Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Activation of Wnt/β-catenin pathway causes insulin resistance and increases lipogenesis in HepG2 cells via regulation of endoplasmic reticulum stress

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ARTICLE INFO

Article history:
Received 8 March 2020
Accepted 25 March 2020
Available online xxx

Keywords:
CP21R7
Endoplasmic reticulum stress
Insulin resistance
Wnt/β-catenin

ABSTRACT

Background: Wnt/β-catenin signaling is involved in glucose and lipid metabolism, but the mechanism is not clear yet.
Aim: The objective is to study mechanisms of Wnt/β-catenin signaling on regulating hepatocytes metabolism.
Methods: Real-time qPCR, Western blot, and Oil-red O staining methods were used.
Results: The Wnt/β-catenin signaling was activated in hepatocytes by CP21R7, and the level of phosphorylated IRS-1 (Ser307) and TRB3 were significantly increased, while the levels of phosphorylated IRS-1 (Tyr612) and phosphorylated Akt were decreased. Moreover, the expression of FGF21, FAS, SCD1, PPARγ and ADRP was significantly increased. The expression of ATF4, ATF5, eIF2α, GRP78, CHOP and phosphorylated level of PERK were also increased. The expression of FGF21 and TRB3 was significantly downregulated, and the lipid droplets were notably reduced after the ER stress was inhibited by TUDCA. The expression of FGF21 was significantly decreased when the IRE1 pathway of the UPR was inhibited by STF-083010.
Conclusions: Activation of Wnt/β-catenin signaling pathway could cause insulin resistance and lipogenesis in hepatocytes via regulation of the IRE1 pathway of the ER stress and UPR, providing new targets for the treatment of metabolic disorders.

1. Introduction

Wnt/β-catenin signaling pathway plays important roles in many biological processes, including embryonic development, cell migration, polarization, and maintenance of stem cells et al. [1,2]. It has also been reported to promote the self-renewal, metastasis, and chemoresistance of cancer stem cells [3]. And, it is involved in the formation and development of cancer by regulation of the cell division cycle, the immune cycle and circadian rhythms [4]. Although activation of the Wnt/β-catenin signaling pathway could lead to cancers, its activation also could inhibit amyloid-β production and tau protein hyperphosphorylation in the brain of Alzheimer's disease (AD) patients, thus activators of Wnt/β-catenin signaling, such as CP21R7 and lithium chloride, might be used for the treatment of AD [5].

Hirabayashi S et al. found that high dietary sugar could increase the expression of Wg protein in *Drosophila*, which could upregulate the expression of insulin receptor and promote insulin sensitivity [6]. Au DT et al. reported that the expression of LRP1 in macrophages could promote hepatic inflammation, glucose intolerance and insulin resistance by modulating Wnt signaling pathway using macrophage-specific LRP1-deficient mice [7]. SFRP5 has been identified as a kind of adipokine, and it could stimulate the

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https://doi.org/10.1016/j.bbrc.2020.03.147
0006-291X/

differentiation of the adipocytes by inhibition of the Wnt/β-catenin signaling pathway [8]. Therefore, except its important roles in the pathological processes of cancers, Wnt/β-catenin signaling pathway is also involved in regulation of metabolism. However, its function on the metabolism of hepatocytes is still unclear.

The endoplasmic reticulum (ER) is one of the critical sites for the synthesis of proteins and lipids, and calcium homeostasis [9–11]. The ER can adapt to the metabolic changes, such as an increase in protein synthesis and accumulation of unfolded proteins and cholesterol, and when unfolded proteins accumulate in the ER, the ER stress and the unfolded protein response (UPR) are activated [11], which interact with several signaling pathways that are essential in metabolic disorders including obesity, insulin resistance, and diabetes [10–12].

Compound 21 (CP21) is an inhibitor of GSK3β, and the treatment of the human granulosa cells with CP21 could result in upregulated β-catenin signal activation [13]. In the present report, HepG2 cells were treated with CP21 to activate the Wnt/β-catenin signaling pathway, and the effects of the Wnt/β-catenin signaling pathway on hepatic metabolism were further studied. The association between Wnt/β-catenin pathway, ER stress and UPR related signaling pathways, and insulin resistance and lipid metabolism was uncovered, stimulating further studies that focus on the Wnt/β-catenin pathway as one of the important targets in the treatment of glucose and lipid metabolic disorders in hepatocytes.

