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Oleuropein, a Secoiridoid Derived from Olive Tree, Inhibits the Proliferation of Human Colorectal Cancer Cell Through Downregulation of HIF-1α

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Oleuropein (OL) is the most prominent phenolic compound in the fruit of olive tree. Although OL has shown powerful anticancer activity the underlying action mechanism remains largely unknown. The present study evaluated the effects of OL on hydroxityrosol (HT)-29 human colon adenocarcinoma cells in comparison to hydroxytyrosol, its hydrolysis product, and to elucidate the underlying anticancer molecular mechanisms involved. Cell proliferation was determined using SRB assay. Cell cycle and apoptosis were assessed by flow cytometry and changes in MAPK cascade protein expression, HIF-1α, p53, PPARγ, and NFKβ signaling pathways by Western blot. Although OL showed less potency than HT, in terms of cell growth inhibition, induced significant changes in cell cycle analysis and caused a significant increase in the apoptotic population. Both compounds produced a remarkable decrease in HIF-1α protein and an upregulation of p53 protein expression. However, no significant changes in IκB-α and MAPK cascade protein expressions were observed. HT produced a significant upregulation in peroxisome proliferator-activated receptor gamma (PPARγ) expression whereas OL failed. PPARγ upregulation may be one of the principal mechanisms of the tumor shrinkage by HT. Our novel findings demonstrate that OL limits the growth and induces apoptosis in HT-29 cells via p53 pathway activation adapting the HIF-1α response to hypoxia.

INTRODUCTION

The fact that only 5%–10% of all cancer cases are due to genetic defects and that the remaining 90%–95% are due to lifestyle factors such as diet, infections, and environmental pollutants provides major opportunities for preventing cancer. The association between the most frequent tumors (colorectal, lung, breast, stomach, and prostate) and several selected food and nutrient factors is clear (1). Mediterranean countries have lower rates of mortality from colon cancer than Northern European or other Western countries. This has been attributed, at least in part, to the so-called Mediterranean diet, which is characterized by large amounts of naturally derived food, such as vegetables, fruits, nuts, fish, and grains and, in opposition to all other healthy diets, has a high content of total fat as its most distinctive feature. This is due to the usual high intake of olive oil, from olive tree, *Olea europaea*, the characteristic culinary fat of the Mediterranean area (2).

In this sense, OL, a heterosidic ester of β-glucosylated oleoside 11-methylester and HT (3, 4-dihydroxyphenylethanol) (Fig. 1) are the most significant phenolic compounds present in the fruit of olive tree. Both compounds are absorbed in a dose-dependent manner in animals and humans and are excreted in the urine mainly as glucuronide conjugates (3). The absorption takes place in the small intestine and colon (4). Finally, after postprandial absorption, they are bonded to circulating human lipoproteins (5).

Regarding to their biological actions, both compounds have demonstrated their powerful antioxidant capacity, as well as antiinflammatory, antiplatelet aggregation, antiatherogenic and cardioprotective, antimicrobial, antiviral, and anticancer activities (6,7).

With regard to the anticancer capacity of OL, there are only few studies. For instance, Hamdi et al. showed that OL inhibited growth on LN-18 cells (8), a poorly differentiated glioblastoma cell line, TF-1a, an erythroleukemia and tumor cell lines derived from advanced-grade human tumors. Later, Menendez et al. and Han et al. showed that OL was the most potent phenolic compound in decreasing breast cell viability inducing strong tumoricidal effects on MCF7 cells and HER2-over2 expressing breast carcinomas (9,10). Similarly, Goulas et al. have reported antiproliferative effects of crude extracts with a high concentration of OL on MCF7 cells, human urinary bladder carcinoma (T-24) and bovine brain capillary endothelial (11). However, the molecular mechanisms underlying the anticancer effects remain to be explored in depth.

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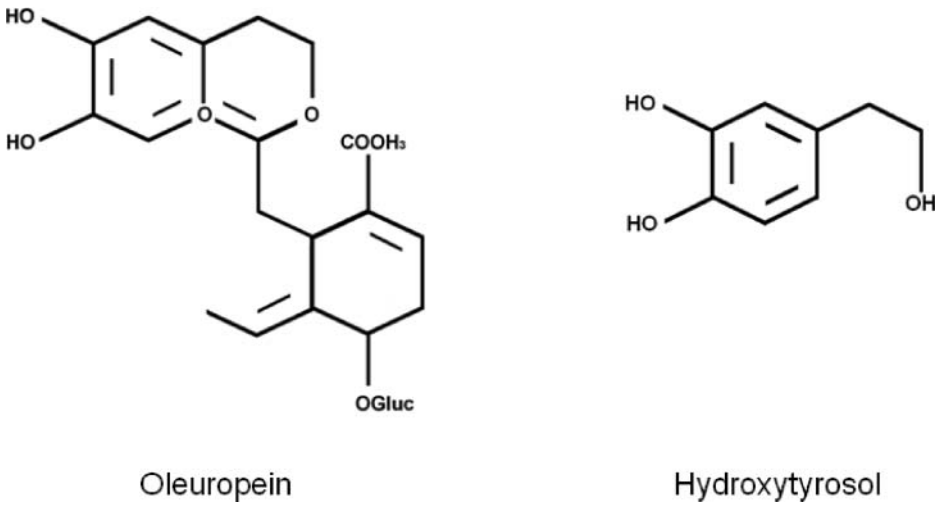

FIG. 1. Chemical structures of oleuropein (OL) and hydroxityrosol (HT).

Taken this background into account, we designed the present study to investigate the effects of OL in comparison to HT on HT-29 human colon adenocarcinoma cells proliferation, cell cycle, and apoptosis as well as to elucidate the underlying anticancer molecular mechanisms involved. In particular, we studied the key role of peroxisome proliferator-activated receptor gamma (PPARγ), hypoxia-inducible factor alpha (HIF-1α), tumor suppressor p53 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 mitogen-activated protein kinase (MAPK) and Jun n-terminal kinase (JNK) signaling pathways, in the anticancer effects of OL ***vs.*** HT on HT-29 human colon adenocarcinoma cells.

