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Modulation of adipogenesis, lipolysis and glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes by hydroxytyrosol acetate: A comparative study

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ABSTRACT

Hydroxytyrosol acetate (Hd-Ac) is a polyphenol that is present in the olive fruit and oil at a concentration similar to that of hydroxytyrosol (Hd). The effects of Hd-Ac on adipogenesis, lipolysis, and glucose consumption in 3T3-L1 cells were investigated. Treatment with Hd-Ac at concentrations of 0–75 μmol/L inhibited 3T3-L1 differentiation and lipid accumulation in a dose-dependent manner. At the same concentration range, no effect on cell viability was observed in the MTT assay. Inhibition of adipogenesis was associated with the downregulation of PPARγ, C/EBPα, SREBP-1c, and their downstream target genes (GLUT4, CD36, and FAS) as revealed by qRT-PCR. On the other hand, Hd-Ac dose dependently activated glycerol release in fully differentiated 3T3-L1 adipocytes, indicating lipolysis. This stimulation of lipolysis was mediated via the activation of hormone-sensitive lipase (HSL) by phosphorylation at Ser563 and Ser660, and the phosphorylation of perilipin. Further investigation of the *in vitro* activities of this polyphenol showed that Hd-Ac has the capability to increase glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes.

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1. Introduction

Obesity, which is caused by both genetic and environmental factors, is a major risk factor for diabetes, hypertension, and heart disease [1–3]. The development of obesity is characterized by an increase in the number of fat cells and their lipid content, as a result of adipogenesis and differentiation [3,4]. Several anti-obesity mechanisms have thus far been proposed: reduction of energy and food intake, decreased preadipocyte proliferation and differentiation, and increased lipolysis and fat oxidation. Currently, studies on obesity focus on discovering plant-derived functional molecules that have the capability of suppressing the proliferation and the differentiation of adipocytes in adipose tissues. For example, resveratrol from red grapes, hydroxytyrosol from olives, and epigallocatechin gallate from tea suppress adipogenesis and fat accumulation [5–7].

Several studies have shown that the Mediterranean diet is endowed with various health benefits. This beneficial effect is due to a number of food components including olive oil as the main source of fats. In addition to oleic acid, the major component of olive oil that can have health benefits, several lines of evidence suggest that the beneficial effect of the oil could also be due to the minor amounts of polyphenols that are present in virgin olive oil [8–10]. This effect may originate from the action of polyphenols on different biochemical pathways, or from the synergism between the effects of polyphenols and fatty acids. Hydroxytyrosol (Hd) is the major polyphenol in virgin olive oil, and can also be found in the leaves and fruits of olive. The beneficial effects of Hd on risk factors for cardiovascular disease such as obesity, blood pressure, inflammation, and oxidative stress have been well discussed in many studies [7,11]. Although Hd is the main polyphenol in virgin olive oil, other polyphenols such as hydroxytyrosol acetate (Hd-Ac) are also present. The concentration of Hd-Ac is approximately equal to that of Hd in some olive varieties such as Arbequina, but is twice as high as that of Hd in the Picual variety, and 3–4 times as high in the Manzanilla and Hojiblanca varieties [12].

The aim of this study was to evaluate the effects of Hd-Ac on the differentiation, lipolysis, and glucose consumption of 3T3-L1 and C2C12 cells and compare these effects to those of Hd.

2. Materials and methods

2.1. Materials

3T3-L1 and C2C12 cells were provided by RIKEN (Institute of Physical and Chemical Research Cell Bank, Tsukuba, Ibaraki, Japan).

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Hd was obtained from Extrasynthese Company (Genay, France). Hd-Ac was obtained from Enzo Life Sciences (Michigan, USA). Dulbecco's modified Eagle's medium (DMEM high-glucose), dexamethasone, 3-iso-butyl-1-methylxanthine, insulin, and isoproterenol hydrochloride were purchased from Sigma–Aldrich (Missouri, USA). The phosphospecific-HSL Ser563, phosphospecific-HSL Ser660, total perilipin, and total β-actin antibodies were obtained from Cell Signaling Technology (Tokyo, Japan).

