Hydroxytyrosol, an ingredient of olive oil, reduces triglyceride accumulation and promotes lipolysis in human primary visceral adipocytes during differentiation

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Abstract

Hydroxytyrosol has various pharmacological properties, including anti-oxidative stress and anti-inflammatory activities, preventing hyperglycemia, insulin resistance, and the metabolic syndrome. The present study is focused on the anti-adipogenic and lipolytic activity of hydroxytyrosol on primary human visceral adipocytes. Pre-adipocytes were analyzed after 10 (P10) and 20 (P20) days of treatment during differentiation and after 7 (A7) days of treatment when they reached mature shape. The treatment with hydroxytyrosol extract significantly ($P < 0.001$) increased apoptosis in P10 and P20 cells in comparison to control and A7 cells; significantly ($P < 0.001$) reduced triglyceride accumulation in P20 cells compared to P10 and control cells; and significantly ($P < 0.001$) increased lipolysis in P20 cells in comparison to control cells and A7 mature adipocytes. Hydroxytyrosol-treated P20 cells significantly ($P < 0.05$) increased expression of genes involved in inhibition of adipogenesis, such as GATA2, GATA3, WNT3A, SFRP5, HES1, and SIRT1. In contrast, genes involved in promoting adipogenesis such as LEP, FGF1, CCND1, and SREBF1 were significantly down-regulated by hydroxytyrosol treatment. These data suggest that hydroxytyrosol promotes lipolysis and apoptotic activity in primary human visceral pre-adipocytes during differentiation and does not affect already mature adipocytes.

Keywords: Human visceral adipocytes, differentiation, gene expression, lipolysis, apoptosis, hydroxytyrosol

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Introduction

Mediterranean diet is known to promote health benefits that partially originate from virgin olive oil, an important component of this diet. One of the most potent phenolic compounds with antioxidant activity present in olive oil is hydroxytyrosol (HT).¹ HT presents anti-inflammatory, anti-thrombotic, and anti-atherogenic properties, improving endothelial dysfunction, hemostatic and lipid profiles, and decreasing oxidative stress and inflammatory cell activation.^{2–4} This hydrosoluble and liposoluble molecule is present either as simple phenol or esterified with elenolic acid to form oleuropein aglycone.⁵

Recently, studies are focused more on functional compounds of plant origin that promote suppression of differentiation of preadipocytes into adipocytes or enhance lipolysis of already mature adipocytes. For example, carnolic acid from rosmarin^{6,7} and rosavines from *Rhodiola rosea*⁸ inhibit triglyceride accumulation and stimulate lipolytic activity in differentiating preadipocytes through a modulation of genes involved in adipogenesis. There is enough

scientific evidence that HT is beneficial in treating hyperglycemia, insulin resistance, and the metabolic syndrome.^{9–11} Its metabolic and anti-inflammatory effect has been recently reported in human Simpson–Golabi–Behmel syndrome (SGBS) preadipocyte cell strain and in 3T3-L1 mouse embryo fibroblasts. It was demonstrated that in both cell model systems, the health-promoting effects of HT were achieved via prevention of adiponectin down-regulation in inflamed adipocytes through an attenuation of JNK-mediated PPAR γ suppression.¹² More recently, it was reported that HT has an anti-adipogenic effect on 3T3-L1 cell differentiation by down-regulating cell proliferation and cannabinoid receptor (CB1) gene expression.¹³

In the present research we analyzed, the effects of HT on primary omental pre-adipocyte and adipocyte viability, apoptosis, adipogenesis, and lipolysis. Furthermore, the expression level of genes involved in pathways affecting the process of human adipogenesis and fat storage were identified and validated using RT² Profiler PCR array.

Material and methods

Materials

3-Hydroxytyrosol, (3,4-dihydroxyphenyl ethanol) was purchased from Sigma (cat. n. H4291 Milan, Italy) with a purity of $\geq 98\%$ (HPLC) and kept in dark at 4°C until further use. Stock solution of 3-Hydroxytyrosol was prepared in 1 ml of 10% dimethylsulfoxide (DMSO), filtered through 0.22- μm pore size nylon membrane (Millipore, Milan, Italy) and kept in dark at -20°C until further analysis.