2. Materials and methods

2.1. Cell culture

HepG2 cells (purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) was cultured in DMEM (GIBCO) medium containing 10% FBS (GIBCO) and 1% penicillin and streptomycin (Solarbio). Cells were cultured at 37 °C and 5% CO_2, and when the cells grew to about 70–80% of the density, the control group was treated with 0.05% DMSO for 24 h; the CP21 group was treated with CP21R7 (5 μM, Selleck) for 24 h; the CP21 and TUDCA group was treated with CP21R7 (5 μM, Selleck) and TUDCA (100 μM, Selleck) for 24 h; the CP21 and GSK2606414 group was treated with CP21R7 (5 μM, Selleck) and GSK2606414 (2 μM, Selleck) for 24 h; the CP21 and STF-083010 group was treated with CP21R7 (5 μM, Selleck) and STF-083010 (50 μM, Selleck) for 24 h.

2.2. RT-qPCR

The detailed procedure has been described in our previous report [14], and the sequences of primers for qPCR are listed in Table S1 (Sangon Biotech (Shanghai) co., Ltd., china).

2.3. Western blot

The cells were washed twice with PBS (Biological Industries), lysed in 50 μl protein lysate (RIPA: Cocktail: PMSF protease inhibitor: phosphatase inhibitor = 100:1:1:2, Meilun Biotechnology Company, China), and centrifuged at 12,000 rmp at 4 °C for 30min, and the supernatant was frozen at −80 °C. The concentration of proteins was tested using BCA (Beyotime), equal amounts of protein (20 μg) were subjected to SDS-PAGE on a 10% or 12% gel. The separated proteins were transferred electrophoretically to a PVDF membrane, after which the PVDF membrane was blocked with 5% nonfat milk at room temperature for 1 h, and incubated with primary antibodies at 4 °C overnight. Subsequently, the membrane was incubated with horseradish peroxidase-labeled antibodies at room temperature for 1 h. The signals were detected using enhanced chemiluminescence reagent (Bio-Rad 170-5060). The primary antibodies included mouse anti-β-catenin (1:1000; Cat. No. 610154; BD Transduction Laboratories™), rabbit anti-IRS-1 (1:1000; Cat. No. 2382; CST), rabbit anti-phospho- IRS1 (Ser307) (1:1000; Cat. No. 2381; CST), rabbit anti-phospho- IRS1 (Tyr608) mouse (1:1000; Cat. No. 09-432; Millipore), rabbit anti-Akt (1:1000; Cat. No. 4685; CST), rabbit anti-phospho- Akt (Ser 473) (1:1000; Cat. No. 4060; CST), mouse anti-GAPDH (1:1000; Cat. No. ab8245; abcam), rabbit anti-TRB3 (1:1000; Cat. No. ab75846; abcam), rabbit anti-PERK (1:1000; Cat. No. 3192; CST), rabbit anti-phospho-PERK (Thr980) (1:1000; Cat. No. 3179; CST), rabbit anti-eIF2α (1:1000; Cat. No. 5324; CST), rabbit anti-phospho-PERK (Ser 51) (1:1000; Cat. No. 3398; CST), rabbit anti-ATF4 (1:500; Cat. No. sc-200; Santa Cruz Biotechnology), rabbit anti-GRP78 (1:1000; Cat. No. 3177; CST), rabbit anti-XBP1s (1:500; Cat. No. sc-7160; Santa Cruz Biotechnology), and rabbit anti-IRE1α (1:1000; Cat. No. 3294; CST). The secondary antibodies included HRP-donkey anti-mouse IgG (1:2,000; Cat. No. ab150105; Abcam) and HRP-goat anti-rabbit IgG (1:2,000; Cat. No. os0701; Earthox Life Sciences).

2.4. Oil-red O staining

The cells were washed with PBS, and fixed with 4% formaldehyde for 30 min. After fixation, the cells were washed with PBS, soaked with 60% isopropanol for about 15s, stained with oil-red O working solution (Sigma) for 20 min, observed and photographed under the inverted microscope (Olympus Corporation; ckx41sf).

