Oleuropein activated AMPK and induced insulin sensitivity in C2C12 muscle cells

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Abstract

Aims: Oleuropein has been recognized as an important medicinal compound because of its various biological properties, including anti-cancer, antidiabetic and anti-atherosclerotic activities. Here, we evaluate the antioxidant activity as well as the mechanism of the hypoglycemic effects of oleuropein in C2C12 cells and we establish the mechanism underlying these effects.

Main methods: To perform this study, C2C12 cells viability was analyzed via MTT assay and the antioxidant activity was investigated by ROS and TARS assays. Also, the effect of oleuropein on AMPK and PI3 kinase signaling pathways was evaluated.

Key findings: Treatment with oleuropein was able to protect cells against H₂O₂ induced stress in cells. On the other hand, the molecular bases of its actions have been scarcely understood. Oleuropein significantly enhanced glucose consumption and the phosphorylation of AMPK (AMP-activated protein kinase/ ACC (acetyl-CoA carboxylase)) and MAPKs (mitogen-activated protein kinases), but not PI3 kinase (Phosphatidylinositol 3-kinase)/Akt. However, the co-treatment of oleuropein and insulin improved the insulin sensitivity *via* insulin-dependent (PI3 kinase/Akt) and insulin independent (AMPK/ACC) pathways. These results could be confirmed from the findings of GLUT4 translocation which was strongly enhanced in the case of oleuropein.

Significance: Our results provide important insights for the possible mechanism of action of oleuropein as a therapeutic agent in diabetic patients.

Keywords: Skeletal muscle cells; AMPK; MAPK; insulin sensitivity; Oleuropein

Introduction

Oxidative stress has been proposed as a cause of metabolic disorders. Many researchers have examined the mechanisms behind free radicals and the possible role of oxidative stress in the pathogenesis of obesity, diabetes mellitus and other diseases in patients and animal models [1,2]. They also suggested that oxidative stress-linked cell signaling is closely associated with abnormal glucose metabolism. Diabetes mellitus is an epidemic disease characterized by high blood glucose levels. The most important factor in maintaining blood glucose levels is glucose transport [3]. Approximately, 70% of postprandial blood glucose clearance takes place in skeletal muscle and two distinct mechanisms are responsible for glucose transport including phosphatidylinositol-3 kinase (PI3 kinase) pathway and AMP-activated protein kinase (AMPK) activation. AMPK activation leads to glucose transporter 4 (Glut4) translocation from the cytosol to the plasma membrane [4]. It plays a central role in metabolism by triggering ATP-generating catabolic pathways, including β -oxidation and glycolysis [5]. In contrast, ATP-consuming anabolic pathways, such as fatty acid synthesis, cholesterol synthesis and protein synthesis are inhibited by AMPK activation [6].

On the other hand, the PI3 kinase pathway includes activation of Akt leading to activation of glycogen synthesis and the other enzymes/proteins necessary for the acute metabolic effects of insulin, which promotes GLUT4 translocation from an intracellular pool to plasma membrane [7]. Although the precise action mechanism of thiazolidinedione compounds, such as troglitazone, ciglitazone, and pioglitazone, has not been fully elucidated, the end results of their physiologic effects are to improve insulin-mediated peripheral glucose disposal and reduce hepatic glucose output, as demonstrated

by in vitro and in vivo studies [8]. However, these thiazolidinedione have several side effects, which, in many cases, may worsen health conditions. Therefore, the search for natural agents that minimize these undesirable effects, and are still effective in regulating the disturbances observed in obesity, has been receiving increasing attention from the scientific community [9]. In this sense, olive leaves have been used as herbal medicine and are known to exert beneficial effects such as antioxidant, anti-cancer and anti-obesity [10]. Active components of olive leaves, such as oleuropein, verbasocside and luteolin, have been studied as possible diabetes-preventive components that decreased blood glucose concentrations, treated hyperglycemic symptoms, and enhanced antioxidant activity [11]. In a recent study, Drira *et al.* 2013 have found that hydroxytyrosol, which is obtained by hydrolysis of oleuropein, activated glucose consumption in a dose-dependent manner in both 3T3-L1 adipocytes and C2C12 myotubes via the expression of HSL [12]. Moreover, Hao showed that hydroxytyrosol activated AMPK kinase in adipocytes and muscle cells [13]. In addition, few studies have investigated the effects of olive leaf extract on the mechanisms of insulin resistance in type 2 diabetes. In this context, Liu *et al.* 2014 have reported that oleuropein improved insulin resistance in high fat diet induced diabetes in rats by reducing tumor necrosis factor-alpha (TNF- α), and interleukin-6 (IL-6) [14]. This study sought to evaluate the antioxidant effect of oleuropein on muscle cells and to explore whether this compound could activate the AMPK and the insulin pathway PI3 kinase.