2.2. Cell culture

C2C12 myoblasts were cultured in DMEM supplemented with 10% FBS at 37 °C, and 5% CO_2. To induce differentiation, media was replaced with DMEM containing 2% horse serum when the cells reached confluence. Experiments were performed in differentiated C2C12 myotubes 7 days after differentiation was induced. 3T3-L1 adipocyte cells were cultured in DMEM medium containing 10% FBS at 37 °C and 5% CO_2. Cells were plated at a density of 3×10^5 cells in a 60 mm culture dish, and 5×10^4 cells in a 24-well plate. Adipocyte differentiation was initiated using DMEM containing 10% FBS supplemented with 10 mg/L insulin, 0.5 mmol/L isobutylmethylxanthine, and 1 μmol/L dexamethasone (DMI) for 2 days. The medium was then replaced with DMEM containing 5 mg/L insulin and the cells were grown for 2 more days, and then replaced with fresh medium every 2 days.

2.3. Oil red O staining

Eight-day-differentiated 3T3-L1 adipocytes were washed twice with phosphate buffered saline (PBS, pH 7.4), fixed with 4% paraformaldehyde (Kantou Chemistry, Tokyo, Japan) at 4 °C for 1 h, and stained with 3 g/L oil red O (in 60% isopropanol) at room temperature for 10 min. The cells were then washed extensively with sterile water, and visualized using a microscope (BioZero BZ-8000; Keyence, Osaka, Japan). The differentiated adipocytes were stained with 0.3 g/L oil Red O, the dye was extracted with isopropanol, and the absorbance was measured at 490 nm by using a Spectra Max microplate reader (Spectra Max 190; Molecular Devices Corporation, CA, USA).

2.4. MTT assay

3T3-L1 cells were cultured in a 24-well plate. After reaching confluence, the cells were incubated for 48 h in the presence of Hd-Ac. Subsequently, the culture medium was removed and replaced by 500 μL of fresh culture medium containing 10% sterile filtered MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich). After 3 h, the insoluble formazan crystals were dissolved in 500 μL/well isopropanol and absorbance was measured at 570 nm, using the 630 nm reading as a reference. The inhibition of growth due to Hd-Ac was expressed as a percentage of viable cells in experimental wells relative to control wells.

2.5. Measurement of lipolysis

Glycerol released into the medium was measured by a colorimetric method using a glycerol assay kit from Sigma–Aldrich (Missouri, USA). Adipocytes were lysed and the results were normalized to the quantity of total protein.

2.6. Glucose consumption

Glucose level in the cell culture media was determined by a calorimetric method using a glucose assay kit from Wako chemical company.

2.7. Western blotting

3T3-L1 adipocytes were lysed in 50 mmol/L Tris–HCl [pH: 6.8], 2% SDS, 6% β-mercapthoethanol, 10% glycerol, 1 mmol/L PMSF and 2 μg/ml leupeptin. Cells lysates were separated using SDS–PAGE and blotted on a PVDF membrane. After blocking in 2% BSA, the membrane was incubated with primary antibody in TBS-T buffer, overnight at 4 °C. Then the membrane was incubated with the secondary antibody for 1 h at room temperature and treated with the LuminoGLO reagent (Cell Signaling Technology, Danvers, MA, USA). Protein bands were analyzed using chemiluminescence (LAS1000, FUJI FILM, Minato, Tokyo, Japan) and Image Gauge Software (FUJI FILM).

2.8. Gene expression analysis

Total RNA was extracted from 3T3-L1 cells by the acid-GTC-phenol method [13]. After DNase I (Takara Bio, Otsu, Shiga, Japan) treatment and RNA re-purification, cDNA was synthesized using M-MLV reverse transcriptase (Takara), and subjected to quantitative RT-PCR using SYBR Premix Ex Taq (Takara). Each cDNA was amplified using specific primers (at 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 15 s, for 40 cycles) (Table 1).