Cell culture and treatment

Human omental pre-adipocyte cells and medium were obtained from ZenBio (USA). According to the data kindly provided by ZenBio, pre-adipocyte cells (OP-F-3) were collected from Caucasian, non-diabetic, and non-smoking female donors ($n=3$) with the mean age of 48.7 ± 9.1 years and the mean BMI of $42.7 \pm 6.9 \text{ kg/cm}^2$.

Visceral pre-adipocytes were plated and proliferated in Omental Preadipocyte Medium (OM-PM) till the passage 3. All the experiments were run in the humidified 37°C incubator with 5% CO_2 . OM-PM was changed each second day till the cells reached full confluence. At this moment, OM-PM was completely removed, cells were washed with phosphate-buffered saline (PBS 1 $\times$) and fed with Omental Differentiation Medium (OM-DM) and further with Omental Adipocyte Medium (OM-AM) added with HT at a concentration of 5, 10, 30, and 70 $\mu\text{g/mL}$ with final concentration of 0.0014% DMSO. The control cells (CTRL) were incubated in OM-DM and OM-AM without HT in the presence of 0.0014% DMSO as well.

HT treatment was performed from the first day of differentiation till 10 days in OM-DM (P10), till 20 days in OM-DM (P20) and during seven days post differentiation in OM-AM (A7). Cells were analyzed at the end of each differentiation period.

Apoptosis, lipolysis, and adipogenesis assays were performed in a 96-well plate with the cell density of 10^4 cells/well, while PCR assay was performed in a 6-well plate with the cell density of 10^5 cells/well.

All analyses were performed using cells of three different donors and each donor was assayed in triplicate.

Cell viability

The cytotoxicity of HT at different concentrations (5, 10, 30, and 70 $\mu\text{g/mL}$) was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. At the end of each differentiation period (P10, P20, A7), cells were washed with PBS 1X, added with MTT solution (5 mg/ml in fresh medium) and incubated for 3 h at 37°C . Then, the mixture was carefully removed from each well followed by addition of 100 μL of DMSO. Absorbance was read with a microplate reader at 570 nm. The percentage of viable cells was calculated by normalizing the absorbance value of the treated cells by the absorbance value of the CTRL cells.

Apoptosis assay

Apoptosis determination in P10, P20, A7 HT-treated cells (30 $\mu\text{g/mL}$), and CTRL cells was performed using ApoStrandTM ELISA apoptosis detection kit (Enzo Life Sciences Inc., NY, USA) according to provider's instructions.⁷ The apoptotic positive control (single stranded DNA in PBS) was also included in the analysis. Data are expressed as the percentage of cells, comparing the optical density of the treated/CTRL cells with the optical density of the positive CTRL.

Oil Red O staining and measurement of lipid accumulation

Intracellular lipid accumulation in P10 and P20 HT-treated and CTRL cells was analyzed by microscopy after staining cells with Oil red O (ORO, Sigma, Milan, Italy), and quantification using a spectrophotometer at 520 nm. Data are expressed as percentage of lipid accumulation relative to the control samples.

Lipolysis assay

Detection of the lipolytic activity in HT-treated P20 and A7 cells was performed using AdipoLyzeTM Lipolysis Detection Kit (Lonza Walkersville Inc., MD, USA), based on the quantification of the glycerol release by cells undergoing lipolysis.⁷ Accumulated glycerol in each sample was determined by comparison with a glycerol standard curve.