Materials and Methods

Chemicals and reagents

Oleuropein was purchased from Extrasynthese Company (Genay, France). C2C12 cells were provided by Sigma Aldrich. Dulbecco's modified Eagle's medium (DMEM high-glucose), foetal bovine serum (FBS), bovines serum albumin (BSA), penicillin, streptomycin, trypsin were obtained from Gibco-BRL (Grand Island, NY, USA were purchased from Sigma-Aldrich (Missouri, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Antibodies for AMPK, Phospho-AMPK α (Thr172), Akt, Phospho-Akt (Ser473), ERK, Phospho-Erk (Thr202), IRS, p-IRS (Ser 308) were obtained from Cell Signaling Technology (Beverly, MA). All other reagents used were of the purest grade available.

Cell culture

C2C12 (mouse myoblasts cell line) were maintained in DMEM supplemented with 10% heat inactivated FBS and 1% penicillin streptomycin, at 37 °C in a humidified atmosphere with 5% CO₂. For differentiation into myotubes, cells were seeded at a density of 2. 10⁴ cells/mL. After reaching 80% of confluence, the medium was switched to DMEM with 2% (v/v) FBS and was replaced after 2, 4 and 6 days of culture. Experiments were performed on day 7 when myotubes differentiation was completed.

MTT viability

Cytotoxicity experiments were performed to determine the nontoxic dose of oleuropein (OL). The viability was evaluated by MTT assay [15]. The cells were seeded in 96-well plates at a density of 2 x 10⁴ cells/well and cultured with or without compounds for 24 h. Then, 100 μ L of MTT solution (5 mg/mL MTT in PBS) was added to each well and incubated for 8 h at 37 °C. Then, the formazan crystal formed was dissolved by adding

100 μ L of SDS and the absorbance was detected at 540 nm. Relative cell viability of treatment was calculated as a percentage of the viability.

Intracellular reactive oxygen species (ROS) production with DCFH-DA assay

The ability of oleuropein to reduce intracellular ROS production was estimated using a fluorescent probe, DCFH-DA. The non-ionic DCFH-DA crosses cell membrane and is hydrolysed to nonfluorescent DCFH by intracellular esterases. In the presence of ROS such as hydrogen peroxide (H_2O_2) and lipid hydroperoxides, DCFH is rapidly oxidized to fluorescent Dichlorofluorescein (DCF) which can be detected using a sensitive fluorescence spectrophotometer [16]. The fluorescence intensity reflects the amount of intracellular ROS formed. C2C12 cells were seeded in a 6-well plate (2×10^4 cells/ml), followed by the pretreatment with or without oleuropein (200 and 400 μ M) for 24 h at 37°C. Cells were gently washed with PBS and then incubated in the dark with 30 μ M DCFH-DA for 30 min at 37°C. Next, the cells were treated with H_2O_2 (400 μ M) for 4 h and after washing with PBS, the intracellular ROS formation was then measured (detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm) over 1 h at 37°C. ROS generation was calculated as an increase in fluorescent signal between the control and H_2O_2 treated cells.

Assessment of TBARS levels by spectrophotometry

For TBARS levels, we used the method proposed by [17]. 400 μ L of cellular suspension or 400 μ L of PBS for blank was mixed with 1 mL thiobarbituric acid (TBA) + TCA reagent (3.7 g/L TBA + 15% TCA in 0.25 mol.L⁻¹HCl). The mixture was heated for 15 min in a boiling water bath. After cooling in an ice bath, the tubes were centrifuged at 15000 g for 15 min. The absorbance of the supernatant at 535 nm was measured, and the

concentration of TBARS in the samples was determined using a standard curve made with freshly prepared 1,1,3,3- tetraethoxypropane. Total protein in cellular suspension was assayed using a Bradford reagent from Wako. TBARS concentrations are expressed as nmol TBARS mg⁻¹ protein.