MATERIALS AND METHODS

Cell Culture and Treatment

The human colon adenocarcinoma grade II cell line HT-29 was obtained from the European Collection of Cell Cultures and was maintained in McKoy's media (PAA, Pasching, Austria) supplemented with 10% of fetal calf serum (FCS) (PAA, Pasching, Austria), in presence of 100 mg/mL streptomycin and 100 U/mL penicillin (PAA®, Pasching, Austria) at 37°C and 5% CO2 atmosphere. The cells were kept subconfluent at a density of 0.5×10^6 cells/mL. In each experiment, viability was always $\geq$95% [as assayed by Trypan Blue exclusion test (Fluka, Barcelona, Spain)]. HT and OL were purchased from Extrasynthese (Genay, France) and were always freshly prepared in dimethylsulfoxide (DMSO) (Panreac, Spain) stock solution at a concentration of 0.15 M and diluted to desired concentration directly in the culture medium. The final concentration of DMSO (Panreac, Barcelona, Spain) in all experiments was always $\leq$ 1%.

Antiproliferative Assay

Cells were seeded at a density of 5000 cell per well (15.000 cell/ cm^2) in 96-well plates. The plates were incubated for 24, 48, and 72 h. At the end of the exposure time, cell growth was analyzed using the sulphorhodamine B (SRB) assay (Sigma-Aldrich, St Louis, MO). Ten μM 5-Fluorouracile (Sigma-Aldrich, St Louis, MO) was used as positive control (data not shown).

After incubation time, adherent cell cultures were fixed *in situ* by adding 50 μl of 50% (wt/v) cold of trichloroacetic acid (Sigma-Aldrich, St Louis, MO, USA) and incubated for 60 min at 4°C. The supernatant was then discarded, and the plates were washed 5 times with deionized water and then dried. 50 μL of SRB solution (0.4% wt/v in 1% acetic acid (Panreac, Spain), was added to each well and incubated for 30 min at room temperature. Plates containing SRB solution were washed 5 times with 1% acetic acid. Then, plates were air dried and 100 μL per well of 10 mmol/L Tris base pH 10.5 (Sigma-Aldrich, St Louis, MO, USA) were added and the absorbance of each well was read on an enzyme-linked immunosorbent assay reader at 510 nm. Finally, cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells). Seventy-two hour DMSO control was used for each of the different concentration assayed.

Cell Cycle Analysis

HT-29 cells (5×10^5 cells/mL) were seeded in 6-well plates. Cells were treated in presence or absence of different concentrations of HT and OL in 2 mL of serum complete media. After 24-h treatment, media were collected and cells were detached by brief trypsinization (0.25% trypsin-EDTA; PAA, Pasching, Austria). Then, cells were centrifugated at 4500 rpm for 3 min at room temperature, washed with 1 mL of ice-cool PBS and resuspended in 1 mL of ice-cold 70% ethanol. Cells were fixed at 4°C overnight and samples were then centrifuged as above and washed with ice-cold PBS. Cell pellets were resuspended in 1 mL PBS containing 100 μg/mL RNase (Sigma-Aldrich, St Louis, MO) and incubated at room temperature for 20 min. After that, 50 μg/mL of propidium iodide (PI; Sigma-Aldrich,

St Louis, MO) was added to each sample and incubated on ice in the dark for 30 min. The percentages of G0/G1, S, and G2/M cells were determined by flow cytometry (FACS Calibur Systen, Becton Dickinson, Franklin Lakes, NJ).

Detection of Apoptosis

Cells were gently detached by brief trypsinization, washed with ice-cold PBS after centrifugation (4500 rpm for 3 min, 4°C) and resuspended in ice-cold 1X binding buffer (BB) to 2 $\times$ 10^6 cells/mL. Samples were incubated with 2.5 μL Annexin V-FITC and 2.5 μL PI solution (Apoptosis Detection Kit I, BD Pharmigen, Franklin Lakes, NJ) for 15 min in the dark. A further 400 μL BB was then added and cell samples were assessed by flow cytometry (FACS Calibur Systen, Becton Dickinson, Franklin Lakes, NJ). Four different groups of cells were obtained based on their stainability: those unstainable with annexin V and PI [annexin(-)/PI(-)]: viable cells (quadrant F3); those stainable with annexin V but unstainable with PI [annexin(+)/PI(-)]: early apoptotic cells (quadrant F4); those stainable with both annexin V and PI [annexin(+)/PI (+)]: late apoptotic or necrotic cells (quadrant F2); and those unstainable with annexin V but staible with PI [annexin(-)/PI(+)]: primary necrotic cells (quadrant F1). The untreated population was used to define the basal level of apoptotic and dead cells. The percentage of cells that have been induced to undergo of apoptotic is then determined by subtracting the percentage of apoptotic cells in untreated population from percentage of apoptotic cells in the treated population.

Immunoblot Analyses

Cells (1 $\times$ 10^6 cells/mL) were rinsed, scraped off, and collected in ice-cold PBS containing a cocktail of protease and phosphatase inhibitors. Protein concentration was measured for each sample using a protein assay reagent (BioRad, Hercules, CA) according to the Bradford method (12) and using γ-globuline as a standard. Equal amounts of cell protein extract (50 μg) were loaded on 7%–10% SDS–polyacrylamide electrophoresis gel, transferred onto nitrocellulose membranes and incubated in the appropriate blocking solution (5% nonfat dry milk, 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.6) for 2 h at room temperature, and then incubated in primary antibody-containing blocking solutions rabbit anti-PPARγ, rabbit anti-NFκB inhibitory protein alpha (IκBα; Cell Signalling, Danvers, MA) (1:1000); rabbit anti-HIF-1α, mouse anti-phospho-JNK, rabbit-Jun n-terminal kinase (JNK), mouse antiphospho p38, rabbit-p38 (Santa Cruz Biotechnology, Barcelona, Spain) (1:200); mouse anti-p53 (1:75) (Calbiochem, San Diego, CA) overnight at 4°C and washed 3 times. After rinsing, the membranes were incubated with a horseradish peroxidase-labelled (HRP) secondary antibody [antirabbit (Cayman Chemical®, Ann Arbor, MI)] at dilution 1:50,000 or antimouse (Dako®, Atlanta, GA, USA Spain) (1:2000) containing blocking solution for 1–2 h at room temperature. Immunodetection was performed using enhanced chemioluminiscence light-detecting kit (Pierce, Rockford, IL). Then, the immunosignals were captured using LAS-3000 Imaging System from Fuji Densitometric and data were studied following the normalization to the housekeeping protein, β-actin (Sigma-Aldrich, St Louis, MO) and assessed by KODAK 1D Image Analysis software.

