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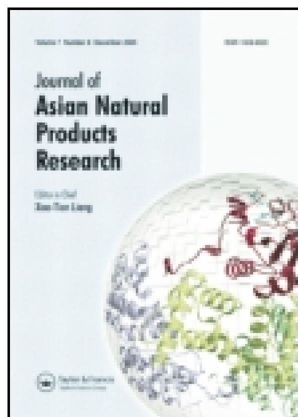

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Rhein, an inhibitor of adipocyte differentiation and adipogenesis

Qian Liu [a], Xiao-Lin Zhang [a], Rong-Ya Tao [a], Ya-Jing Niu [a], Xiao-Guang Chen [a], Jin-Ying Tian [a] & Fei Ye [a]

[a] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050, China

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Rhein, an inhibitor of adipocyte differentiation and adipogenesis

Qian Liu, Xiao-Lin Zhang, Rong-Ya Tao, Ya-Jing Niu, Xiao-Guang Chen, Jin-Ying Tian and Fei Ye*

Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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Rhein (RH), a compound purified from *Radix et Rhizoma Rhei*, has been used to alleviate liver and kidney damage. It is found that RH inhibited the differentiation of 3T3-L1 preadipocytes induced by differentiation medium in a time- and dose-dependent manner. It was revealed that RH downregulated the expression of adipogenesis-specific transcription factors PPARγ and C/EBPα, as well as their upstream regulator, C/EBPβ. Furthermore, the PPARγ target genes that are involved in adipocyte differentiation, such as CD36, aP2, acyl CoA oxidase, uncoupled protein 2, acetyl-CoA carboxylase, and fatty acid synthase, were reduced after to RH. In addition, high-fat diet-induced weight gain and adiposity were reversed by RH in C57BL/6 mice. Consistent with the cells' results, RH downregulated the mRNA levels of PPARγ and C/EBPα, and their downstream target genes in C57BL/6 mice. Taken together, adipocyte differentiation and adipogenesis were inhibited by RH in cultured cells and in rodent models of obesity. The evidence implied that RH was a potential candidate for preventing metabolic disorders.

Keywords: rhein; 3T3-L1; adipocyte differentiation; adipogenesis

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1. Introduction

Obesity, one of the metabolic diseases, is an important risk factor for diabetes and cardiovascular diseases [1,2]. Increases in either adipocyte cell number or the size of individual adipocytes due to increased lipid accumulation result in more fat deposition [3,4]. The process of adipocytes differentiation from preadipocytes has been studied using 3T3-L1 and 3T3-F422A cells, two immortalized cell lines that are already committed to the adipocyte lineage [5,6]. In the presence of a hormonal cocktail consisting of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (referred to as MDI), 3T3-L1 preadipocytes differentiate into adipocyte-like cells, expressing adipocyte-specific genes and accumulating triacylglycerol-rich lipid droplets [7]. Expression of CCAAT-enhancer-binding protein-β (C/EBPβ) is induced in the early stage of 3T3-L1 differentiation that is required for mitotic clonal expansion and for the expression of downstream transcription factors such as peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein-α (C/EBPα) [8,9]; PPARγ and C/EBPα are the key transcription factors for adipogenesis, lipogenesis, and glucose metabolism [10,11]. PPARγ and C/EBPα mediate the transcription of a group of genes, such as CD36, adipose fatty acid-binding

*Corresponding author. Email: yefei@imm.ac.cn

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protein (ap2), acyl CoA oxidase, uncoupled protein 2 (UCP-2), acetyl-CoA carboxylase α (ACCα), fatty acid synthase (FAS), related to fatty acid synthesis, oxidation, transport, storage, or energy expenditure [12]. Suppression of PPARγ and C/EBPα expression blocks adipogenesis and lipogenesis.

Rhein (RH), one of the main active components of Radix et Rhizoma Rhei, is widely used in Chinese medicine to alleviate liver and kidney damage [13–15], and reportedly has beneficial biological effects such as lowering serum cholesterol and improving diabetic nephropathy, as well as protecting pro-oxidation [16,17]. RH increases insulin sensitivity by enhancing insulin-stimulated glucose uptake in 3T3-L1 adipocytes [18]. However, little documentation is available to explain the mechanisms of RH on adipocyte biology and on the pathology of obesity. Here, the inhibition effects of RH on adipocyte differentiation and adipogenesis by evaluation in 3T3-L1 preadipocytes and in C57BL/6 mice fed with high-fat diet (HFD) were investigated.

