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Kirenol inhibits adipogenesis through activation of the Wnt/β-catenin signaling pathway in 3T3-L1 adipocytes

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ABSTRACT

Kirenol, a natural diterpenoid compound, has been reported to possess anti-oxidant, anti-inflammatory, anti-allergic, and anti-arthritic activities; however, its anti-adipogenic effect remains to be studied. The present study evaluated the effect of kirenol on anti-adipogenesis through the activation of the Wnt/β-catenin signaling pathway. Kirenol prevented intracellular lipid accumulation by down-regulating key adipogenesis transcription factors [peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding proteins α (C/EBPα), and sterol regulatory element binding protein-1c (SREBP-1c)] and lipid biosynthesis-related enzymes [fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC)], as well as adipocytokines (adiponectin and leptin). Kirenol effectively activated the Wnt/β-catenin signaling pathway, in which kirenol up-regulated the expression of low density lipoprotein receptor related protein 6 (LRP6), disheveled 2 (DVL2), β-catenin, and cyclin D1 (CCND1), while it inactivated glycogen synthase kinase 3β (GSK3β) by increasing its phosphorylation. Kirenol down-regulated the expression levels of PPARγ and C/EBPα, which were up-regulated by siRNA knockdown of β-catenin. Overall, kirenol is capable of inhibiting the differentiation and lipogenesis of 3T3-L1 adipocytes through the activation of the Wnt/β-catenin signaling pathway, suggesting its potential as natural anti-obesity agent.

1. Introduction

Obesity can lead to an energy imbalance in which energy intake exceeds energy expenditure, resulting in an over-accumulation of lipid in adipocytes and increased number of adipocytes [1,2]. Obesity is considered to be a metabolic syndrome and relate with increased risk of type 2 diabetes, hypertension, hyperlipidemia, and atherosclerosis [3].

Adipogenesis is a multi-faceted process involving preadipocyte proliferation, differentiation, and intracellular lipid accumulation, and involves changes in the expression of genes in the adipogenesis pathway [4]. This differentiation process is controlled by key adipogenic transcriptional factors, such as peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding proteins α (C/EBPα), and sterol regulatory element binding protein-1c (SREBP-1c). The transcriptional factors regulate the expression of several lipid metabolizing enzymes including fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) [2].

The wingless-type MMTV integration site (Wnt)/β-catenin signaling pathway has been reported to be a negative regulator of adipogenesis. In the canonical Wnt/β-catenin signaling pathway, Wnt ligands bind to frizzled receptors and low density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors. Following this interaction, disheveled (DVL) becomes phosphorylated and the glycogen synthase kinase 3 (GSK3)-AXIN-adenomatous polyposis coli (APC) complex is disrupted, which results in the stabilization and nuclear translocation of β-catenin. In the nucleus, β-catenin induces the activation of target genes including cyclin D1 (CCND1), which down-regulates major adipogenic transcription factors, such as PPARγ and C/EBPα [5–7].

Kirenol (Fig. 1), a main *ent*-pimarane diterpenoid isolated from *Herba Siegesbeckia*, has been reported to possess anti-oxidant, anti-inflammatory, immunoregulatory, wound healing, anti-arthritic, and anti-photoaging activities [8–10]; however its anti-adipogenic effect has not yet been investigated. The present study reports the anti-adipogenic effect of kirenol through the activation of the Wnt/β-catenin signaling pathway in 3T3-L1 adipocytes.

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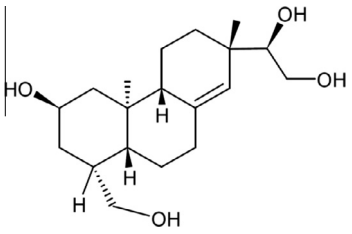

Fig. 1. Chemical structure of kirenol.