Statistical Analysis

All values in the figures and text are expressed as mean value $\pm$ standard error (S.E.M.). Data were evaluated with Graph Pad Prism Version 2.01 software. Comparison was done using 1-way analysis of variance (ANOVA) followed by TukeyeKramer or Dunnett's test when appropriate. P values of <0.05 were considered statistically significant. In the experiment involving antiproliferative assay, cell cycle analysis, detection of apoptosis,

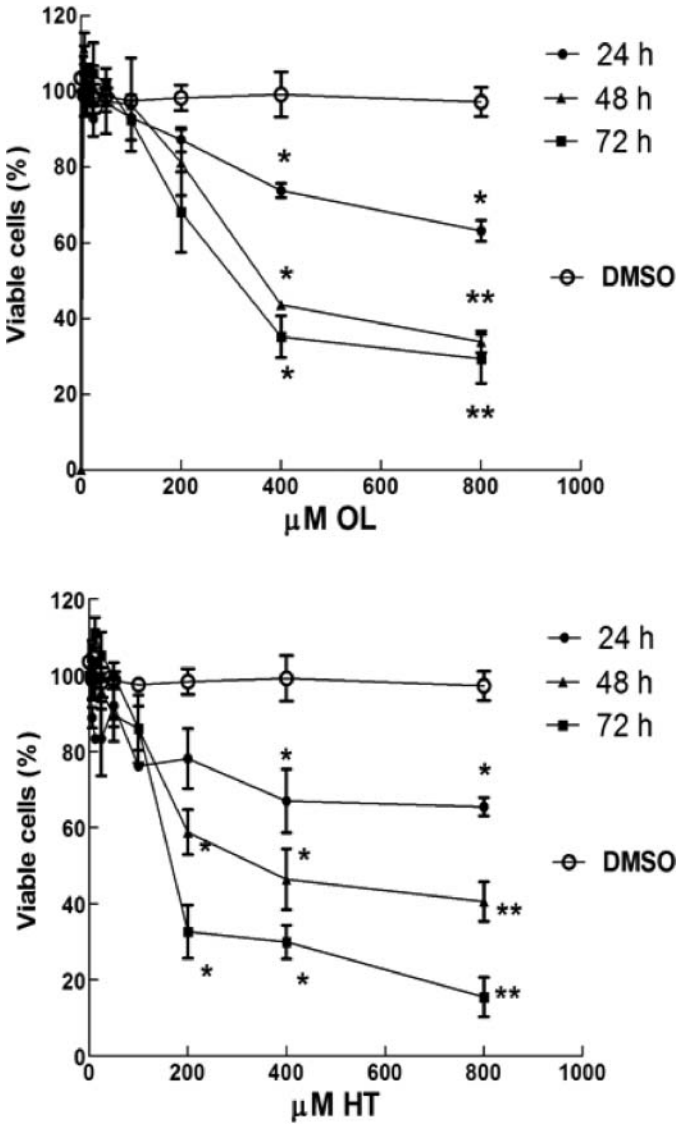

FIG. 2. Growth inhibitory effect of oleuropein (OL) and hydroxityrosol (HT) on HT-29 human colon cancer cells. Cells were seeded in 96-well plates (5 $\times$ 10^3/well) and treated with serial concentrations of OL and HT. After 24, 48, and 72 h of treatments, growth inhibition was measured by sulphorhodamine B (SRB). Data represent mean $\pm$ standard error of mean (S.E.M.). * $P <$ 0.05, ** $P <$ 0.01 vs. dimethylsulfoxide (DMSO).

and immunoblot analyses, the figures shown are representative of at least 3 different experiments performed on different days.

RESULTS

Inhibitory Effect of Hydroxytyrosol and Oleuropein on the Proliferation of Human Colon Cancer Cells

OL and HT inhibited proliferation of human colon cancer HT-29 cells at micromolar (μM) range. Cell viability was determined using the SRB assay. As shown in Fig. 2 there was a significant reduction in cell viability after 24-h treatment with 400 μM OL and HT ($^*P$ <0.05 vs. DMSO control). Likewise, 400 μM and 200 μM OL and HT respectively reduced significantly the growth of colon cancer cells after 48-h treatment ($^*P$ < 0.01 vs. DMSO control). Similar results were obtained after treatment for 72 h ($^*P$ < 0.05 DMSO control).

Effects of Hydroxytyrosol and Oleuropein on Cell Cycle Distribution of Human Colon Cancer Cells

To deep into the action mechanisms by which HT and OL inhibit the growth of human colon cancer HT-29 cells, we studied their effects on the cell cycle distribution. Based on the relative potency of these compounds to inhibit cell growth, we selected the same concentration range to evaluate their effects on the cell cycle distribution. As Fig. 3 shown, 400 μM HT treatment caused a significant cell arrest at the S phase stage ($^*P < 0.05$ vs. DMSO control) without affecting the total GO/G1 population. However, after 24-h treatment, 400 and 800 μM OL caused a significant decrease in the S phase stage ($^*P$ <0.05 vs. DMSO control) as well as a significant cell accumulation in G0/G1 phase ($^*P < 0.05$ vs. DMSO control) (Fig. 3).

HT and OL Induced Apoptosis in Human HT-29 Cells

In attempt to determine the extent by which apoptosis contributes to the growth inhibition caused by OL and HT, we studied the effect of these both polyphenolic compounds on cellular apoptosis in human colon cancer cells after 24-h treatment. The apoptotic cells were quantified by Annexin-V/PI double staining assay. A significant dose-dependent induction of early and late apoptotic cells was observed after HT treatment (Fig. 4). However, only the highest dose of OL assayed was able to

