Tanshinone IIA Improved Non-alcoholic Fatty Liver Disease via A β2-AR-LKB1-AMPK Signaling Axis

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Abstract: No therapeutic options currently exist for nonalcoholic fatty liver disease (NAFLD). Although commonly prescribed for cardiovascular diseases, Danshen (Salvia miltiorrhiza Bunge) has been used historically in folk medicines against chronic liver diseases, but its potential in NAFLD therapy remains uncertain. Here we reported that Tanshinone IIA (TAN-IIA), a principal constituent of Danshen, effectively ameliorated experimental NAFLD via transactivation of AMP-activated protein kinase. Using a mouse model, we established that tanshinone IIA effectively attenuated high-fat-diet induced obesity, hepatomegaly and liver steatosis. Mechanistically, we found that β2-adrenergic receptor (β2-AR) was down-regulated in liver tissues of obese mice. Tanshinone IIA might function as a β2-AR agonist, increase the intracellular cAMP levels, and turn on liver kinase B1 (LKB1)-AMP-activated protein kinase (AMPK)-acetyl-CoA carboxylase 2 (ACC2) signaling axis to promote fatty acid oxidation. Collectively, tanshinone IIA might merit investigation as a potential therapeutic agent for NAFLD especially in those patients with obesity.

Key words: NAFLD;Tanshinone IIA; β2-adrenergic receptor; AMP-activated protein kinase

0 Introduction

Nonalcoholic fatty liver disease (NAFLD) is now a growing public health issue in western society [1]. The clinical implications of NAFLD are mainly from its high prevalence rate and potential to serious liver diseases in latter stage [2]. For example, NAFLD now represents the most common live disease in the United States, and affect up to 25% of general population. Although the etiology of NAFLD remains largely unknown, the associated risk factors include obesity, hyperlipidemia, and type 2 diabetes mellitus. Unfortunately, NAFLD is often asymptomatic and thus historically draw little research interest. Although it can be partly reversed by exercise or weight loss, the patient compliance is still rather low. Worse, no effective therapeutic options currently exist for NAFLD. As such, there is an urgent need for development of new pharmacotherapies for NAFLD management [3-5].

In addition to cardiovascular diseases, Danshen (Salvia miltiorrhiza Bunge) has been widely prescribed for chronic liver diseases in Traditional Chinese Medicine, but such potential has been all but forgotten by modern biomedicine science [6]. During the process of identifying novel hepatic protective agents, Tanshinone IIA, the major active component of Danshen, drew our attention. Lipid peroxidation and the following oxidative injury have long been implicated in NAFLD. In nature, NAFLD is a group of chronic inflammatory liver diseases. In this regards, Tanshinone IIA exhibits excellent antioxidant and anti-inflammatory properties. Patients with hyperlipidemia are at increased risk of NAFLD. More recently, several studies reported its potential beneficial effects on hyperlipidemia [7]. Nonetheless, research efforts so far have centered on metabolic benefits and liver injury, its therapeutic efficacy in NAFLD remains largely unknown. In this study, we investigated the therapeutic efficacy of Tanshinone IIA against NAFLD and clarified the underlying molecular mechanism(s) of action.

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1 Materials and Methods

1.1 Chemicals and Reagents

Primary antibodies against CaMKKβ (sc-271674) and β2-AR (sc-271322) were from Santa Cruz Biotechnology (Santa Cruz, CA). All other primary antibodies were obtained from Cell Signaling Technology (Beverly， MA). Hairpin small interfering RNA (siRNA) template oligonucleotides (β2-AR siRNA, sc-39866; control siRNA, sc-36869) were chemically synthesized by Santa Cruz Biotechnology. Calcium Fluorescent Probe was purchased from Beyotime Biotechnology (Nanjing, China). All chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

1.2 Cell Culture

Human hepatocytes (HepG2) cells were obtained from ATCC and maintained following their instructions. For RNA interference, HepG2 cells at 60–80% confluence were transiently transfected with β2-AR or control siRNA by using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA).