2.5. Statistical analysis

Statistical differences were determined using the SPSS software (23.0). One-way ANOVA was used to determine the difference between different groups. The data are presented as the mean ± standard deviation. $P < 0.05$ was considered to be significant.

3. Results

3.1. Activation of Wnt signaling pathway by CP21R7 could induce insulin resistance in HepG2 cells

To investigate the function of Wnt/β-catenin signaling pathway on regulating in hepatocytes, HepG2 cells were incubated with CP21R7 to active this signaling pathway. After 24 h, the expression of β-catenin protein was significantly increased (Fig. 1A), and the mRNA expression of c-Jun, Cyclin D1, the target genes of the Wnt/β-catenin signaling pathway was also significantly increased (Fig. 1B and C), indicating that Wnt/β-catenin signaling pathway was activated.

The compositions of insulin signaling pathway was further checked, and the results showed that the phosphorylated level of IRS-1 (Ser307) was increased, while the levels of phosphorylated IRS-1 (Tyr612) and phosphorylated Akt were decreased (Fig. 1D). In addition, the expression of TRB3 was significantly increased at both mRNA and protein levels (Fig. 1E). These results indicated that the activation of Wnt/β-catenin signaling pathway might lead to insulin resistance.

3.2. Activation of Wnt signaling pathway could increase the lipogenesis in HepG2 cells

After activation of Wnt signaling pathway in HepG2 cells, the mRNA level of FGF21, the biomarker of hepatic lipid accumulation and NAFLD [15,16], showed significantly increase (Fig. 2A). The expression of lipid metabolism related genes on mRNA level was

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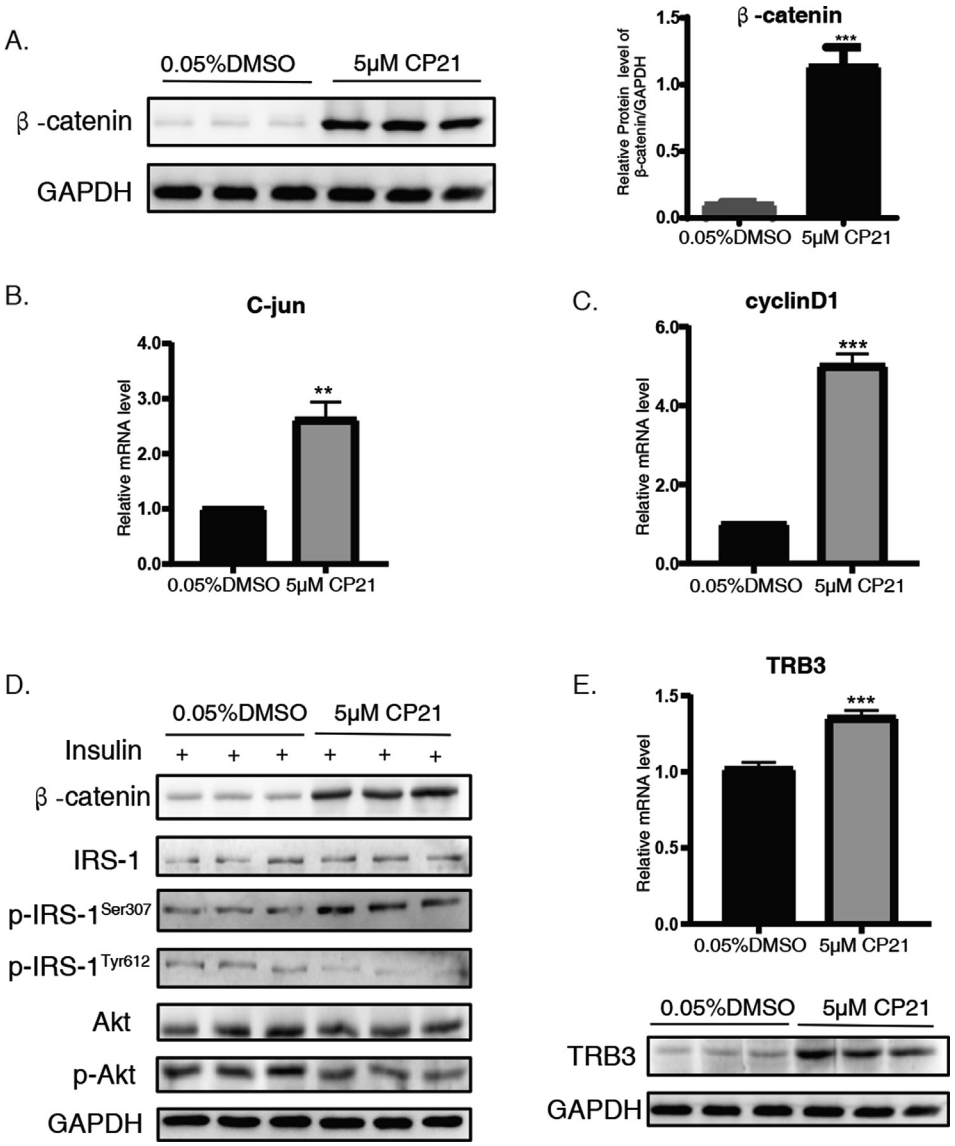

