Adiponectin receptor agonist AdipoRon suppresses adipogenesis in C3H10T1/2 cells through the adenosine monophosphate-activated protein kinase signaling pathway

SHU-JUAN WANG, WEN-YI LU and KAI-YAN LIU

Beijing Key Laboratory of Hematopoietic Stem Cell Transplantation, Peking University People's Hospital and Institute of Hematology, Beijing 100044, P.R. China

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Abstract. The aim of the present study was to investigate the effects of AdipoRon, an adiponectin receptor agonist, on adipogenesis in C3H10T1/2 cells and to explore the underlying mechanisms. C3H10T1/2 cells were treated with increasing doses of AdipoRon for 8 days, and Oil Red O staining was used to assess lipid accumulation. The protein and mRNA expression levels of adipogenic transcription factors and adipocyte-specific genes were examined by western blotting and reverse transcription quantitative polymerase chain reaction, respectively. AdipoRon treatment inhibited lipid accumulation in C3H10T1/2 cells in a dose-dependent manner and significantly suppressed the expression of adipogenic transcription factors, including peroxisome proliferator-activated receptor γ, CAAT/enhancer binding protein (C/EBP)-β and C/EBPα. In addition, cells treated with AdipoRon exhibited a significant decrease in the expression of adipocyte-specific genes, including fatty acid binding protein 4, fatty acid synthase, leptin, adiponectin, and stearoyl-CoA desaturase-1. Notably, AdipoRon significantly increased the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). The results indicated that AdipoRon exerted an inhibitory effect on adipogenesis in C3H10T1/2 cells by downregulating the expression of adipogenic transcription factors and adipocyte-specific genes and by promoting the phosphorylation of AMPK and ACC, which suggested that AdipoRon may be a potential drug to prevent and treat diseases caused by abnormal adipogenesis, such as obesity.

Correspondence to: Professor Kai-Yan Liu, Beijing Key Laboratory of Hematopoietic Stem Cell Transplantation, Peking University People's Hospital and Institute of Hematology, 11 Xi-Zhi-Men South Street, Beijing 100044, P.R. China
E-mail: liukaiyan@medmail.com.cn

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Introduction

Obesity is a common public health problem and is a significant risk factor for a number of metabolic disorders, including type 2 diabetes, hypertension and cardiovascular disease (1). The development of obesity is characterized by an increase in the number and size of mature adipocytes that are produced by differentiation and mitogenesis (2). Adipogenesis is controlled by many transcription factors, including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) (3-5). At the initial stage of adipogenesis, C/EBPβ is activated, followed by the sequential activation of C/EBPα and PPARγ, two major late-adipogenic transcription factors. Activation of C/EBPα and PPARγ subsequently leads to the expression of a number of genes involved in adipocyte differentiation, including genes encoding lipid metabolizing enzymes [such as fatty acid binding protein (FABP)-4 and fatty acid synthase (FAS)] and adipokines (such as adiponectin and leptin) (6).

Adenosine monophosphate-activated protein kinase (AMPK) is an important cellular energy sensor, and its activation is closely related to the balance between lipid accumulation and carbohydrate metabolism (7). AMPK phosphorylation inhibits metabolic enzymes involved in fatty acid synthesis, leading to suppressed adipogenesis (8,9).

Adiponectin is an adipokine that is mainly produced by adipocytes. Unlike other adipokines, plasma adiponectin levels are reduced in obese subjects (10). Adiponectin binds to the adiponectin receptors, AdipoR1 and AdipoR2, and reduces obesity-related insulin resistance (11). AdipoRon, a newly discovered, orally active small molecule, is an adiponectin receptor agonist that binds to and activates both AdipoR1 and AdipoR2, and has been previously reported to improve obesity-related insulin resistance and type 2 diabetes by activating AMPK and PPARα pathways (12). However, the effects of adiponectin on obesity itself and on adipogenesis are controversial. Adiponectin may promote adipogenesis induced by weight gain (11,13). By contrast, elevated adiponectin levels were recently reported to reduce lipid content and lipid metabolism in 3T3-L1 cells (14). However, the effects of AdipoRon on adipogenesis have not yet been investigated. The present study investigated the effects of AdipoRon on

adipogenesis and explored the underlying mechanism in C3H10T1/2 cells.

