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Radicicol, a heat shock protein 90 inhibitor, inhibits differentiation and adipogenesis in 3T3-L1 preadipocytes

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ABSTRACT

Heat shock protein 90 (Hsp90) is involved in various cellular processes, such as cell proliferation, differentiation and apoptosis. As adipocyte differentiation plays a critical role in obesity development, the present study investigated the effect of an Hsp90 inhibitor radicicol on the differentiation of 3T3-L1 preadipocytes and potential mechanisms. The cells were treated with different concentrations of radicicol during the first 8 days of cell differentiation. Adipogenesis, the expression of adipogenic transcriptional factors, differentiation makers and cell cycle were determined. It was found that radicicol dose-dependently decreased intracellular fat accumulation through down-regulating the expression of peroxisome proliferator-activated receptor γ ($PPAR_\gamma$) and CCAAT element binding protein α (C/EBP_α), fatty acid synthase (FAS) and fatty acid-binding protein 4 (FABP4). Flow cytometry analysis revealed that radicicol blocked cell cycle at G1-S phase. Radicicol redcued the phosphorylation of Akt while showing no effect on β-catenin expression. Radicicol decreased the phosphorylation of phosphoinositide-dependent kinase 1 (PDK1). The results suggest that radicicol inhibited 3T3-L1 preadipocyte differentiation through affecting the PDK1/Akt pathway and subsequent inhibition of mitotic clonal expansion and the expression/activity of adipogenic transcriptional factors and their downstream adipogenic proteins.

1. Introduction

Obesity is a worldwide public health threat and a central cause of metabolic disorders, including type-II diabetes, insulin resistance, hyperlipidemia, hypertension and coronary heart disease [1,2]. Obesity develops as a result of imbalance between energy intake and expenditure, and is characterized by increases in the number (hyperplasia) and size (hypertrophy) of adipocytes in adipose tissue [3,4]. Adipocyte hyperplasia can be mimicked *in vitro* by 3T3-L1 preadipocyte differentiation and thus the model of adipocyte differentiation has been widely used in obesity research. Adipocyte differentiation is regulated by peroxisome proliferator-activated receptor γ ($PPAR_\gamma$) and CCAAT element binding protein (C/EBP) transcriptional factors [5]. Activation of these factors leads to an increase in the gene expression of fatty acid synthase (FAS) and fatty acid-binding protein 4 (FABP4), which are responsible for fat synthesis, transport and deposit. Therefore, it is of interest to modulate adipocyte differentiation through regulating these transcriptional factors.

Heat shock proteins Hsp27, Hsp70 and Hsp90 are molecular chaperones, which are localized in different cell compartments. These proteins mediate the folding of newly synthesized proteins and translocation through membranes as well as the maturation and stability of a variety of other proteins [6]. Hsp90 is a constitutively abundant chaperone, accounting for 1–2% of cytosolic proteins, and plays various roles in the regulatory network of cell cycle and signal transductions [7]. This protein is required for the

Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; BCS, bovine calf serum; C/EBP_α, CCAAT element binding protein α; FABP4, fatty acid-binding protein 4; FACS, fluorescence-activated cell sorting; FAS, fatty acid synthase; FBS, fetal bovine serum; Hsp90, heat shock protein 90; IBMX, 3-isobutyl-1-methylxanthine; PDK1, phosphoinositide-dependent kinase 1; Rnase, ribonuclease; $PPAR_\gamma$, peroxisome proliferator-activated receptor γ; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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differentiation of several types of cells, such as leukemia K562 cells, HL-60 cells and muscle cells [8,9]. The loss-of-function studies have verified the role of Hsp90 in cell differentiation and development [10]. It has been shown that Hsp90 inhibitors block the differentiation of C2C12 myoblasts [9]. In Kasumi-1 cells, Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), induces a retinoblastoma-dependent G1 block [11]. However, to our knowledge, there is no information available regarding the role of Hsp90 inhibitors on adipocyte differentiation. The present study was conducted to determine the effect of radicicol, a naturally-occurring compound that binds to Hsp90, on adipocyte differentiation and potential mechanisms in 3T3-L1 preadipocytes. We report here for the first time that radicicol has a dramatic anti-adipogenic effect via inhibiting the key transcriptional factors that regulate adipogenesis and mitotic clonal expansion.