Fig. 1. Activation of Wnt/β-Catenin signaling pathway by CP21R7 induced insulin resistance. A: the expression of β-catenin protein was significantly increased; B, C: the expression of c-Jun and Cyclin D1 mRNA respectively; D: the expression of total protein levels of IRS1and Akt, and the phosphorylated levels of IRS-1 (Ser307), IRS-1 (Tyr612), and Akt were checked by Western blot; E: the expression of TRB3 mRNA and protein were increased. **P < 0.01, ***P < 0.001. IRS-1, insulin receptor substrate-1; TRB3, tribble 3.

further checked, and results demonstrated that the expression FAS, SCD1 and PPARγ was significantly increased, but the expression of ChREBP, ACCα and SREBP1 had no significantly change (Fig. 2A). There was no significant change of genes related with the β-oxidation of fatty acid, such as PPARα, ACOX1 and CPT1 (Fig. 2B). For genes related with lipid droplet formation, the expression of ADRP was significantly increased (Fig. 2B). The results of oil-red O staining showed that after the treatment of CP21, there were more fat droplets in the HepG2 cells (Fig. 2C).

3.3. Activation of Wnt signaling pathway could cause the endoplasmic reticulum stress and unfolded protein response (UPR)

After treatment with CP21, the mRNA levels of ATF4, ATF5, eIF2α, GRP78 and CHOP were significantly increased (Fig. 3A). On protein level, although the total protein expression of PERK and eIF2α had no change, the phosphorylated levels of PERK and eIF2α were significantly increased, and the expression of GRP78 was also significantly increased (Fig. 3B). The expression of ATF4 was obvious increased, though not significant in statistics (Fig. 3B). These results demonstrated that activation of Wnt/β-catenin signaling pathway could cause ER stress and UPR in HepG2 cells.

3.4. Activation of Wnt/β-catenin signaling pathway leaded to the abnormal metabolism of hepatocytes through regulation of IRE1α pathway

Tauroursodeoxycholic acid (TUDCA), an inhibitor of ER stress [17], was added during the activation of Wnt/β-catenin signaling pathway in HepG2 cells, and the results showed that the expression of CHOP and ATF6 was significantly increased after the treatment of CP21, while significantly decreased after the treatment of TUDCA (Fig. 4A and B).

The expression of FGF21 and TRB3 was significantly increased after the activation of Wnt/β-catenin signaling pathway, but when the ER stress was inhibited, the expression of them was significantly down-regulated (Fig. 4C–E), and the lipid droplets were also notably reduced compared with the HepG2 cells treated with only