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2. Results and discussion

2.1 Inhibition of RH on 3T3-L1 adipocyte differentiation

It had been reported that RH (Figure 1A) as a major compound of Rhei Rhizoma extracts reduced the accumulation of triglycerides (TG) in adipocyte [18]. This suggested that RH might be an antagonist in adipocyte differentiation. To address this issue, the effect of RH on adipocyte differentiation was detected in 3T3-L1. First, an acceptable dose of RH in 3T3-L1 adipocyte was selected. The cells were exposed to RH in different concentrations as indicated in Figure 1B, and MTT was performed to detect the cell viability. As shown in Figure 1B, RH exerted cellular toxicity at doses of 20–160 μM. To observe the inhibition of RH on adipocyte differentiation, 3T3-L1 preadipocytes were induced to differentiation in the presence or the absence of RH at day 0. The cells were stained with Oil Red at day 8. The adipocyte differentiation was completely blocked by RH in a dosage-dependent manner (Figure 1C). Consistently, lipid content in cells also showed a decrease after RH treatment (Figure 1E). Furthermore, similar inhibition was also observed at day 8 in a dosage-dependent manner in the cells, which had differentiated for 4 days then exposed to RH for 4 days (Figure 1D and F). These results gave the evidence that RH showed an inhibition on adipocyte in both early and late stages of differentiation.

2.2 Down-regulation of RH on critical adipogenic transcription factors in 3T3-L1 cells

During differentiation period, C/EBPβ has a temporal rise, followed by the stimulation of expression of PPARγ and C/EBPα, which play an important role in adipogenesis. To investigate whether RH suppresses adipogenesis through these transcription factors, total RNA was purified in different stages of differentiated 3T3-L1 cells and polymerase chain reaction (PCR) was carried out. As shown in Figure 2A, the mRNA levels of C/EBPβ decreased at day 1 in the presence of RH, which is expressed in the early stage of 3T3-L1 differentiation and serves as the regulator of PPARγ and C/EBPα as well as clonal expansion. Also, PPARγ and C/EBPα are strongly inhibited by RH at day 8 at the transcriptional level (Figure 2B). Furthermore, the protein levels of PPARγ and C/EBPα were reduced in a dosage-dependent manner in adipocytes' exposure to RH (Figure 2C and D). Taken together, these results indicated that RH inhibited differentiation via downregulating the expression of transcriptional factors C/EBPs and PPARγ in adipocytes.