2. Materials and methods

2.1. Chemical reagents

Kirenol was purchased from Institute for Korea Traditional Medical Industry (Daegu, Korea). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Bovine calf serum (BCS) was purchased from Gibco (Grand Island, NY, USA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), Oil Red O, troglitazone, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St Louis, MO, USA). Antibodies against phosphorylated GSK3β, PPARγ, and α-tubulin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against C/EBPα and SREBP-1c were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An antibody against β-catenin was purchased from BD Biosiences (Franklin Lakes, NJ, USA). β-Catenin small interfering RNA (siRNA) and control siRNA were purchased from Santa Cruz Biotechnology, Inc., Lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture and differentiation

3T3-L1 preadipocytes cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown in DMEM with antibiotics (100 units/mL penicillin A and 100 μg/mL streptomycin) and 10% BCS in an atmosphere of 5% CO_2 at 37 °C. Two days after achieving post-confluence (day 0), 3T3-L1 cells were induced to differentiation in DMEM with 10% FBS and MDI (IBMX 0.5 mM, DEX 0.2 μM, and insulin 1.7 μM) for 2 days (from day 0 to day 2). The culture medium was replaced with DMEM containing 10% FBS and insulin (from day 2 to day 8), which was changed every 2 days. To examine the effect of kirenol on the adipogenesis, the cells were cultured with the differentiation medium in the presence of various concentrations (10–40 μM) of kirenol.

2.3. Cell viability

Cell viability was determined using the MTT assay. The 3T3-L1 preadipocytes were treated with various concentrations (10–60 μM) of kirenol for 24 h, and MTT solution (0.5 mg/mL) was added. After a 3 h incubation at 37 °C for MTT-formazan formation, the supernatant was removed, and dimethyl sulfoxide (DMSO) was added. Absorbance at 540 nm was determined spectrophotometrically by using a VERSAmax tunable microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). Cell viability showed no significant toxicity at various concentrations of kirenol: 10, 20, and 40 μM for 24 h (data not shown). Further studies were carried out at kirenol concentrations under 40 μM.

2.4. Oil Red O staining

Eight days following adipocyte differentiation induction, the cells were washed with phosphate-buffered saline (PBS) and fixed with 10% buffered formalin for 1 h. After washing with 60% isopropanol, the cells were stained with Oil Red O solution (0.5 g in isopropanol) for 1 h. Excess stain was removed by washing with distilled water, and the stained cells were dried. The stained lipid droplets in the cells were visualized by microscopy (Nikon TE-200U, Tokyo, Japan). Quantitation was carried out by extracting Oil Red O-stained lipid droplets with 100% isopropanol, and OD was measured at 540 nm with a VERSAmax tunable microplate reader (Molecular Devices Inc.). Lipid accumulation for each experimental group is expressed relative to that of MDI-differentiated cells (designated as 100%).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 3T3-L1 adipocytes using Trizol reagent (Invitrogen). The cDNA was converted in a 20 μL reaction containing 2 μg of total RNA, oligo (dT), and Reverse Transcription Premix (ELPIS-Biotech, Daejeon, Korea). PCR amplification of the cDNA products (3 μL) was performed with PCR premix (ELPIS-Biotech) and the following primer pairs (Bioneer, Daejeon, Korea): PPARγ forward 5′-CCT GTT GAC CCA GAG CAT GG-3′ and PPARγ reverse 5′-CGA GTG GTC TTC CAT CAC GC-3′ (147 bp); C/EBPα forward 5′-CTG CCC CTC AGT CCC TGT C-3′ and C/EBPα reverse 5′-GTT CCT TCA GCA ACA GCG G-3′ (352 bp); SREBP-1c forward 5′-GCG CTA CCG GTC TTC TAT CA-3′ and SREBP-1c reverse 5′-TGC TGC CAA AAG ACA AGG G-3′ (335 bp); FAS forward 5′-GGT TCG GAA TGC TAT CCA GG-3′ and FAS reverse 5′-CTG CGG AAA CTT CAG GAA AT-3′ (301 bp); ACC forward 5′-TGA CCG TGG GCA CAA AGT T-3′ and ACC reverse 5′-AGG AGG AAC CGC ATT TAT CGA-3′ (351 bp); LRP6 forward 5′-ACC TCA ATG CGA TTT GTT CC-3′ and LRP6 reverse 5′-GGT GTC AAA GAA GCC TCT GC-3′ (215 bp); DVL2 forward 5′-GCT TCC ACA TGG CCA TGG GC-3′ and DVL2 reverse 5′-TGG CAC TGC TGG TGA GAG TCA CAG-3′ (195 bp); β-catenin forward 5′-GCC AAG TGG GTG GTA TAG AG-3′ and β-catenin reverse 5′-CTG GGT ATC CTG ATG TGC-3′ (329 bp); CCND 1 forward primer 5′-AAA ATC GTG GCC ACC TGG AT-3′ and CCND1 reverse primer 5′-CAT CCG CCT CTG GCA TTT TG-3′(346 bp); β-actin forward 5′-CCA CAC CTT CTA CAA TGA GC-3′ and β-actin reverse 5′-TGAGGTAGTCAGTCAGGTC-3′ (308 bp); All primers were denatured at 94 °C for 5 min prior to performing PCR amplification. Amplification consisted of 28–30 cycles as follows: denaturing at 94 °C for 30 s, annealing at 56 °C for 1 min, and extending at 72 °C for 1 min, followed by a final 5 min extending phase at 72 °C. PCR was performed using a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR products were electrophoresed by 1.5% agarose gel electrophoresis and visualized using a G:BOX EF imaging system (Syngene, Cambridge, UK) and the Gene Snap program. β-Actin was used as an internal control.