2.9. Statistical analysis

Results are expressed as mean ± SD. Comparisons between groups were made using Student's *t* tests. Differences were considered as significant at a P value <0.05.

3. Results

3.1. Hd-Ac inhibited 3T3-L1 adipogenesis

3T3-L1 preadipocytes were differentiated for 8 days in the absence or presence of different concentrations of Hd-Ac. As shown in Fig. 1A, Hd-Ac attenuated most of the adipocyte differentiation at 75 μmol/L. Oil red O lipid quantification showed that Hd-Ac decreased lipid accumulation in a dose-dependent manner. This inhibitory effect on lipid metabolism was independent of the cytotoxic effect of Hd-Ac on 3T3-L1 preadipocytes, which was observed starting at a higher concentration (150 μmol/L [Fig. 1B]). Similarly, at 75 μmol/L, Hd-Ac exhibited a greater inhibitory effect on 3T3-L1 lipid accumulation than Hd (Fig. 1C).

3.2. Hd-Ac downregulated adipogenesis-related transcription factors and their target genes

In order to understand the mechanisms underlying Hd-Ac induced suppression of 3T3-L1 differentiation, the expression of transcription factors PPARγ, C/EBPα and SREBP-1c, and their target genes were examined by qRT-PCR. When used at the same range of concentration as in the differentiation experiment described above, Hd-Ac decreased the expression of PPARγ, C/EBPα, and SREBP-1c transcription factors mainly in a dose-dependent manner. Moreover, the expression of CD36, GLUT4 and FAS were significantly reduced during adipocyte differentiation (Fig. 2).

3.3. Hd-Ac activated lipolysis in fully differentiated adipocytes

Differentiated 3T3-L1 adipocytes were treated with Hd-Ac and lipolysis was quantified by measuring the amount of released glycerol. Hd-Ac stimulated glycerol release into the culture medium in a dose and time-dependent manner. Glycerol release increased by

Table 1
Primers used in qRT-PCR analysis. PCR was performed using the primers indicated in the table, under optimal amplification conditions (50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 15 s, for 40 cycles) for each gene. The PCR amplification of each cDNA was performed in triplicate, in 3 independent samples.

Name	Forward	Reverse
G3PDH	5′-TGGTGAAGGTCGGTGTGAACGG-3′	5′-TGCCGTTGAATTTGCCGTGAGT-3′
PPARγ	5′-AAACTCTGGGAGATTCTCCT-3′	5′-TGGCATCTCTGTGTCAAC-3′
C/EBPα	5′-GCCAAACTGAGACTCTTC-3′	5′-GGAAGCCTAAGTCTTAGC-3′
GLUT4	5′-TGCTGGGCACAGCTACCC-3′	5′-CGGTCAGGCGCTTTAGAC-3′
CD36	5′-AAACCCAGATGACGTGGC-3′	5′-AAGATGGCTCCATTGGGC-3′
SREBP-1c	5′-GCTTAGCCTCTACACCAACTGGC-3′	5′-ACAGACTGGTACGGGCCACAAG-3′
FAS	5′-TGGAGCCTGTGTAGCCTTCGAG-3′	5′-ACAGCCTGGGGTCATCTTTGCC-3′

approximately 2, 3, and 4-fold at 25, 50, and 75 μmol/L of Hd-Ac, respectively (Fig. 3A).

PKA is known to activate phosphorylation of perilipin and that of HSL at 3 different sites; Ser563, Ser659, and Ser660 and this is known to be the limiting step of lipolysis [29]. Western blot analysis using an antibody against perilipin revealed an increase in the molecular weight of perilipin and a clear electrophoretic shift from 65 to 67 kDa at 75 μmol/L of Hd-Ac (Fig. 3B). The same effect was observed when isoproterenol was used as a positive control. Moreover, the exposure of adipocytes to Hd-Ac enhanced significantly the phosphorylartion of HSL at Ser563 and Ser660. No significant difference was observed between the effects of Hd and Hd-Ac on glycerol release at a concentration of 75 μmol/L and both polyphenols exhibited similar effects on lipolysis in 3T3-L1 fully differentiated adipocytes after a short treatment that lasted 24 h (Fig. 3C).