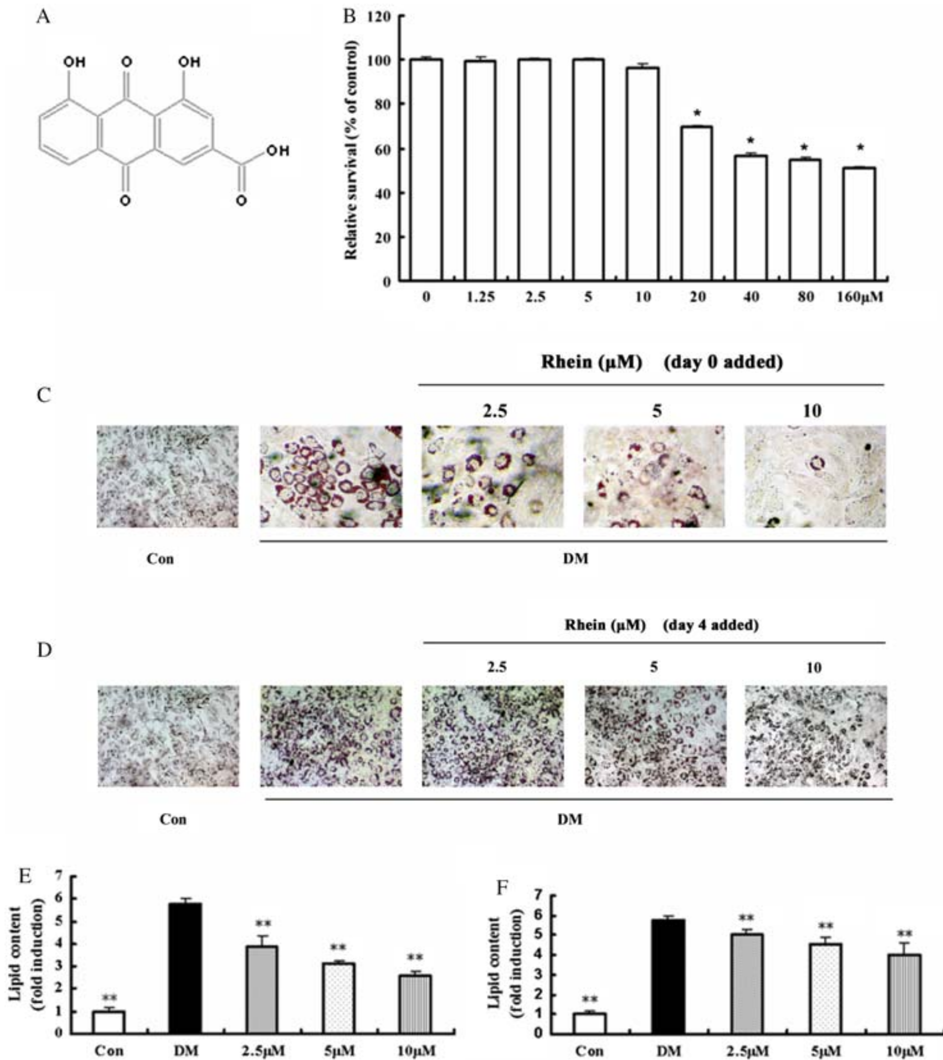

Figure 1. Effect of RH on 3T3-L1 adipocyte differentiation. The structure of RH (A). For the measurement of intracellular cytotoxicity, cells were treated with different concentrations of RH in the presence of hormonal cocktail for 8 days, and then intracellular toxicity was measured by MTT assay (B). A total of 2.5, 5, and 10 μM of RH were used at day 0, 3T3-L1 cells were stained with oil red O at day 8 (C). Also stained lipid content was quantified by measuring absorbance (E). A total of 2.5, 5, and 10 μM RH were used at day 4, 3T3-L1 cells were stained with oil red O at day 8 (D) and stained lipid content was quantified by measuring absorbance (F). The data are presented as the statistical significance $**P < 0.01$ vs. DM.

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2.3 Regulation of RH on PPARγ target and lipogenic gene expressions

As RH downregulated C/EBPα and PPARγ, it was conceivable that RH would affect the expression of adipogenic- and lipogenic-related genes. Thus, mRNA levels of adipogenic and lipogenic specific genes including FAS, ACC, aP2, UCP-2, and CD36 were tested in RH-treated 3T3-L1 cells. All target genes were assayed at day 8 of differentiation medium (DM)-induced differentiation in 3T3-L1 cells. As