2. Materials and methods

2.1. Chemicals and reagents

Radicicol, insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, protease inhibitor, propidium iodide, ribonuclease (Rnase) and chemiluminescent peroxidase substrate-1 were bought from Sigma (St. Louis, MO, USA). High glucose Dulbecco's modified Eagle's medium (DMEM) was from Cellgro Mediatech Inc. (Manassas, VA, USA). Fetal bovine serum (FBS) was bought from PAA Laboratories (Etobicoke, ON, Canada). Bovine calf serum (BCS) and adipogenesis assay kits were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). The BCA protein assay kit was from Thermo Scientific (San Jose, CA, USA). RIPA lysis buffer was from Millpore (Billerica, MA, USA). Protein loading buffer was obtained from Bio-Rad laboratories (Montreal, QC, Canada). Antibodies against fatty acid-binding protein 4 (FABP4) and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against $PPAR_{\gamma}$ and CCAAT element binding protein α (C/EBP_{α}), phosphoinositide-dependent kinase 1 (PDK1), phospho-PDK (pPDK), Akt, phospho-Akt (pAkt), β-catenin and phospho-β-catenin (pβ-catenin) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Fatty acid synthase (FAS) antibody was purchased from Novus Biologicals (Oakville, ON, Canada).

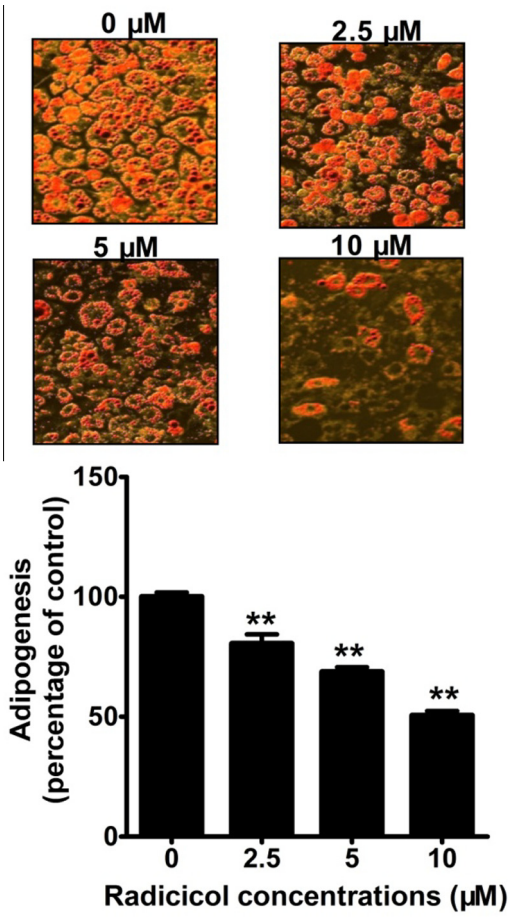

Fig. 1. Effect of radicicol on lipid accumulation in 3T3-L1 adipocytes. After reaching confluency, 3T3-L1 preadipocytes were induced to differentiate in the absence or presence of radicicol for 8 days. The morphological changes associated with cell differentiation were photographed after Oil Red O staining. Then, the stained lipids were extracted and quantified by measuring absorbance at 520 nm. Data are expressed as means ± SD ($n = 3$). $^{**}P < 0.01$ vs. the control.

2.2. Cell culture and differentiation

3T3-L1 preadipocytes were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM containing 10% BCS until confluent and maintained in the same medium for additional 2 days. Then, differentiation was induced by adding 0.5 mM IBMX, 1 μM dexamethasone, 5 μg/mL insulin in DMEM with 10% FBS. After 3 days, the medium was replaced with fresh DMEM containing 10% FBS and 5 μg/mL insulin. Two days later, the medium was changed to DMEM with 10% FBS and kept for additional 2 or 3 days.

2.3. Measurement of lipid accumulation

The 3T3-L1 cells were grown in 96-well plates and differentiated for 8 days in the presence of different concentrations of radicicol. Fat content was measured using a commercial assay kit following the kit's instructions (Ann Arbor, Michigan, USA). Briefly, adipocytes were fixed with the fixative solution for 15 min, stained with Oil Red O for 20 min, and washed with distilled water. Images were captured under a inverted microscope (Nikon ECLIPSE TE200) after washing with distilled water. Lipids were extracted with the extraction solution for 15–30 min and read for absorbance at 520 nm on a Varioskan Flash spectral scanning multimode plate reader (Thermo Fisher Scientific, Waltham, MA).