3.4. Hydroxytyrosol acetate activated glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes

3T3-L1 adipocytes and C2C12 myotubes were treated with Hd-Ac for 12 h, and the quantity of glucose consumed by the cells was measured using a glucose oxidase assay kit. As shown in the Fig. 4A and B, this polyphenol has the capacity to dose-dependently stimulate glucose consumption in adipocytes and myotubes. After treating adipocytes for 12 h with Hd-Ac or Hd at the same concentration (75 μmol/L), no significant difference was detected in glucose consumption of cells (Fig. 4C).

4. Discussion

The study presented here has shown that Hd-Ac inhibits preadipocyte differentiation induced by DMI treatment of 3T3-L1 cells.

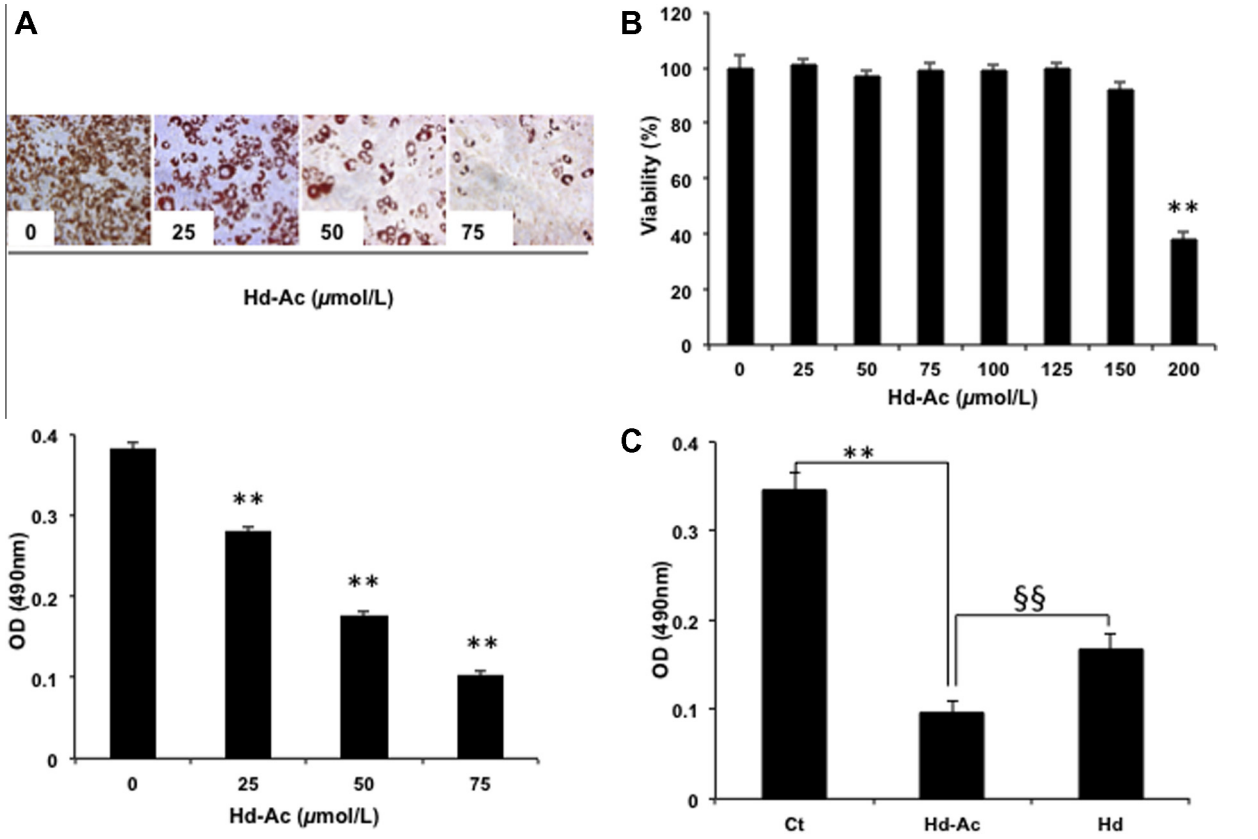

Fig. 1. Effect of Hd-Ac on triglyceride accumulation and viability. 