxidative stress. The effect of HTy was dependent on stimulation of the PI3K/AKT and ERKs pathways followed by the nuclear translocation of Nrf2 and the subsequent activation of antioxidant/detoxifying enzymes such as GPx, GR and GST. Interestingly, activation of upstream signalling pathways by HTy was essential in determining the susceptibility of HepG2 cells to cell injury elicited by t-BOOH. Therefore, together with the inherent radical scavenging activity, the effect of HTy modulating signalling pathways involved in antioxidant/detoxifying enzymes regulation should be considered as an additional mechanism of the olive oil phenol action contributing to prevent oxidative stress-associated cell damage.

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