Materials and methods

Reagents. AdipoRon, 5-aminoimidazole-4-carboxamide-1-β -D-ribofuranoside (AICAR), dexamethasone (Dex), 3-isobutyl-1-methylxanthine (IBMX), indomethacin, insulin and Oil Red O were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Minimum essential Eagle's medium with Earle's Balanced Salts (MEM-EBSS) was purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Monoclonal antibodies, including rabbit anti-PPARγ (cat no. 2443), rabbit anti-FABP4 (cat no. 3544), rabbit anti-C/EBPβ (cat no. 3087), rabbit anti-C/EBPα (cat no. 2295), rabbit anti-AMPKα (cat no. 5831), rabbit anti-phosphorylated-acetyl-CoA carboxylase (p-ACC; cat no. 11818), rabbit anti-adiponectin (cat no. 2789) and rabbit anti-GAPDH (cat no. 5174) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal anti-p-AMPKα1/2 antibody (cat no. sc-33524) and goat polyclonal anti-AdipoR1 antibody (cat no. sc-46748) and anti-AdipoR2 antibody (cat no. sc-46751) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and adipocyte differentiation. The C3H10T1/2 mouse embryonic mesenchymal stem cell line was purchased from the Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in MEM-EBSS supplemented with 10% FBS at 37˚C in a 5% CO_2 atmosphere until adipocyte differentiation. Two days after reaching confluence (day 0), the C3H10T1/2 cells were cultured in differentiation medium (DM; MEM/EBSS containing 10% FBS, 1 μM Dex, 0.5 mM IBMX, 50 mM indomethacin and 1 μg/ml insulin) for 2 days to induce differentiation. Following incubation, MEM/EBSS supplemented with 10% FBS and 1 μg/ml insulin was added for 2 days as a differentiation medium and was changed every 2 days.

Cell viability assay. Cell viability was detected by using a CCK-8 kit, according to the manufacturer's instructions. Briefly, C3H10T1/2 cells were seeded in 96-well plates at a density of $5x10^4$ cells/ml at 37˚C in a humidified 5% CO_2 atmosphere and treated with increasing doses of AdipoRon (1, 5, 10, 20 and 40 μM) for 24, 48 or 72 h. Subsequently, 10 μl of kit reagent was added to each well and incubated at 37˚C for 2 h. The plates were scanned with a microplate reader (Benchmark; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Oil Red O (ORO) staining. Cells were cultured in 24-well plates with a seeding density of $5x10^4$ cells/ml and differentiated as described above. On day 8, the cells were stained with ORO as previously described (2). Briefly, the cells were fixed with 10% formalin (Solarbio, Beijing, China) at 4˚C for 30 min and stained with ORO at room temperature for 30 min following washes with phosphate-buffered saline. Oil droplets in the cells were observed under an inverted microscope (Olympus-CKX41; Olympus Corporation, Tokyo, Japan). The ORO-stained plates were then washed and treated with 100% isopropanol (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and lipid accumulation was detected by measuring the absorbance at 490 nm using a Benchmark microplate reader (Bio-Rad Laboratories, Inc.).

Western blot analysis. Cells were cultured in 6-well plates with a seeding density of $5x10^4$ cells/ml and differentiated as described above. Cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with a protease inhibitor cocktail and phosphatase inhibitors (Roche Molecular Diagnostics, Pleasanton, CA, USA) for 20 min and centrifuged at 12,000 x g for 20 min at 4˚C. Protein concentration was determined using the Bicinchoninic Acid Protein Assay reagent kit (Beijing Solarbio Science & Technology Co., Ltd.). A total of 30 μg of protein lysates from each sample were separated on 10 or 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBST) (both from Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature, followed by hybridization with primary antibodies against PPARγ (1:1,000), C/EBPβ (1:1,000), C/EBPα (1:1,000), FABP4 (1:2,000), adiponectin (1:500), AMPKα (1:1,000), p-ACC (1:500), GAPDH (1:1,000), p-AMPKα1/2 (1:200), AdipoR1 (1:200) and AdipoR2 (1:200) overnight at 4˚C. Following three washes with TBST, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat no. sc2004; 1:10,000; Santa Cruz Biotechnology, Inc.) or a horseradish peroxidase-conjugated rabbit anti-goat IgG secondary antibody (cat no. ZB-2306; 1:10,000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. Following three washes with TBST, the bands were visualized with an Enhanced Chemiluminescence Substrate (Thermo Fisher Scientific, Inc.). Densitometry of the western blot bands was performed using ImageJ software version 1.31 (National Institutes of Health, Bethesda, ML, USA).