1.3 Intracellular Ca^{2+} Concentration Determination

The intracellular Ca^{2+} concentration was measured as described [8]. After drug treatment for 1 h, HepG2 cells were incubated with a Ca^{2+}-sensitive fluorochrome Fluo-4/acetoxymethyl ester (Fluo-4/AM, 2.5 μM, 30 min) at 37 °C in the dark, rinsed and suspended with PBS, and immediately analyzed by a BD FACSCalibur Flow Cytometer (San Jose, CA). The excitation and emission wavelengths were 488 nm and 516 nm, respectively. The intracellular Ca^{2+} concentration was finally calculated from the fluo-4 fluoresce intensity.

1.4 Intracellular Cyclic AMP Determination

Briefly, 1.0×106 HepG2 cells had been treated Tanshinone IIA (20 μmol/L) with or without a highly selective β2-AR antagonist ICI118-551 (1 μmol/L) for 30 minutes. The cAMP level was measured by a cAMP ELISA Kit (ImmunoWay Biotechnology Company, Newark, DE). The cAMP level was finally expressed as picomoles per milligram of protein [9].

1.5 Western Blot

After drug treatment, cells were harvested in RIPA buffer, and protein concentration of cell lysates was determined by Bradford assay. Protein samples (20 ug) were analyzed by Western blot. And final protein bands were visualized using an enhanced chemiluminescence reagent (GE Healthcare, Pittsburgh, PA).

1.6 Mouse Model Studies

All animal studies were performed by following the guidelines of the Institutional Animal Ethics Committee of Jiangnan University (Protocol number JN20181115c0400430). A high-fat-diet (HFD) mouse model was adopted to induce NAFLD as described previously [10]. The 6-week-old male C57BL/6 mice were fed ad libitum with control diet (1022, Beijing HFK Bioscience Co., Ltd.) or high-fat diet (DIO-H10060, Beijing HFK Bioscience Co., Ltd.) for 14 weeks. The HFD-fed mice were then divided into three groups (n = 8), and treated orally once daily with TAN-IIA (50 mg/kg), metformin (100 mg/kg) or the vehicle (0.5%, v/v,

carboxymethylcellulose sodium in normal saline) from 15-19 weeks. Metformin has been reported to attenuate NAFLD and thus used as positive control [2]. Body weights and fasting blood glucose were measured once a week. The oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed at week 18, and the metabolic rate and activity of mice were evaluated at week 19. At the end of the experiment, mice were euthanized after 12 h of fasting. Liver tissues were collected for either western blot or histology study (hematoxylin and eosin staining). Blood samples were centrifuged at 2000*g* for 15 min, and the supernatant was designated as plasma. Concentration of plasma non-esterified fatty acid (NEFA) was determined using a commercial kit (A042-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plasma total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by an automatic biochemistry analyzer (Beckman CX7, Chaska, MN).

1.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 Software. The Student's t test was used to compare data between two groups. To measure the strength of association between two variables, Pearson correlation was used. Values are expressed as the means ± SD. Differences were regarded as statistically significant if $P \leqslant 0.05$.

2 Results

2.1 TAN-IIA Ameliorates Diet-induced Obesity

Patients with obesity are at increased risk of NAFLD. Using HFD mouse model, the development of obesity and metabolic syndrome was successfully induced at week 14. Once treated with TAN-IIA, obese mice failed to gain additional body weight from HFD (Figure 1A). TAN-II also significantly lowered the accumulation of epididymis fat in obese mice (Table 1). After 5 weeks of TAN-II treatment, the fasting blood glucose levels of obese mice became normal (Figure 1B). TAN-IIA treatment resulted in an improvement either in glucose tolerance or insulin tolerance (Figure 1C and D). More importantly, TAN-IIA even exhibited comparable anti-hyperglycemic effect to metformin, a first-line therapeutic drug for type-2 diabetes.