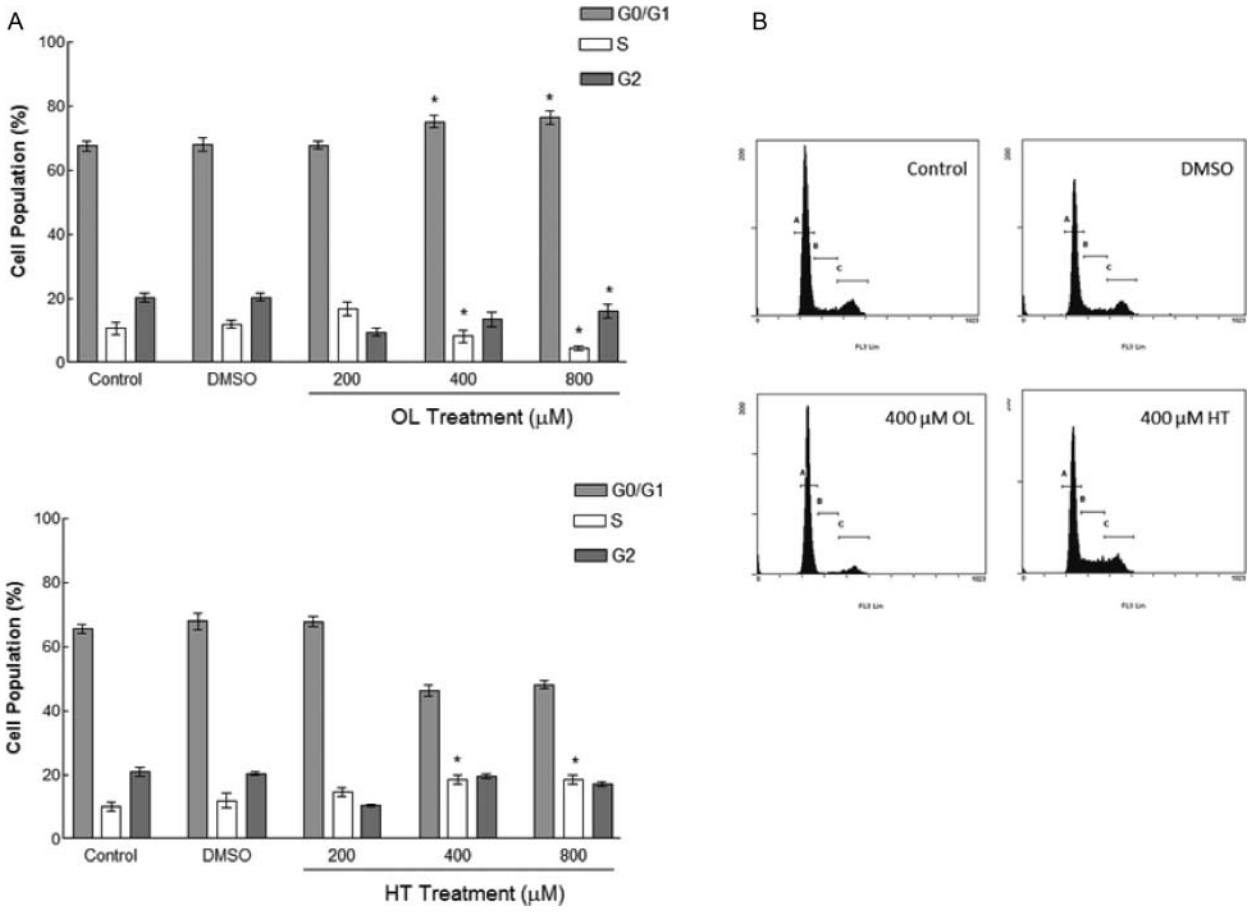

FIG. 3. Cell cycle distribution of HT-29 cells after oleuropein (OL) and hydroxityrosol (HT) treatments. Cells were seeded in 6-well plates at a density of 5 × 10^5 cells per well in presence of 200, 400, and 800 μM HT and OL or dimethylsulfoxide (DMSO) (vehicle control) or left untreated (control) for 24 h. After 24 h of HT and OL treatment, cells were harvested and the DNA was stained with propidium iodide. Data are represented as mean ± S.E.M. $^*P <$ 0.05 vs. DMSO.