RNA extraction and adipogenesis PCR array

Prior to the total RNA extraction, HT-treated P20 and CTRL cells were rinsed with ice-cold PBS followed by the RNA extraction with miRNeasy kit and QIAzol Lysis Reagent (Qiagen, Milan, Italy) according to the manufacturer's recommendations. Synthesis of the first strand cDNA was performed using RT² First Strand kit (Qiagen, Milan, Italy).⁸

The gene expression profile of adipogenesis was determined using ready to use human Adipogenesis RT² Profiler PCR array (PAHS-049Z; Qiagen, Milan, Italy) containing primers for 84 tested, five housekeeping genes and controls for RT and PCR reactions. The expression of target genes was normalized and ΔCt s were calculated by the difference between Ct of target genes and the geometric mean of the four housekeeping genes. Differences between HT samples and CTRL were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method,^{14,15} where $2^{-\Delta\Delta\text{Ct}}$ represents the difference of a given target gene in HT-treated cells versus CTRL. The n -fold expression of a given target gene was calculated as $\log_2(2^{-\Delta\Delta\text{Ct}})$.

Statistical analysis

In the cell viability assay, differences between HT-treated cells were determined by one-way ANOVA with HT concentration (4 levels) as fixed factor.¹⁶ Significant effects of the HT treatment on cell apoptosis, triglyceride accumulation, and lipolysis were assessed with two-way ANOVA¹⁶ considering the fixed effect of treatment (HT-treated and CTRL cells) and the fixed effect of period of differentiation (for apoptosis and lipolysis assay: P20 and A7 cells; for lipid

accumulation assay: P10 and P20 cells). The effect of interaction (treatment $\times$ period of differentiation) was tested in two-way ANOVA as well.

PCR array data, expressed as $\log_2(n\text{-fold})$, were analyzed using one sample T test.¹⁶ Results are displayed in a form of a volcano plot, which graphically shows the relationships between fold-change estimates and statistical significance values, reported as the negative $\log_{10}(P\text{ value})$. The horizontal red line on the plot indicates a significance cutoff of $P=0.05$.

Results

Cell viability

The cell viability assay was performed to determine the highest dose of HT that did not reduce the cell viability for more than 40% compared to CTRL cells. Treatment of P10 and P20 cells with 5, 10, 30, and 70 $\mu\text{g/mL}$ of HT showed a significant ($P < 0.001$) decrease in cell viability in a dose-dependent manner (Table 1). Cell viability of P10 and P20 remained over 70% with HT concentration

till 30 $\mu\text{g/mL}$ and dramatically decreased ($P < 0.001$) at a concentration of 70 $\mu\text{g/mL}$. On the contrary, HT treatment of A7 cells did not influence so much the cell viability. For A7, cell viability remained over 90% even at the concentration of 70 $\mu\text{g/mL}$. According to these observations and in accordance with Drira *et al.*,¹⁷ the HT concentration of 30 $\mu\text{g/mL}$ was chosen for the further experiments.

Effect of HT on apoptosis

The apoptotic effect of the HT was examined on P10, P20, and A7 cells treated with 30 $\mu\text{g/mL}$ of HT and on corresponding CTRL cells. The results are reported as the percentage of apoptotic cells, calculated as the ratio of apoptotic positive cells, provided by the commercial ELISA kit, and of the treated/CTRL cells (Figure 1). The percentage of apoptosis induced by HT extract was significantly ($P < 0.001$) higher in P10 and P20 cells respect to CTRL cells ($48.5 \pm 2.5\%$ vs. $19.7 \pm 8.7\%$ and $48.4 \pm 0.8\%$ vs. $20.3 \pm 2.5\%$, respectively). Conversely, in A7 cells, the percentage of apoptosis did not significantly vary between treated and CTRL cells. The interaction between effect of treatment (HT-treated and CTRL cells) and effect of period of differentiation (P10, P20 and A7) showed high significance ($P < 0.001$).

