Promoting effect of triterpenoid compound from *Agrimonia pilosa* Ledeb on preadipocytes differentiation via up-regulation of PPARγ expression

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ABSTRACT

Background: *Agrimonia Pilosa Ledeb* (APL), a traditional Chinese medicine, has been reported a variety of biological activities, including treating T2DM. **Objective:** Triterpenoid compound (TC) was collected from APL. The aim of this study was to investigate the effects of TC on 3T3-L1 preadipocytes differentiation and genes related to differentiation and IR. **Materials and Methods:** Column chromatography was used to collect TC from ALP. 3T3-L1 cell differentiation was induced typically in the presence of various concentrations of TC or pioglitazone. Oil red O staining and measurement of intracellular TG content were performed on the seventh day of differentiation. Then quantitative polymerase chain reaction (Q-PCR) was used to test the expressions of three transcription factors （PPARγ, CCAAT enhancer binding protein-α (C/EBP-α), and sterol regulatory element-binding protein 1 (SREBP-1)） and the target genes of PPARγ including glucose transporter (GLUT4), lipoprotein lipase (LPL), fat acid binding protein (AP2), and adiponectin in 3T3-L1 cells. **Results:** At the concentration of 5, 25 and 125 μg/mL, TC significantly promoted triglyceride accumulation. Further study showed that TC could promote the expression of PPARγ, C/EBPα and ADD1/SREBP1 significantly at 125 μg/mL. As for downstream genes controlled by PPARγ, TC at 25 and 125 μg/mL could significantly promote the expression of GLUT4 and adiponectin. However, the expression of aP2 related to lipid metabolism and adiposity in the TC group was significantly lower than that in the pioglitazone group. **Conclusion:** TC could promote preadipocytes differentiation through activating PPARγ and downstream controlled genes. TC has the ideal insulin sensitization with lower adipogenic action than classical TZDs *in vitro*. So TC from *Agrimonia Pilosa* Ledeb has a good prospect as a natural drug for IR and T2DM.

Key words: *Agrimonia Pilosa* Ledeb, aP2, PPARγ, triterpenoid compound, Type 2 diabetes mellitus

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a systemic metabolic disease, whose occurrence and development involved in many factors and signal pathways including hyperglycaemia, dyslipidaemia and oxidative damage. Although the etiology mechanism is complex, the main pathological features are insulin resistance (IR) and β-cell function damage.[1] IR, a physiological condition, is that cells fail to respond to the normal actions of insulin. In muscle and fat cells, IR reduces the uptake of glucose and the storage of muscle glycogens and triglycerides, while IR in liver cells results in reduced liver glycogen synthesis and storage.

Now the treatments of T2DM mainly focus on improving IR. The most effective drugs which improve IR are thiazolidinedione (TZDs), such as rosiglitazone, pioglitazone and so on.[2] As a ligand of peroxisome proliferator-activated receptor gamma (PPARγ), TZDs can promote the transcriptional regulation of downstream genes related to IR selectively, and suppress inflammatory factors, such as TNF-α, NF-κB and so on. Though promoting differentiation of preadipocytes, they gather more intracellular lipids. So TZDs are associated with safety issues including weight gain, edema and cardiovascular disease while in treatment.[3,4] In 2010, the use of rosiglitazone had been restricted by the Food and Drug Administration and European Medicines Evaluation Agency. In order to

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resolve the current predicament of T2DM treatment, some alternative cure measures should be considered. There is a growing need in searching for natural bioactive components with benefits of improving IR or treating T2DM from vegetables, fruits, tea, spice, and medical herbs.[5,6]

