Laquinimod Prevents Adipogenesis and Obesity by Down-Regulating PPAR-γ and C/EBPα through Activating AMPK

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ABSTRACT: Background and Purpose: obesity is defined as excessive accumulation of adipose tissues and is becoming one of the main global severe public health issues. The present study aims to investigate the anti-adipogenesis of laquinimod and the underlying mechanism.

Methods: a differentiation cocktail was used to differentiate 3T3-L1 cells, and mice were fed with high fat food to establish the obesity animal model. Oil red O staining, glycerol production assay, and the release of triglyceride were used to evaluate the differentiation degree of 3T3-L1 cells. The expression level of sterol regulatory element binding transcription factor 1 (Srebp1), fatty acid binding protein-4 (FABP4), glucose transporter 4 (GLUT4), peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT enhancer-binding proteins (C/EBPα), and phosphorylation of adenosine 5′-monophosphate (AMP)-activated protein kinase α (p-AMPKα) was determined by quantitative real time PCR and western blot analysis. The pathological state of adipose tissues was evaluated by hematoxylin and eosin staining. Results: the amount and UV absorption of oil red O, glycerol production, release of triglyceride, and the expression of SREBP1, FABP4, and GLUT4 in differentiated 3T3-L1 cells were decreased by the administration of laquinimod. PPAR-γ and C/EBPα were down-regulated, and p-AMPKα was up-regulated by laquinimod. The down-regulated PPAR-γ and C/EBPα, as well as the inhibited lipid accumulation functioned by laquinimod, were reversed by the coinubation with the AMPK inhibitor compound C. Decreased body weight, visceral adipocyte tissue weight, and size of adipocytes were observed in in vivo obesity mice after administration with laquinimod. Conclusion: laquinimod might prevent adipogenesis by down-regulating PPAR-γ and C/EBPα through activating AMPK.

1. INTRODUCTION

Obesity is mainly defined as excessive accumulation of adipose tissues in our body, which is mainly resulted from increased size and amounts of adipocytes. Currently, body mass index (BMI) is used to identify obesity. BMI = body weights (kg)/height² (m) is utilized to calculate the value of BMI. In Asia, the body with BMI larger than 23 is regarded as overweight and larger than 25 is regarded as obesity.1-3 As the living standards improve, excessive uptake of food with high energy, such as deep fried food and puffed food, contributes to the sharp increase of obese people, which makes obesity one of the global severe public health issues. Not only chronic diseases, such as type II diabetes, cardiovascular-related diseases, and steatohepatitis, but also malignant tumor can be derived from obesity.4-6 Therefore, it is of great significance to investigate the pathological mechanism of obesity and explore the potential therapeutic food or drugs.

Adipose tissues are composed of multiple types of cells, such as endothelial cells, blood cells, fibroblasts, pre-adipocytes, macrophages, and other lymphocytes, among which mature adipocytes are mostly involved.6 The transformation from pre-adipocytes to mature adipocytes is mediated by plenty of transcription factors with both inhibitory and activation effects. CCAAT enhancer binding proteins (C/EBPs), peroxisome hyperplasia activated receptors (PPARs), and sterol response element-binding protein-1 (SRENP-1) are reported to be main transcription factors that regulate the transformation. C/EBPs belong to an alkaline leucine zipper transcription factor family, which is named because of their activation on the CCAAT sequence of enhancer for a specific target gene. McKnight first reported the effects of C/EBPs on lipolysis.7 By binding with the ligands, PPARγ can interact with retinoid X receptor to form a heterodimer, which subsequently binds with the peroxisome proliferator response elements to activate PPARγ. In this way, the transcription of targets genes that are involved in the metabolism of energy and lipids, inflammation, and insulin sensitivity will be regulated.8,9 In addition, the activation of the AMPK signal pathway is also reported to be involved in the progress of adipogenesis. Wang reported that the osteogenesis was promoted and the adipogenesis was
inhibited by the activation of AMPK through the AMPK-G61-OPN axis.\textsuperscript{10} To suppress obesity, effective improvement might be achieved by targeting these transcription factors or AMPK signal pathway.

Laquinimod, the structure of which is shown in Figure 1, is an oral effective immune regulator verified by III phase clinical trials,\textsuperscript{11} which is reported to up-regulate the population of type 1 T helper lymphocytes (Th1) and down-regulate the population of type 2 T helper lymphocytes (Th2) to mediate the immune system.\textsuperscript{12} In the present study, the inhibitory effects of laquinimod against adipogenesis, as well as the underlying mechanism, will be investigated to claim the potential therapeutic effects of laquinimod on obesity.

