



# Lentinan depresses 3T3-L1 fat cell formation by inhibiting PPAR $\gamma$ /AKT signaling pathway

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**ABSTRACT.** We investigated the mechanism of the effect of lentinan on 3T3-L1 fat cell formation by inhibiting the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )/protein kinase B (AKT) signaling pathway. 3T3-L1 fat cells were treated with 80  $\mu$ M lentinan with or without the PPAR $\gamma$  activator, 100  $\mu$ M rosiglitazone for 24 h. Reverse transcription-polymerase chain reaction was applied to detect PPAR $\gamma$  and AKT mRNA expression levels. Western blotting was used to detect AKT protein expression level. Compared with the control group, 80  $\mu$ M lentinan increased PPAR $\gamma$  mRNA expression and downregulated AKT mRNA expression. After treatment with rosiglitazone, PPAR $\gamma$  mRNA expression increased by 78% ( $P < 0.05$ ), while AKT mRNA expression decreased by 71% ( $P < 0.05$ ). Lentinan treatment decreased AKT protein expression by 33%, and AKT protein expression in the lentinan and rosiglitazone co-treatment group was reduced by 28% compared with the lentinan treatment group. We found that 80  $\mu$ M lentinan increased PPAR $\gamma$  mRNA expression and reduced AKT mRNA. Combination treatment with rosiglitazone increased this effect. This suggests that lentinan can depress 3T3-L1 fat cell formation by inhibiting the PPAR $\gamma$ /AKT signaling pathway.

**Key words:** Lentinan; 3T3-L1 fat cells; Peroxisome proliferator-activated receptor gamma/protein kinase B signaling pathway

## INTRODUCTION

Lentinan is an effective active ingredient extracted from high-quality mushroom fruiting bodies. This compound contains branched  $\beta$ -(1-3)-D-glucan with the main chain composed of  $\beta$ -(1-3)-linked glucosyl. The  $\beta$ -(1-6)-linked glucosyl is distributed randomly along the main chain as a comb structure. Recent experiments have indicated that lentinan has inhibitory effect on adipose differentiation (Attia et al., 2013), though the mechanism of this action is unclear. Obesity is common and mainly caused by an imbalance in energy metabolism in the body, while white adipose tissue plays an important role in balancing energy metabolism. Moderate fat levels are essential for normal development. The occurrence of obesity and related diseases are caused by excessive fat deposition. Fat has important effects on insulin levels, and fat generation is very closely related to the insulin signaling pathway. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) and protein kinase B (AKT) play important roles in adipocyte differentiation and lipid formation, though the exact mechanism remains unclear. In this study, we examined the effect of lentinan on PPAR $\gamma$  and AKT expression, investigated the possible signal transduction pathway involved in inhibiting fat differentiation, and suggest a scientific basis for obesity and related disease treatment.

## MATERIAL AND METHODS

### Materials

Lentinan was purchased from Shanghai Pharmaceutical Company (Shanghai, China), fetal bovine serum was purchased from Zhejiang Tianhang Biotechnology Co. Ltd. (Huzhou, China). 3T3-L1 preadipocytes were from American Type Culture Collection (Manassas, VA, USA). Dexamethasone, insulin, 1-methyl-3-isobutyl-xanthine, pioglitazone, fatty acid-free bovine serum albumin, and rosiglitazone were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Trizol reagent and the reverse transcription-polymerase chain reaction kit were purchased from Takara (Shiga, Japan). DMEM high-glucose medium was from Bioss Biotechnology Co., Ltd. (Beijing, China). RIPA cell lysis solution was purchased from Gibco (Grand Island, NY, USA). Primary antibodies were from Millipore (Billerica, MA, USA). The secondary antibody was from and the electrochemiluminescence imaging agent was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Culture, differentiation, and identification of 3T3-L1 preadipocytes were conducted as described in a previous report (Oster et al., 2010). Cells were maintained in high-glucose DMEM medium supplemented with 10% fetal bovine serum in a humid atmosphere containing 5% CO<sub>2</sub> at 37°C. Cells were then maintained in high-glucose inducing DMEM medium (containing 0.5 mM 3-isobutyl-1-methylxanthine, 10% fetal bovine serum, 1  $\mu$ M dexamethasone, and 10  $\mu$ g/mL insulin) at 48 h after contact inhibition for 48 h. The cells were maintained in high-glucose DMEM medium (containing only 10  $\mu$ g/mL insulin) for another 48 h. Finally, the cells were maintained in complete medium (with no inducer) for 8-10 days. More than 90% of the 3T3-L1 preadipocytes differentiated into mature fat cells.

## Lentinan and rosiglitazone solution treatment

### *Preparation of lentinan solution*

First, 80 mg lentinan was dissolved in 1 mL anhydrous as stock solution. The stock solution was dissolved in 0.1 M NaOH and mixed with fatty acid-free bovine serum albumin in a 3:1 ratio (Yu et al., 2009; Yang, 2010).

### *Preparation of rosiglitazone solution*

The final concentration of rosiglitazone solution was 100  $\mu$ M and the preparation method was the same as that used for the lentinan solution.