Tab. 1 Effect of Tan-IIA on Epididymal Fat and Serum Lipid Parameters

Parameter	NC	HFD	HFD+ TAN-IIA	HFD+ MET
Number of animals	8	8	8	8
Epididymis fat weight (g)	0.48(0.08)	2.58(0.30)	1.61(0.28) ***	1.34(0.43) ***
Epididymis fat /body weight (%)	1.83(0.26)	6.13(0.97)	4.87(0.73) *	4.08(0.97) **
NEFA (mmol/L)	0.21(0.03)	0.67(0.11)	0.33(0.08) ***	0.32(0.06) ***
LDL-C (mmol/L)	0.24(0.06)	0.69(0.19)	0.45(0.08) ***	0.38(0.06) ***

Data are presented as the mean ± SD (n = 8). The asterisks indicate a significant difference compared to HFD vehicle group (* , $p < 0.05$; * * , $p < 0.01$; * * * , $p < 0.001$). NEFA, non-esterified fatty acid; LDL-C, low-density lipoprotein cholesterol.

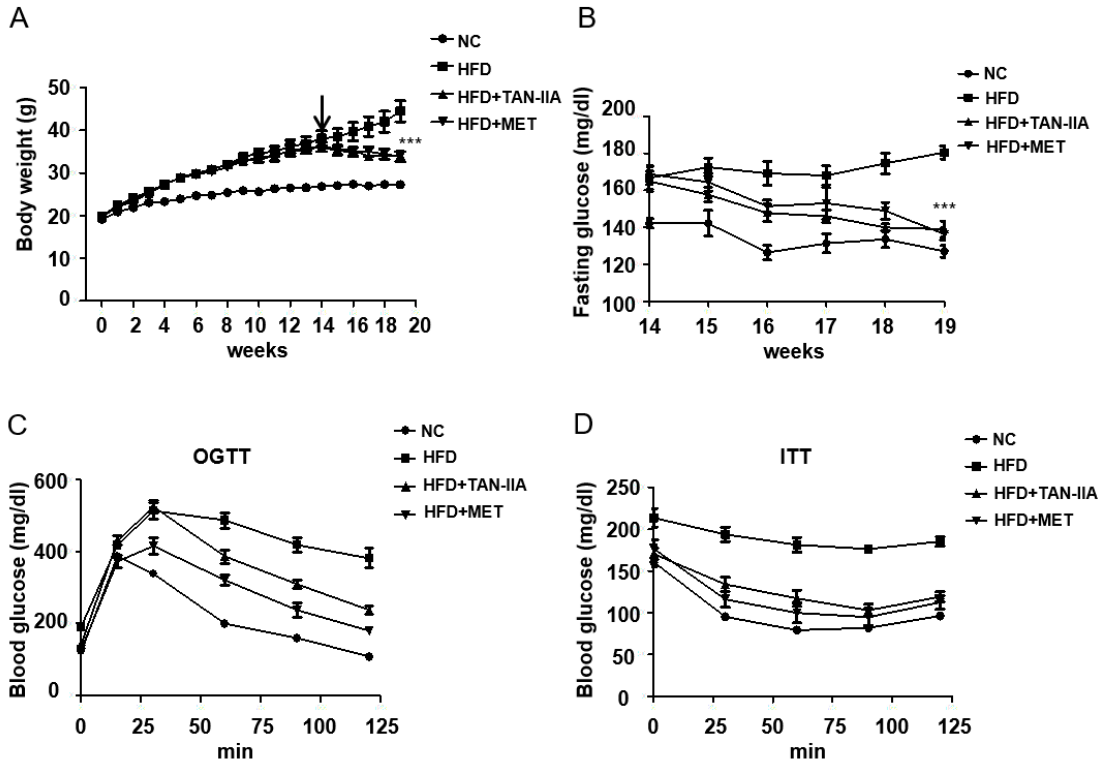

Figure 1. TAN-IIA Treatment Improves Diet-induced Obesity and Metabolic Syndrome. Effects of TAN-IIA treatment for 5 weeks on HFD mice. (A) Body weight; (B) fasting glucose; (C) GTT; (D) ITT. Data are presented as the mean ± SD (n= 8). The asterisks indicate a significant difference compared to each respective vehicle group (* * * , $p < 0.001$).