3T3-L1 preadipocytes were treated for 8 days (A), or 48 h (B) with several concentrations of Hd-Ac. Total accumulated lipid was extracted and quantified by oil red O (A), and cell viability was determined by using the MTT assay (B). Cells were treated with either Hd-Ac or Hd at 75 μmol/L for 8 days and lipid accumulation was quantified (C). Values represent mean ± SD. Results are representative of 3 different experiments with $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, versus the control group. $^{§§}P < 0.01$, versus the Hd group.

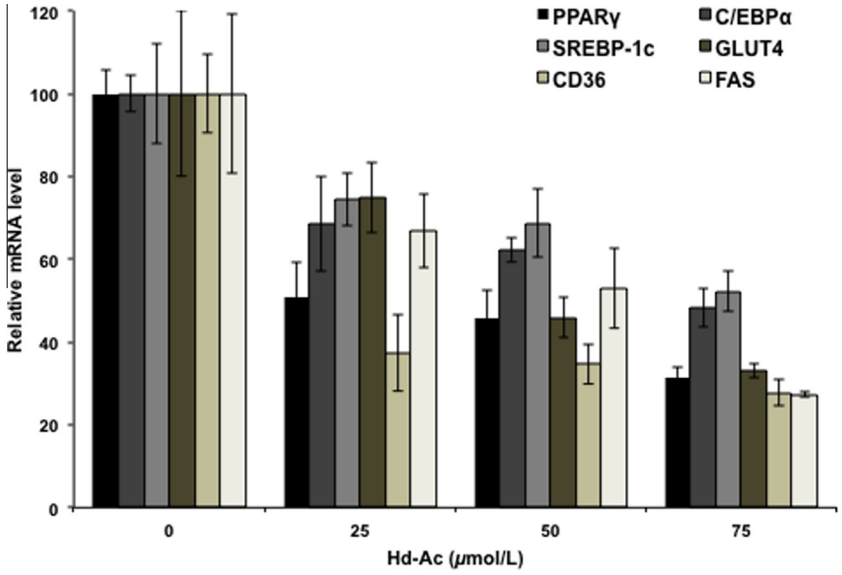

Fig. 2. Effect of Hd-Ac on PPARγ, C/EBPα, SREBP-1c, CD36, GLUT4 and FAS mRNA levels. 3T3-L1 preadipocytes were differentiated for 8 days with 0–75 μmol/L of Hd-Ac. After treatment, total RNA was extracted, and gene expression was quantified by qRT-PCR analysis. PPARγ, C/EBPα, SREBP-1c, CD36, GLUT4 and FAS gene expression levels were normalized to G3PDH mRNA levels. Values represent mean ± SD. Results are representative of 3 independent experiments with $n = 3$.