HT decreases triglyceride accumulation

According to the results of the apoptosis assay, P10 and P20 cells were significantly affected by HT treatment and differed from the mature adipocytes (A7). To evaluate the ability of HT to affect adipogenesis through the prevention of the triglyceride accumulation in differentiating pre-adipocytes, the total amount of lipid accumulation in HT-treated P10 and P20 cells respect to untreated CTRL cells was reported (Figure 2). These data indicate that HT treatment inhibits adipogenesis of differentiating P10 and P20 cells,

Table 1 Modulation of MTT metabolism by HT in human omental pre-adipocytes. Cells were treated with 5, 10, 30, and 70 $\mu\text{g/mL}$ HT

Dose ($\mu\text{g/mL}$)	P10	P20	A7
5	$90.25^A \pm 7.42$	$92.95^A \pm 2.97$	$97.97^A \pm 2.02$
10	$74.39^B \pm 6.97$	$79.50^B \pm 2.75$	$96.13^A \pm 2.39$
30	$71.16^B \pm 7.82$	$70.68^C \pm 2.86$	$95.02^A \pm 2.44$
70	$48.13^C \pm 4.46$	$45.72^D \pm 1.88$	$90.97^B \pm 2.00$

HT: hydroxytyrosol.

Note: P10 differentiating pre-adipocytes treated for 10 days; P20 differentiating pre-adipocytes treated for 20 days; A7, mature adipocytes treated for 7 days. Data are means $\pm$ standard deviation (SD). Different superscript capital letters indicate significant differences ($P < 0.001$) within treatments at different concentrations.

Figure 1 Modulation of apoptosis by HT in human omental pre-adipocytes. Cells were treated with 30 $\mu\text{g/mL}$ HT extract. P10, differentiating pre-adipocytes treated for 10 days; P20, differentiating pre-adipocytes treated for 20 days; A7, mature adipocytes treated for 7 days. Data are reported as the percentage of apoptotic cells in HT-treated/CTRL cells vs positive CTRL. Data are mean $\pm$ standard deviation (SD). Asterisks on the plain line indicate the significant difference between treatments, $P < 0.001$. Asterisks on the dotted line indicate the significant difference between different differentiation periods (P10, P20, A7), $P < 0.001$

Figure 2 Effect of HT (30 µg/mL) on triglyceride accumulation during pre-adipocyte differentiation. Data are expressed as percentage of triglyceride accumulation in differentiating HT-treated pre-adipocytes (P10 and P20) relative to the control cells. Results are depicted as mean ± standard deviation (SD). Asterisks indicate the significant difference between treatments for $P < 0.001$

Figure 3 Detection of the lipolytic activity in differentiating and mature adipocytes after HT treatment (30 µg/mL). Glycerol release was measured after 20 days (P20) of treatment during pre-adipocytes differentiation and after seven days of treatment of already mature cells (A7). Accumulated glycerol in each sample was determined by comparison with a glycerol standard curve. Glycerol content is expressed in µM and depicted as mean ± standard deviation (SD). Asterisks indicate the significant difference between treatments for $P < 0.001$

decreasing significantly ($P < 0.001$) the level of triglycerides. The percentage of triglyceride accumulation in HT-treated cells (P10 and P20) respect to CTRL cells was significantly ($P < 0.001$) lower ($100.1 \pm 19.6\%$ in P10 cells and $100.5 \pm 8.1\%$ in P20 cells). The percentage of triglyceride accumulation in HT-treated P20 cells was significantly ($P < 0.001$) lower respect to HT-treated P10 cells ($51.1 \pm 10.2\%$ in P10 cells and $22.1 \pm 3.4\%$ in P20 cells). The interaction between the effect of treatment (HT-treated and CTRL cells) and the effect of period of differentiation (P10, P20) was significant ($P < 0.001$) as well.

HT treatment increases glycerol release

To determine whether HT treatment affected lipolytic responsiveness of cells, we measured glycerol release in

P20 pre-adipocytes and in mature adipocytes (A7). HT-treated P20 pre-adipocytes were chosen as at this stage of differentiation cells are not fully mature but still in active development and at the same time already reached the reduction of TG in comparison to HT-treated P10 cells. The effect of HT treatment on glycerol release in A7 adipocytes was performed as these cells were considered fully mature, showed rounded shape, and contained large lipid droplets in the cytoplasm (data not shown).