2.4. Western blotting

3T3-L1 preadipocytes were grown in 75 cm^2 flasks and differentiated in the presence of 0, 2.5, 5.0, and 10.0 μM of radicicol. The cells were washed with ice-cold PBS and lysed in ice-cold RIPA lysis buffer. Protein concentrations were determined using a BCA protein assay kit. Proteins were separated a SDS–PAGE and transferred to nitrocellulose membrane, with β-actin being used as the loading control. Protein bands were detected by enhanced chemiluminescence (ECL) reagents and quantified using the Molecular Imager (Bio-Rad).

2.5. Flow cytometric analysis of cell cycle

Two days after reaching confluence, 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of 2.5, 5, and 10 μM of radicicol, respectively. Twenty-four hours later, the cells were harvested and fixed overnight with 70% ethanol at 4°C. Ethanol was removed and the cells were washed with cold PBS and stained with propidium iodide solution containing 20 μg/ml RNase for 30 min. Fluorescence-activated cell sorting (FACS) analysis was performed on a Becton–Dickinson FACScan system and data were analyzed using the Flowjo software (Version 7.6.1, Tree Star Software, San Carlos, CA, USA).

2.6. Statistical analysis

The treatment effect was determined using one-way ANOVA and followed by a post hoc Dunnett's or Bonferroni's test for multiple comparisons if a significant treatment effect was detected. Data are presented as means ± SD, where P values less than 0.05 were considered significant. All analyses were performed using SPSS (version 13.01S; Stats Data Mining Co. Ltd., Beijing, China).

3. Results

3.1. Radicicol inhibits lipid accumulation in 3T3-L1 adipocytes

Following the addition of induction medium, 3T3-L1 preadipocytes underwent morphological changes from the spindle-like features to round shape and accumulation of intracellular lipids. The addition of radicicol reduced intracellular lipids in a dose-dependent manner, resulting in significantly lower lipid contents in cells treated with 2.5, 5, and 10 μM of radicicol, respectively (Fig. 1). Most of the radicicol-treated cells remained the preadipocyte features, especially those treated with 10 μM of radicicol.

3.2. Radicicol decreases the protein expression of PPAR$_\gamma$ and C/EBP$_\alpha$

PPAR$_\gamma$ and C/EBP$_\alpha$ are two transcriptional factors that tightly regulate preadipocyte differentiation. As shown in Fig. 2A and B, radicicol significantly decreased the protein expression of PPAR$_\gamma$ at the concentrations of 2.5, 5, or 10 μM and C/EBP$_\alpha$ protein expression at 5 or 10 μM compared to the control on day 8 post the induction of differentiation. It was suggestive that radicicol affected adipocyte differentiation through inhibiting the expression of these transcriptional factors.

3.3. Radicicol affects the gene and protein expressions of lipogenic genes

FAS and FABP4 are downstream enzyme/protein targets of adipogenic transcription factors PPAR$_\gamma$ and C/EBP$_\alpha$ and control fatty acid biosynthesis and deposition during adipocyte differentiation. Since PPAR$_\gamma$ and C/EBP$_\alpha$ were downregulated, it was hypothesized that FAS and FABP4 were affected by radicicol. The results indeed showed that radicicol decreased the expression of FAS and FABP4 as compared with the control (Fig. 2C and D). Radicicol at 10 μM downregulated FAS and FABP4 protein expressions by 75% and 90%, respectively.