2.6. Western blot analysis

3T3-L1 adipocytes were lysed using RIPA lysis buffer (Elpis-Biotech) with a protease inhibitor cocktail (Sigma). The lysate protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (35–50 μg) in each sample were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Whatman GmBH, Dassel, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature and then incubated overnight with primary antibodies against PPARγ, C/EBPα, SREBP-1c, β-catenin, phosphorylated GSK3β, and α-tubulin (1:1000 dilution) at 4 °C. After washing three times in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:5000 dilution;

Bethyl Laboratories, Inc., Montgomery, TX). Proteins were detected with the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK) and visualized with the G:BOX EF imaging system (Syngene) and the Gene Snap program.

2.7. β-Catenin knockdown by siRNA transfection

β-Catenin knockdown by siRNA transfection was performed as previously described [5], with a slight modification. Two days after achieving confluence, 3T3-L1 cells were cultured in serum-free medium for 1 h and transfected with 60 nM of β-catenin siRNA or control siRNA using the Lipofectamine RNAiMAX transfection reagent. After 9 h, the transfected cells were differentiated according to the culturing method previously described. After 8 days, total RNA extract was prepared for RT-PCR.

2.8. Statistical analysis

All experiments were repeated at least three times, and each experiment was performed in triplicate. Results are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Group differences were assessed by unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by a Scheffe's test. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Kirenol inhibits lipid accumulation and the expression of adipogenic transcription factors

Kirenol treatment decreased the amount of intracellular lipid droplets in a dose-dependent fashion when compared to the MDI control, as revealed by microscopic examination following Oil Red O staining in differentiated 3T3-L1 adipocytes (Fig. 2A). The quantification of Oil Red O showed that lipid accumulation resulting from adipocyte differentiation was significantly reduced by kirenol treatment (Fig. 2A). Adipogenic differentiation-related lipid accumulation is accompanied by the induction of key adipogenic transcription factors, including PPARγ, C/EBPα, and SREBP-1c [2]. Kirenol treatment significantly suppressed the protein and mRNA expression of PPARγ, C/EBPα, and SREBP-1c as compared to the MDI control (Fig. 2B and C). Taken together, kirenol treatment inhibited lipid accumulation and differentiation of 3T3-L1 preadipocytes into adipocytes through down-regulation of adipogenic transcription factors.