Agrimonia Pilosa Ledeb (APL), belonging to Rosaceae, is a traditional Chinese medicine and a wild vegetable for tonic function. It is used to treat tumor, T2DM and blood, gastrointestinal, genitourinary, and gynecological diseases in traditional Chinese medicine. Now clinical practices had shown that *Agrimonia Pilosa* Ledeb had good therapeutic effect on treating T2DM. A growing body of research found that the major chemical components in *Agrimonia Pilosa* Ledeb are triterpenoids,[7] flavonoids,[8-10] coumarin compounds and agrimonolides. Triterpenoids from some plants have been confirmed to promote differentiation of 3T3-L1 preadipocytes and to improve IR,[11,12] retinopathy, nephropathy and diabetic vascular dysfunction.[13] In previous study, we found that triterpenoids was one of the most abundant components in *Agrimonia Pilosa* Ledeb. In order to clarify whether TC from *Agrimonia Pilosa* Ledeb is the component with therapeutic effect on IR or T2DM, we collected TC from *Agrimonia Pilosa* Ledeb, and then studied its effects on the differentiation of 3T3-L1 preadipocytes and the expressions of genes related to preadipocytes differentiation and IR, including PPARγ, C/EBPs, adipocyte determination differentiation dependent factor1/sterol regulatory element binding protein1 (ADD1/SREBP1), aP2, GLUT4, LPL and adiponectin. Our study will lay the foundation for developing drugs for IR and T2DM from *Agrimonia Pilosa* Ledeb.

MATERIALS AND METHODS

Materials

3T3-L1 preadipocytes were purchased from ATCC (American Type Culture Collection). The dried entire plants of *Agrimonia Pilosa* Ledeb purchased from Western Medicine City (Chongqing, China) in 2011. Pioglitazone was purchased from Chongqing Taiji Industry (Group) Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were obtained from HyClone. Bovine calf serum was obtained from Gibco. Paraformaldehyde, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5 -di-phenytetrazoliumromide (MTT), Oil Red O, 3-isobutylmethylxanthine (IBMX), dexamethasone (DEX), dimethylsulfoxide (DMSO) and insulin were obtained from Sigma-Aldrich (St Louis, MO). Trizol reagent was purchased from Invitrogen and reverse transcription system from Takara. SYBR Green was purchased from BIO-RAD. All other reagents were of analytical grade.

The triterpenoid compound (TC) from *Agrimonia Pilosa* Ledeb was prepared by ourself in lab. The collected procedure and the constituents analysis of TC have been described in detail in reference.[14] TC has a high level of total triterpenoids with a value of 415.97 ± 5.15 mg/g and is abundant of 1β, 2β, 3β, 19α-tetrahydroxy-12-en-28-oic acid (265.2 mg/g) and corosolic acid (100.9 mg/g).[14]

Cell culture and preadipocyte differentiation

3T3-L1 preadipocytes were cultured at 37°C, 5% CO_2. According to the standard protocols, 3T3-L1 preadipocytes were differentiated into adipocytes for 8 days.[15] The preadipocytes were maintained in DMEM (10% bovine calf serum, 1% penicillin-streptomycin). For the differentiation protocols, after reaching confluency (defined as day 0), the preadipocytes were cultured in differentiation medium DMEM (0.5 mM IBMX, 1 μg/ml insulin, 0.25 μM DEX and 10% FBS) for 2 days. After 2 days, the differentiation medium was replaced to DMEM (1 μg/ml insulin and 10% FBS) for 2 days. In day 5 to 8 days, the differentiation medium was changed again with fresh DMEM (10% FBS). The pioglitazone as positive control group and serial dilutions of TC from *Agrimonia Pilosa* Ledeb were administered at the initiation of differentiation and with every medium change. At 8th day, the differentiated cells (3T3-L1 adipocytes) were collected for analysizing mRNA expression level and Oil Red O staining.