2.2 TAN-IIA Reversed Experimental NAFLD

TAN-IIA treatment improved hepatomegaly, liver steatosis, fatty infiltration, and aminotransferase abnormalities in obese mice (Figure 2). A net retention of lipids within hepatocytes is accepted as not only a prerequisite but also a hallmark of NAFLD [1]. Our data showed that it was virtually cleaned up after TAN-IIA treatment for 5 weeks (Figure 2E). Taken together, TAN-IIA might hold promise for NAFLD therapy especially in obese humans.

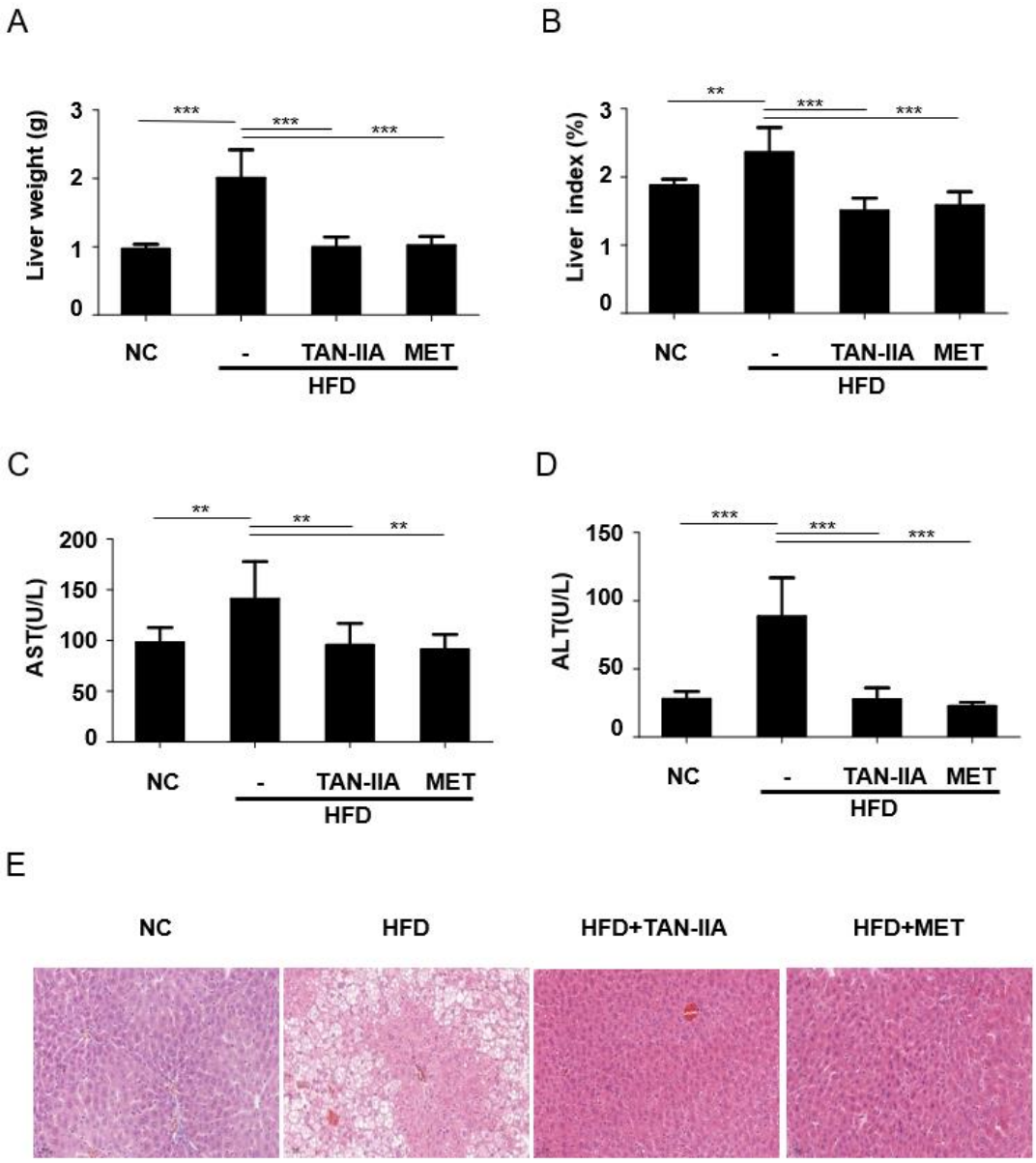

Figure 2. TAN-IIA Treatment Reverses Obesity-induced Hepatic Steatosis. Effects of TAN-IIA on experimental non-alcoholic fatty liver diseases. (A) Liver weight. (B) Liver index. (C) Plasma ALT activity. (D) Plasma AST activity. Data are presented as the mean ± SD (n = 8). The asterisks indicate a significant difference compared to each respective vehicle group (＊＊, $p < 0.01$; ＊＊＊, $p < 0.001$). (E) Hepatic histology. Histological studies were based on H&E staining, as described. Original magnification = 200×.

2.3 TAN-IIA Transactivated AMPK to Improve Lipid Metabolism