Glucose consumption

Glucose level in the cell culture media was determined by a calorimetric method using a glucose assay kit from sigma Aldrich.

Effect on insulin sensitivity

C2C12 cells were incubated with insulin (100 nM) for 30 min in the presence or absence of oleuropein at 200 and 400 µM. Total cell lysates were prepared for each condition and Western blot analysis was performed for phospho-Akt (Ser 473) and p-IRS (Ser 307) as described above.

Western blot

Cells were washed twice with cold PBS and then scraped with 500 µL of RIPA buffer (according the manufacturer's instructions) with a protease inhibitor cocktail (Gen DEPT, TX). Protein concentration was determined by the Bradford reagent (REF). Twenty micrograms of protein from each sample was separated by electrophoresis on 12% SDS-PAGE. The gel was blotted onto a PVDF membrane by semi-dry transfer (Bio-Rad, CA). The membranes were blocked with 2% BSA in TBS-T buffer (10 mM Tris-Cl pH 8.00), 100 mM NaCl, and 0.1% Tween 20) for 2 h and then incubated overnight with a specific primary antibody for each protein. Subsequently, the membrane was washed with a TBS-T buffer and was probed with a goat anti-mouse Ig G-horseradish peroxidase conjugate (Santa Cruz Biotechnology, CA). The protein band signal was detected by a

chemiluminescence (ECL) detection kit (Amersham Bioscience, NJ), and the image of the signal was visualized by the LAS 4000 mini-imaging system (Fuji Life Sciences, CT).

Statistical analysis

The results of four experiments were pooled and expressed as mean $\pm$ standard deviation (SD). Data were subjected to a one-way analysis of variance (ANOVA) using SPSS 19 statistical package (SPSS Ltd., Woking, UK) followed by Tukey post hoc tests. Differences were considered significant when $p < 0.05$.

Results

Effect of oleuropein on C2C12 cell viability

In order to assess the non-cytotoxic concentration of oleuropein, the viability of C2C12 cells was tested at different concentrations varied from 0 - to 600 μ M using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Our results illustrated that oleuropein had no significant effect ($p < 0.05$) on the cell viability and the concentrations used in subsequent experiments are up 400 μ M for oleuropein (**fig.1**).

Oleuropein activated glucose consumption in C2C12 myotubes

C2C12 myotubes were treated with oleuropein for 24 h, and the quantity of glucose consumed by the cells was measured. As shown in the figure 2, Oleuropein was able to stimulate glucose consumption in myotubes cells in a dose dependent manner (**fig.2**).

The effect of oleuropein on ROS production

The production of free radicals in organisms causes oxidative stress which is associated with several diseases. This process may cause peroxidative tissue damage in

inflammation, cancer, toxicity of xenobiotics, and aging. Hydrogen peroxide toxicity leads to severe oxidative stress in human C2C12 cells [2].

The myotube cells were treated with various concentrations of H₂O₂ (0-900 µM), for 4 h, to determine the H₂O₂ concentration needed for inducing oxidative damage in the C2C12 cells (data not shown). The H₂O₂ induced cytotoxicity was concentration dependent. Lower concentrations of H₂O₂ (0-300 µM) reduced the cell viability but the effect was not significant (P>0.05). However, higher concentrations of H₂O₂ (500-900 µM) significantly decreased the cell viability (P<0.05). In the presence of 400 µM H₂O₂ a moderate reduction in cell viability was observed. Therefore, 400 µM H₂O₂ was used as the ideal concentration during the following experiments for assessing the effect of olive leaves compounds on ROS and TBARS levels in C2C12 cells.

The ability of these compounds to reduce H₂O₂ induced ROS production was evaluated using the fluorescence probe DCFH-DA. The myotubes cells exposed to H₂O₂ showed an increase in the intracellular level of ROS compared to the untreated cells used as a control (**Fig. 3a**). However, this effect was reduced significantly (p<0.05) in C2C12 pretreatment with oleuropein at 200 and 400 µM and the ROS rate was of about 15 % and 34 %, respectively, in comparison with muscle cells treated with H₂O₂ only.

Effects of oleuropein compounds on TBARS levels in C2C12 cells

The results showed that H₂O₂ treatment increased significantly TBARS (a marker of lipid peroxidation) levels by 29% compared to control. Pretreatment of cells with oleuropein at 200 and 400 µM before H₂O₂ administration led to a significant decrease in TBARS levels by 18.4 % and 30.7% respectively (**Fig. 3b**).