The effect of TC on cell viability assay (MTT assay)

The MTT assay was performed according to the method of Mosmann.[16] 3T3-L1 preadipocytes were digested, centrifuged and prepared into single cell suspension, then calculated cell numbers in 1 mL by cell counting chamber. Then 3T3-L1 preadipocytes were plated into each well of a flat 96-well plate at a density of 2000 cells/well. After 24 h, the culture medium was replaced by pioglitazone and serial dilutions of TC from *Agrimonia Pilosa* Ledeb, and the cell cultures were incubated for 24 h or 48 h. Culture solutions were then removed and replaced by 80 μL of culture medium containing DMEM with 10% bovine calf serum and 20 μL filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH 7.4). After 4 h, solutions were removed and replaced by DMSO (150 μL/well) and the microtiter plate was closed by silver paper, and then measured spectrophotometer on microplate reader at 492 nm.

Oil Red O staining

At 8th day, discarding the culture and washing with PBS for three times. Then cells were fixed with 10% paraformaldehyde for 50 min, washed with PBS, and dyed with Oil Red O working dye (sealed, avoid light) for 50 min. The differentiated cells were photographed under a microscope (100× magnification) after washing with PBS twice.

Triglyceride Mass

3T3-L1 preadipocytes were cultured in 24-well microtiter plates, then stained with oil red O on 8th day. The redundant dye was washed twice by 60% isopropanol alcohol and then tri-distilled water for three times, and replaced by 1 mL 100% isopropanol alcohol, then vibrated 20 min by electronic oscillator. The absorbance was measured at 490 nm using a spectrophotometer to calculate TG mass.[17]

RNA preparation and quantitative real-time PCR

By Trizol reagent, total RNA was isolated from 3T3-L1 differentiated cells in 6 well plates. Then 1 µg RNA of each sample was reverse-transcribed to cDNA. At last, RT-PCR was performed with iQTM SYBR Green supermix according to the protocol. The PCR conditions were 1 cycle of 95°C for 3 min, followed by 39 cycles of 95°C for 10 s and 60°C for 30 s. The primer sequences were as follows: PPAR-γ, 5'-GCC CAC CAA CTT CGG AAT C-3' and 5'- TGC GAG TGG TCT TCC ATC AC -3'; ADD1/SREBP1, 5'- TGG CTT GGT GAT GCT ATG TTG -3' and 5'-GAC CAT CAA GGC CCC TCA A-3'; C/EBP-α, 5'-AGC TGA GTT GTG AGT TAG CCA TGT-3' and 5'-ACC CCA CAA AGC CCA GAA A -3'; GLUT4, 5'-CAT GGC TGT CGC TGG TTT C-3' and 5'- AAA CCC ATG CCG ACA ATG A-3'; aP2, 5'-CAA CCT GTG TGA TGC CTT TGT G-3' and 5'-CTC TTC CTT TGG CTC ATG CC-3'; adiponectin, 5'-GAC ACC AAA AGG GCT CAG GAT-3' and 5'-TGG GCA GGA TTA AGA GGA ACA-3'; LPL, 5'- GGA CTG AGG ATG GCA AGC AA -3' and 5'-GCC ACT GTG CCG TAC AGA GA-3'.

Statistical analysis

Data are presented as means ± SD. A paired-samples *t*-test was used for the difference analysis between groups by using SPSS 19.0 software. Difference with a value of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of TC on the cell viability of 3T3-L1 preadipocytes