RNA preparation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were cultured in 6-well plates with a seeding density of $5x10^4$ cells/ml and differentiated as described above. RNA was extracted from C3H10T1/2 cells on day 8 using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize the cDNA, according to the manufacturer's protocol. mRNA expression levels were determined using an ABI 7500 Fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers were obtained from Genotech (Shanghai, China) and the sequences are provided in Table I. GAPDH was used as the internal control. qPCR was performed using the following previous described cycling conditions (15): Initial denaturation at 95˚C for 10 min, followed by 40 cycles

at 95˚C for 15 sec and 60˚C for 1 min. Each experiment was performed in triplicate. Gene transcript levels were calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Statistical analysis. All experiments were performed at least three times. The results are expressed as the mean ± standard deviations (SD). The data were analyzed using Student's t-test or one-way analysis of variance followed by least significance difference test. All analyses were performed with GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

AdipoRon suppresses lipid accumulation in C3H10T1/2 cells without cytotoxicity. Following treatment for 24, 48 or 72 h, AdipoRon exhibited no significant effects on C3H10T1/2 cell viability at concentrations ≤40 μM (Fig. 1A). Therefore, 5-20 μM AdipoRon was used in all subsequent experiments. The antiadipogenic effects of AdipoRon treatment were not due to cytotoxicity. C3H10T1/2 cells were induced into adipocytes for 8 days in the presence or absence of various doses of AdipoRon (0-20 μM) to investigate the effects of AdipoRon on adipocyte differentiation. Based on the results of ORO staining, lipid accumulation in C3H10T1/2 cells was dose-dependently decreased by AdipoRon (Fig. 1B and C). Protein expression of the adiponectin receptors, AdipoR1 and AdipoR2, were detected in C3H10T1/2 cells (Fig. 1D).

Effects of AdipoRon on adipogenic transcription factors expression in C3H10T1/2 cells. Adipogenesis is controlled by numerous transcription factors; the present study examined the effects of various concentrations of AdipoRon (0-20 μM) on the mRNA expression levels C/EBPβ, C/EBPα and PPARγ, to investigate whether AdipoRon inhibits adipogenesis by downregulating the expression of these adipogenic transcription factors. AdipoRon treatment significantly reduced the expression levels of the C/EBPβ, C/EBPα and PPARγ mRNAs in a dose-dependent manner (Fig. 2A-C). In addition, treatment with 20 μM AdipoRon was used to examine the expression levels of the C/EBPβ, C/EBPα and PPARγ proteins. The expression levels of the C/EBPβ, C/EBPα and PPARγ proteins were decreased by 20 μM AdipoRon compared with those of the controls (Fig. 2D).

Effects of AdipoRon on the expression levels of adipogenesis-related genes in C3H10T1/2 cells. As AdipoRon treatment significantly downregulated the expression of adipogenic transcription factors, the effects of various concentrations of AdipoRon on the expression levels of adipogenesis-related genes were also examined. AdipoRon exposure dose-dependently suppressed the mRNA expression of FAS, leptin, stearoyl-CoA desaturase (SCD)-1, adiponectin and FABP4 (Fig. 3A-E). Consistent with the changes in mRNA expression levels, 20 μM AdipoRon decreased the expression levels of the adiponectin and FABP4 proteins (Fig. 3F).

Effects of AdipoRon on AMPK phosphorylation in C3H10T1/2 cells. AdipoRon treatment significantly increased the levels of p-AMPK and p-ACC, which is the downstream target of AMPK (Fig. 4A-C). The AMPK activator AICAR was used as a positive control to further explore the association between the activation of AMPK signaling and the antiadipogenic effects mediated by AdipoRon. As shown in Fig. 4D and E, both AICAR (1 mM) and AdipoRon (10 and 20 μM) were able to activate the AMPK signaling pathway. Both AICAR and AdipoRon treatments were also able to significantly reduce lipid accumulation and the expression of adipogenesis-related genes, PPARγ and adiponectin, in C3H10T1/2 cells (Fig. 4F-H).