### *Fat cell treatment*

Mature fat cells were treated with 80  $\mu$ M lentinan with or without 100  $\mu$ M PPAR $\gamma$  agonist rosiglitazone and cultured for 24 h. Each experiment was repeated 3 times.

## PCR detection

Total RNA was extracted using Trizol reagent (Akca et al., 2011; Xu et al., 2011). Next, 1  $\mu$ g total RNA was reverse-transcribed into cDNA using Oligo (dt) as a primer for PCR amplification. The primers used are listed in Table 1.

**Table 1.** Primers for PPAR $\gamma$ , AKT, and  $\beta$ -actin.

	Forward	Reverse
PPAR $\gamma$	5'-ACAGACCTCAGGCAGATCGT-3'	5'-GGGTGAAGGCTCATGTCTGT-3'
AKT	5'-GGAACGTGTGCAGGTGGAT-3'	5'-TCTCCAGCAGTGCCATCTCT-3'
$\beta$ -actin	5'-GATCAATTGCTCCTCTGAGC-3'	5'-ACATCTGCTGGAAGGTGGAC-3'

The cycling conditions consisted of an initial, single cycle for 2 min at 95°C, followed by 40 cycles for 5 s at 95°C and 30 s at 60°C. The fold-change in expression for each mRNA was calculated using the  $2^{-\Delta\Delta Ct}$  method (Sun et al., 2011).

## Western blot

The cells were harvested and homogenized in lysis buffer. Total protein was separated by denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Detection was performed using electrochemiluminescence. Antibody dilutions were 1:1000 for AKT. Protein levels were normalized to  $\beta$ -actin.

## Statistical analysis

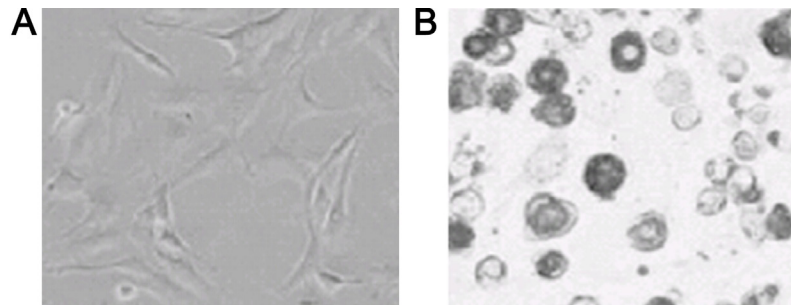
Numerical data are reported as means  $\pm$  standard deviation (means  $\pm$  SD). All statistical analyses were performed using the SPSS13.0 software (SPSS, Inc., Chicago, IL, USA).

Differences between means were analyzed using Student *t*-test. *P* values < 0.05 were considered to be statistically significant.

## RESULTS

### Induction, differentiation, and identification of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were expressed, similar to fibroblasts, with no lipid drops in the cell before induction (Figure 1A). After induction, intracellular lipid drops gradually increased and the cells became larger. By the 8th day, Oil Red O staining showed that small lipid droplets had fused into larger droplets in more than 90% of the cells and the cells contained bright red granules (Figure 1B).



**Figure 1.** Oil Red O staining. **A.** 3T3-L1 preadipocytes (400X). **B.** 3T3-L1 mature fat cells (400X).

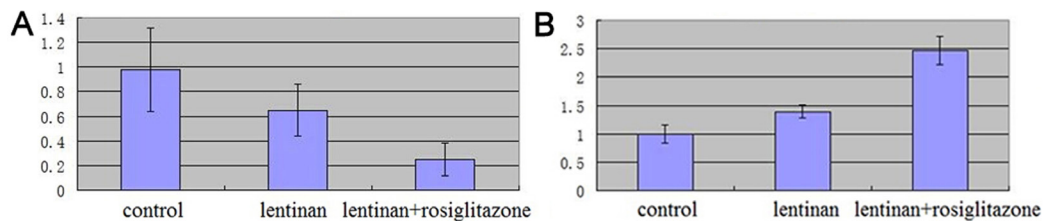
### Effect of lentinan and rosiglitazone on PPAR $\gamma$ and AKT mRNA expression

After treatment with lentinan alone, PPAR $\gamma$  mRNA expression was significantly higher than in the control group, while AKT mRNA expression was lower. When the cells were co-treated with lentinan and rosiglitazone, PPAR $\gamma$  mRNA was overexpressed and AKT mRNA decreased more dramatically (Table 2 and Figure 2).

**Table 2.** Effect of lentinan and rosiglitazone on PPAR $\gamma$  and AKT mRNA expression (means  $\pm$  SD).

Group	Control	Lentinan	Lentinan + rosiglitazone
PPAR $\gamma$	1.00 $\pm$ 0.16	1.39 $\pm$ 0.12 <sup>Δ</sup>	2.47 $\pm$ 0.25*
AKT	0.98 $\pm$ 0.34	0.65 $\pm$ 0.21 <sup>Δ</sup>	0.25 $\pm$ 0.13*

<sup>Δ</sup>Compared with control, *P* < 0.05; \*compared with <sup>Δ</sup>, *P* < 0.05.