To assess cytotoxic effect of TC, cell viability in 3T3-L1 preadipocytes was evaluated using MTT assay [Figure 1]. At concentrations of 1-1000 µg/mL (1, 10, 50, 100, 500 and 1000 µg/mL) for 24 h and 1-500 µg/mL (1, 10, 50, 100 and 500 µg/mL) for 48 h, TC had no significant inhibitory effects on cell viability compared with the control group. At the concentration of 1000 µg/mL, TC showed inhibition activity on cell growth in 48 h with a value of 22.33%. The cell viability in pioglitazone group had no obvious change in 24 h, but decreased to 91.30% in 48 h. The results showed that TC had slight influence on cell viability in 48 h at 1-500 µg/mL.
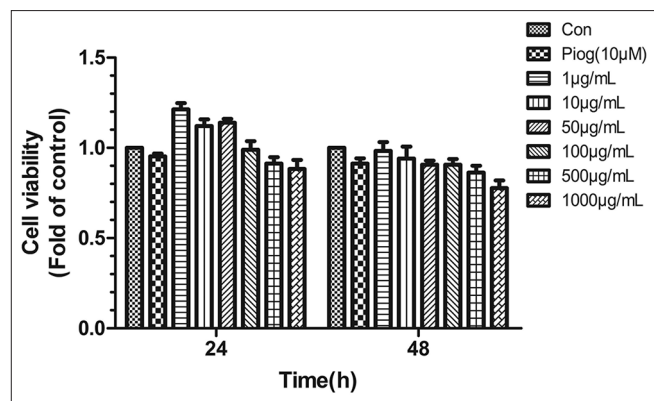

Figure 1: The effect of TC from *Agrimonia Pilosa* Leded on the cell viability of 3T3-L1 preadipocytes. The values are means ± SD of three independent experiments

TC promotes 3T3-L1 cells differentiation and triacylglycerol accumulation

Now, adipose tissue is recognized as not only an energy-storage tissue but also an endocrine tissue that secretes a variety of bioactive substances to participate in the occurrence and development of IR and obesity. The key link between these symptoms is the proliferation and differentiation of preadipocytes.[18,19] Under stimulation of specific hormones, the cell had fundamental changes in form and function through the expression of different genes and regulation of various kinds of cytokines.[20] As an azo dye, red oil O can combine with triacylglycerol (lipid droplet) to give out jacinth lipid droplets. When preadipocytes differentiate into adipocytes, large numbers of jacinth lipid droplets will be observed. In this paper, we used 3T3-L1 preadipocytes as cell model to investigate the effect of TC on the preadipocytes differentiation through the cellular morphology and triacylglycerol accumulation.

Preadipocytes were irregular form, but adipocytes turned round after induction [Figure 2]. Pioglitazone, as a PPARγ agonist, can promote the preadipocytes differentiation and triacylglycerol accumulation.[21] So we used pioglitazone as positive control. We found TC could induce differentiation obviously in dose-dependent manner. In addition, pioglitazone had similar effect [Figure 3a]. To further characterize the effects of TC on differentiation, we measured intracellular TG content. The results showed that TC groups could increase TG content in cells with a dose-dependent manner. Compared with control, TG content in cells treated with 1, 5, 25 and 125 µg/mL TC were about 1.25, 1.43, 1.76 and 2.01 fold greater, respectively [Figure 3b].