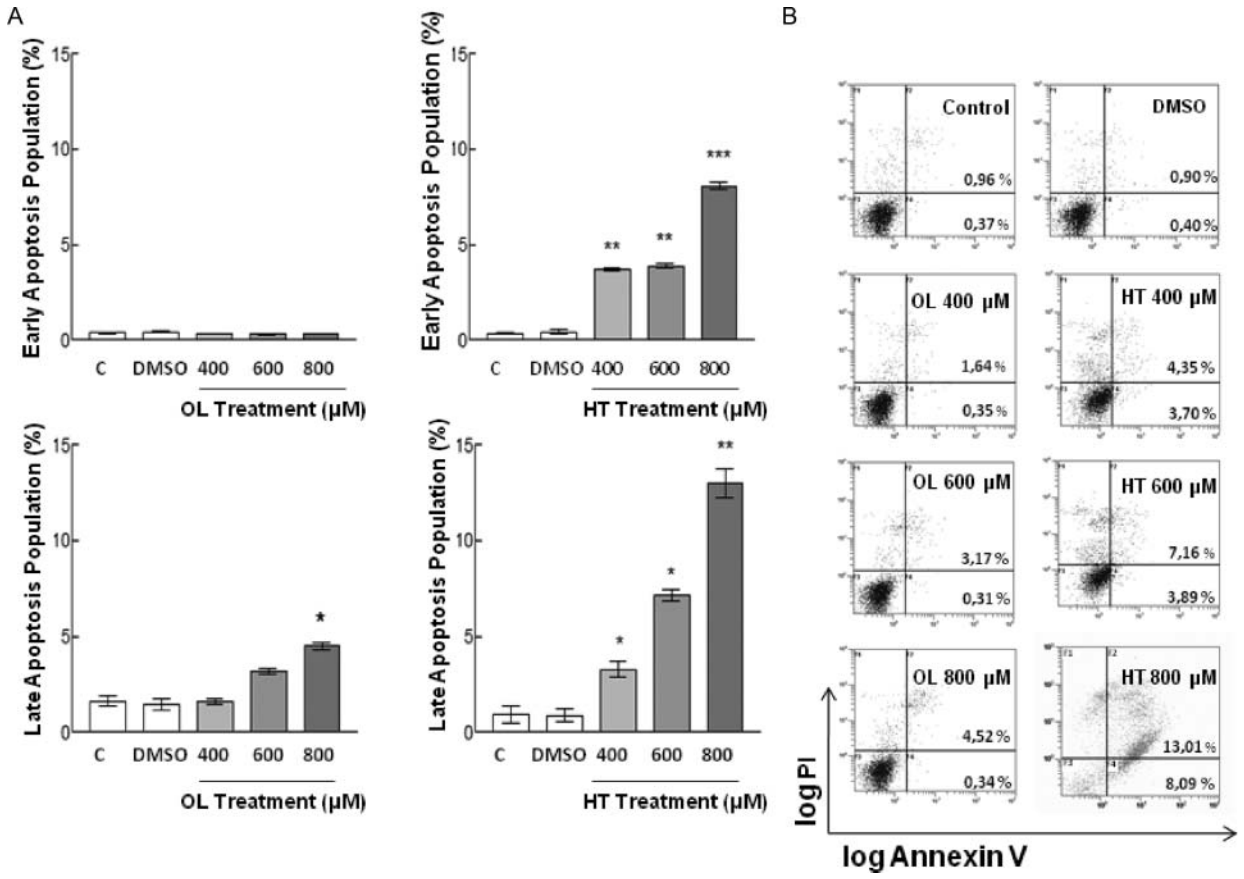

FIG. 4. Oleuropein (OL) and hydroxityrosol (HT)induce cell death in HT-29 cells. The cells (0.5×10^6/well) were incubated in complete McKoy medium for 24 h in the absence (control) and in presence of dimethylsulfoxide (DMSO; vehicle) or HT and OL (400, 600, and 800 μM). Viable cells are seen in quadrant F3, early apoptotic cells in F4, late apoptotic cells in F2 and primary necrotic in F1. The controls used to set up compensation and quadrants were unlabeled (unstained), cells stained with Annexin V or with propidium iodide (PI) alone. Histogram for each treatment group represents the percentage of early and late apoptotic cells (quadrant F4). The results are expressed as mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. DMSO.

induced a significant increase in late apoptotic population after 24-h treatment ($^*P$ <0.05 vs. DMSO control).

Time-Dependent Effect of OL and HT on the Expression of HIF-1α, p53, PPARγ, IκB-α, pJNK, and p38 MAPK Proteins in HT-29 Colon Cancer Cells

To obtain further insights into the HT and OL molecular action mechanisms, we evaluated their effects on key proteins related with colon cancer cell progression and apoptosis in HT-29 cells. Western blotting was performed on the whole-cell lysates after HT and OL treatment at different time points.

HIF-1α plays a crucial role in the regulation of genes involved in processes of cell motility adhesion and angiogenic cytokines. As shown in Fig. 5A, HIF-1α protein expression decreased significantly after HT and OL treatments. HIF-1α protein downregulation was detectable 2 h following the addition of 400 and 800 μM OL treatment when compared to the vehicle control ($^*P < 0.05$ vs. DMSO control). On the contrary, the HIF-1α downregulation induced by 400 μM and 800 μM HT treatment was significantly detectable lately after 24 and 48 h ($^*P < 0.05$ vs. DMSO control).

Induction of p53 is thought to be the major cause for apoptosis by triggering Bax activation. As shown in Fig. 5B, p53 protein expression was significantly increased after 800 μM OL treatment for 48-h ($^*P < 0.05$ vs. DMSO control, respectively). Similarly, we detected a significant p53 protein upregulation in 400 μM HT-treated cells after 24 and 48 h ($^*P < 0.05$ and $^{***}P < 0.001$ vs. DMSO control) (Fig. 5B).

On the other hand, it is known that PPARγ regulates cellular proliferation and differentiation and induces apoptosis in the gastrointestinal tract, and may therefore play a regulatory role in carcinogenesis. These findings led us to test whether OL and HT stimulated the expression of PPARγ in HT-29 cells. As our results showed, a significant and dose-dependent PPARγ protein upregulation was observed after 48-h incubation in presence of 400 and 800 μM HT ($^*P < 0.05$ vs. DMSO control). However, OL failed to stimulate PPARγ protein expression (Fig. 6A).

IκB-α is normally in the cytoplasm sequestering the NF-κB transcription factor, preventing the activation of antiapoptotic