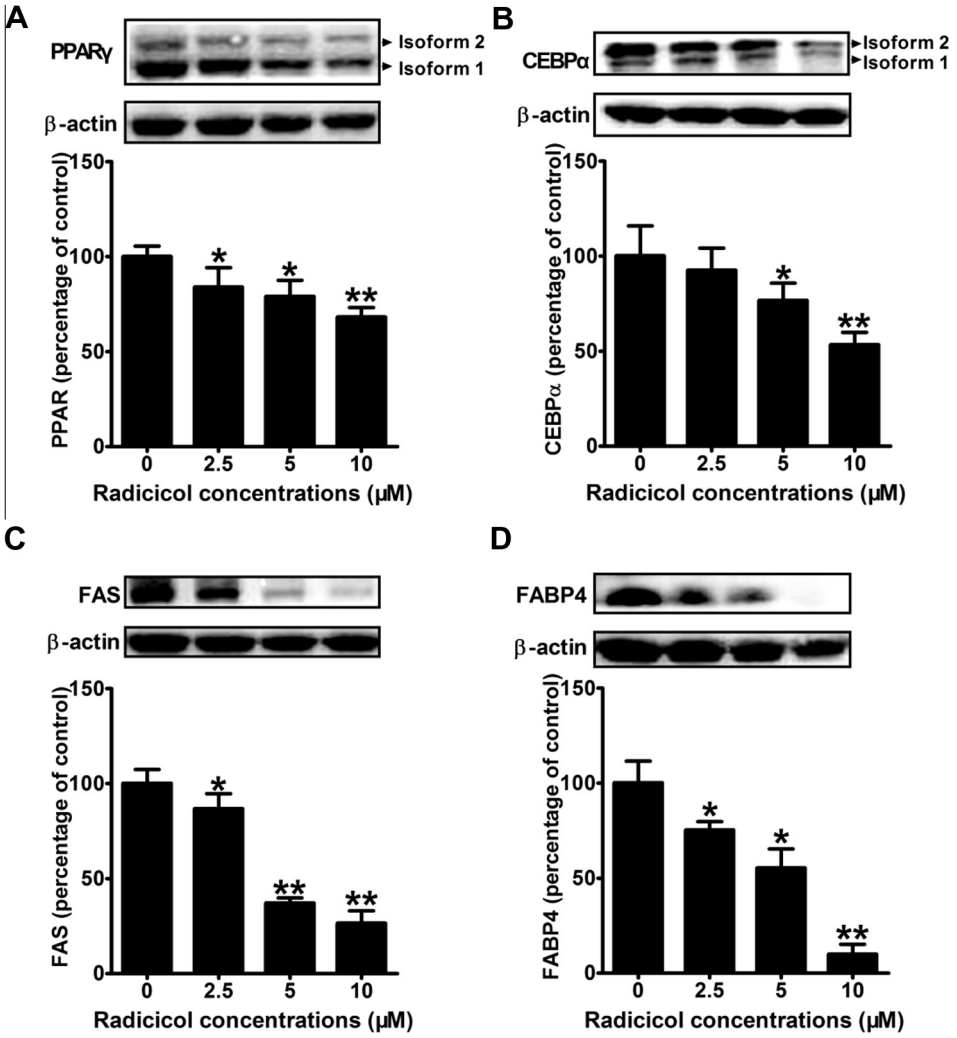

Fig. 2. Effect of radicicol on the protein expression of differentiation-related transcriptional factors and lipogenic proteins. 3T3-L1 preadipocytes were incubated in differentiation medium without or with the indicated concentrations of radicicol for 8 days. The expression of PPAR$_\gamma$, C/EBP$_\alpha$, FAS and FABP4 were detected by Western blotting. Data are expressed as means ± SD (n = 3). $^*P < 0.05$ and $^{**}P < 0.01$ vs. the control.