TC activates the expressions of genes related to preadipocytes differentiation

To explicit whether TC had effect on expression of target genes related to differentiation, we performed RT-PCR to analysis target genes related to differentiation, including
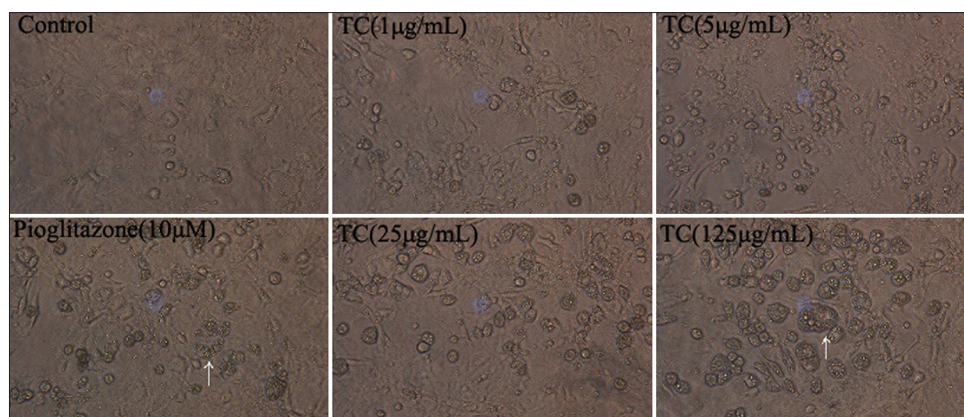
Figure 2: The morphology of 3T3-L1 cells treated without or with Pioglitazone (10 μM) or TC (1 μg/mL, 5 μg/mL, 25 μg/mL, or 125 μg/mL) for 7 days (×200)
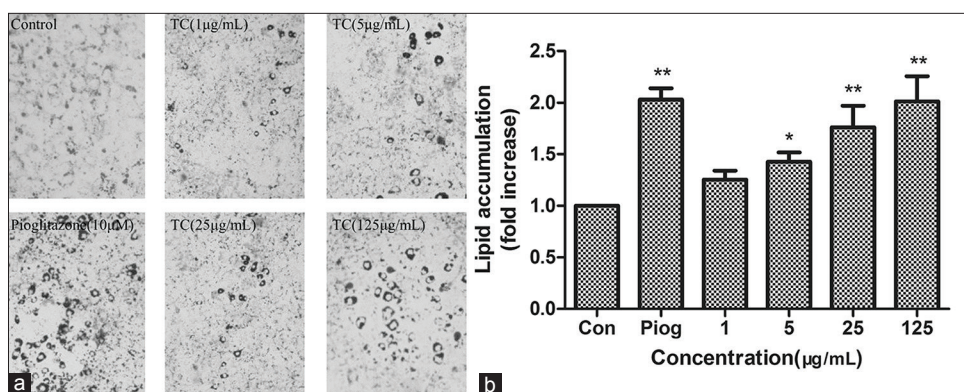

Figure 3: (a) The Red Oil staining of 3T3-L1 cells treated with various concentrations of TC for 7 days (×100). (b) The triglyceride content of 3T3-L1 cells treated with various concentrations of TC for 7 days. Data are expressed as means ± SD (*n*= 6). *P < 0.05, **P< 0.01, TC or Piog vs Control

C/EBP-α, PPARγ and ADD1/SREBP1.[22,23] C/EBPs can regulate differentiation of preadipocytes through multiple paths, mainly by aP2 and GLUT4.[24] PPARγ plays a critical role in regulation of glucose, lipid homeostasis and downstream genes including aP2, GLUT4 and adiponectin.[25-27] Overexpression of PPARγ can induce and accelerate adipocyte differentiation.[28] As shown in Figure 4, TC could promote the expressions of PPARγ, C/EBP-α and ADD1/SREBP-1 in a dose-dependent manner. The expressions of PPARγ and ADD1/SREBP-1 after treatment with TC at 125 μg/mL were comparable to that of pioglitazone and significant higher than that of the control group (P < 0.05), suggesting that TC might have ideal insulin sensitizer effect as pioglitazone. As for C/EBP-α, TC at 125 μg/mL promoted its expression significantly compared with the control group (P < 0.05), but the promotion was not as strong as the pioglitazone group.

The gene aP2 plays an important role in adiposity and other side effects after activating PPARγ.[29-31] We found the expression of aP2 increased significantly in cells treated with TC at 25 and 125 μg/mL (P < 0.05),