Table I. Primers used for reverse transcription quantitative polymerase chain reaction.

Gene	Primer sequence (5'→3')
C/EBPα	F: CGGGAACGCAACAACATCGC
C/EBPα	R: CGGTCATTGTCACTGGTCAACTC
C/EBPβ	F: GTTTCGGGAGTTGATGCAATC
C/EBPβ	R: AACAACCCCGCAGGAACAT
PPARγ	F: CATTCGCATTCCTTTGAC
PPARγ	R: CGCACTTTGGTATTCTTGGAG
FAS	F: CAAGTGTCCACCAACAAGCG
FAS	R: GGAGCGCAGGATAGACTCAC
FABP4	F: TGTGCGAAACTGAATTTCCTGC
FABP4	R: GAGATCGGTCCTGAGCCAGC
Adiponectin	F: GCCGCTTATGTGTATCGCTCAG
Adiponectin	R: GCCAGTGCTGCCGTCATAATG
Leptin	F: TCGCCTTTCTCCTGATGACG
Leptin	R: GCAATCACACGGATGGCTTC
SCD-1	F: CGCTGGCACATCAACTTCAC
SCD-1	R: AGGAACTCAGAAGCCCAAAGC
GAPDH	F: TCAATGACAACTTTGTCAAGCTCA
GAPDH	R: GTGGGTGGTCCAGGGTTTCTTACT

C/EBP, CAAT/enhancer binding protein; FABP, fatty acid binding protein; FAS, fatty acid synthase; PPAR, peroxisome proliferator-activated receptor; SCD-1, stearoyl-CoA desaturase 1.

Discussion

Obesity and its associated complications have become common problems worldwide. AdipoRon, an adiponectin receptor agonist, is a promising new orally administered drug for the treatment of obesity-related diabetes (12). However, its effects on obesity itself and adipogenesis have not yet been investigated. Results from the present study demonstrated that AdipoRon treatment inhibited the differentiation of C3H10T1/2 cells into adipocytes by downregulating the expression of adipogenic transcription factors and regulating the AMPK signaling pathway.

Abnormal adipogenesis and lipid accumulation have previously been associated with the development of obesity (2). A potential treatment strategy for obesity is to inhibit adipogenesis. The presents study results indicated that AdipoRon dose-dependently inhibited lipid accumulation in