Oleuropein activated AMPK and MAPKs signaling but not by PI3 kinase/Akt pathway

215 To investigate the mechanism of action of oleuropein, we first examined its effect on
216 AMPK and ACC phosphorylation in C2C12 myotubes that were exposed to various
217 concentrations for 24 hours. As shown in **Figure 4**, the oleuropein induced significantly
218 the phosphorylation of AMPK in a dose-dependent manner. The phosphorylation of
219 ACC-Ser79, which is the best-characterized downstream molecule of AMPK, also
220 showed a similar activation response. This phosphorylation level could be observed at the
221 lowest concentration and was maintained up to 24 h (**Fig. 4a and b**).

222 On the other hand, AMPK activation has been reported to be associated with the
223 activation of several kinases such as MAPK [18,19]. Additionally, MAPK has been
224 proposed to facilitate the glucose transporter. Therefore, we explored whether oleuropein
225 has the ability to induce glucose uptake through this pathway. Our results showed that
226 oleuropein promoted MAPK phosphorylation in a dose dependent manner (**Fig. 4a and**
227 **b**).

228 In addition to AMPK, the insulin signaling pathway is also involved in glucose uptake.
229 Akt is (the major) a serine/threonine protein kinase that leads to a translocation of insulin-
230 sensitive glucose transporter (GLUT4) to plasma membrane *via* the activation of signal
231 transduction cascade involving insulin receptor substrate (IRS) family, and PI3-kinase
232 [20]. Therefore, we examined whether oleuropein, administered alone, could affect the
233 basal activity of PI3Kinase/Akt on glucose metabolism in comparison with those of
234 insulin. As shown in Figure 4, insulin caused a phosphorylation of Akt, whereas
235 oleuropein treatment at a dose up to 400 μ M had no stimulatory effect on Akt. These
236 results indicate that mechanism of action underlying the oleuropein induced glucose

uptake in muscle cells is linked to the activation of AMPK but don't require the PI3 kinase/Akt activation.

3.7. Oleuropein improves insulin sensitivity *via* activation of PI3 kinase/Akt.

Insulin is an important endocrine adipokine which plays a key role in the regulation of glucose metabolism in liver, adipocyte, and muscle cells. Insulin has a major regulatory function for glucose metabolism in liver, adipocyte and skeletal muscle by redistributing GLUT4 from intracellular vesicles to the cell surface. Thus, it was important to elucidate the interaction between oleuropein and insulin on Akt pathway. The treatment of oleuropein separately did not affect Akt phosphorylation. Surprisingly, when oleuropein was combined with insulin, the phosphorylation levels of Akt and IRS were significantly ($p < 0.05$) much higher than the individual effects of each active compound (Fig. 5a and b). To confirm this result, we performed a GLUT4 Western blotting. The result illustrated that the co-treatment of oleuropein and insulin further increased the GLUT4 (Fig. 5c and d).

Discussion

Oxidative stress is a result of imbalance between antioxidant defense mechanism and free radical production which lead to a damage of target molecules. Among this, diabetes mellitus which is a worldwide increasing disease. The main causes of hyperglycemia can be associated with metabolic stress conditions and a defective glucose circulation in insulin target tissues as liver, skeletal muscle and adipose tissue. The skeletal muscle is the most important site of energy production and it is responsible for more than 70% of glucose uptake and oxidation.

259 In muscle cells, an amount of ROS is necessary for physiological processes, including
260 phagocytosis [21], intracellular signaling [22], cell proliferation, metabolism, apoptosis
261 and muscle contraction [23]. However, excessive formation of ROS leads to muscle
262 damage, fatigue and mediated insulin resistance. ROS can be induced by various agents.
263 Superoxide ($O_2^{\bullet}$) and nitric oxide ($NO^{\bullet}$) are the primary species produced by
264 mitochondrial respiration. H_2O_2 is a precursor of the formation of ROS leading to the
265 destruction of the antioxidant defense in various tissues [24,25]. It readily penetrates into
266 cells and reacts with intercellular ions such as iron and copper, to generate highly reactive
267 hydroxyl radicals that successively attack cellular components [26].