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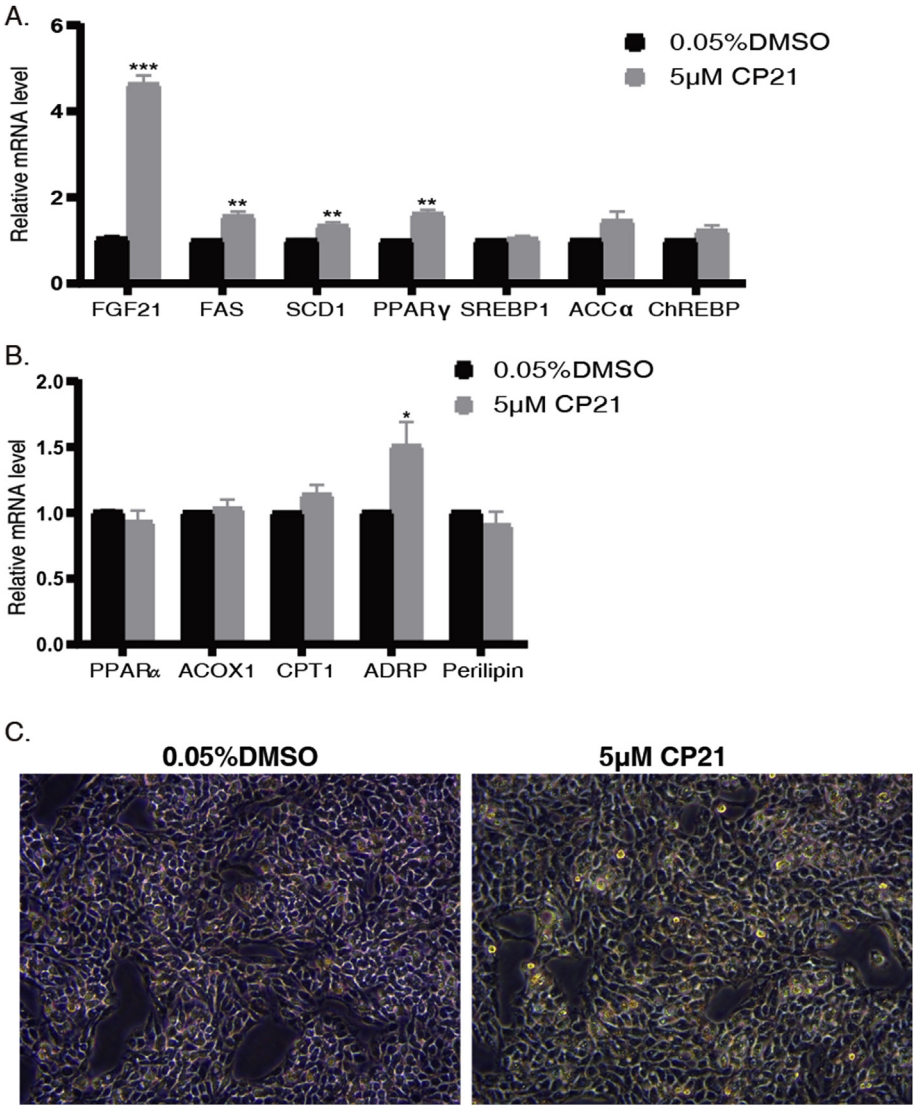

Fig. 2. Activation of Wnt/β-catenin signaling regulated the lipogenesis in HepG2 cell line. A: the expression of fatty acid related genes on mRNA level: FGF21, FAS, SCD1, PPARγ, SREBP1, ACCα and ChREBP; B: the expression of genes related with the β-oxidation of fatty acid and lipid droplet formation on mRNA level: PPARα, ACOX1, CPT1, ADRP and Perilipin; C: the results of oil-red O staining showed that there were more fat droplets in the HepG2 cells after the treatment of CP21. *P < 0.05, **P < 0.01, ***P < 0.001. FGF21, fibroblast growth factor 21; FAS, fatty acid synthase; SCD1, Stearoyl-coA desakturase-1; PPARγ, peroxisome proliferator-activated receptor γ; SREBP1, sterol regulatory element-binding protein 1; ACCα, acetyl-CoA carboxylase α; ChREBP, Carbohydrate response element-binding protein; PPARα, peroxisome proliferator-activated receptor α; ACOX1, acyl-CoA oxidase 1; CPT1, carnitine palmitoyltransferase I; ADRP, Adipose differentiation related protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CP21 (Fig. 4F).