3.2. Kirenol modulates the expression of lipogenic enzymes and adipocytokine

Kirenol treatment decreased the mRNA expression of lipogenic enzymes, including FAS and ACC, in a dose-dependent manner (Fig. 3A). The levels of FAS and ACC mRNA expression were significantly inhibited at 40 μM of kirenol, by 59.6% and 68.2%, respectively, as compared to the MDI control. The levels of adiponectin and leptin mRNA expression in 3T3-L1 differentiated adipocytes were markedly decreased following treatment with 40 μM kirenol by 74.9% and 50.4%, respectively, as compared to the MDI control (Fig 3B). Kirenol effectively inhibited the expression of lipogenic enzymes and adipocytokine genes during 3T3-L1 adipocyte differentiation.

3.3. Kirenol stimulates the expression of the Wnt/β-catenin signaling pathway components

The mRNA expression of genes in the Wnt/β-catenin signaling pathway, including LRP6, DVL2, β-catenin, and CCND1, were significantly up-regulated by kirenol treatment (Fig. 4A). Furthermore, kirenol treatment efficiently activated β-catenin but inactivated GSK3β by increasing its phosphorylation (Fig. 4B). This finding demonstrates that kirenol up-regulated some components of the

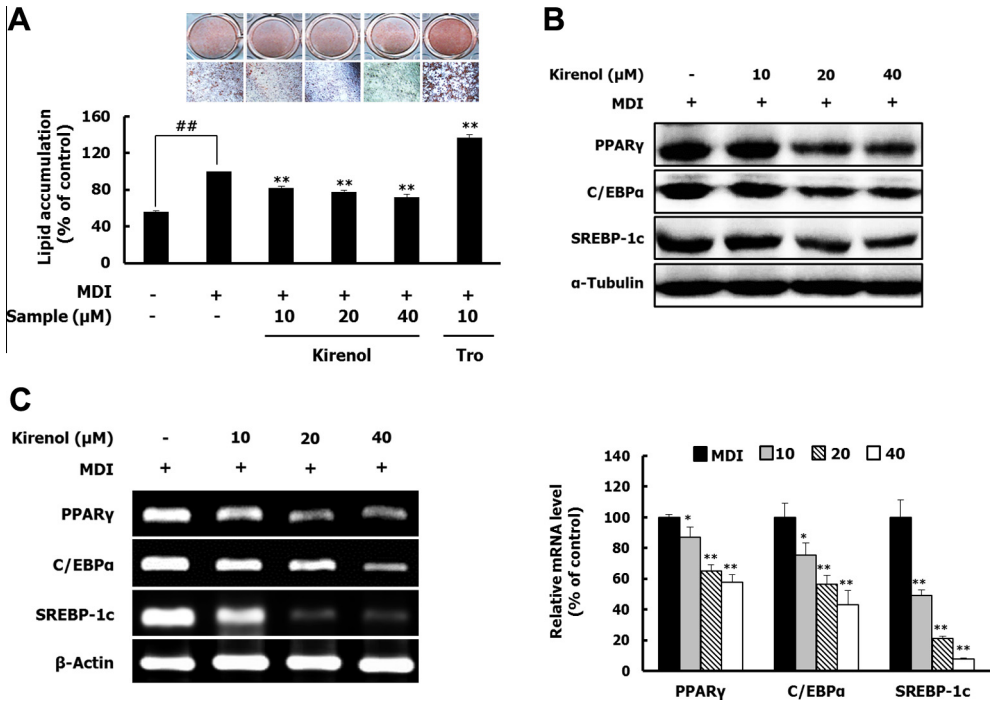

Fig. 2. Effects of kirenol treatment on lipid accumulation and the expression of differentiation-related transcriptional factors in 3T3-L1 adipocytes. (A) Inhibitory effect of kirenol on intracellular lipid droplet formation. (B) Effects of kirenol treatment on the protein and mRNA expression (C) of PPARγ, C/EBPα, and SREBP-1c. α-Tubulin and β-actin were used as internal controls. The results are expressed as mean ± SD (% control) of three independent experiments. $^{\#\#}P < 0.01$ (untreated control vs. MDI control); $^{*}P < 0.05$ and $^{**}P < 0.01$ (MDI control vs. sample-treated cells).