Respect to CTRL cells, an HT-induced increase ($P < 0.001$) of free glycerol in the culture medium was observed both in P20 ($93.2 \mu\text{M} \pm 4.0$ in CTRL and $199.5 \mu\text{M} \pm 36.1$ in P20 cells) and A7 cells ($2.5 \mu\text{M} \pm 0.6$ in CTRL cells and $153.8 \mu\text{M} \pm 73.7$ in A7 cells) (Figure 3). The interaction between effect of treatment and period of differentiation in this analysis was not significant.

Figure 4 Volcano plot of significantly ($P < 0.05$) up- and down-regulated genes in HT-treated ($30 \mu\text{g/mL}$) P20 cells in comparison to untreated P20 CTRL cells. Human RT² Profiler PCR array data are reported as $\log_2(n\text{-fold})$ values plotted against negative $\log_{10}(P \text{ value})$. Red indicator = significantly up-regulated gene; Green indicator = significantly down-regulated gene. Red line = indicates $-\log_{10}(P \text{ value})$, $P < 0.05$. (A color version of this figure is available in the online journal.)

Effects of HT treatment on the adipogenesis-related gene expression

According to the results of the apoptosis, adipogenesis and lipolysis assays, P20 cells, as the most responsive to the HT treatment, were chosen for the determination of the expression pattern of genes involved in the adipogenesis pathways. RT² Profiler PCR array data are reported in a form of a volcano plot as $\log_2(n\text{-fold})$ values of significantly ($P < 0.05$) up- and down-regulated genes in comparison to CTRL cells (Figure 4). The results revealed that HT treatment at a dose of $30 \mu\text{g/mL}$ significantly ($P < 0.05$) up-regulated the expression of 19.0% (16/84) of genes and down-regulated the expression of 4.7% (4/84) of genes involved in adipogenesis pathways (Figure 4; SM Table 1).

Among the significantly ($P < 0.05$) up-regulated genes that inhibited adipogenesis in P20 HT-treated cells are ADRB2, DDIT3, GATA2, GATA3, HES1, LPL, PPARGC1A, PRDM16, SIRT1, SFRP5, WNT1, and WNT3A. Four significantly ($P < 0.05$) down-regulated genes in P20 HT-treated cells included CCND1, FGF1, LEP, and SREBF1.

Discussion

The present study aimed to characterize the effects of HT, known as an antioxidant phenolic olive-derived compound typically present in the virgin olive oil, on human visceral pre-adipocytes differentiation, lipolysis, and apoptosis.

The significant decrease in triglyceride accumulation by HT treatment (Figure 2) is in accordance with the results obtained on the 3T3-L1 cells treated with five times more concentrated HT.¹⁷ Drira *et al.*¹⁷ proposed that the

anti-adipogenic activity of HT at early stage of cell differentiation is due to a delay in the cell cycle progression and to a down-regulation of the SREBP-1c expression. Our data demonstrated that SREBF1 (also known as SREBP-1c) and CCND1 are among the down-regulated genes in HT-treated P20 cells (Figure 4 and SM Table 1), suggesting the same involvement in the cycle arrest and attenuated lipogenic activity. Interestingly, HT treatment on P20 cells significantly reduced the expression of FGF1, which in turn is related to CCND1 via ERK signaling.

It was previously reported¹⁸ that the high-fat diet, supplemented with the analogues antioxidant olive-derived molecule known as oleuropein, decreases mRNA levels of FGF1-mediated signaling molecules in liver of mice.

Drira and Sakamoto¹⁹ showed that HT treatment of 3T3-L1 cells stimulated glycerol release in addition to the increased rate of HSL and perilipin phosphorylation via PKA and also through ERK phosphorylation.