but was far lower than that in the pioglitazone group [Figure 5]. Compared with the control group, the expression of aP2 of 25 μg/mL TC group, 125 μg/mL TC group and pioglitazone group was about 2.15, 2.41 and 5.62 fold greater, respectively. The expression of GLUT4 was also significantly up-regulated by TC with concentrations of 25 μg/mL and 125 μg/mL (P < 0.05). What's more, the activation of PPARγ can trigger the expression of LPL which can produce free fatty acid (FFA), and then accumulate TG in cells and promote differentiation further. In this research, TC could increase the expression of LPL about 1.34 fold greater and pioglitazone can increase about 1.89 fold greater than the control group [Figure 5]. Adiponectin, a crucial adipokine secreted by adipocyte, can regulate metabolism of lipid and glucose,[32] which associates with anti-diabetic, anti-inflammatory and anti-atherogenic effects of PPARγ.[33] In this paper, adiponectin [Figure 6] was in agreement with other genes expression. Treating with TC at 25 μg/mL and 125 μg/mL, the expression of adiponectin in cells was significantly increased by 1.86 and 1.98 fold greater than that of the control group.
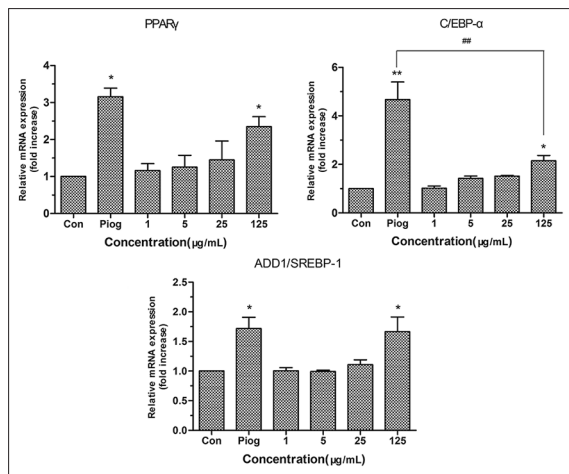
Figure 4: Effects of TC on the expression of main genes related to differentiation in 3T3-L1 adipocytes. The values are expressed as means ± SD of three independent tests.*P<0.05 and **P<0.01 as compared with 1% DMSO-treated control group; ##P<0.01 TC *vs* Piog
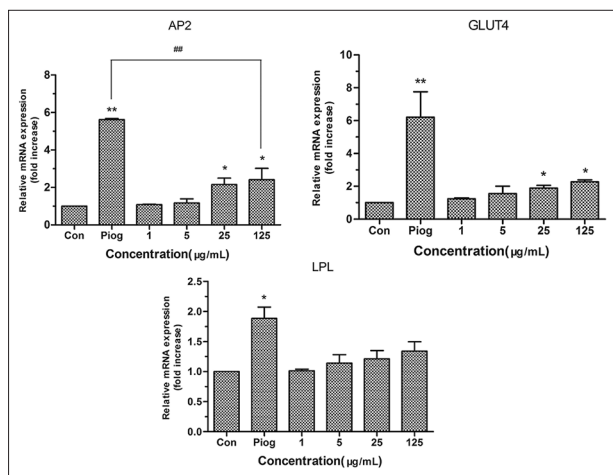

Figure 5: Effects of TC on the expression of main downstream genes related to differentiation in 3T3-L1 adipocytes. The values are expressed as means ± SD (*n*= 3). *P<0.05 and **P<0.01 TC *vs* 1% DMSO-treated control group; ##P<0.01 TC *vs* Piog

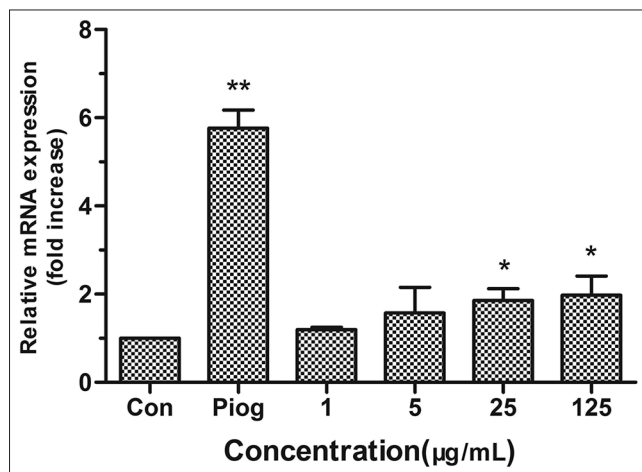**Figure 6:** Effects of TC on the mRNA expression of adiponectin in 3T3-L1 adipocytes. The values are expressed as means ± SD (*n*= 3). *P<0.05 and **P<0.01 as compared with control group