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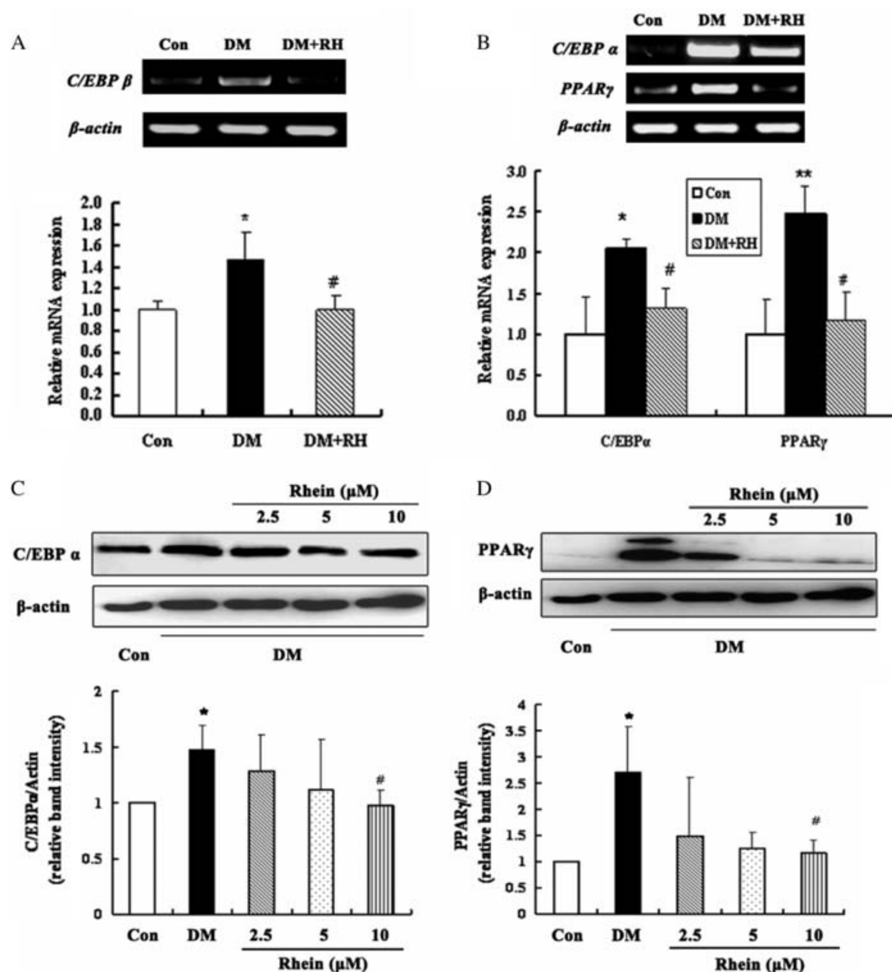

Figure 2. RH suppresses protein level of PPARγ and gene expression of C/EBPα, C/EBPβ, and PPARγ in DM-induced 3T3-L1 adipocytes. Western blot showed that PPARγ protein was reduced in RH-treated 3T3-L1 cells at day 8 of differentiation. 1, 3T3-L1 control; 2, DM induction cells: 3–5, DM + RH 2.5; 5, 10 μM-treated cells (A). PCR showed that mRNA levels of PPARγ and C/EBPα at day 8 (B) and C/EBPβ at day 1 (C) in differentiated 3T3-L1 cells were suppressed by 10 μM of RH. For Western blot or PCR experiments, β-actin protein or mRNA was measured as an internal control. Data are presented as means ± SD; $*P < 0.05$, $**P < 0.01$ vs. Con, $^{\#}P < 0.05$ vs. DM; $n = 3$.

expected, these gene transcripts were obviously suppressed by RH (Figure 3).

2.4 *Reversion of RH on HFD-induced obesity*

The results of RH on adipocyte differentiation indicated that RH might have a therapeutic role against HFD-induced obesity. To test this hypothesis, biological effects of RH in C57BL/6 mice were explored. During the 14-week feeding period, before the drug treatments, body weights (BWs) of the mice fed with HFD were much higher than those of mice on normal diet ($P < 0.001$, data not shown). At the end of the study, BWs of the HFD group increased by 68% compared with the controls (Table 1). White adipose

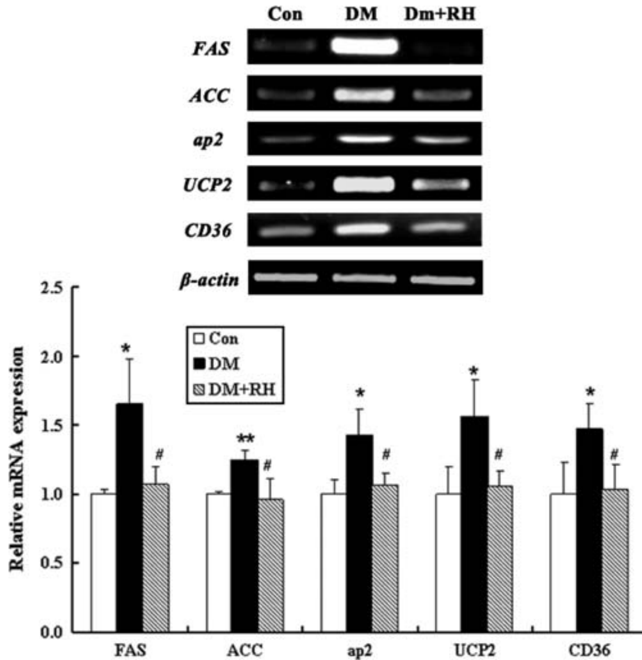

Figure 3. RH blocks adipogenic- and lipogenic-related genes. PCR results of 10 μM RH-treated 3T3-L1 cells at day 8 indicated that the mRNA levels of FAS, ACC, aP2, UCP-2, and CD36 were inhibited. The results represented at least three independent experiments, and β-actin was used as an internal control. Data are presented as means ± SD; $*P < 0.05$, $**P < 0.01$ vs. Con, $^{\#}P < 0.05$ vs. DM.