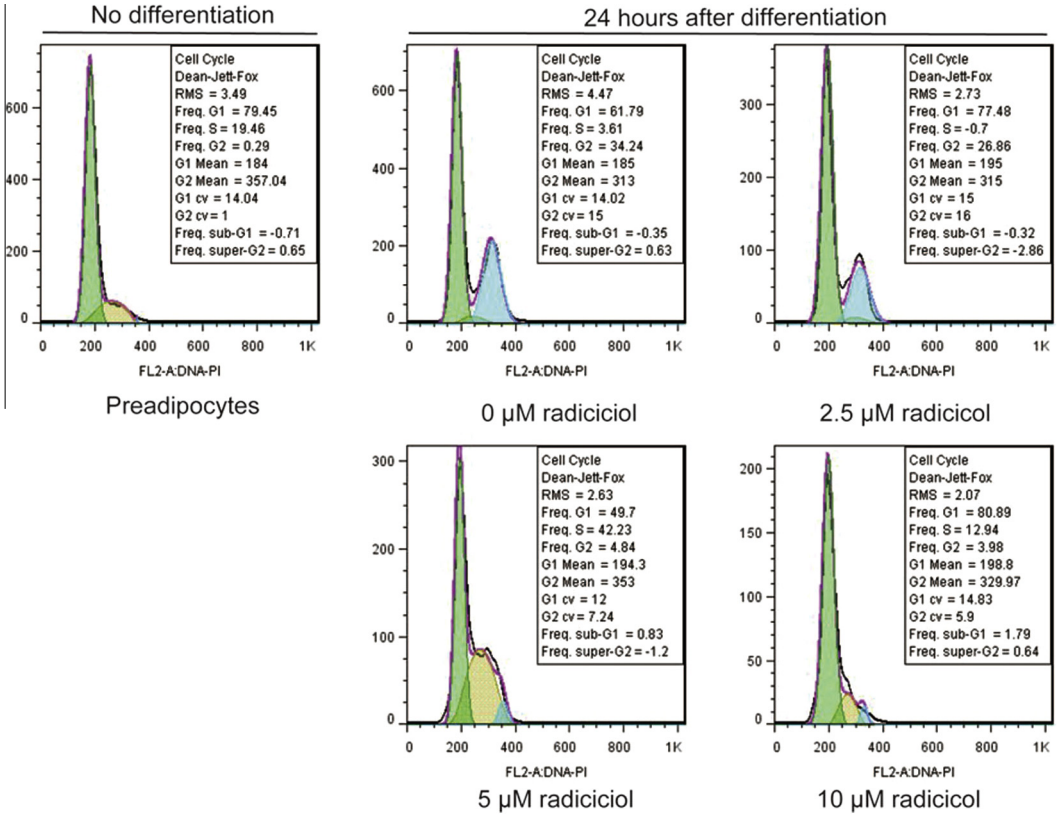

Fig. 3. Effect of radicicol on cell cycle progress during the mitotic clonal expansion. Postconfluent 3T3-L1 preadipocytes were cultured for 24 h after the induction of differentiation in the presence of several concentrations of radicicol. The changes of cell cycle were determined on Becton–Dickinson FACScantoII instrument and data were analyzed using the Flowjo software.The experiment was repeated 3 times and similar results were obtained.

3.4. Radicicol blocks cell cycle progression during 3T3-L1 preadipocyte differentiation

Cell cycle progression was initiated by differentiation medium but blocked by radicicol, especially at the phase of G1/S transition. Radicicol at 5 or 10 μM markedly blocked the progression at G2/M phase (Fig. 3). In cells treated with 10 μM of radicicol, the cell cycle was nearly the same as the normal growth-arrested preadipocytes. These results suggest that the effect of radicicol on cell cycle arrest might be another factor responsible for the observed inhibition of preadipocyte differentiation.

3.5. Radicicol inhibits preadipocyte differentiation through the PDK1/Akt pathway

To elucidate the signaling pathway through which radicicol affects preadipocyte differentiation, the PDK1/Akt and Wnt/β-catenin pathways were examined because these two signaling pathways have been reported to control cell cycle and preadipocyte differentiation. The activity of Akt and β-catenin was first determined and the results showed that the phosphorylation of Akt but not β-catenin was suppressed by radicicol (Fig. 4A and B), indicating that radicicol exerted its effect on the cell cycle and differentiation of 3T3-L1 preadipocytes via Akt rather than β-catenin pathway. Thus, the expression of Akt upstream regulator PDK1 was further determined. Consistently, the phosphorylation of PDK1 was suppressed by the radicicol at 5 μM and 10 μM, respectively (Fig. 4C).

4. Discussion

Hsp90 is a highly conserved stress protein and expressed in all eukaryotic cells. It controls the activity, turnover, and trafficking of a variety of proteins [12]. Of which many are involved in signal transduction. In the past, most studies focused on the effect of Hsp90 on oncogenic differentiation in cancer cell lines [10,13–15] while a few investigating the differentiation of other types of cells, such as myoblasts, C2C12 cells, nerve cells and mouse germ cells [9,16–19]. Radicicol inhibits the activity of Hsp90 by interacting with the structural ATP-binding motif [20]. To our knowledge, however, there is no report on the effect of radicicol on preadipocyte differentiation. We report here for the first time that Hsp90 inhibitor radicicol dose-dependently inhibited adipogenesis in 3T3-L1 adipocytes. Because adipogenic differentiation is critical in the development of obesity, the inhibition of adipocyte differentiation by radicicol may provide a means to develop therapeutics for the prevention and treatment of obesity.