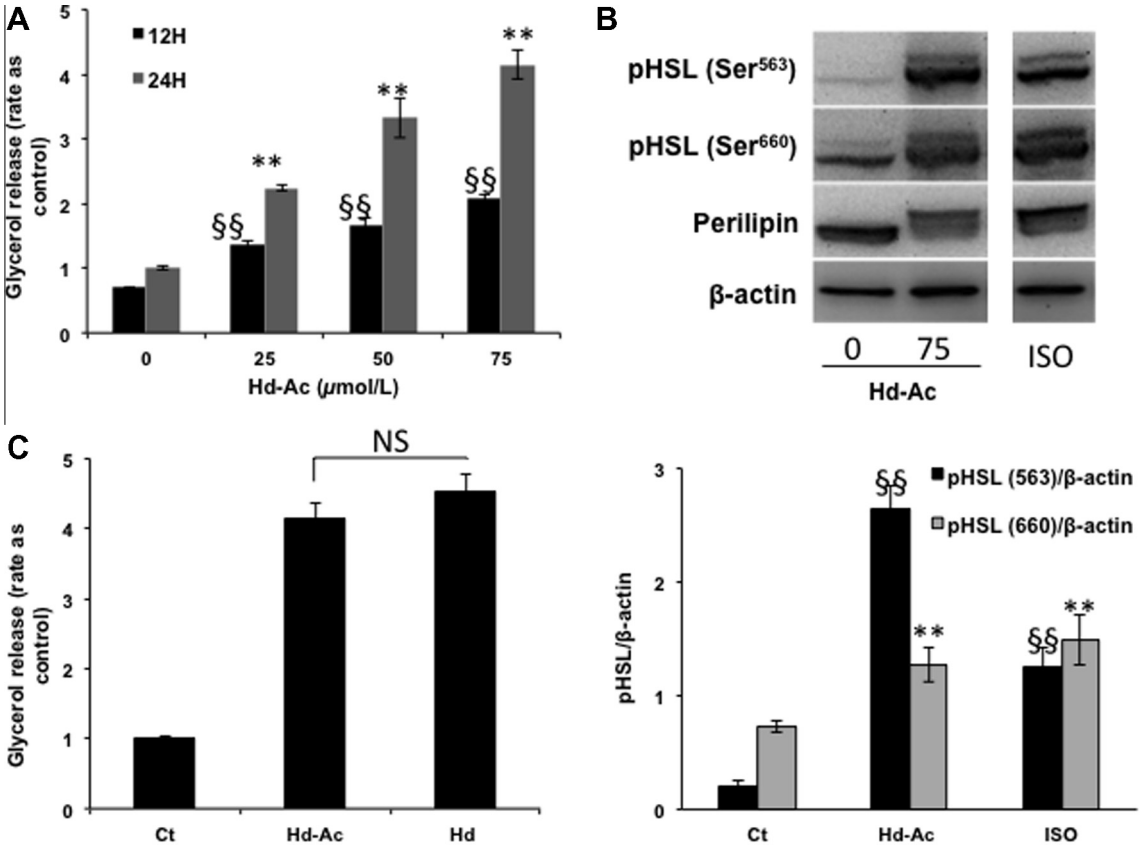

Fig. 3. Stimulation of lipolysis by Hd-Ac in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with 0–75 μmol/L Hd-Ac for 12 and 24 h. Glycerol released into the medium was quantified, and the results were normalized to protein quantity (A). Cells were cultured with 0–75 μmol/L Hd-Ac or 30 nmol/L Isoproterenol (ISO), then sonicated in lysis buffer, and proteins were subjected to Western blotting against HSL and HSL phosphorylated at Ser563 and Ser660 (B). Densities were quantified with ImageJ software, and the result is shown in the graph as a relative value against total β-actin. Adipocytes were treated with either Hd-Ac or Hd at 75 μmol/L for 24 h, and glycerol release was measured (C). Values represent mean ± SD. Results are representative of 3 different experiments with $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\S\S}P < 0.01$, versus the control group.

In fact, the differentiation of 3T3-L1 cells in the presence of Hd-Ac decreased adipogenesis, as measured by total lipid accumulation, and downregulated key adipogenesis-related transcription factors and their target genes.