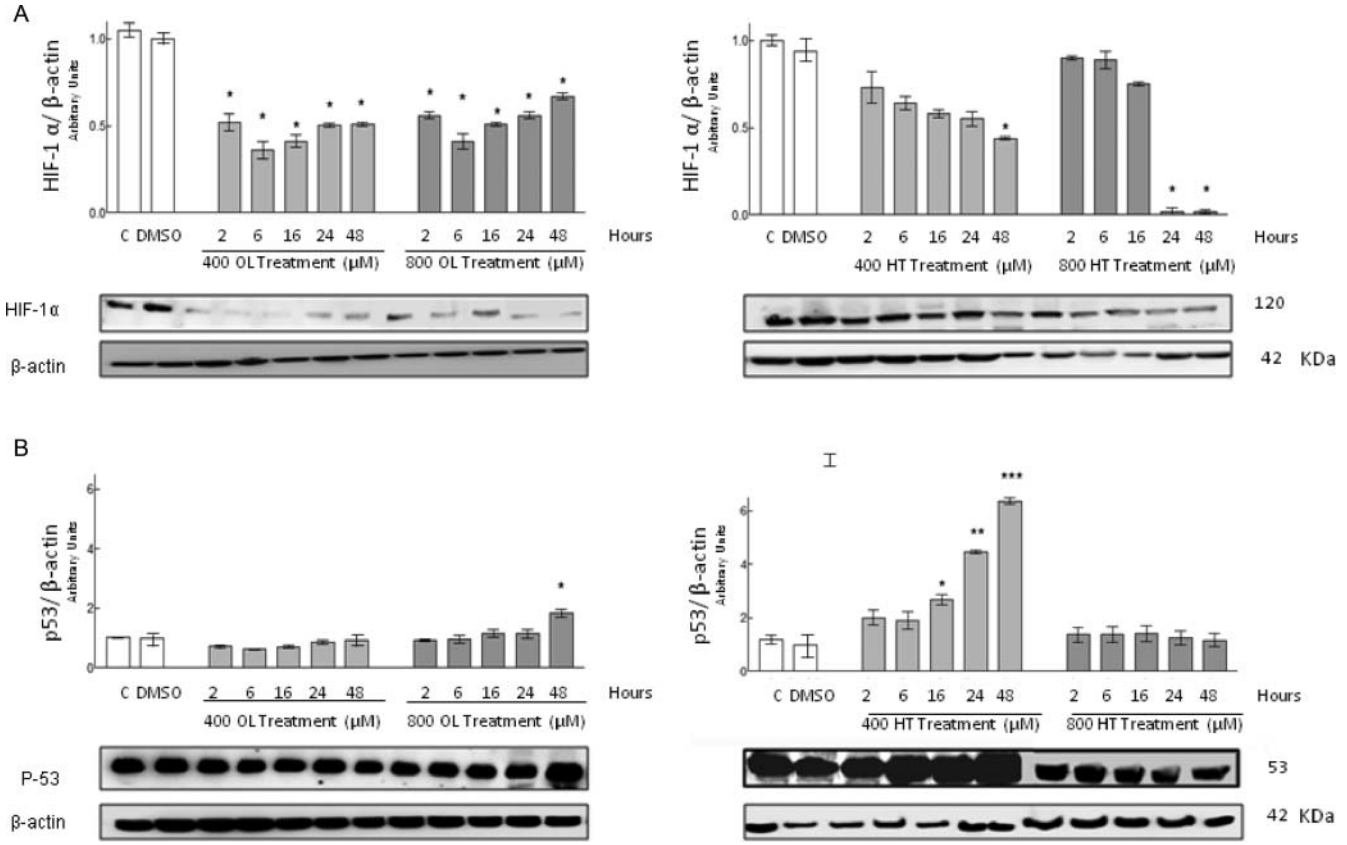

FIG. 5. Representative Western blotting analysis of HIF-1α and p53 after oleuropein (OL) and hydroxityrosol (HT) treatments in HT-29 cells. Cells were incubated with 400 and 800 μM OL or HT at various time points (2, 6, 16, 24, and 48 h) after the cells were harvested. As controls, cells were also treated with dimethylsulfoxide (DMSO; solvent control) or left untreated. The plots represent band intensity were measured by Kodak 1D software. β-Actin was served as an equal loading control for normalization. Data are represented as mean $\pm$ S.E.M. $^*P < 0.05$ vs. DMSO.

genes such as cIAP (inhibitor of apoptosis proteins)-1 and -2 that blocks a proapoptotic response by inhibition of caspase-8. According to our results, treatment with HT and OL (400 and 800 μM) did not cause a significant change in IκB-α protein expression (Fig. 6A). Thus, the NFκB signaling pathway does not seem to be involved in the anticancer effects of HT and OL in HT-29 cells.

Finally, JNK pathway can promote either proliferation or apoptosis. Conversely, the p38 MAPK pathway is activated upon cellular stress and often engages pathways that can block proliferation or promote apoptosis. According to the results, there were no significant differences neither for pJNK and pp38 protein expression after OL and HT treatment when compared to the vehicle control (Figs. 7A and B).

DISCUSSION

In this study we have demonstrated for first time that OL inhibits human HT-29 cancer cell proliferation and induces apoptosis at a micromolar range in a dose-dependent manner modulating the tumor repressor protein p53 and hypoxia inducible factor expression.

Our results showed that OL inhibited cell proliferation, blocking the G1 phase of the cycle with a proportional increase of cells in the G0/G1 phase and a concomitant decline in S and G2/M phases. However, HT stopped the cell cycle at phases S and G2/M at the dose of 400 μM. These results are in agreement with those obtained by Della Ragione et al. and Guichard et al. indicating that HT caused S-phase and G2/M-phase arrest thus preventing the cells from entering into mitosis without affecting significantly the cell population at G0/G1-phase (13,14). Similar results have been described by Han et al. in MCF-7 breast cancer cells in presence of OL and HT, showing a G1 to S phase transition blockade and an increase in the number of cells in the G0/G1 phase (10). Likewise, Obied et al. and more recently, Notarnicola et al. have described similar effects of OL and HT on proliferation, cell cycle, and apoptosis on HT-29 cells (15,16). However, the exact mechanisms by which these polyphenolic compounds exert their antiproliferative and proapoptotic effects remain unidentified. Thus, to further elucidate the responsible mechanism of OL inhibitory effect on colon cancer cells we decide to study the contribution of NFkB signaling pathways and many others such as MAPK, HIF, and p53.