2. RESULTS

2.1. Effects of Laquinimod during 3T3-L1 Adipogenesis. Two concentrations (5, 10 μM) of laquinimod were incubated with differentiated 3T3-L1 cells. Treated cells were stained with Oil Red O on the Day 8. As shown in Figure 2A, compared with 3T3-L1 cells, more Oil Red O staining was observed in the mature adipocytes. The amount of Oil Red O staining was attenuated significantly as the dosage of laquinimod increased from 0 to 10 μM. Figure 2B shows that by differentiating 3T3-L1 cells, the absorbance at 540 nm of Oil Red O of mature adipocytes was increased greatly, which was inhibited by the treatment with laquinimod in a dose-dependent manner.

2.2. Triglyceride Content was Inhibited, and Lipolysis was Induced by Laquinimod during 3T3-L1 Adipogenesis. To explore the effects of laquinimod on triglyceride release and lipolysis, 5 and 10 μM laquinimod was used to incubate with differentiated 3T3-L1 cells, and the concentrations of triglyceride and glycerol were detected. As shown in Figure 3A, the total contents of triglyceride in 3T3-L1 cells, differentiated 3T3-L1 cells, 5 μM Laquinimod-treated differentiated 3T3-L1 cells, and 10 μM Laquinimod-treated differentiated 3T3-L1 cells were 9.6, 31.5, 22.3, and 15.1 nmol/ng protein, respectively. Significant increased release of triglyceride was observed in differentiated 3T3-L1 cells, compared with 3T3-L1 cells (***, $P < 0.0001$ vs vehicle group), which was suppressed by laquinimod at a dose-dependent manner. The release of glycerol was recorded in Figure 3B. The release speeds of glycerol in 3T3-L1 cells, differentiated 3T3-L1 cells, 5 μM laquinimod-treated differentiated 3T3-L1 cells, and 10 μM laquinimod-treated differentiated 3T3-L1 cells were 6.3, 12.6, 17.5, and 19.8 nmol/mg protein/h, respectively. These data indicate that the lipolysis was promoted during the progress of differentiation of 3T3-L1 cells, which was suppressed by laquinimod at a dose-dependent manner.

2.3. Effects of Laquinimod on the Expression of Adipogenic and Lipogenic Genes. Two concentrations (5, 10 μM) of laquinimod were incubated with differentiated 3T3-L1 cells, 5 μM Laquinimod-treated differentiated 3T3-L1 cells, and 10 μM Laquinimod-treated differentiated 3T3-L1 cells were 9.6, 31.5, 22.3, and 15.1 nmol/ng protein, respectively. Significant increased release of triglyceride was observed in differentiated 3T3-L1 cells, compared with 3T3-L1 cells (***, $P < 0.0001$ vs control), which was suppressed by laquinimod at a dose-dependent manner. The release of glycerol was recorded in Figure 3B. The release speeds of glycerol in 3T3-L1 cells, differentiated 3T3-L1 cells, 5 μM laquinimod-treated differentiated 3T3-L1 cells, and 10 μM laquinimod-treated differentiated 3T3-L1 cells were 6.3, 12.6, 17.5, and 19.8 nmol/mg protein/h, respectively. These data indicate that the lipolysis was promoted during the progress of differentiation of 3T3-L1 cells, which was suppressed by laquinimod at a dose-dependent manner.
binding protein-4 (FABP4), and glucose transporter 4 (Glut4) detected by quantitative real time PCR (qRT-PCR) is shown in Figure 4A—C. SREBP1 was significantly down-regulated on Day 3 by the introduction of laquinimod and remained the same level as control on Day 6 and 8. However, FABP4 and Glut4 were greatly down-regulated on Day 3, 6, and 8 by the introduction of laquinimod. These data indicated that laquinimod has an inhibitory effect on the expression of adipogenic and lipogenic genes.

2.4. Expression of Adipogenic and Lipogenic Transcriptional Factors Was Inhibited by Laquinimod. To explore the effects of laquinimod on the expression of adipogenic and lipogenic transcriptional factors, 5 and 10 μM laquinimod were used to incubate with differentiated 3T3-L1 cells. The gene and protein expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT enhancer-binding proteins (C/EBPα) were evaluated by qRT-PCR and western blot analysis. As shown in Figure 5A—D, PPAR-γ and C/EBPα were significantly up-regulated during the differentiation of 3T3-L1 cells, which were inhibited by the interference of laquinimod at a dose-dependent manner.