**Figure 2.** mRNA expression. **A.** PPAR $\gamma$  mRNA expression in 3 groups. **B.** AKT mRNA expression in 3 groups.

### Effect of lentinan and rosiglitazone on AKT protein expression

AKT protein expression was reduced in the lentinan treatment group compared with the control group, while lentinan and rosiglitazone co-treatment decreased AKT protein more significantly compared with the control group and lentinan treatment group (Figure 3).

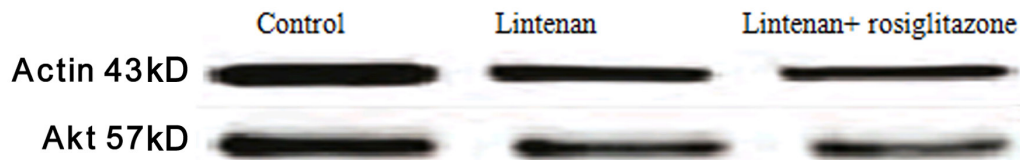


Figure 3. AKT protein expression.

### DISCUSSION

Obesity can cause serious damage to human health; it is common and mainly caused by an imbalance of energy metabolism in the body. Adipose tissue is primarily composed of fat cells. Preadipocytes can differentiate into mature fat cells to provide space for lipid accumulation. The regulation and control preadipocyte differentiation has vital significance for preventing or treating obesity and related diseases.

Adipose tissue is an active endocrine organ that can secrete a variety of cytokines and is very important for maintaining the steady state of glucolipid metabolism. This tissue secretes PPAR $\gamma$ , AKT, and many other cytokines that affect insulin sensitivity. Obesity, particularly central obesity, is positively correlated with insulin resistance. Because fat cells are terminally differentiated, we used the 3T3-L1 preadipocyte cell line in our experiment. 3T3-L1 preadipocytes are isolated from mesoblastic pluripotent stem cells from mouse embryos and can differentiate into mature fat cells under insulin induction. We used lentinan and rosiglitazone solution to intervene in the process of differentiation to study the effect of lentinan on inhibiting fat cells formation.

PPAR $\gamma$  is a member of the nuclear transcription factor superfamily. It is mainly expressed in fat tissue and can regulate glucolipid metabolism and fat cell differentiation. AKT, also known as protein kinase B, is a 57-kD serine/threonine protein that plays an important role in regulating anti-apoptosis (Shultz et al., 2010; Liu et al., 2011; Gray et al., 2013). It plays an important role in regulating apoptosis, proliferation, differentiation, metabolism, and cancer. It exhibits enzymolysis directly or indirectly on the proteins necessary for apoptosis, leading to apoptosis (Huang et al., 2011). The 3 subtypes of AKT include AKT1, AKT2, and AKT3, which show different expression levels in different tissues. AKT1 is expressed high in most normal tissues; AKT2 is overexpressed in insulin target tissues (for example, adipose tissue, liver, and muscle), and AKT3 is overexpressed in the brain. Adipocyte differentiation is closely related to insulin regulation, while AKT2 plays an important role in regulating the insulin response. Thus, we speculated that AKT2 may be important in adipocyte differentiation. It is also an important downstream target kinase in the PPAR $\gamma$ /AKT signaling pathway (Lancaster et al., 2003; Daniels et al., 2011).

PPAR includes PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ . Upregulating PPAR $\gamma$  activity can reduce AKT expression in fat cells (Kong et al., 2011; Zhang and Qian, 2012). The PPAR $\gamma$ /

AKT signaling pathway can regulate cell proliferation, differentiation, and apoptosis, and has an important role in promoting cell proliferation, anti-apoptosis, angiogenesis, fat cell differentiation, and fat particle accumulation (Kawano and Arora, 2009; Wang et al., 2009; Cheng and Leung, 2011). In this study, treatment with lentinan alone caused significant upregulation of PPAR $\gamma$  mRNA expression, indicating that lentinan acting on fat cells can promote PPAR $\gamma$  mRNA expression. AKT mRNA expression was lower after the fat cells were treated with lentinan, suggesting that lentinan may inhibit AKT mRNA expression indirectly by promoting PPAR $\gamma$  mRNA expression. PPAR $\gamma$  mRNA was upregulated significantly after co-treatment with lentinan and rosiglitazone compared with lentinan treatment alone, while AKT mRNA expression was downregulated. Western blotting further confirmed that the AKT protein level was lower in the lentinan treatment group compared to the control group, and was much lower in the co-treatment group compared to the lentinan treatment group. These results support the role of lentinan in the PPAR $\gamma$ /AKT signaling pathway and are consistent with the results of previous reports (Xue et al., 2011; Jang et al., 2012).

Our results showed that lentinan may impact PPAR $\gamma$  and AKT expression and inhibit lipid deposition. We suggest that lentinan may inhibit adipocyte differentiation and lipid deposition through the PPAR $\gamma$ /AKT signaling pathway, as well as promote fat cell apoptosis indirectly. If this pathway can be effectively inhibited by an active inhibitor, our results may lead to new treatments for obesity and related diseases.

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