tissue weights of the HFD group were about twofold greater than those of the control mice, indicating that diet-induced obesity was achieved in this study. After 4 h of fasting, the model mice demonstrated hypercholesterolemia, but not hypertriglyceridemia.

RH attenuated BW gain in HFD mice (Table 1). By week 4, mice administrated with RH-M (60 mg/kg BW) or RH-H (120 mg/kg BW) had significantly lower BW than HFD-fed mice receiving water alone. Compared with those in the HFD group, BWs were reduced by 6 and 12% in RH-M and RH-H groups, respectively. Moreover, RH treatment groups all had decreased white fat tissue mass relative to HFD group. RH-H also had significantly decreased adipose index ($P < 0.01$ vs. HFD group). There was,

Table 1. Effects of RH on lipid profile in HFD-induced C57BL/6 mice.

	Con	HFD	RH-L	RH-M	RH-H
Body weight (g)	33.2 ± 4.18	48.9 ± 1.3**	46.9 ± 5.1	45.9 ± 4.1$^{\#}$	43.1 ± 3.5$^{\#\#}$
White adipose tissue (g)[a]	1.07 ± 0.45	2.61 ± 0.46**	2.11 ± 0.37$^{\#}$	2.15 ± 0.22$^{\#}$	1.70 ± 0.29$^{\#\#}$
Adipose index (%)[b]	3.27 ± 1.00	5.44 ± 1.01**	4.59 ± 0.76	4.76 ± 0.61	4.04 ± 0.74$^{\#\#}$
TC (mg/dl)	48.3 ± 6.7	117.8 ± 21.1**	114.0 ± 18.1	98.8 ± 16.3$^{\#}$	87.6 ± 15.2$^{\#}$
TG (mg/dl)	93.7 ± 19.1	92.3 ± 14.5	84.5 ± 22.0	75.8 ± 7.0$^{\#\#}$	69.4 ± 9.4$^{\#\#}$

Note: $n = 10$. Each value is expressed as mean ± SD; $*P < 0.05$, $**P < 0.01$, vs. Con. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. HFD.

[a] Adipose tissue: epididymal fat pad and abdominal adipose tissue weight.

[b] Adipose index was calculated as white adipose tissue weight (g)/BW (g) × 100.

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however, no significant difference in food intake among all HFD mice with or without drug treatment (data not shown). Then, the effects of RH on the serum TG and total cholesterol (TC) levels were examined in C57BL/6 mice fed with a HFD. Both RH-M (60 mg/kg BW) and RH-H (120 mg/kg BW) had decreased serum TG levels by 18 and 25%, and downregulated serum TC levels by 16 and 17% (Table 1).

2.5 RH alters the expression of metabolic genes in adipose tissue **in vivo**

To investigate the mechanism of RH action on obesity, the effects of RH on the expression of transcription factors that are known to play a critical role in adipogenesis and energy balance were assessed. As shown in Figure 4, the expression of PPARγ and C/EBPα was reduced significantly in white adipose