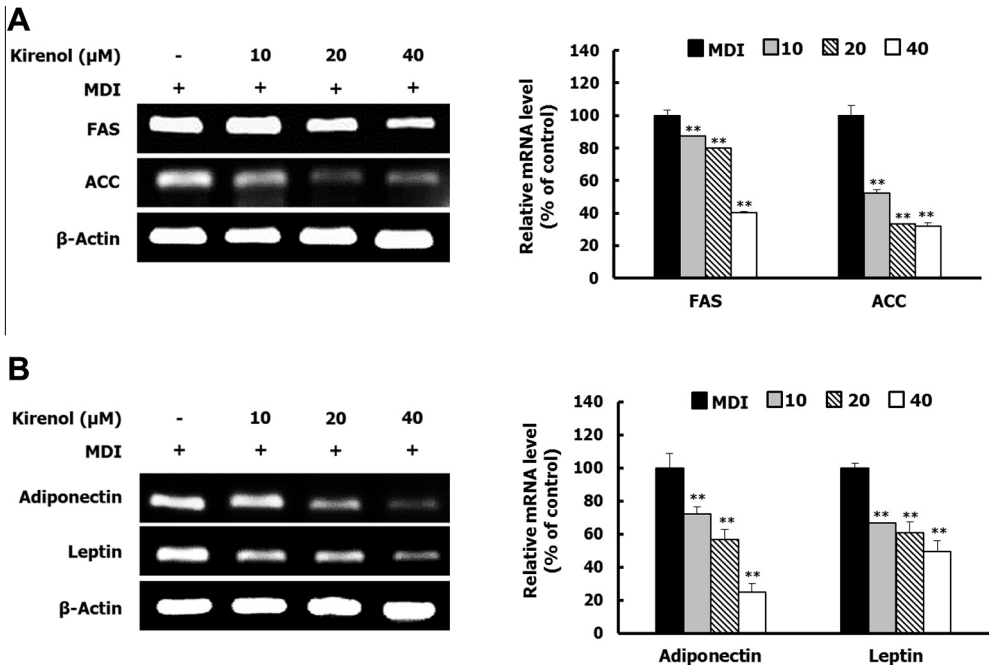

Fig. 3. Effects of kirenol treatment on the expression of lipogenic enzymes and adipocytokine in 3T3-L1 adipocytes. (A) Effect of kirenol treatment on the mRNA expression of lipogenic enzymes, such as FAS and ACC. (B) Effect of kirenol treatment on the mRNA expression of adipocytokines, such as adiponectin and leptin. β-Actin was used as an internal control. The results are expressed as mean ± SD (% control) of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (MDI control vs. sample-treated cells).

Wnt/β-catenin signaling pathway, leading to the reduced expression of adipogenesis-mediated markers.

3.4. siRNA-mediated knockdown of β-catenin attenuates the anti-adipogenic effect of kirenol

To evaluate the essential aspects of β-catenin function in relation to the anti-adipogenic effects of kirenol, β-catenin siRNA was transfected into 3T3-L1 adipocytes with or without kirenol treatment. The mRNA expression of β-catenin and CCND1 were effectively decreased by the siRNA, as compared to cells transfected with control siRNA, in the presence or absence of kirenol. In addition, kirenol treatment down-regulated the mRNA expression of the adipogenic transcription factors PPARγ and C/EBPα, which were effectively recovered by treatment with β-catenin siRNA (Fig. 4C). The results confirm that β-catenin is involved in the anti-adipogenic effects of kirenol.