There are three branches of the UPR: IRE1 pathway, PERK pathway and ATF6 pathway [11]. GSK2606414 was used to inhibit the PERK pathway [18], but the increased expression of FGF21 induced by CP21 had no significant change (Fig. 4G). STF-083010 was used to inhibit the IRE1α pathway [19] (Fig. 4H), and the increased expression of FGF21 induced by CP21 was significantly reduced after the treatment (Fig. 4I).

4. Discussion

In the present report, Wnt/β-catenin signaling pathway was activated in HepG2 cells by treatment with CP21. Behari J et al. reported that high-fat diet (HFD) fed hepatocyte-specific β-catenin transgenic mice developed obesity and insulin resistance rapidly, and Wnt signaling in hepatocytes was essential for the development of diet-induced fatty liver and obesity, but they found β-catenin indirectly affected hepatic insulin signal [20]. However, decrease of hepatic and adipose tissue expression of β-catenin could ameliorate hepatic steatosis, increase insulin-stimulated glucose metabolism, and improve hepatic insulin sensitivity [21]. Indirubin-3′-oxime (I3O), an activator of the Wnt/β-catenin signaling pathway, could inhibit the differentiation of 3T3-L1 cells into mature adipocytes and decrease the expression of adipocyte related genes [22]. Therefore, the effects of the Wnt/β-catenin signaling pathway on glucose and lipid metabolism are complicated. Our results demonstrated that the level of phosphorylated IRS-1 (Ser307) was increased, while the phosphorylated levels of IRS-1 (Tyr612) and p-Akt were decreased after activation of Wnt signaling in HepG2 cells. The increase of IRS-1 serine phosphorylation, decrease in IRS-1 tyrosine phosphorylation and p-Akt were typical in the situation of insulin resistance [23,24]. TRB3 was the endogenous inhibitor of Akt [25], and our result showed that the expression of TRB3 protein was significantly increased after

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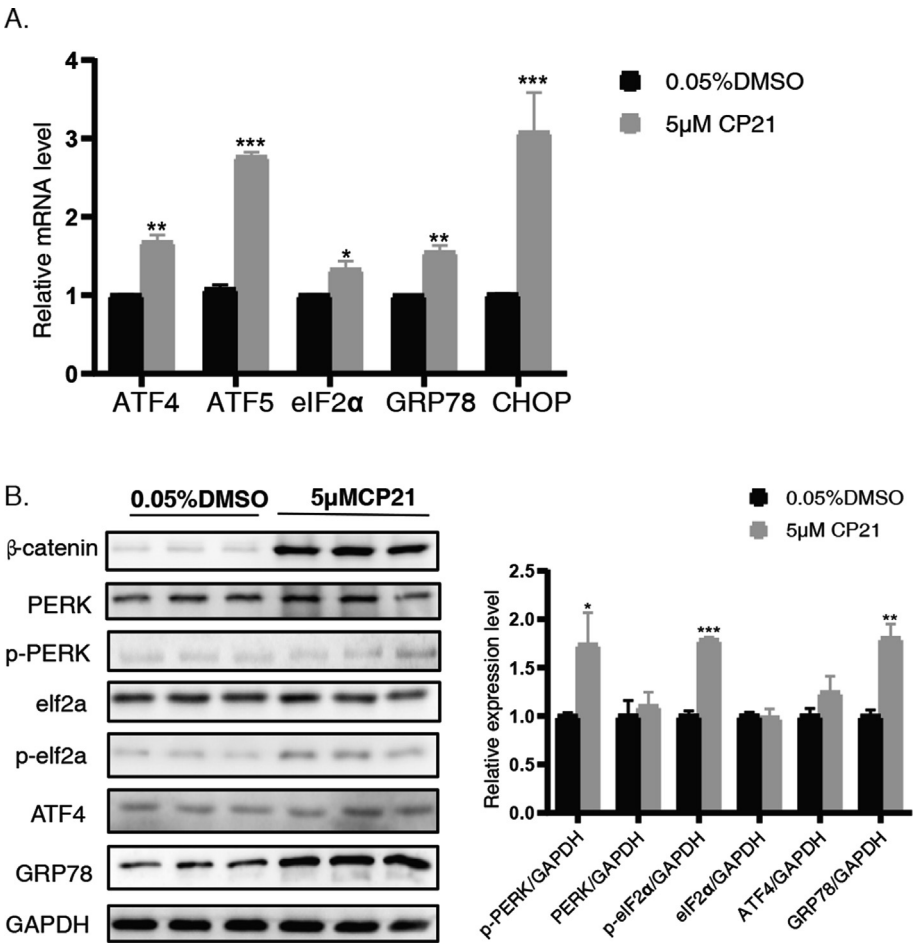