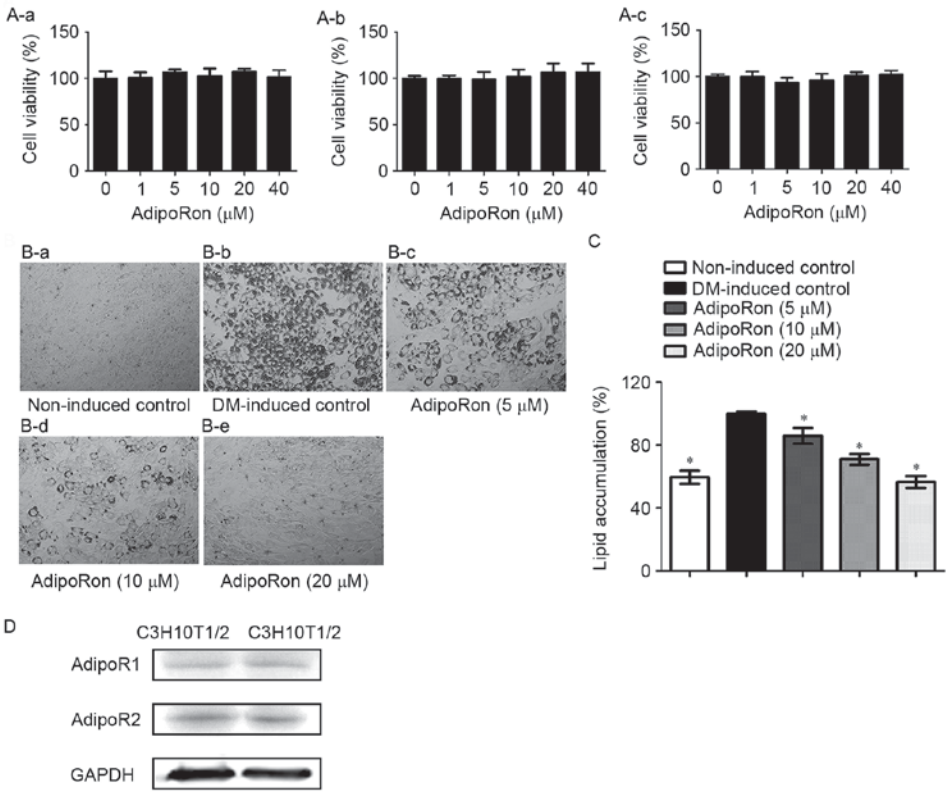

Figure 1. Effects of AdipoRon on viability and adipocyte differentiation of C3H10T1/2 cells. (A) The effects of AdipoRon on the viability of C3H10T1/2 cells were examined at (A-a) 24, (A-b) 48 and (A-c) 72 h. (B) Images of the ORO staining at day 8 of adipocyte differentiation; (B-a) non-induced; (B-b) DM-induced adipocytes; DM-induced and treated with (B-c) 5 μM, (B-d) 10 μM or (B-e) 20 μM AdipoRon; magnification, x200. (C) Lipid accumulation was determined by measuring the absorbance at 490 nm. (D) The protein expression levels of AdipoR1 and AdipoR2 in C3H10T1/2 cells. Results are presented as the mean ± standard deviation; n=3; *P<0.05 vs. DM-induced control). AdipoR, adiponectin receptor; DM, differentiation medium; ORO, Oil Red O.

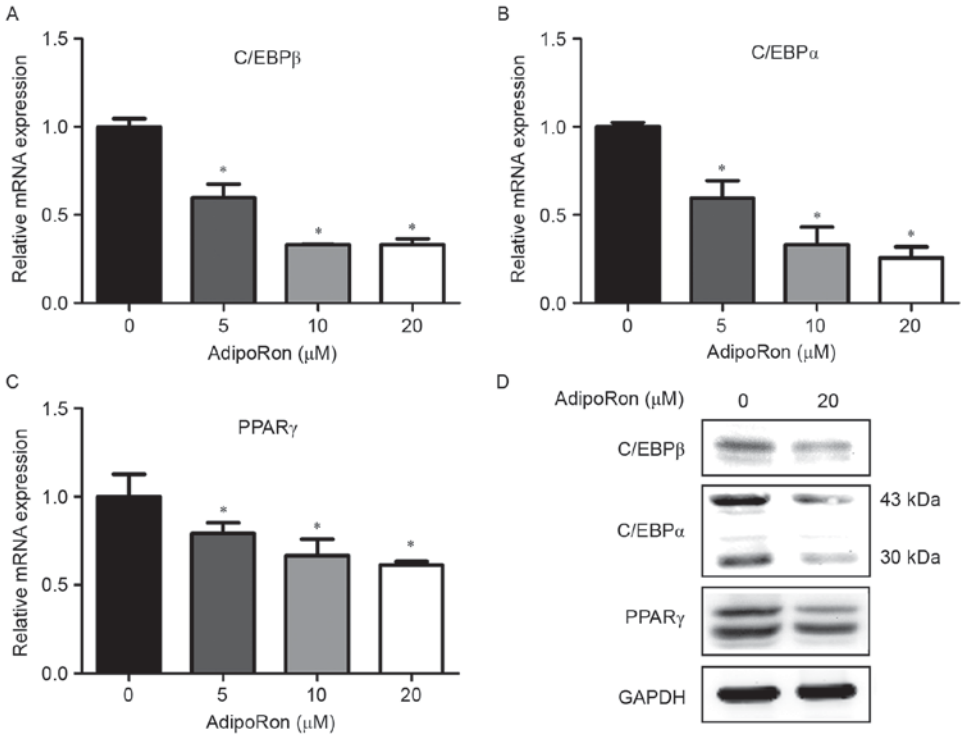

Figure 2. Effects of AdipoRon on the expression levels of adipogenic transcription factors in C3H10T1/2 cells. C3H10T1/2 cells were incubated with AdipoRon during adipogenesis. Reverse transcription-quantitative polymerase chain reaction was used to analyze mRNA expression, and western blotting was used to analyze protein expression at day 8 post-treatment. Effects of AdipoRon (0-20 μM) on the mRNA expression levels of the (A) C/EBPβ, (B) C/EBPα and (C) PPARγ. (D) Effects of AdipoRon (20 μM) on the protein expression levels of the C/EBPβ, C/EBPα and PPARγ. Results are presented as the mean ± standard deviation; n=3; *P<0.05 vs. 0 μM AdipoRon. C/EBP, CAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor.