NAFLD is frequently associated with lipid metabolic abnormalities. Accordingly, we checked the effect of TAN-IIA on plasma lipid parameters (Table 1). In this connection, we found that the concentrations of LDL-C and free fatty acid (NEFA) were significantly lowered after TAN-IIA treatment. The levels of cholesterol and triglycerides dropped as well, but they did not reach statistical significance.

In addition to its pivotal role in lipotoxicity, FFA might participate in cellular energy homeostasis by serving as either a direct energy source or a precursor for ketone body formation [11]. We then questioned whether TAN-IIA affected hepatic lipid metabolism (Figure 3). Adenosine monophosphate activated protein kinase (AMPK) is known as a key cellular sensor as

well as master regulator in energy homeostasis [12,13]. As expected, TAN-IIA treatment activated AMPK and its downstream targeting enzyme acetyl-CoA carboxylase 2 (ACC2) [14,15]. Meanwhile, TAN-IIA treatment increased phosphorylation of Ser428 on liver kinase B1 (LKB1). As to another upstream kinase of AMPK, we observed that TAN-IIA enhanced the cytosolic free Ca2+ levels in HepG2 cells, while TAN-IIA-induced AMPK phosphorylation could be partly attenuated by a STO-609, a highly selective inhibitor of Ca/Calmodulin-dependent protein kinase kinase β (CaMKKβ). Taken together, TAN-IIA might promote hepatic fatty acid oxidation via transactivation of LKB1-AMPK-ACC signaling axis.