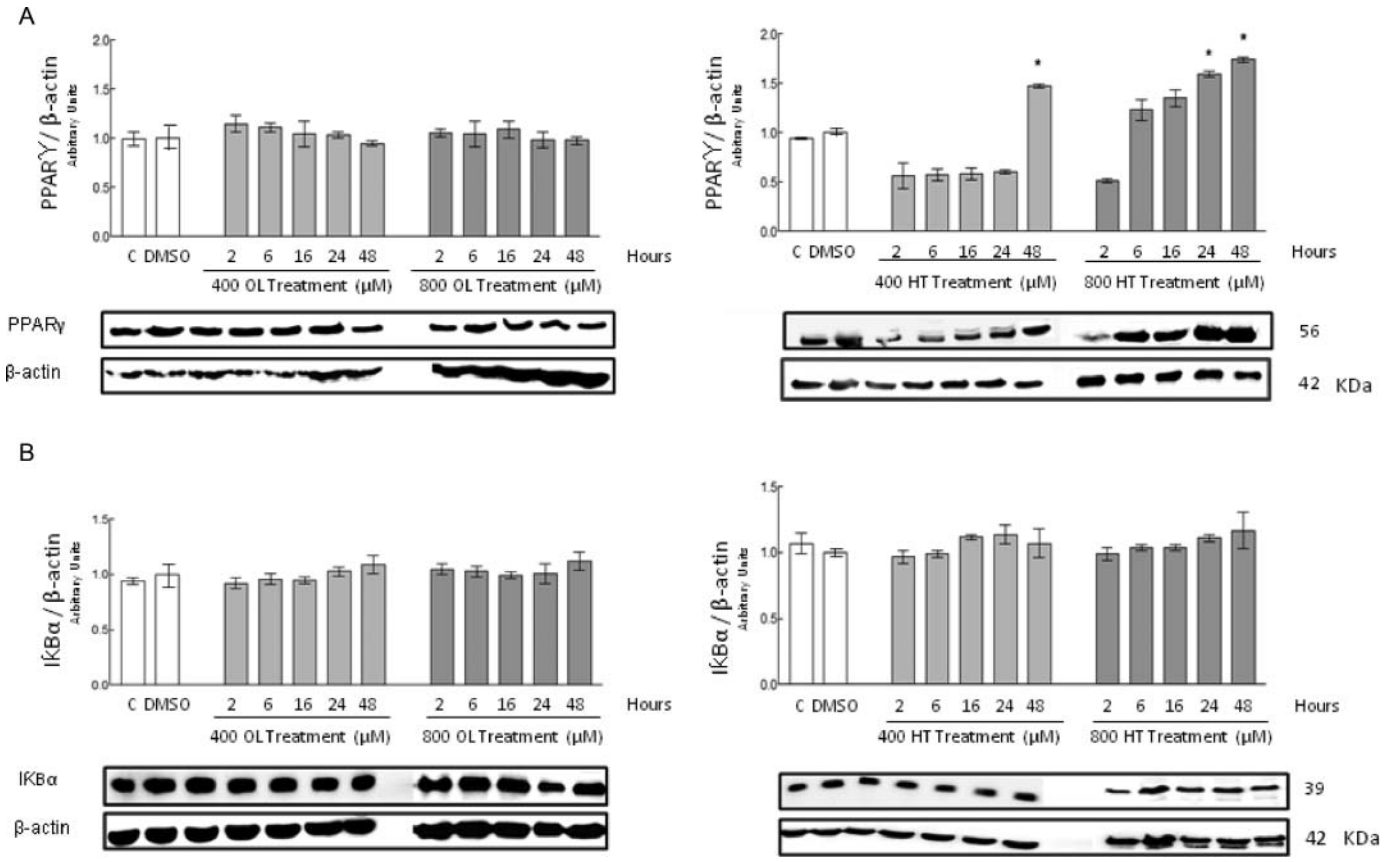

FIG. 6. Expression of peroxisome proliferator-activated receptor gamma (PPARγ) and anti-NFκB inhibitory protein alpha (IκBα) proteins after oleuropein (OL) and hydroxityrosol (HT) treatment in HT-29 cells by Western blotting. Cells were incubated with 400 and 800 μM OL or HT at various time points (2, 6, 16, 24, and 48 h) after the cells were harvested. As controls, cells were also treated with dimethylsulfoxide (DMSO; solvent control) or left untreated. The plots represent band intensity were measured by Kodak 1D software. β-Actin was served as an equal loading control for normalization. Data are represented as mean ± S.E.M. $^{*}P < 0.05$ vs. DMSO.

HIF-1α is a transcriptional activator that mediates adaptive responses to hypoxia. HIF-1α activity is increased in the majority of human cancers as a result of genetic alterations and intratumoral hypoxia. HIF-1α activates the transcription of genes that increase $O(_2)$ availability by stimulating angiogenesis and consequently inducing vascular endothelial growth factor expression, which is closely associated with the induction of the neovasculature in human cancer or that reprogram cellular metabolism to adapt to reduced $O(_2)$ availability (17,18). It also plays a crucial role in gen regulation implicated in process of cell motility and adhesion. In the present study, we examined the effects of OL and HT on HIF-1α protein expression. Our data showed that HIF-1α protein expression decreased after both treatments. These results are in line with those obtained by Terzuoli et al. who showed the antiinflammatory and antiangiogenic activities of HT in a HT-29 xenograft model resulting in a downregulation of HIF-1α (19). Thus, our results imply that HIF-1α-mediated deregulation may be one of the principal mechanisms of the tumor shrinkage by both OL and HT.

The tumor suppressor p53 plays a central role in cellular stress response pathways, as regulates DNA repair, cell cycle progression, and apoptosis and consequently is subject to tight regulatory control in cells (20). p53 protein upregulation of leads to an inhibition of growth and proliferation in cancer cells. Normally, p53 protein is maintained at a low level through the MDM2-mediated ubiquitination and degradation pathway. However, when cells are exposed to stress including genotoxic stress, p53 protein is rapidly accumulated and activated for downstream biological functions (21). As shown in Fig. 5B, OL and HT treatments significantly increased the upregulated p53 apoptosis-related protein up to 48 h. Current evidence indicates that the mode of action of p53-mediated apoptosis involves transactivation of target genes and direct signaling events are transcription independent (22). It has been proposed that p53 may induce 2 sets of genes upon stress signals. One set, such as p21/waf-1 and GADD45, mainly functions in cell growth control, and the other, such as Bax and Bcl-2, acts on apoptosis (23). In addition, it has been proposed that p53 may interact with the HIF system (24). p53 activation by hypoxia