4. Discussion

Kirenol is the major bioactive component of species of *Herba Siegesbeckia,* such as *Siegesbeckia. orientalis*, *Siegesbeckia glabrescens*, and *Siegesbeckia pubescens* [8]. Kirenol has been reported to confer anti-rheumatism and anti-inflammation activities by up-regulating nuclear Annexin-1, inhibiting the NF-κB activity in the synovium, and reducing cytokine expression in synovial fluid to attenuate synovial inflammation of collagen-induced arthritis in a rat model [10,12]. In addition, kirenol regulated immunosuppressive effects in autoimmune arthritis by modifying the T-cell balance [10,11]. However, its possible anti-obesity effect remains uncharacterized.

PPARγ, C/EBPα, and SREBP-1c are the master adipogenic transcription factors, which are mainly found in adipose tissue [3]. PPARγ is a ligand-activated transcription factor and promotes the expression of several adipogenic and lipogenic genes in adipocytes [13,14]. C/EBPα plays important roles in the early phase of preadipocyte differentiation, inducing and maintaining PPARγ expression [4,15]. SREBP-1c is involved in lipid metabolism and regulates genes required for fatty acid and lipid production [15]. This study demonstrated that kirenol effectively inhibited the induction of these transcriptional factors, resulting in reduced adipocyte differentiation (Fig. 2). The results suggest that kirenol inhibits preadipocyte differentiation by suppressing the expression of adipogenic transcriptional factors involved at different differentiation stages.

The adipogenic transcription factors coordinately regulate the expression of adipocyte-specific genes, including FAS and ACC. It determines the later stages of adipogenesis and the key lipogenic enzymes controlling biosynthesis of fatty acids and triacylglycerols [15]. Kirenol significantly decreased the expression of FAS and ACC (Fig. 3A), suggesting that kirenol inhibited lipid accumulation through controlling these biosynthesis-related genes in differentiated 3T3-L1 adipocytes. Adiponectin and leptin are the most abundant circulating adipocytokines [16]. Adipocytokines decrease the expression of PPARγ and C/EBPα in mature adipocytes [17]. Kirenol reduced the expression of adiponectin and leptin in differentiated 3T3-L1 adipocytes (Fig. 3B). Thus, the inhibition of lipid accumulation by kirenol treatment may attenuate the expression of adiponectin and leptin in differentiated 3T3-L1 adipocytes.

The Wnt/β-catenin signaling pathway suppresses adipocyte differentiation by blocking induction of PPARγ and C/EBPα [18]. The components of the Wnt/β-catenin signaling pathway, including β-catenin and CCND1, were up-regulated by kirenol in differentiated 3T3-L1 adipocytes (Fig. 4A and C). It is known that β-catenin is a negative regulator of adipogenesis and directly decreases the activity of PPARγ through functional interaction between the TCF/LEF-binding domain of β-catenin and the catenin-binding domain of PPARγ [19,20]. In addition, β-catenin activates the transcription of target genes, such as CCND1, which suppresses the activity of PPARγ and C/EBPα. Accordingly, the anti-adipogenic function of kirenol can be induced through the regulation of β-catenin and CCND1 expression.