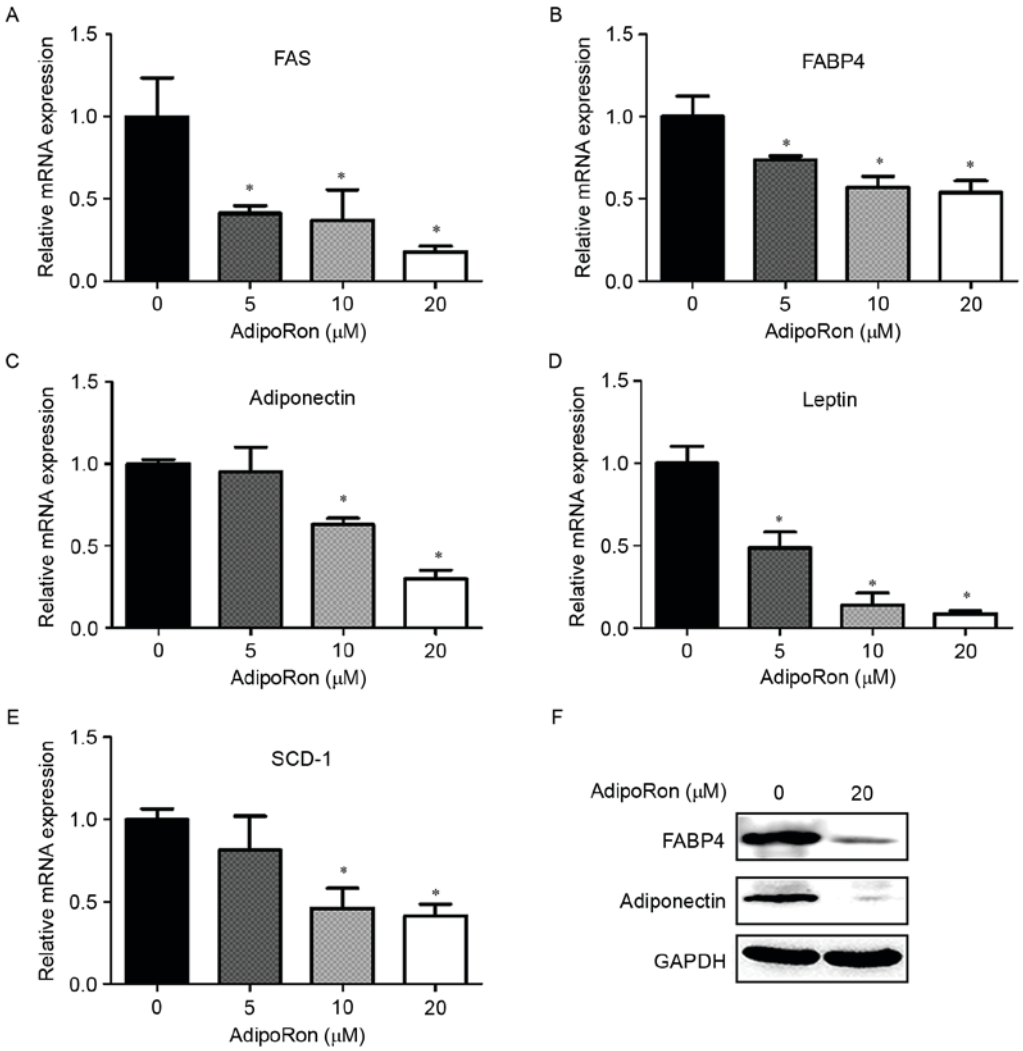

Figure 3. Effects of AdipoRon on the expression levels of adipocyte-specific markers in C3H10T1/2 cells. C3H10T1/2 cells were incubated with AdipoRon during adipogenesis. Reverse transcription-quantitative polymerase chain reaction was used to analyze mRNA expression levels, and western blotting was used to analyze protein expression levels at day 8 post-treatment. The effects of different concentrations of AdipoRon on the mRNA expression levels of (A) FAS, (B) FABP4, (C) adiponectin, (D) leptin and (E) SCD-1. (F) The effects of 20 μM AdipoRon on the protein expression levels of FABP4 and adiponectin. Results are presented as the mean ± standard deviation; n=3; *P<0.05 vs. 0 μM AdipoRon. FABP, fatty acid binding protein; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase 1.