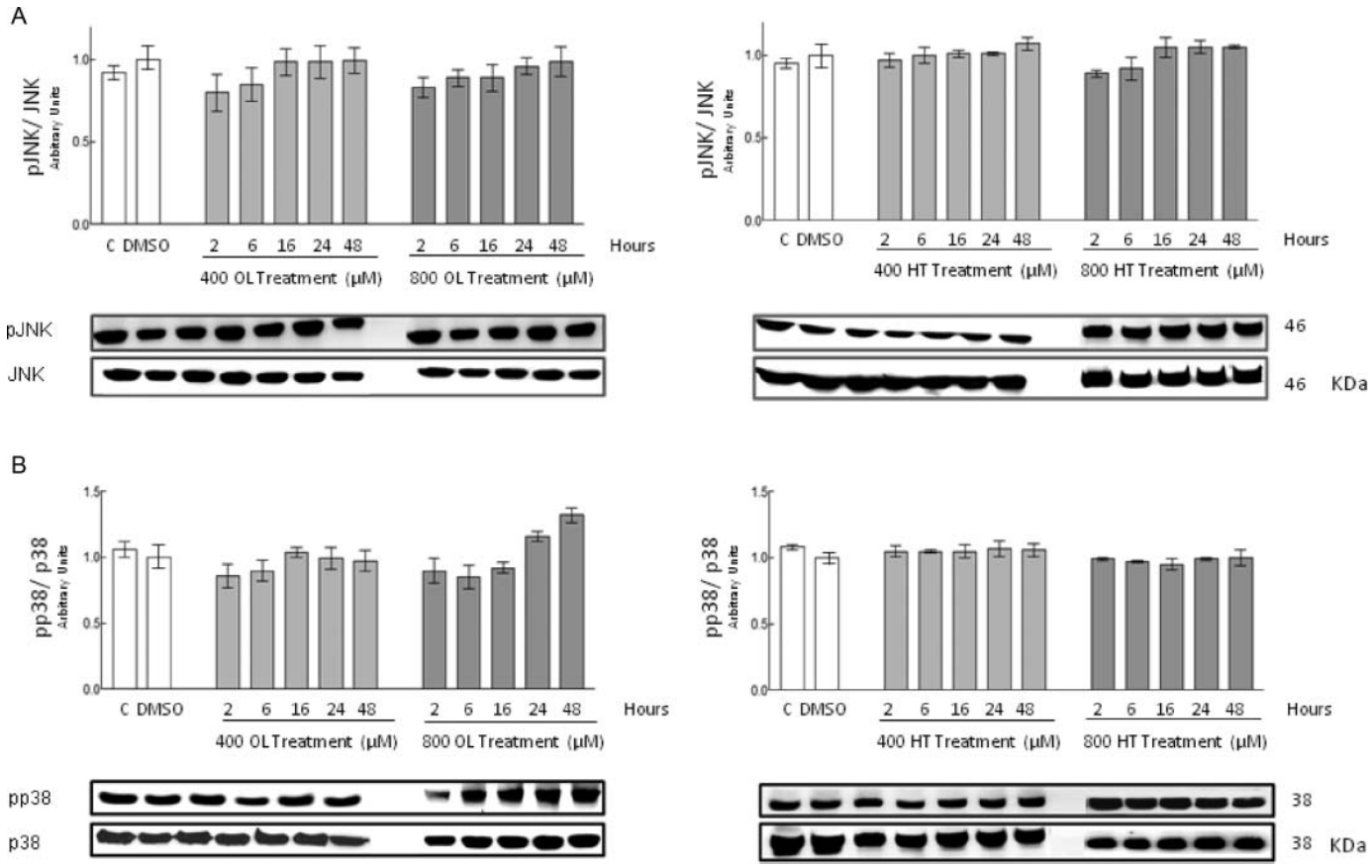

FIG. 7. No changes in pJUNK and pp38 proteins after oleuropein (OL) and hydroxityrosol (HT) treatment in HT-29 cells. Cells were incubated with 400 and 800 μM OL or HT at various time points (2, 6, 16, 24, and 48-h) after the cells were harvested. As controls, cells were also treated with dimethylsulfoxide (DMSO; solvent control) or left untreated. The plots represent band intensity were measured by Kodak 1D software. β-Actin was served as an equal loading control for normalization. Data are represented as mean $\pm$ S.E.M.

induces cell death in normal cells but could also be involved in angiogenesis regulation. Indeed, within a tumor, cells are exposed to ischemia that leads to acidosis, hypoxia and hypoglycaemia. Under these conditions, p53 activation may play a role in controlling angiogenic signaling, either by acting directly on the balance of pro and antiangiogenic effectors or signaling molecules, or on modulating the HIF-1α response to hypoxia. Consequently, our results confirm for the first time that both OL and HT induce apoptotic cell death in HT-29 colon tumor cells by p53-dependent apoptotic cell death mechanism adapting the HIF-1α response to hypoxia.

There is increasing evidence supporting the role of PPARγ as a regulator of proliferation and a cell growth modulator (25). Recent studies have demonstrated that activators of PPARγ suppress the growth response of colon cancer cells and inhibit colon tumorigenesis in animal models. Cell growth inhibition is partly due to the induction of apoptosis upon PPARγ activation. In addition, several reports describe a caspase-3 induction by PPARγ ligands in a large variety of cancer cells. Conversely, several reports suggest an association between loss of function mutations of PPARγ and the development of colorectal cancer. In fact, PPARγ agonists may promote colon cancer under certain circumstances (26). Surprisingly, as our results shown, OL failed to stimulate PPARγ protein expression. In contrast, in presence of HT, a dose-dependent and significantly upregulation of PPARγ expression was induced. Our findings are in agreement with those obtained by Hao et al. who recently described that HT upregulates gene expression of PPARγ but has no PPAR agonistic activity in 3T3-L1 adipocytes (27).

Uncontrolled tumor cell proliferation requires upregulation of multiple intracellular signaling pathways, including cascades involved in survival, proliferation, and cell cycle progression. The most significant effects of oxidants on signaling pathways have been observed in the MAPK/AP-1 and NF-κB pathways. NF-κB regulates several genes involved in cell transformation, proliferation, and angiogenesis (28). The transcription factor NFκB consists of p50 and p65 heterodimer, which is retained in the cytoplasm by masking nuclear localization signal (NLS) by the inhibitor IκBα. Upon activation, IκBα kinase phosphorylates IκBα, promoting its ubiquitination and degradation, thus allows p50–p65 being translocate to the nucleus, binds to its consensus sequence, and induces transcription of essential genes

concerned in cell proliferation and survival (29). Our data are paralleled with those obtained by Guichard et al. who determined whether HT modulated the NF-kB transcriptional activity in HT-29 colon cells (14). Surprisingly, HT treatment produced dose-dependent TNF-α downregulation and induced NF-kB activation whereas HT alone (400 μM) failed to stimulate NF-kB transcriptional activity. Our results revealed that IκBα activation was not impaired in presence of OL or HT at 400 and 800 μM within 2–48 h.

On the other hand, the JNK pathway activated by chemical and radiation induced stresses and by inflammatory cytokines, is involved in the regulation of cell proliferation, apoptosis, and autophagy (30). Nevertheless, JNK pathways show an interesting dichotomy, having displayed both an oncogenic and a proapoptotic function. In the former context, the JNK cascade seems to initiate cellular transformation, whereas its proapoptotic function seems to be required for the apoptosis induced. JNK also seems to play a significant role in tumor development. An important aspect of tumor development is the suppression of apoptosis, and human tumors seem to use several different mechanisms to evade cell suicide, so a normal function of JNK might be to suppress tumor formation by activating apoptosis (31). Our data show that OL treatment did not produce any significant changes in JNK protein expression. Although previous data suggest that HT might mediate JNK pathway activation (14) we found a normal function of JNK expression respect to the control after HT incubation.

The p38 MAPK pathway is involved in inflammation, cell growth, cell death, and cell differentiation. Activated by a similar array of stimuli, including pro-inflammatory mediators, growth factors, and environmental factors, it has a complex role in cancer (32). However, OL and HT treatment did not modulate p38 MAPK activity.

This is the first study demonstrating that OL limits the growth and induces apoptosis of HT-29 colon cells via p53 pathway activation adapting the HIF-1α response to hypoxia. In addition, our results imply that PPARγ upregulation may be one of the principal mechanisms of the tumor shrinkage by HT. On the contrary, p38 MAPK, JNK, and NF-κB signaling pathways do not seem to be involved in the antiproliferative and proapoptotic effects of these both polyphenols in HT-29 human colorectal cancer cells.

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