It is known that catecholamines may influence both PKA and ERK pathways to induce lipolysis²⁰ through the activation of β -adrenoceptors. Although we did not measure the downstream activation of HSL nor ERK phosphorylation followed by HT treatment of P20 cells, a significant increase of adrenoceptor beta 2 (ADRB2) expression was observed (Figure 4 and SM Table 1). This observation is matching well with the fact that ADRB2 expression in adipose tissue is conversely down-regulated in obese subjects.²¹

Of note, a significant ADRB2 up-regulation could contribute to the enhanced lipolysis observed in 3T3-L1 cells by an increase of autolysosome-targeted lipid droplets.²²

Interestingly, HT treatment effectively ($P < 0.001$) induced apoptosis only in P20 cells, whereas the treatment of A7 adipocytes did not lead to any difference in comparison to CTRL cells (Figure 1). Similar results were obtained previously with *Rosmarinus officinalis*⁷ and *Rhodiola rosea*⁸ extracts. The apoptotic effect of HT, through the G0/G1 phase arrest and a concomitant decrease of cells in the S and G2/M phases, was already demonstrated in different cell lines, including human colon cancer cells²³ and MCF-7 human breast cancer cells.²⁴

As already evidenced, different biological compounds affect adipogenesis through the wingless-type (WNT) signaling.^{8,25} In the present study, a significant ($P < 0.05$) increase of canonical expression of WNTs, such as WNT1, WNT3A, and WNT pathway inhibitor gene SFRP5, was observed.²⁶ The significantly decreased expression of CCND1, a downstream target gene of β -catenin,²⁷ can be also related to the up-regulation of SFRP5 (Figure 4), which prevents activation of frizzled receptors and in this way attenuating the non-canonical WNT signaling.²⁸

SIRT1 gene is known to reduce fat synthesis and fat cell differentiation²⁹ and to improve free fatty acid release from fat cells.³⁰ Its up-regulation ($P < 0.05$) in HT-treated P20 cells is consistent with the down-regulation of PPARG (although not significantly modulated; SM Table 1) and with the significantly enhanced cell lipolysis. Previously it was already reported that PPARG expression is down-regulated by SIRT1 in 3T3-L1 adipocytes.³⁰ Moreover, HT has recently been shown to up-regulate the SIRT1 gene expression in the heart tissue of senescence-accelerated mouse.³¹

Two isoforms, GATA-2 and GATA-3, are specifically expressed in pre-adipocytes and down-regulated in mature adipocytes³² and it was suggested that GATAs activity is mediated through reduced PPARG promoter activity.³³ In the present study, GATA-2 was significantly ($P < 0.05$) up-regulated in HT-treated P20 cells in comparison to CTRL cells and this is coherent with an inhibitory effect on adipogenesis, observed in P20 cells treated with 1% salidroside plus 3% rosavines.⁸

The over-expression of HES1 ($P < 0.05$) in HT-treated P20 cells can be related to a reduction of intracellular lipid accumulation. It was found by several research groups that HES1 transcription factor is down-regulated during adipogenesis *in vitro* and *in vivo*,³⁴ inhibiting the early phase of adipogenesis in 3T3-L1 cells³⁵ and porcine mesenchymal stem cells.³⁶

Expression of PPARGC1A, a major regulator of mitochondrial biogenesis and an activator of mRNA expression of uncoupling protein 1 (UCP1) in white adipocytes, were up-regulated by HT treatment in P20 cells (Figure 4 and SM Table 1). The same conclusions were reached by Hao *et al.*³⁷ at lower HT concentration and 3T3L1 cells. PPARGC1A was found to be activated also by grape skin and seed extracts, that are rich in antioxidant phenolic compounds.³⁸ PRDM16, bounded by PPARGC1A, was also positively regulated in HT-treated P20 cells and its expression has also been shown to protect against weight gain on high-fat diets.³⁹

Expression of genes involved in adipocyte function and development, such as LEP, were significantly ($P < 0.05$) decreased during differentiation of pre-adipocytes in the presence of HT extract.

Conclusions

In conclusion, we have demonstrated that HT extract induced apoptosis in differentiating pre-adipocytes (P10 and P20), but not in mature adipocytes (A7). HT extract inhibited differentiation of visceral pre-adipocytes and promoted lipolytic activity in pre-adipocytes as well as in already mature adipocytes. Molecular analysis showed that HT treatment modulated the expression of several genes associated with adipogenesis.

Authors' contribution: BS was the supervisor; MC was the originator and participated in experimental and analytical work, ran a majority of the statistics and wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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