DISCUSSION

T2DM is characterized by persistent hyperglycemia, IR and β-cell dysfunction, leading to macro- and micro-vascular diseases, such as diabetic retinopathy, nephropathy, neuropathy and sexual dysfunction.[34] In the clinic treatment of T2DM, TZDs, as PPARγ agonists, have an outstanding effect. But on the other hand, TZDs are associated with numerous safety issues.[35,36] So the research on exploring some new alternatives is urgent. In Chinese folk medicine, *Agrimonia Pilosa* Ledeb has showed good therapeutic effect on treating T2DM. However, the bioactive components and therapeutic mechanism are still obscure. Understanding these problems could be of value in developing natural drugs to fight IR and T2DM. Considering TC is one of the most abundant components in *Agrimonia Pilosa* Ledeb, we collected TC from *Agrimonia Pilosa* Ledeb and studied its effect on the differentiation of 3T3-L1 preadipocytes to develop new insulin sensitizing agent. Our results indicate that TC can promote 3T3-L1 preadipocytes differentiation and TG accumulation in the differentiated protocol of 3T3-L1 cells. Further studies show that TC can up-regulate the mRNA expression of genes related to insulin sensitization significantly, including PPARγ, SREBP-1 and C/EBP-α. As for downstream genes controlled by PPARγ, TC did not have drastic promoting effects on aP2 and LPL comparing with pioglitazone. But TC could significantly activate the mRNA expression of adiponectin and GLUT4.

PPARγ has an outstanding function in the process of cell differentiation and lipid metabolism. Its agonists significantly ameliorate insulin sensitivity in patients with T2DM.[37] aP2 plays an important role in adiposity, is a key controller for peripheral insulin sensibility and glucose and lipid metabolism.[38] Overexpression of aP2 causes accumulation of lipid and metabolism disorder, so it has a very close relationship with lots of metabolic disorders and has been recognized as a potential marker of undesirable effects.[29-31,39] In our study, induction of aP2 expression by TC was only about 30% compared with pioglitazone. These data suggest that TC can promote expression of PPARγ without activating aP2 sharply.

LPL plays an important role in providing energy and metabolizing lipoprotein. When PPARγ is activated, LPL will express and produce FFA. In turn, FFA can promote PPARγ expression. This process can promote cell differentiation, but also cause accumulation of triacylglycerol in the meantime. In this study, we find that TC can promote LPL gene expression, but less than pioglitazone. The results illustrated TC might reduce the main side effects such as weight gain. Adiponectin, an adipokine, is produced when 3T3-L1 preadipocytes

differentiate to be mature adipocytes. *In vivo*, the level of circulating adiponectin has positive relationship with IR generally and higher adiponectin in fatter people. In normal physiological condition, it improves absorbption of glucose, oxidation of fatty acid and sensibility of peripheral insulin,[40,41] acting as anti-diabetic, anti-atherogenic and anti-inflammatory.[42,43] Our results show that TC plays an obvious role in promoting its expression.

CONCLUSIONS

In conclusion, TC from *Agrimonia Pilosa* Ledeb can promote preadipocytes differentiation through activating the mRNA expression of PPARγ to improve IR. Excitingly, though TC could activate the expression of PPARγ sharply, which was comparable to the effect of pioglitazone, the promotion of TC on the expression of aP2 and LPL did not have drastic effects compared with that of pioglitazone. It implied that TC cannot induce over-production of FFA or worsen IR. In addition, mRNA expression of GLUT4 and adiponectin can also be up-regulated significantly. So TC from *Agrimonia Pilosa* Ledeb has a good prospect as a natural drug for IR and T2DM.

ACKNOWLEDGMENTS

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