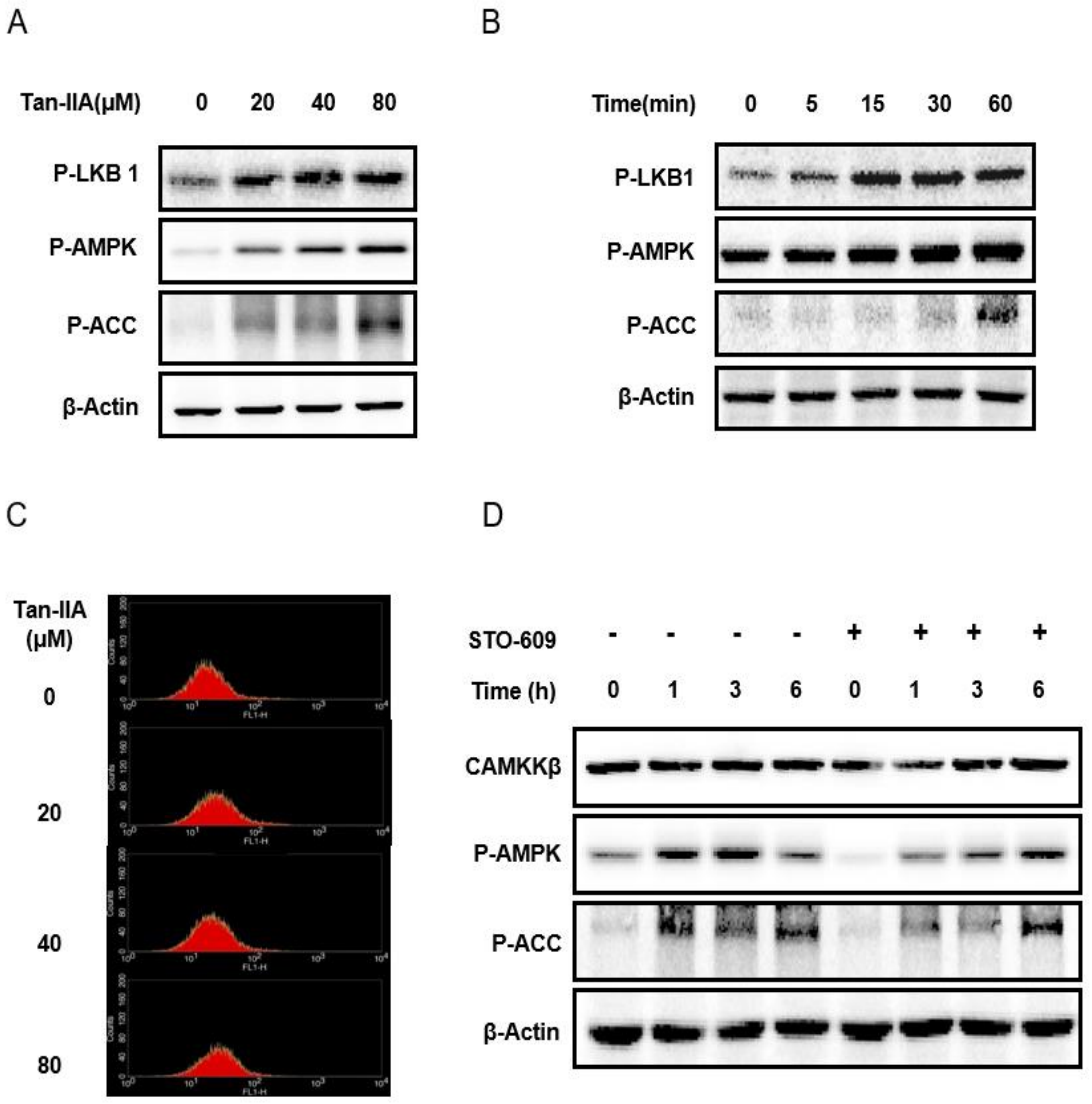

Figure 3. TAN-IIA Transactivates AMPK-ACC Signaling Axis. (A) TAN-IIA transactivates AMPK in a dose-dependent manner. HepG2 cells was treated with TAN-IIA at different doses for 1h. (B) AMPK transactivation by TAN-IIA is a rather early event. HepG2 cells were treated with TAN-IIA (20 μM) for different times. (C) Effects of TAN-IIA on cytosolic free Ca2+ levels in HepG2 cells. (D) STO-609, a selective CaMKKβ inhibitor attenuated AMPK transactivation by TAN-IIA. HepG2 cells were pretreated in the presence or absence of STO-609 (25 μM) for 2h, then treated with TAN (20 μM) for different times.

2.4 TAN-IIA Targets β2-Adrenergic Receptor Signaling Pathway

Although commonly prescribed as an anti-asthmatic drug, ephedrine is well studied for body fat loss [16]. Mechanistically, ephedrine functions as a non-selective adrenoreceptor agonist to

increase metabolic rate as well as heat expenditure. In this study, we surprisingly noticed that TAN-IIA treatment seemed share similar metabolic phenotypes to those of ephedrine. More importantly, β2-adrenergic receptor (β2-AR) was recently implicated in the pathogenesis of NAFLD [17-19]. Those findings prompted us to examine the possibility that TAN-IIA might affect on β2-AR signaling pathway. In this connection, TAN-IIA might affect fatty acid oxidation in a β2-AR-dependent manner (Figure 4A and B). The sensitivity of HepG2 hepatocytes to TAN-IIA was greatly impaired by either β2-AR knockdown or ICI 118-551, a β2-AR selective antagonist. The second message cyclic AMP (cAMP) level for β2-AR activation was elevated by TAN-IIA treatment. Such stimulatory action of TAN-IIA was abolished by either β2-AR knockdown or ICI 118-551 (Figure 4C and D). Taken together, TAN-IIA might confer metabolic benefit by modulating β2-AR signaling pathway.