Adipocyte differentiation is controlled by a complex transcriptional cascade involving peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein (C/EBP) [5]. Radicicol inhibited the differentiation of 3T3-L1 cells. Further experiments revealed that radicicol downregulated PPARγ and C/EBPα protein expressions. The expression of C/EBPα can be induced by PPARγ and is required for the expression of adipogenic genes [21]. It is not known whether radicicol inhibited the expression of C/EBPα directly or via downregulating PPARγ expression.

FABP4 facilitates the influx of fatty acids across the plasma membrane [22]. FAS catalyzes the synthesis of fatty acids from

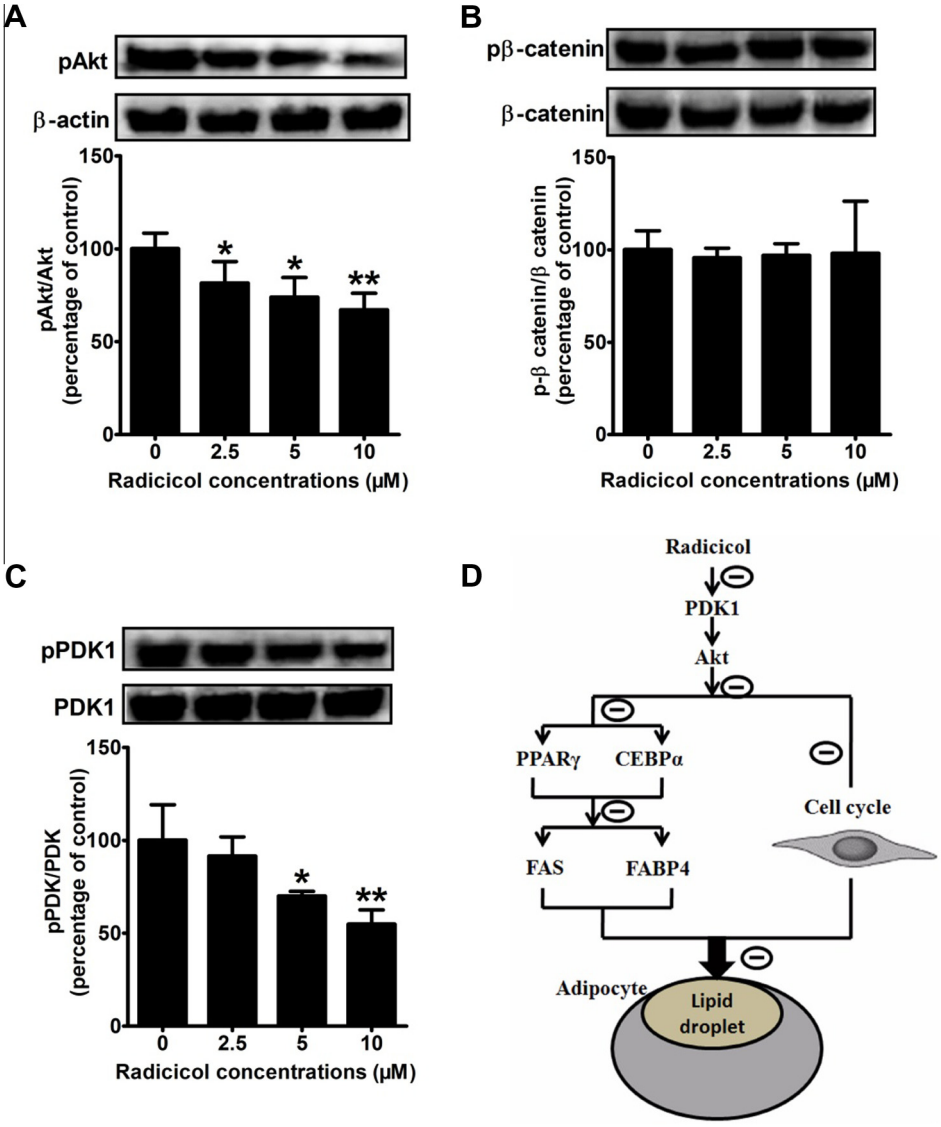

Fig. 4. Effect of radicicol on the activity of Akt, β-catenin and PDK1, and mechanisms of the anti-adipogenesis effect of radicicol. (A–C) 3T3-L1 preadipocytes were incubated in differentiation medium with or without the indicated concentrations of radicicol for 8 days. The expression of Akt, pAkt, β-cateninm, pβ-catenin, PDK1 and pPDK1 were detected by Western blotting. Data are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control. (D) – indicates an effect of inhibition.