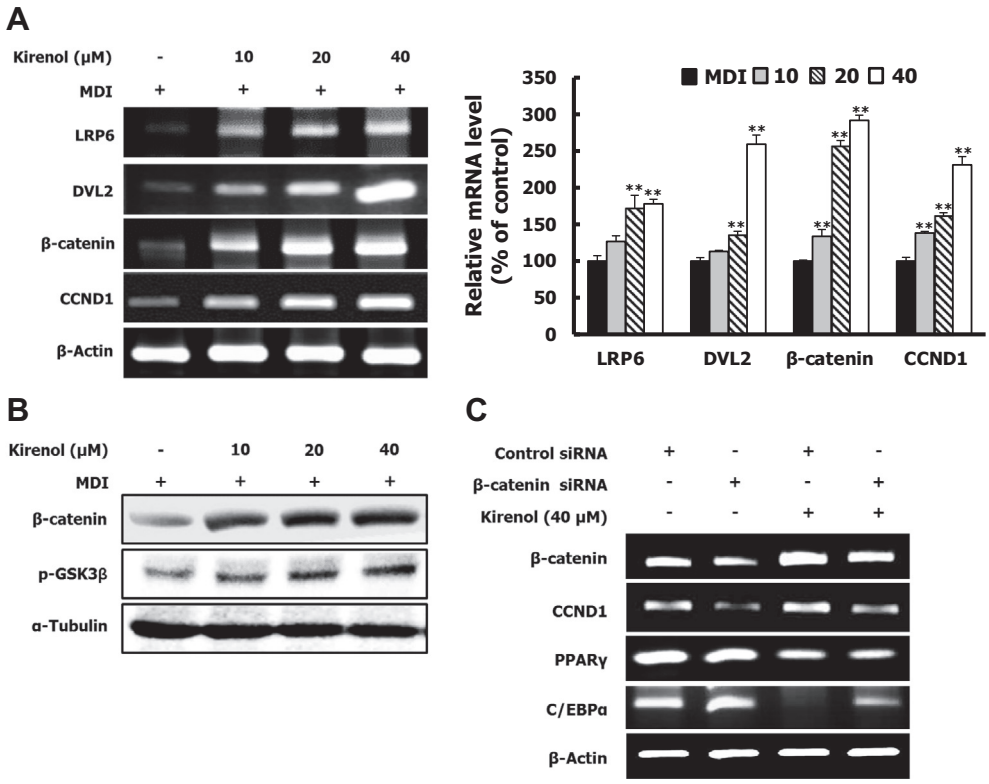

Fig. 4. Effect of kirenol treatment on the Wnt/β-catenin pathway in 3T3-L1 adipocytes. (A) Effects of kirenol treatment on the mRNA expression of LRP6, DVL2, β-catenin, and CCND1. (B) Effects of kirenol treatment on the protein expression of β-catenin and p-GSK3β. (C) Effects of β-catenin siRNA on the mRNA expression of β-catenin, CCND1, PPARγ, and C/EBPα in the presence and absence of kirenol. β-Actin and α-tubulin were used as internal controls. The results are expressed as mean ± SD (% control) of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (MDI control vs. sample-treated cells).

The Wnt/β-catenin signaling pathway is activated when the secreted glycoprotein Wnt binds to a cell surface receptor, such as the frizzled receptor and LRP5/6 co-receptors [21]. Kirenol significantly increased the mRNA expression of LRP6 in differentiated 3T3-L1 adipocytes (Fig. 4A). LRP6 inhibits adipogenesis and its deficiency spontaneously leads to adipogenic differentiation [19]. Kirenol significantly activated DVL2, while it inactivated GSK3β by increasing its phosphorylation (Fig. 4A and B). DVL2 is the only activated member of the DVL family and regulates the disruption of Axin–GSK3β–APC complex. GSK3β is known to be a negative regulator of the WNT/β-catenin pathway, and disruption of GSK3β stabilizes β-catenin and reduces elevating adipocyte differentiation [5,18]. These results confirm that modulation of the Wnt/β-catenin signaling pathway by kirenol treatment up-regulates β-catenin through inactivation of GSK3β and the expression of its target gene CCND1, which can inhibit PPARγ and C/EBPα. β-Catenin siRNA transfection showed that β-catenin knockdown resulted in recovered expression of PPARγ and C/EBPα, indicating that kirenol down-regulated PPARγ and C/EBPα through β-catenin up-regulation (Fig. 4C). These results support the conclusion that the anti-adipogenic effect of kirenol modulates components of the Wnt/β-catenin signaling pathway.

Overall, kirenol inhibited adipocyte differentiation and lipid accumulation by regulating adipogenic transcriptional factors and their downstream lipogenic enzymes through activating the Wnt/β-catenin signaling pathway. Thus, it is anticipated that kirenol can be employed as a natural anti-obesity agent for the prevention and treatment of obesity. Further studies are necessary to elucidate its anti-obesity activity *in vivo* and characterize the underlying mechanisms in an obese mouse model.

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