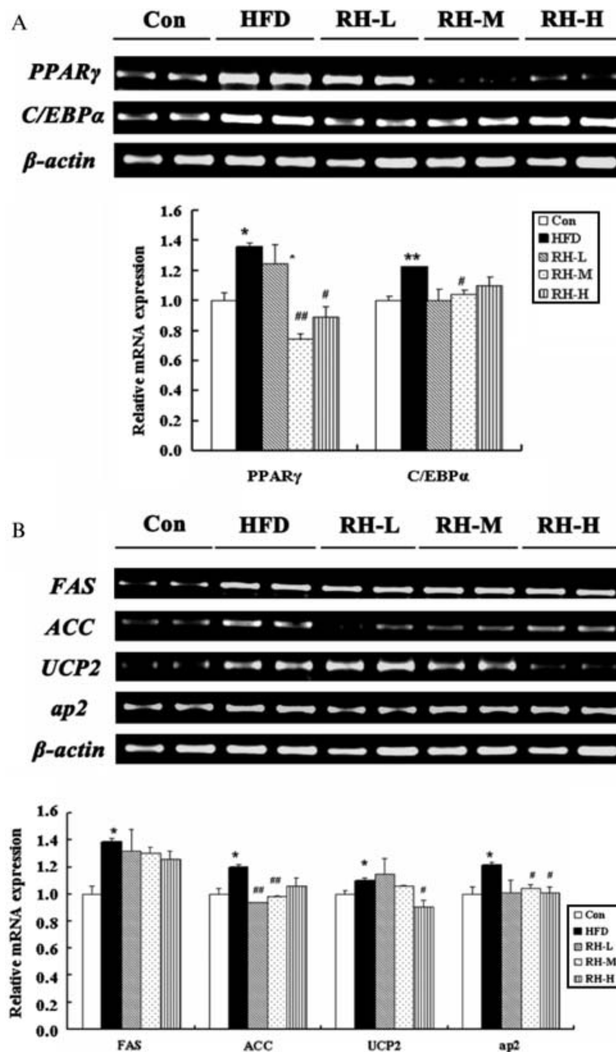

Figure 4. RH alters the expression of metabolic genes in fat *in vivo*. PCR results of RH-treated HFD at day 28 indicated that PPARγ and C/EBPα mRNA levels were downregulated (A); the downstream genes of PPARγ and C/EBPα involved in lipogenesis, such as FAS, ACC, aP2, and UCP-2 mRNA levels, were inhibited (B). β-actin was used as an internal control. Data are presented as means ± SD; $*P < 0.05$, $**P < 0.01$ vs. Con, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. HFD.

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tissue of RH-treated groups. Most genes involved in lipogenesis were downregulated by RH treatment. The expression of a number of adipocyte-specific genes including FAS, ACC, UCP2, and aP2 was lowered too. Similarly with the cells' results, these observations demonstrated that RH treatment resulted in an altered gene expression profile that would promote catabolism of high energy intermediate *in vivo*.

2.6 Discussion

Blocking of adipocytes differentiation is one of the anti-obesity strategies falling under the category of modulating fat storage. Utilization of anti-adipogenic compounds from natural sources could be helpful in the prevention of obesity and reducing side effects. It is reported that RH as a major compound of Rhei Rhizoma extracts increased glucose uptake in 3T3-L1 adipocytes [19], improved dyslipidemia in hypercholesterolemic patients and mice [16], reduced the accumulation of TG in adipocyte [18], and inhibited lipid synthesis [13]. This suggested that RH might be an inhibitor in adipocyte differentiation. To address this issue, the effect of RH on adipocyte differentiation was detected in 3T3-L1 adipocytes. The inhibitory effect on 3T3-L1 adipose differentiation is caused by the suppression of differentiation, and/or proliferation, or both. The results from our study indicate that RH inhibits differentiation of 3T3-L1 adipocytes in a dose- and time-dependent manner. It has been confirmed that the inhibitory effect of RH was observed not only in the early use (day 0) of RH in 3T3-L1 differentiation induction, but also in the late use (day 4) of RH when the mitotic clonal expansion had been completed, suggesting that RH suppressed both 3T3-L1 differentiation and mitotic clonal expansion.

During differentiation period, C/EBPβ has a temporal rise, followed by the stimulation of the expression of PPARγ and C/EBPα, which plays an important role in adipogenesis. C/EBPα and PPARγ were two key transcriptional factors for adipogenesis, which drive the expression of genes that are necessary for the generation and maintenance of adipogenic phenotype. In this study, it was found that RH significantly downregulated the mRNA and protein levels of PPARγ and C/EBPα induced by DM in 3T3-L1 cells. This could also be explained in two ways: RH inhibited PPARγ and C/EBPα or suppressed the upstream molecules. It was shown that RH reduced the mRNA level of C/EBPβ at day 1 in 3T3-L1 cells, suggesting that the inhibitory effects of RH on PPARγ and C/EBPα were independent on the C/EBPβ signal. After mitotic clonal expansion is complete, the RH still inhibits the adipogenesis when upregulation of C/EBPβ has taken place, and thus it seems that RH also directly inhibits the mRNA and protein levels of PPARγ.