acetyl-CoA and malonyl-CoA in the presence of NADPH [23]. The expressions of FAS and FABP4 are controlled coordinately by $PPAR_{\gamma}$ and C/EBP_{α} during adipocyte differentiation [24,25]. To verify the inhibitory effect of radicicol on lipogenesis, the expression of FAS and FABP4 was determined in 3T3-L1 cells at the later stages of differentiation. In line with the effect on $PPAR_{\gamma}$ and C/EBP_{α}, radicicol markedly decreased the expression of FAS and FABP4 protein expressions. These results further demonstrated that radicicol exerted the inhibitory effect on adipogenesis by suppressing the expression and activity of transcriptional factors and subsequently the expression of their downstream lipogenic genes during adipocyte differentiation.

It is well established that hyperplasia and hypertrophy of adipose tissue contribute to the development of obesity [26]. The latter is due to the processes of mitogenesis that occurs during the induction period of cell differentiation. During cell differentiation, growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle, undergo mitotic clonal expansion, and then express adipocyte-specific proteins [27]. Accordingly, identifying factors that affect the mitogenesis of preadipocytes to adipocytes becomes important in obesity research. In the current study, radicicol showed a significant effect of blocking mitotic clonal expansion at the G1/S phase, particularly in cells treated with 10 μM radicicol. This finding is in accordance with the observed inhibitory effect on adipocyte differentiation, indicating that cell cycle arrest might be another mechanism through which radicicol inhibited adipogenesis.

Insulin and extracellular signal-regulated kinase signaling pathways are involved in the regulation of adipocyte differentiation. Insulin produces proadipogenic effect through the receptor (IR)/Akt signaling pathway [28]. Contradictory to the effect of insulin signaling, Wnt/catenin pathway negatively regulates adipocyte differentiation. It represses adipogenesis when activated and by contrast initiates adipogenesis when turned off [29,30]. Although the phosphorylation of β-catenin was not changed, the activity of Akt was suppressed by radicicol. Consistently, the activity of PDK1, an upstream protein kinase of Akt in the insulin signaling pathway, was inhibited by radicicol through suppressing phosphorylation. It is suggestive that insulin signaling was involved in the inhibitory action of radicicol on adipocyte differentiation, being consistent with the inhibitory effect on $PPAR_{\gamma}$.

Although radicicol has shown potential to reduce fat deposition by reducing the expression of PPAR$_\gamma$ and its downstream adipogenic genes, its potential side effect should be evaluated prior to the clinical application, especially in obese patients who are also diabetic. It is well established that there is cross-talk between PPAR$_\gamma$ and insulin signaling. PPARγ is the master regulator of adipogenesis and the activation of PPARγ in adipocytes induces the expression of a number of genes involved in the insulin signaling cascade and thereby improving insulin sensitivity [31]. Thus, the activation of PPAR$_\gamma$ is an accepted medical approach to improving insulin resistance and PPAR$_\gamma$-agonists have been used to treat type-2 diabetes since 1997. As obesity and dyslipidemia constitute two overwhelming factors in the development of insulin resistance and type-2 diabetes, the inhibition of PPAR$_\gamma$ by radicicol may cause adverse effect when used in obese patients with diabetes. The extended activation of PPARγ may be deleterious as it promotes adipogenesis and results in further fat and weight gain. A moderate activation of PPARγ is believed to improve insulin sensitivity without affecting fat mass and other side effects [32]. Therefore, the benefit and safety of radicicol as a potential anti-obese therapy await further investigation although a number of studies demonstrated PPARγ antagonism as a potential remedy for obesity and diabetes [33].

In summary, the present study demonstrated that radicicol was a potent inhibitor of preadipocyte differentiation and adipogenesis. It exerted anti-adipogenic effect by suppressing the PDK1/Akt pathway, downregulating the expression/activity of transcription factors PPARγ and CEBPα and their downstream lipogenic targets FAS and FABP4, and blocking cell cycle during adipocyte differentiation (Fig 4D). Additionally, findings of the present may suggest that the inhibition of Hsp90 protein open a new avenue for developing therapeutics of obesity.

Acknowledgments

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