2.5. Laquinimod Suppressed Autophagy in 3T3-L1 Cells. Autophagy plays an important role in adipocyte differentiation. The ratio of LC3-II to LC3-I has been considered as a marker of autophagy. It has been shown that the LC3-II:LC3-I ratio increased significantly by approximately 4-fold in 3T3-L1 cells cultured in DMI, whereas co-treatment with laquinimod (5, 10 μM) significantly prevented the increase in LC3-II:LC3-I ratio in a dose dependent manner (Figure 6).

2.6. Inhibitory Effects of Laquinimod on 3T3-L1 Adipogenesis Is Mediated by AMPK. To investigate the potential mechanism on the inhibitory effects of laquinimod on 3T3-L1 adipogenesis, the expression level of p-AMPKα was evaluated following the introduction of laquinimod and the AMPK inhibitor compound C was used. As shown in Figure 7A, the expression level of p-AMPKα was inhibited during the differentiation of 3T3-L1 cells, which was restored by the incubation of laquinimod at a dose-dependent manner. The effects of AMPK inhibitor compound C on the inhibitory function of laquinimod on 3T3-L1 adipogenesis are recorded in Figure 7B—D. The down-regulated PPAR-γ and C/EBPα by laquinimod were significantly up-regulated by the co-incubation of the AMPK inhibitor. The absorbance at 540 nm of oil red O was decreased from 1.68 to 0.62 by the administration of laquinimod, which was promoted to 1.55 after the co-incubation of AMPK inhibitor compound C. These findings suggest that blockage of AMPK with its inhibitor compound C abolished the protective effects of laquinimod against adipogenesis. Further in vivo study with a AMPK deficiency rodent model will further verify this mechanism.

2.7. Laquinimod Decreased the Weight of Visceral Adipocyte Tissues and the Body Weight of HFD-Induced Obese Mice. To evaluate the inhibitory effects of laquinimod on adipogenesis in vivo, obese mice model was established by feeding the animals with high fat diet (HFD). As shown in Figure 8A, the average body weight of mice was significantly increased by feeding the animals with HFD, which was decreased by administration with 12.5 and 25.0 mg/kg laquinimod in a dose-dependent manner. The average visceral adipose tissue weight (Figure 8B) was promoted from 0.36 to 3.72 g by feeding the animals with HFD, which was suppressed to 2.57 and 1.53 g by administrating with 12.5 and 25.0 mg/kg laquinimod, respectively. The hematoxylin–eosin stainings on the adipose tissues are shown in Figure 8C. Obvious larger size and amounts of adipocytes were observed in HFD fed mice, compared with control. The size of adipocytes shrank and the amounts of adipocytes decreased by the administration of 12.5 and 25.0 mg/kg laquinimod, respectively. The hematoxylin–eosin stainings on the adipose tissues are shown in Figure 8C. Obvious larger size and amounts of adipocytes were observed in HFD fed mice, compared with control. The size of adipocytes shrank and the amounts of adipocytes decreased by the administration of 12.5 and 25.0 mg/kg laquinimod, respectively. The hematoxylin–eosin stainings on the adipose tissues are shown in Figure 8C. Obvious larger size and amounts of adipocytes were observed in HFD fed mice, compared with control. The size of adipocytes shrank and the amounts of adipocytes decreased by the administration of 12.5 and 25.0 mg/kg laquinimod, respectively.

3. DISCUSSION

Obesity is becoming more and more popular because of over-uptake of high-energy food and lacking of durable exercise as the growth in the living standard. Although obesity is not defined as a threatening disease, it is one of the main factors that resulted in type II diabetes, cardiovascular diseases, hypertension and other chronic metabolism related diseases. Excess energy will be transformed to fat when energy expenditure is exceeded by nutrient uptake, which finally contributes to obesity. The differentiation from pre-adipocytes to mature adipocytes is reported to play an important role in the progress of obesity. In the present study, a differentiation cocktail was used to induce the differentiation from 3T3-L1 cells to mature adipocytes, which was verified by the increased number and absorption
of Oil Red O staining, promoted triglyceride and glycerol release, and up-regulated adipogenic and lipogenic genes. In addition, high fat food was used to feed mice to establish the obesity model in mice, which was verified by increased body weight and visceral adipocyte tissue weight, promoted amount, and size of adipocytes. The in vitro and in vivo obesity models were used in the present study to evaluate the effects of laquinimod on adipogenesis. By the introduction of laquinimod, the decreased number and absorption of Oil Red O staining, inhibited triglyceride and glycerol release, and down-regulated adipogenic and lipogenic genes were observed in the differentiated 3T3-L1 cells, and the decreased body weight, visceral adipocyte tissue weight, and amount and size of adipocytes were observed in in vivo obesity mice. These data indicated that laquinimod exerts promising anti-adipogenesis effects both in vitro and in vivo.