268 In the first part of this study, we investigated the potential protective activity of
269 oleuropein against H_2O_2 induced oxidative stress in muscle cells. The treatment with
270 H_2O_2 increased significantly ROS levels by 39%. The pretreatment with oleuropein at
271 200 and 400 μM led to a significant decrease in ROS levels by 15.7% and 35.8%,
272 respectively. Several previous reports have linked the ROS formation with glucose
273 uptake. In this context, Evans *et al.* have demonstrated that an increase in ROS level
274 contributes directly to the activation of serine/threonine. The phosphorylation of IRS
275 reduced the ability to activate tyrosine phosphorylation and accelerated the degradation
276 of IRS-1 causing impaired glucose uptake in muscle cells [27].

277 Moreover, the effect of oleuropein on TBARS levels was studied (a marker of lipid
278 peroxidation). Treatment of C2C12 cells with H_2O_2 increased significantly TBARS levels
279 by 40%. H_2O_2 reacts with Fe^{2+} leading to the formation of $OH^{\bullet}$ and hydroxyl radical
280 ($OH^{\bullet}$). The generation of $[OH^{\bullet}]$ has been associated with lipid peroxidation [28]. The
281 pretreatment of muscle cells C2C12 with oleuropein decreased TBARS levels.

282 Interestingly, in alloxan-induced diabetic rats and rabbits, oleuropein rich extract
283 significantly reduced plasma glucose and TBARS levels [29,30].

284 In muscle cells the regulation of glucose uptake is quasi important to maintain the energy
285 balance. 5'-AMP-activated protein kinase (AMPK) and phosphatidylinositol-3 kinase
286 (PI3 kinase) are two distinct pathways. AMPK is an independent insulin signaling that
287 regulated intracellular energy metabolism [31]. Activation of AMPK leads to the
288 phosphorylation and regulation of a number of downstream targets that are involved in
289 diverse pathways, including acetyl-CoA carboxylase (ACC) in the body [32]. PI3 kinase,
290 is a key molecule, and its main downstream target is Akt, that is important in insulin-
291 stimulated glucose transport and metabolism [33].

292 The principal finding of the present study is that oleuropein stimulated glucose
293 consumption and increased insulin sensitivity in skeletal muscle cells *via* multiple
294 mechanisms. The results of the glucose consumption assay indicated that oleuropein can
295 enhance glucose uptake in a dose-dependent manner in differentiated C2C12 cells (Fig
296 2A), indicating its possible regulatory role in the glucose metabolism of skeletal muscle
297 cells. To understand the mechanism of action related to oleuropein, we carried out
298 Western blot analysis using specific antibodies for the signaling molecules associated
299 with glucose metabolism. Our data demonstrated that oleuropein was able to activate
300 AMPK in a dose dependent manner. However, in insulin signaling pathway, oleuropein
301 treatment had no significant effect on Akt comparing to control treated with insulin that
302 revealed a robust phosphorylation. We can conclude that oleuropein alone had no
303 stimulatory effect on PI3/Akt pathway.

304 AMPK and insulin pathways had distinct mechanisms but these two pathways meet at
305 MAPK. MAPK regulates the activity and translocation of GLUT4 and as a consequence,
306 facilitates glucose absorption [19,34]. For example, p38 MAPK pathway is activated in
307 skeletal muscle cells under various conditions, including hypoxia, hypertonicity and
308 ischemia, and has been shown to stimulate glucose uptake *via* GLUT4 translocation [18].
309 Thus, there is increasing evidence that p38 MAPK is a downstream molecule of AMPK
310 and could be a possible target in glucose metabolism. In order to confirm this
311 relationship, the effect of oleuropein on MAPK was studied and we showed that the ERK
312 expression was enhanced in the presence of oleuropein compared to untreated control.
313 Few studies have investigated the effect of oleuropein on insulin sensitivity. Several
314 molecular mechanisms have been proposed to underlie its hypoglycemic effect by
315 reducing the inflammatory cytokines levels such as TNF- α , IL-6, and enhanced insulin
316 sensitivity *via* IRS activation. It was reported that increased pro-inflammatory cytokine
317 production in diabetes increases phosphorylation of insulin receptor substrate, causing
318 antagonistic properties in insulin, which leads to insulin resistance [35]. In this context,
319 Liu *et al.*, have demonstrated that oleuropein rich extract reduced the secretion of the pro-
320 inflammatory cytokine such as TNF- α , IFN- γ , IL-2, IL-6 and IL-1 β , whereas it increased
321 the levels of the anti-inflammatory cytokine IL-10 and enhanced the expression of insulin
322 receptor substrate-1 (IRS-1) mRNA expression in streptozotocin induced diabetic mice
323 [14,36].
324 From another perspective, previous findings suggested that there is a link between
325 AMPK and PI3K signaling pathway [37]. In agreement to these reports, when cells were
326 co-treated with oleuropein and insulin, PI3/Akt pathway was more enhanced comparing