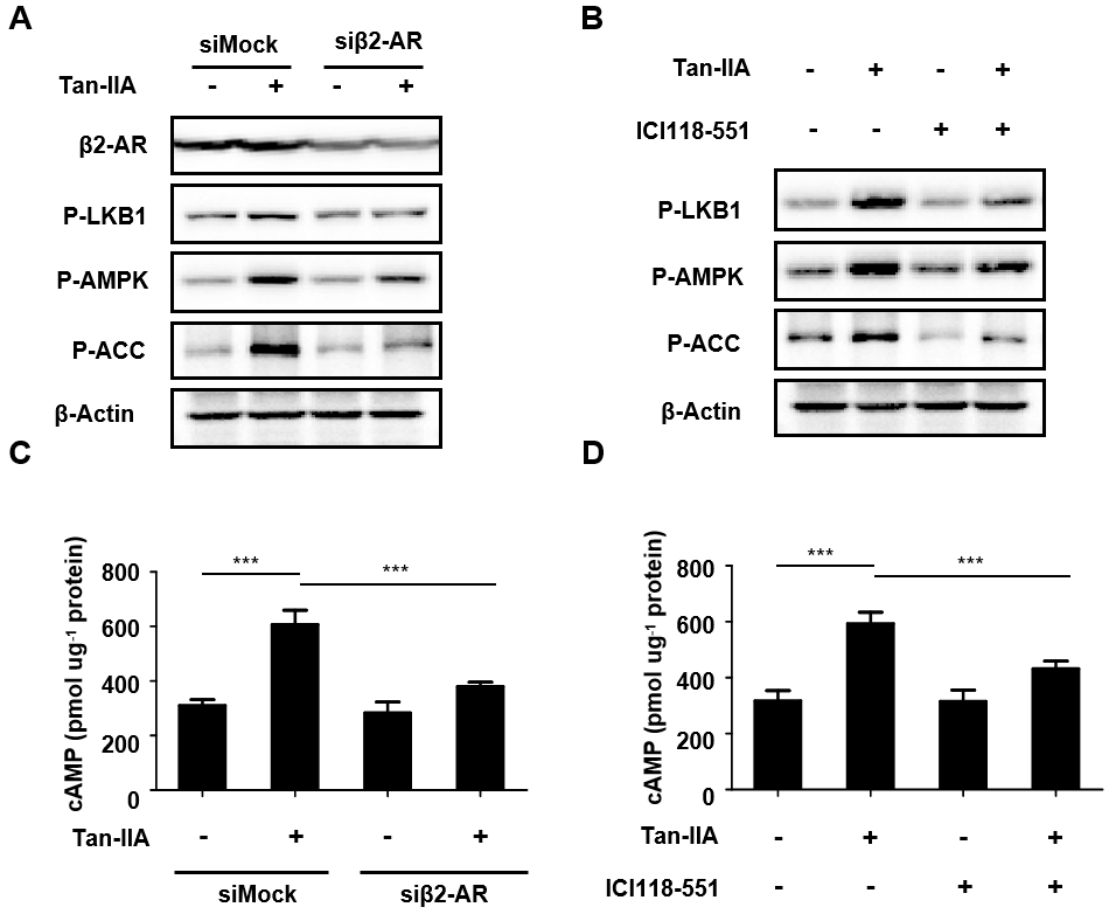

Figure 4. TAN-IIA Transactivates AMPK in a β2-Adrenergic Receptor Dependent Manner. (A) Effects of β2-AR knockdown on TAN-IIA induced AMPK transactivation. HepG2 cells were transfected with siβ2-AR or control siRNA, then treated with TAN (20 μM) for 1h. (B) Effects of β2-AR pharmacological inhibition on TAN-IIA induced AMPK transactivation. HepG2 cells were pretreated with a β2-AR selective antagonist (ICI 118-551, 1 μM) for 30 mins, then treated with TAN (20 μM) for 1 h. (C) Effects of β2-AR knockdown on TAN-IIA induced intracellular cAMP acclamation. (D) Effects of β2-AR pharmacological antagonist on TAN-IIA induced intracellular cAMP acclamation. Data are presented as the mean ± SD (n = 4). The asterisks indicate a significant difference compared to each respective vehicle group (＊＊＊, $p < 0.001$).