C3H10T1/2 cells without cytotoxicity. Adipogenesis is coordinated by many transcription factors, such as C/EBPs and PPARs; AdipoRon exposure downregulated the expression levels of the C/EBPβ mRNA, which subsequently suppressed the expression of the C/EBPα and PPARγ mRNAs. PPARγ and C/EBPα are major regulators of adipogenesis by activating the transcription of terminal adipocyte differentiation marker genes, including FABP4, FAS, leptin, SCD-1 and adiponectin (3-5); the expression levels of these mRNAs were demonstrated to be significantly decreased by AdipoRon in the present study. Therefore, AdipoRon treatment suppressed the expression of transcription factors during adipogenesis, which then inhibited adipocyte differentiation of C3H10T1/2 cells.

Obesity occurs when total energy intake exceeds total energy expenditure. AMPK is a metabolic energy sensor that senses nutritional stress for the purpose of regulating glucose and lipid metabolism and may be a potential target for the treatment of obesity (17). AMPK has been reported to serve an important role in inhibiting adipogenesis in 3T3-L1 cells that have been induced by a number of different natural compounds (2,6,8,18-20). Activated AMPK inhibits the differentiation of adipocytes and the expression of pro-adipogenesis transcription factors PPARγ and C/EBPα (21). AMPK phosphorylation induces the phosphorylation of its substrate, ACC, which functions to strictly regulate the enzymes that are involved in the fatty acid synthesis pathway that produces malonyl-CoA. Notably, p-ACC lacks the ability to synthesize fatty acids (22). AdipoRon was previously reported to activate AMPK signaling in muscle and liver of obese mice (12). To elucidate whether AdipoRon inhibited adipogenesis through the AMPK signaling pathway, the present study measured the levels of p-AMPK and p-ACC; AdipoRon treatment significantly increased the phosphorylation of AMPK and ACC. In addition, the AMPK activator AICAR was used as a positive control to further elucidate the association between the anti-adipogenesis ability of AdipoRon and the activation of the AMPK signaling pathway. AICAR was