Fig. 3. Activation of Wnt/β-catenin signaling pathway could cause the ER stress. A: the expression of ATF4, ATF5, eIF2α, GRP78 and CHOP on mRNA level; B: Western blot results of the level of total PERK protein, phosphorylated PERK, total eIF2α protein, phosphorylated eIF2α, ATF4 protein and GRP78 protein. *P < 0.05, **P < 0.01, ***P < 0.001. ATF4, activating transcription factor 4; ATF5, activating transcription factor 5; eIF2α, eukaryotic initiation factor 2 alpha; GRP78, glucose-related protein 78; CHOP, C/EBP homologous protein; PERK, PKR-like endoplasmic reticulum kinase; ER, endoplasmic reticulum.

treatment with CP21. Therefore, the activation of Wnt/β-catenin signaling in HepG2 cells could lead to insulin resistance directly.

The expression of lipid metabolism related genes, including FGF21, FAS, SCD1 and PPARγ, was significantly increased. It has been reported that serum FGF21 level and FGF21 mRNA expression in the liver were increased in NAFLD patients, and FGF21 was a biomarker of hepatic lipid accumulation in obesity [15,16]. FAS is an essential enzyme that catalyzes the de novo synthesis of long chain fatty acids [26]. SCD1 is a critical lipogenic enzyme that catalyzes the synthesis of oleate and palmitoleate, from the saturated fatty acids stearate and palmitate respectively [27]. PPARγ is a key regulator of adipogenesis [28]. ADRP, also known as perilipin 2 or adipophilin, is a molecule involved in lipid droplet formation in the liver and peripheral tissues, and usually highly expressed in differentiated adipocytes [29]. Our result showed that the expression of ADRP was significantly increased after the activation of the Wnt/β-catenin signaling. Moreover, the results of oil-red O staining demonstrated that there were more lipid droplets in the HepG2 cells treated with CP21. So our results demonstrated that the expression of fatty acid synthesis related genes was up-regulated, and the lipid droplets formation was increased after the activation of Wnt/β-catenin signaling in HepG2 cells.

It has been reported that ER stress and activation of the UPR are linked to many human disorders and pathological processes, including obesity, type 2 diabetes, and hepatic steatosis [30]. Our results demonstrated that the expression of activating ATF4, ATF5, eIF2α, GRP78 and CHOP mRNA was significantly increased. The expression of GRP78 protein, and phosphorylated levels of PERK and eIF2α were also significantly increased. ATF4, phosphorylated PERK and eIF2α, GRP78 and CHOP are all ER stress markers [31–33]. It seemed that the activation of the Wnt/β-catenin signaling induced the ER stress and UPR in HepG2 cells.

It has been reported that TUDCA could inhibit ER stress via the PERK/eIF2α/ATF4/CHOP signaling pathway [34]. In the present study, the expression of CHOP and ATF6 was significantly increased in HepG2 cells treated with CP21 only, but their expression was significantly decreased when the HepG2 cells were treated with CP21 and TUDCA, indicating that TUDCA inhibited the ER stress induced by the activation of Wnt/β-Catenin signaling pathway successfully. The expression of FGF21 and TRB3 was significantly increased after the activation of the Wnt/β-catenin signaling, but significantly decreased in the HepG2 cells treated with CP21 and TUDCA. In addition, the lipid droplets were also notably reduced compared with the HepG2 cells treated with only CP21, indicating that inhibition of ER stress could alleviate the insulin resistance and abnormal lipid metabolism caused by the activation of Wnt signaling pathway, and the activation of Wnt signaling pathway might lead to abnormal liver metabolism by inducing ER stress.