PPAR-γ is mainly expressed in adipose tissues and a transcription factor that regulates the specific differentiation of adipocytes. Multiple types of biological reactions will be initiated by the activation of PPAR-γ, such as morphology changes, adipose accumulation, and the allergy to insulins. It is verified in the recent adipogenesis deficient reports that PPAR-γ is essential and sufficient on inducing the differentiation of adipocytes. PPAR-γ-deficient mice achieved by traditional homozygous deletion of related genes died in 10 days because of placental developmental defect, and the adipocytes were undetectable in the 10 days. Rosen reported that chimeric mice were achieved by inserting the wild type ES cells and PPAR-γ deficient ES cells. The PPAR-γ deficient ES cells were found to be differentiated into multiple types of tissues except for adipose tissues in mice, which indicated that PPAR-γ was essential for the differentiation of adipose tissues. In the present study, PPAR-γ was found to be highly expressed in differentiated 3T3-L1 cells and adipose tissues from high fat food fed mice. By administering laquinimod, PPAR-γ was found to be down-regulated in both differentiated 3T3-L1 cells and adipose tissues from high fat food fed mice. These data indicated that PPAR-γ might exert
an important role in the anti-adipogenesis effects of laquinimod.

C/EBPα is reported to play important roles in the advanced stage of adipocytes differentiation.22 Plenty adipocytes differentiation-related genes are found to be up-regulated as soon as the expression level of C/EBPα is promoted. The differentiation of 3T3-L1 cells could be induced by the overexpression of C/EBPα under the situation of lacking of hormones,23 which could be reversed by the introduction of C/EBPα siRNA.24 In the present study, we also found that C/EBPα was highly expressed in differentiated 3T3-L1 cells and adipose tissues from high fat food fed mice. By the treatment of laquinimod, C/EBPα was found to be down-regulated in both differentiated 3T3-L1 cells and adipose tissues from high fat food fed mice. These data indicated that C/EBPα was significantly involved in the anti-adipogenesis effects of laquinimod. Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) is an AMP-dependent protein kinase, which was found to be inactivated in the process of adipogenesis and regulate the expression of PPAR-γ and C/EBPα.25,26 In the present study, we found that p-AMPKα was down-regulated in the differentiated 3T3-L1 cells, the expression level of which was elevated greatly by the introduction of laquinimod. However, the expression level of PPAR-γ and C/EBPα was promoted by co-incubation of the differentiated 3T3-L1 cells with both laquinimod and AMPK inhibitor, as well as the increased lipid accumulations. These data indicated that the biological effects of laquinimod on the expression level of PPAR-γ and C/EBPα and the adipogenesis might be related to the activation of AMPK. However, the deep molecular relationship should be investigated between laquinimod and AMPK, as well as the interaction between laquinimod and AMPK in obesity animal models.

Taken together, our data indicated that laquinimod might prevent adipogenesis by down-regulating PPAR-γ and C/EBPα through activating AMPK.

4. MATERIALS AND METHODS

4.1. Cell Culture and Adipocyte Differentiation. 3T3-L1 adipocytes were purchased from Wuhan Punosai Life Science and Technology co. LTD and were incubated in the DEME medium containing 5% fetal calf serum. Two days after
the cells were completely fused, the cultural medium was
changed to complete differentiation cocktail (DMI) medium
and dexamethasone were removed 48 h later and the cells were
incubated with medium only containing 5 μg/mL insulin. 3T3-
L1 cells were differentiated by incubation in cell culture
medium by adding a differentiation cocktail (DMI) with
laquinimod (5, 10 μM) [TEVA Pharmaceuticals Industries,
Ltd (Israel)] for 8 days. The mature adipocytes were used in
the subsequent studies.27

4.2. Oil Red O Staining. The differentiated adipocytes
were stained with oil red O according to the instructions of the
manufacturer (Abcam, USA). Briefly, the mature adipocytes
were washed with phosphate buffer saline (PBS) and fixed
with formalin solution for 20 min, which was then stained with oil
red O solution for 30−40 min at room temperature. The red
oil droplets staining in the differentiated adipocytes were
pictured by a microscope (Olympus).