The inhibition of RH on lipogenesis and adipogenesis was also confirmed on HFD-induced C57BL/6 mice. The fatty liver was improved, and the fat mass with lower lipogenesis genes expression in adipose tissue was decreased by RH. In combination with the results *in vitro*, RH showed a remarkably suppressed effect on lipogenesis and adipogenesis.

In summary, RH can inhibit 3T3-L1 adipocyte differentiation and lipid accumulation. These effects may work on multiple molecular targets and complex mechanisms. RH not only significantly downregulated the mRNA and protein levels of C/EBPα and PPARγ, but also suppressed their upstream molecules-C/EBPβ. The expressions of C/EBPα and PPARγ target genes such as *FAS*, *ACC*, *ap2*, *UCP2*, and *CD36* were inhibited by RH. As RH is not a PPARγ ligand and does not change endogenous PPAR ligand synthesis or binding activities, the possible explanation is that the inhibition of C/EBPα and PPARγ transcription is the

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consequence of the reduction of C/EBPα and PPARγ protein levels, and the low C/EBPα and PPARγ protein levels in RH-treated cells are not enough to activate target gene expression. RH decreased HFD-induced BW gain and adiposity, downregulated the expression of genes involved in lipogenesis and upregulated the genes involved in energy expenditure in adipose tissue. Combined with the studies showing lipid- and glucose-lowering effects of RH in db/db diabetic mice [16], these results suggest that RH might have multiple therapeutic effects and is a potential candidate drug to treat metabolic syndrome, such as obesity, type 2 diabetes, and dyslipidemia.

3. Experimental

3.1 Compound

RH was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China, purity $>$ 99%).

3.2 Cells culture

3T3-L1 cell was kindly gifted by Servier Pharmaceutical Research Center (Neuilly-sur-Seine, France), and was grown and maintained in DMEM containing 10% (v/v) fetal bovine serum (Hyclone, Logan, UT) at 37°C in a humidified atmosphere of 5% CO_2. For adipocyte differentiation, cells were grown to full confluence; then, DM, containing 10 μg/ml insulin (Sigma, St Louis, MO), 1 μM dexamethasone (Sigma), 0.5 mM isobutylmethyl xanthine (IBMX, Sigma), and 10% fetal bovine serum was added. After 4 days induction, the medium was changed to DMEM with 10% fetal bovine serum, and RH was dissolved in DMEM and added to the medium at an indicated concentration.

3.3 MTT assay

Cells were cultured in 24-well plates with each stimulus, and then incubated with 30 μl of MTT solution (5 mg/ml in PBS) for 3 h at 37°C. After discarding the medium, cells were dissolved with DMSO and then relative cell viability was determined by spectrophotometry (490 nm).

3.4 Oil red O staining

The cells were washed with PBS twice, fixed with 10% formalin at room temperature for 10 min. Oil red O (Sigma, 0.5% in isopropanol) was diluted with water (3:2), filtered twice with a 0.45 μm filter, and added to fixed cells at 60°C for 10 min. Cells were washed with water, and then visualized by light microscopy and photographed. The pictures were taken using an Olympus microscope (Tokyo, Japan). The stained lipid droplets were dissolved in 1 ml isopropanol and quantified by spectrophotometry (520 nm).

3.5 Animals

Four-week-old male C57BL/6 mice were obtained from the Animal Center of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, housed 4 per cage and maintained under standardized conditions of temperature (21–22°C) and humidity (40–60%), with light from 06:00 to 18:00. One group of mice used for control (Con) was allowed to eat normal rodent chow obtained from the Animal Center of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. The rest of the mice were fed with HFD *ad libitum* [20] to induce obesity. After 14-week feeding, based on the BWs the model mice were randomly divided into four groups, HFD (the model control), RH-L (RH 30 mg/kg BW/day), RH-M (RH 60 mg/kg BW/day), and RH-H (RH 120 mg/kg BW/day) with 10 mice per group. RH was dissolved in water at indicated concentrations. RH groups were fed by gavage for 4 weeks. The HFD and

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Con groups received distilled water, respectively. The BWs and food uptake were recorded weekly. All mice were sacrificed by decapitation after 4-h of food deprivation. Blood samples were collected for the assays of lipid profiles. White adipose tissues were carefully removed and used to determine the adiposity index, calculated as white adipose tissue weight (g)/BW (g) × 100, and then were snap frozen in liquid nitrogen, and stored at −70°C. All animal experiments were approved by the Ethics Committee of Laboratory Animals of Beijing Municipality.