to its individual treatment. Moreover, the activation of AMPK on glucose uptake occurs independently of insulin, and stimulates the translocation of GLUT4 which can lead to the improvement of insulin stimulated glucose uptake [38]. We demonstrated that levels of total GLUT4 protein were up regulated by oleuropein. The increased GLUT4 level might contribute to reversing the state of increased circulating anti-insulin sensitivity factor, as stated above and improve peripheral insulin sensitivity. (Fig. 5c). In this context, Hao *et al.* showed that hydroxytyrosol activated AMPK after exposure for 30 min [13]. However, the effect of this compound on the Akt expression and the translocation of GLUT1 as well as GLUT4 have not been studied thoroughly.

Conclusion

The data obtained in the present study suggest that oleuropein plays a beneficial role in glucose metabolism of differentiated C2C12 skeletal muscle cells .Oleuropein treatment activated AMPK/ACC and MAPKs independently of insulin. Moreover, oleuropein stimulated insulin sensitivity, which is coupled to basal glucose translocation of GLUT4, through both of insulin-independent (AMPK/ACC and MAPKs) and insulin-dependent (PI3 kinase/Akt) pathways. These findings support a therapeutic potential of oleuropein to the treatment of diabetes and its complications. Further investigations are needed to study the effect of oleuropein on 3T3-L1 cells another target of insulin response.

Conflict of interests

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1: Effect of oleuropein (OL) on viability of C2C12. Values represent mean $\pm$ SD. Results are representative of 3 different experiments. [#]statistically significant compared to control.

Fig. 2: Stimulation of glucose consumption oleuropein in C2C12 myotubes. Seven-day-differentiated myotubes were treated with oleuropein for 24 h. Glucose consumed by the cells was quantified and the results were normalized to the protein quantity. Values represent mean $\pm$ SD. Results are representative of 3 different experiments. [#]statistically significant compared to control.

Fig. 3: Effects of oleuropein on ROS levels (a) and TBARS levels (b) in C2C12 myoblasts. Cells were studied under three conditions: under normal conditions (control), under treatment with H₂O₂ (0.4mM) for 30 min and under the combination of oleuropein (200 and 400 μ M) for 24 h and H₂O₂ (0.4mM) for 30 min.

ROS levels were assessed by fluorescence intensity using DCFH-DA. TBARS levels were evaluated by spectrophotometer. All values are presented as the mean $\pm$ of 3 experiments. * Statistically significant compared to H₂O₂ alone ($P < 0.05$). [#]statistically significant compared to control.

Fig. 4: (a) effect of oleuropein on glucose transport is dependent upon AMPK/ACC and MAPKs pathways in C2C12 cells but not insulin signaling pathway. Differentiated C2C12 cells were incubated with oleuropein at various concentrations (200 and 400 μ M) for 24 h. Protein extracts were prepared and subjected to Western blot assay using the primary antibodies for phospho-acetyl-CoA carboxylase (Ser79), phospho-AMPK α (Thr172), p-Akt (Ser 473), β -actin protein levels were used as a control for equal loading.

The protein band signal was detected by a chemiluminescence (ECL) detection kit (Amersham Bioscience, NJ), and the image of the signal was visualized by the LAS 4000 mini-imaging system (Fuji Life Sciences, CT).(b). The results are expressed as mean $\pm$ S.D. for three independent experiments.

Fig.5: Oleuropein improves insulin sensitivity in C2C12 cells. Differentiated C2C12 cells were treated with oleuropein (200 and 400 μ M) for 24 h and then 100 nM of insulin was added for 30 min. Phospho-AMPK α (Thr172), phospho-Akt (Ser473), phospho-IRS (Ser 307) and GLUT4 were detected (a and c) and quantified (b and d).[#]statistically significant compared to control.

Fig. 1

Fig. 2

Fig. 3

a)

b)

Fig. 4

a)

b)

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Fig. 5

a)

b)

c)

d)