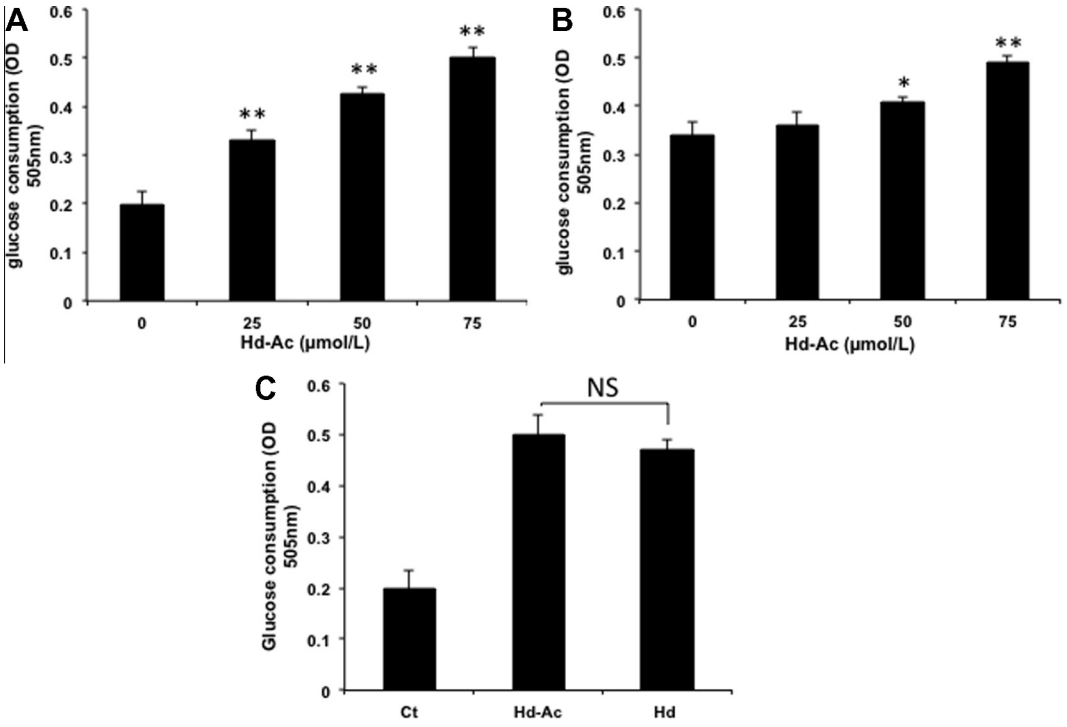

Fig. 4. Stimulation of glucose consumption by Hd-Ac in 3T3-L1 adipocytes and C2C12 myotubes. Ten-day-differentiated 3T3-L1 adipocytes (A), or seven-day-differentiated C2C12 myotubes were treated with Hd-Ac for 12 h (B). Fully differentiated adipocytes were incubated with either Hd-Ac or Hd for 24 h (C). Glucose consumed by the cells was quantified, and the results were normalized to the protein quantity. Values represent mean ± SD. Results are representative of 3 different experiments with $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, versus the control group.

Of the several transcription factors that have been identified as directly influencing adipocyte differentiation, C/EBPα, PPARγ, and SREBP-1c were found to play a major role in hydroxytyrosol acetate induced effects. It is known that induction of preadipocyte differentiation increases the expression of transcription factors C/EBPβ and C/EBPδ that are essential during an early step of differentiation. Both factors upregulate PPARγ and C/EBPα, which in turn increase the expression of their downstream target genes that are involved in triacylglycerol metabolism, including the fatty acid transporter CD36 and glucose transporter GLUT4 [14–16]. Our findings indicate that Hd-Ac treatment remarkably reduced the gene expression levels of PPARγ and C/EBPα, and their downstream target genes CD36 and GLUT4, suggesting that Hd-Ac suppresses triglyceride synthesis. Additionally, previous studies have demonstrated the importance of SREBP-1c in the regulation of mRNA expression of genes involved in adipocyte differentiation and fatty acid synthesis [17]. qRT-PCR PCR showed that Hd-Ac downregulated the expression of SREBP-1c and its downstream target gene FAS.