3.6 Biochemical assays

Serum was separated by centrifugation at 4°C and analyzed immediately or stored at −20°C. Serum levels of TG and TC were determined by spectrophotometry.

3.7 Reverse transcription polymerase chain reaction analysis of adipogenesis genes

Total RNA was isolated from 3T3-L1 cell lines and adipose tissue of mice with Trizol (Invitrogen, Carlsbad, CA, USA), respectively, and reverse transcribed by two-step method with the SuperScript First-Strand Synthesis System. The resulting single-stranded cDNA (2 μl) was denatured at 94°C for 5 min and, after the addition of the polymerase, subjected to 28–32 cycles of amplification, each consisting of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C, with a 2-min final extension at 72°C during the last cycle. Each PCR mixture (50 μl) contained the cDNA template, 1 μM of the primers, 200 μM of dNTPs, 1.5 mM $MgCl_2$, and 1.25 U Platinum Taq polymerase (Invitrogen Corp., USA). The expressions of genes including PPARγ, C/EBPα, C/EBPβ, ACCα, FAS, CD36, ap2, and UCP2 were analyzed. The assay of every gene in each sample was replicated three times. The mouse β-actin gene was amplified as a loading control. The PCR products were separated by electrophoresis on 1% agarose gel. The genes and their forward and reverse primers are listed in Table 2.

3.8 Western blotting

3T3-L1 adipocytes were washed once with cold PBS (pH 7.4) and scraped into lysis

Table 2. Sequences of the primers used in the PCR measurements.

Gene	Sense	Sequence (5′–3′)	Gene bank no./ref.
PPARγ	Forward	GCAAGACATAGACAAAACACCAGTGTGA	NM011146
	Reverse	AGCAACCATTGGGTCAGCTCTTGTGA	
C/EBPα	Forward	CTGCCCCTCAGTCCCTGTC	BC058161
	Reverse	GTTCCTTCAGCAACAGCGG	
C/EBPβ	Forward	AACCTGGAGACGCAGCACAA	NM_009883
	Reverse	TGCATCAAGTCCCGAAACCC	
ACCα	Forward	AGGAGGACCGCATTTATCGAC	NM133360
	Reverse	TGACCGTGGGCACAAAGTT	
FAS	Forward	CTGCGGAAACTTCAGGAAATG	NM007988
	Reverse	GGTTCGGAATGCTATCCAGG	
CD36	Forward	TGTACCTGGGAGTTGGCGAG	NM007643
	Reverse	CTGCTGTTCTTTGCCACGTC	
ap2	Forward	TGGAAGACAGCTCCTCCTCG	NM024406
	Reverse	CCGCCATCTAGGGTTATGATG	
UCP-2	Forward	GCTGGTGGTGGTCGGAGATA	NM_011671
	Reverse	ACTGGCCCAAGGCAGAGTT	
β-Actin	Forward	CCCATC TACGAGG GCTAT	NM007393
	Reverse	TGGCC AGTAA TGTCC AGG	

buffer containing 1% SDS and 60 mM Tris–HCl (pH 6.8). Lysates were clarified by centrifugation, and protein concentration was measured using the Bradford protein assay kit (Bio-Rad, Richmond, CA), and samples were boiled at 100°C for 10 min. Equal amounts of protein were subjected to SDS-PAGE and immunoblotted with anti-PPARγ, anti-C/EBPα, or β-actin antibody (Santa Cruz, CA), respectively. Immunoblot analysis was performed with enhanced chemiluminescence reagent and detected by a gel analysis system (Flurochem 5500, Alpha Innotech, San Leandro, CA, USA).

3.9 Statistical analysis

Data are expressed as means ± SD. Differences between groups were estimated by one-way ANOVA (SPSS version 10.0, USA). *P* values of 0.05 (or less) were considered statistically significant.

Acknowledgements

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