4.3. Glycerol Production Assay. A Glycerol Assay Kit
(Thermo Fisher Scientific) was used to detect the release of
glycerol according to the protocol of the manufacturer. Briefly,
the supernatants of the differentiated adipocytes were collected
to be mixed with the reagents and added into the a 96-well
plate. The absorption at 570 nm was determined by a
microplate reader (Thermo Fisher Scientific, USA).

4.4. Measurement of Total Content of Triglyceride in
Cells. The differentiated adipocytes were incubated with the
test articles for 72 h and collected to be lysed with lysis buffer
(1% Triton X-100 in PBS) for 45 min. A commercial kit
(Zenbio) was used to evaluate the triglyceride content.
Reagent A, reagent B, glycerol standard (10 mM), and diluent
were involved in the commercial kit and were added step by
step according to the protocol. A microtiter plate reader was
used to read the absorption values, which was recorded as
glycerol reading and used to calculate the triglyceride level
from the standard curve.

4.5. qRT-PCR. Total RNA was isolated from cells and tissue
samples using TRIzol reagent (Thermo Fisher Scientific,
USA). Then, 2 μg of the isolated total RNA was used to
synthesize cDNA with the PrimeScript kit (Thermo Fisher
Scientific, USA). PCR was performed with the TransStart Tip
Green qPCR SuperMix kit (GenScript). The 2−ΔΔCt method
was used to compare the relative expression levels of target
genes.28

4.6. Western Blotting. Cells were collected and lysed in
RIPA buffer (Thermo Fisher Scientific) to generate crude
extracts, and the proteins in the extracts were separated by 15%
SDS−PAGE. Subsequently, the separated proteins were
transferred to PVDF membranes (Bio-Rad). The membranes
were blocked by incubation with 5−10% BSA solution for 1−2
h. Then, the membranes were incubated with primary
antibodies against PPAR-γ, C/EBPα, AMPKα, and β-actin
(Abcam, USA) at 25 °C for 2 h. After washing, the blot was

Figure 8. Laquinimod decreased the weight of visceral adipocyte tissues and the body weight of HFD-induced obese mice. (A) Growth curve; (B) visceral adipocyte tissue weight; (C) histological sections of visceral adipocyte tissue in mice; and (D) quantification of adipocyte size (****, p < 0.0001 vs vehicle group; $$, $$$$, P < 0.01, 0.0001 vs DMI treated group).
incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000, Abcam) at 25 °C for 1–2 h. Finally, the blots were incubated with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and visualized with an Amersham Imager 600 (GE).

4.7. High-Fat Diet Mice Model and Administration of Laquinimod. Male C57BL/6 4-week-old mice were acclimatized on a normal diet (ND) for 1 week. Mice were randomly subdivided into 4 groups of 8 mice which were treated as follows for 16 weeks: Normal group mice were fed a ND (composed of 12%-fat, 23%-protein, 65%-carbohydrates based on caloric content); the HFD group mice were fed an HFD (composed of 40%-fat, 20%-protein, 40%-carbohydrates based on caloric content). The HFD + laquinimod group mice were fed an HFD diet and administered orally every day by oral gavage in a volume of 100 µL (12.5, 25 mg/kg laquinimod). The normal and HFD group mice were orally gavaged with water. Mice were maintained on 12:12 h light-dark cycles (lights on at 06:00).

4.8. Hematoxylin–Eosin Staining. Adipocyte tissues of each animal were collected and washed over by sterile water for a couple of hours. The tissue was dehydrated by 70, 80, and 90% ethanol and xylene. After 15 min incubation, the tissue was sectioned, and stained with hematoxylin and eosin. Pictures were taken using an inverted microscope (Olympus).

4.9. Statistical Analysis. Data are shown as the mean ± standard deviation (SD). GraphPad prism 7 was used to analyze the data. Analysis of variance followed by the Bonferroni’s post-hoc test was used to compare experimental data in different groups. A difference with a P value less than 0.05 was considered as statistically significant.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

Srebp1, sterol regulatory element binding transcription factor 1; FABP4, fatty acid binding protein-4; GLUT4, glucose transporter 4; PPAR-γ, peroxisome proliferator-activated receptor-γ; C/EBPα, CAAT enhancer-binding proteins; AMPK, AMP phosphorylation of adenosine 5′-monophosphate; p-AMPKα, activated protein kinase α; qRT-PCR, quantitative real time PCR; PBS, phosphate buffer saline; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SD, standard deviation; ANOVA, analysis of variance; HE, hematoxylin–eosin; RXR, retinoid X receptor

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