Several studies have proposed that, in obesity, fat accumulation is correlated with systemic oxidative stress in humans and mice [18]. In fact, reactive oxygen species (ROS) are increased during adipogenesis, and its production is important in regulating mitotic clonal expansion and enhancing the C/EBPβ DNA-binding activity, which is required for the terminal differentiation of an adipocyte. By extension, reducing intracellular ROS levels by antioxidant treatments affects C/EBPβ DNA-binding activity and cell cycle progression, which in turn decreases adipocyte differentiation [19,20]. According to our data, Hd-Ac has a higher anti-adipogenic effect than Hd. In a previous study carried out on the transport of radiolabeled Hd using Caco-2 cells, Manna et al. [21] demonstrated that this polyphenol was transported across the membrane of the human enterocytes by a bidirectional passive diffusion mechanism. Also, Caco2/TC7 cell monolayers have been used to study the metabolism of other olive oil polyphenols, such as Hd-Ac, results showed that acetylation of Hd significantly increases its transport across the small intestinal epithelial cell barrier, and then largely converted to free Hd and subsequently metabolized to homovanillyl alcohol. Both polyphenols were transferred across human Caco-2/TC7 cell monolayers, but the acetylated compound exhibited an apparent permeability 2.1-fold higher than free Hd [22]. To study the cellular metabolism of olive oil phenolic acids, human hepatoma HepG2 cells were incubated for 2 and 18 h with tyrosol, Hd and Hd-Ac. Whereas, extensive uptake and metabolism of Hd and Hd-Ac were observed, Hd-Ac was converted into free Hd and then metabolized to glucurono- and methyl-conjugates inside the cells [23]. This difference in activity, observed on 3T3-L1 adipogenesis, is probably related to the different rate of transport across the cells. Although, previous findings have shown that Hd and its acetylated form present *in vivo* and *in vitro* have antioxidative stress protective effects, Hd-Ac might have a better antioxidant activity against ROS generation than Hd, probably due to its stronger lipophilic nature, that allows it better penetration into cells [24,25].

Many compounds of plant origin including genistein, quercetin, fisetin, and grape seed proanthocyanidins are known to activate lipolysis [26–28]. Until recently, HSL, which is endowed with triacylglycerol, diacylglycerol, and cholesterol ester hydrolase activities was considered to be the rate-limiting enzyme in adipocyte lipolysis. HSL is activated by numerous hormones and metabolic signals, and can be phosphorylated at 5 different sites. Mutagenesis experiments have shown that Ser563, Ser659, and Ser660 are the major PKA phosphorylation sites responsible for increasing HSL catalytic activity and its translocation from the cytosol to the lipid droplet [29,30]. The hydrolytic action of HSL is regulated by perilipin, a lipid droplet-associated protein. Upon hormonal stimulation, PKA phosphorylates perilipin at six serine residues and this may facilitate the translocation of phosphorylated HSL into the lipid droplet and globally regulate lipolysis in adipocytes [31–34].

Our study has shown that Hd-Ac activated glycerol release in a dose-dependent manner in 3T3-L1 adipocytes. This effect was

similar to that displayed by Hd. Moreover, Hd-Ac enhanced the phosphorylation of HSL at Ser563 and Ser660, and caused an increase in the molecular weight of perilipin, indicating its phosphorylation. Phosphorylation of both perilipin and HSL efficiently catalyzed the conversion of triacylglycerol into glycerol and free fatty acids.

We also found that Hd-Ac activated glucose consumption in a dose-dependent manner both in 3T3-L1 adipocytes and C2C12 myotubes. No significant difference was observed between the effects of Hd-Ac and Hd in fully differentiated adipocytes. This preliminary *in vitro* report, needs to be followed by a detailed investigation to identify the factors directly responsible for the observations made here. In a recent study, Hao et al. [35] showed that 5′ AMP-activated protein kinase or AMPK, which is closely correlated with an increase in glucose uptake in adipocytes and muscle cells, was activated with Hd after only 30 min of exposure. Irrespective of the activation state of AMPK, the kinase AKT and the translocation of GLUT1 and GLUT4 in the presence of Hd-Ac, should be studied more thoroughly.

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