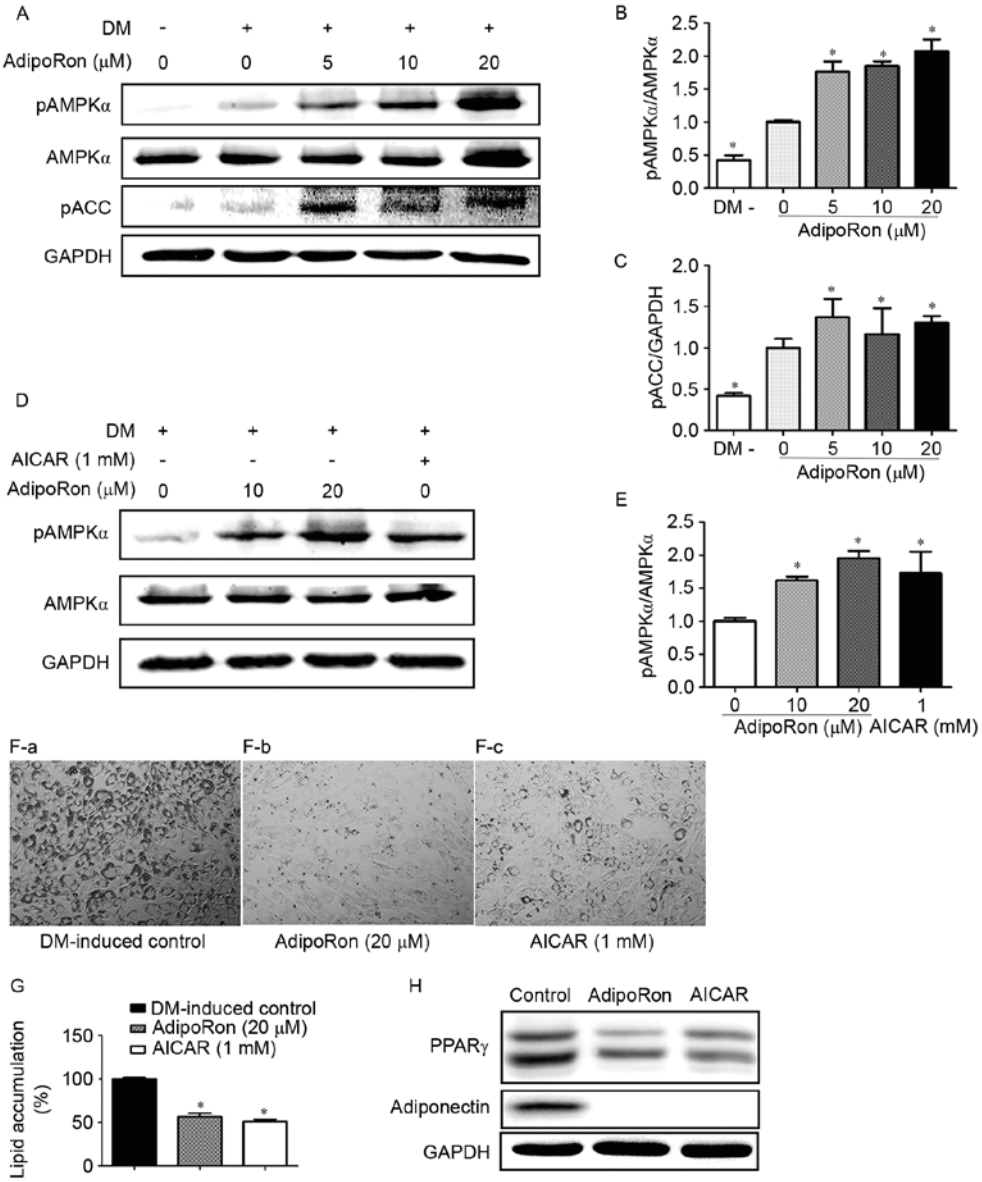

Figure 4. Effects of AdipoRon on the phosphorylation of proteins in the AMPK signaling pathway. (A) C3H10T1/2 cells were treated with or without DM in the presence or absence of AdipoRon for 15 min. Protein expression levels of p-AMPK, AMPK and p-ACC were determined. (B and C) Densitometric analyses for (B) AMPK phosphorylation and (C) ACC phosphorylation from part (A). (D) C3H10T1/2 cells were treated with DM in the presence or absence of AdipoRon (10 or 20 μM) or AICAR (1 mM) for 15 min. The expression levels of the p-AMPK and AMPK proteins were determined. (E) Densitometric analyses for AMPK phosphorylation from part (D). (F) Images of ORO staining at day 8 of adipocyte differentiation; (F-a) DM-induced adipocytes; (F-b) DM-induced adipocytes treated with 20 μM AdipoRon; (F-c) DM-induced adipocytes treated with 1 mM AICAR; magnification, x200. (G) Lipid accumulation was determined by measuring the absorbance at 490 nm. (H) Effects of AdipoRon (20 μM) or AICAR (1 mM) on the expression levels of the PPARγ and adiponectin proteins. Results are presented as the mean ± standard deviation; n=3; *P<0.05 vs. DM-induced control. ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimi dazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, adenosine monophosphate-activated protein kinase; DM, differentiation medium; ORO, Oil Red O; p, phosphorylated; PPAR, peroxisome proliferator-activated receptor.

previously reported to inhibit adipogenesis in 3T3-L1 adipocytes and restore metabolic alterations in a diet-induced mouse model of obesity (23-25). Results from the present study revealed that both AICAR and AdipoRon were able to activate the AMPK signaling pathway and markedly reduce lipid accumulation in C3H10T1/2 cells, which indicated that the anti-adipogenic effects of AdipoRon may be related to the activation of the AMPK signaling pathway. As C3H10T1/2 is a mouse embryonic mesenchymal stem cell line, the anti-adipogenesis effects of AdipoRon require further validation in human-sourced mesenchymal stem cell line.

In conclusion, AdipoRon, an adiponectin receptor agonist, suppressed adipocyte differentiation of C3H10T1/2 cells, probably by activating the AMPK signaling pathway and downregulating the expression of key regulators of adipogenesis. Therefore, AdipoRon may be a drug with the potential to prevent and treat obesity.

Acknowledgements

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