3 Conclusion

In this study, we showed that TAN-IIA, a natural component of Danshen, effectively mitigated obesity-induced NAFLD as well metabolic syndrome in mice. Our data indicated that activation of β2-AR, and the consequent cytosolic cAMP and Ca^{2+} elevation coupled with the

downstream LKB1-AMPK-ACC signaling axis, is perhaps an important mechanistic cascade in hepatic fatty acid oxidation. TAN-IIA might function as a β2-AR agonist. Danshen, the herbal parent of Tan-IIA, has been in medicinal use against liver diseases since ancient times [6]. Collectively, TAN-IIA might hold promise for NAFLD therapy.

β2-AR was recently implicated in the pathogenesis of NAFLD [17-19]. For example, common diseases associated with gain-or loss-of function mutations in β2-AR were nocturnal asthma and obesity. Obesity is known as a risk factor for NAFLD. Ephedrine, a first-line drug for asthma, has been used for body fat loss for many years. Interestingly, ephedrine was proposed to exert anti-lipolytic effects via activation of AR [16]. More recently, specific β2-AR variants have been associated with hyperlipidemia and NAFLD susceptibility [17]. Impotently, either genetic deletion of β2-AR or β2-AR specific antagonist exacerbated experimental NAFLD [18,19]. In this study, we noticed that β2-AR expression as well as fatty acid oxidation under fasting condition was down-regulated in obese mice. All studies above indicated that β2-AR might a conserved switch to LKB1-AMPK axis in energy metabolism. Taken together, β2-AR might be a relevant target for NAFLD therapy.

Although our findings are intriguing, several issues still need to be addressed. For example, to clarify the role of β2-AR in the etiology of NAFLD, future study should be carried out using conditional β2-AR knockout mice. The molecular nature by which β2-AR governs lipid metabolism remains elusive. Insulin resistance functionally mediates hepatic fat accumulation during NAFLD progression [20]. It remains unknown whether a cross-talk between β2-AR and insulin receptor exists. Another issue is how to translate our basic research findings into clinical use. Our findings were mainly derived from based on cell and mouse models. And thus, the efficacy of TAN-IIA against NAFLD must to be evaluated in human trials. Furthermore, NAFLD could be partly reversed by exercise or physical activity, but the patient compliance is still low. Although it pointed to alternate mechanisms at play, a full understanding of such phenomenon is still missing.

In summary, the present study indicated the clinical potential of TAN-IIA in NAFLD therapy. TAN-IIA might ameliorate obesity-related metabolic phenotypes, at least in part, by targeting β2-AR-LKB1-AMPK-ACC signaling axis.

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丹参酮 IIA 通过β2-AR-LKB1-AMPK 信号通路改善非酒精性脂肪性肝病

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摘要：非酒精性脂肪肝病（NAFLD）目前还没有理想的治疗方式。尽管丹参通常用于治疗心血管疾病，在历史上也作为治疗慢性肝病的民间药物，但其在 NAFLD 治疗中的潜力尚不清楚。本研究发现了丹参酮 IIA 通过激活 AMPK 有效地改善了非酒精性脂肪肝病。使用小鼠模型，确定丹参酮 IIA 可有效减轻高脂饮食诱导的肥胖，肝肿大和肝脂肪变性。从机理上讲，我们发现肥胖小鼠的肝组织中 β2-肾上腺素受体被下调，而丹参酮 IIA 可能起 β2-肾上腺素受体激动剂的作用，增加细胞内 cAMP 的水平，并激活 LKB1-AMPK-乙 ACC 信号通路来促进脂肪酸氧化。总而言之，丹参酮 IIA 可能具有作为非酒精性脂肪肝病的潜在治疗剂的研究价值，尤其是在那些肥胖患者中。

关键词：非酒精性脂肪肝病；丹参酮 IIA；β2-肾上腺素受体；AMP 活化的蛋白激酶
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