The UPR includes IRE1 pathway, PERK pathway and ATF6 pathway [11]. When the ER stress happens, these pathways are

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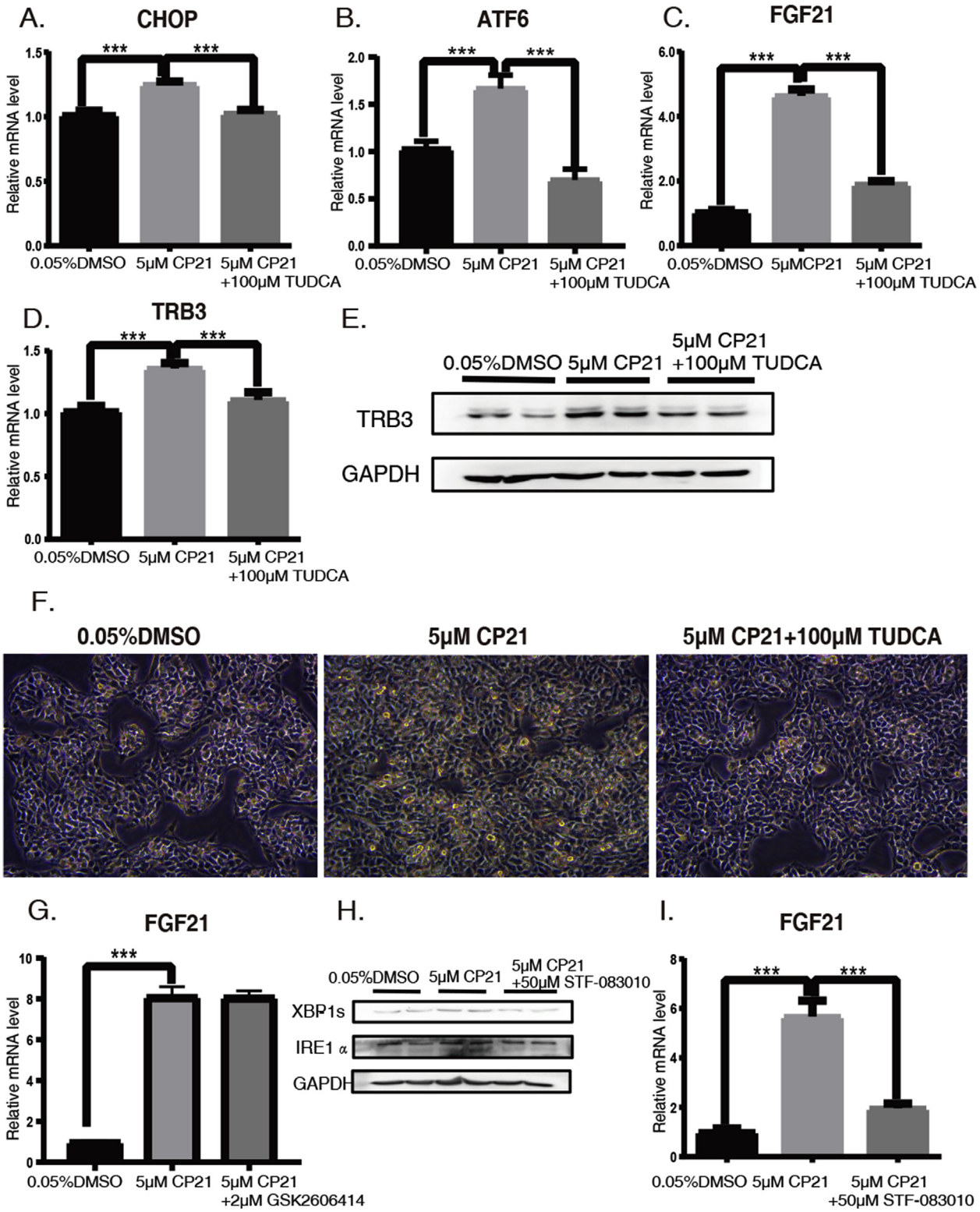

Fig. 4. Activation of Wnt/β-catenin signaling pathway leaded to the abnormal metabolism of hepatocytes by regulation of the IRE1 pathway of the ER stress. A–D: the expression of CHOP, ATF6, FGF21 and TRB3 mRNA respectively; E: the expression of TRB3 protein; F: the results of oil-red O staining showed that the lipid droplets were notably reduced when HepG2 cells were treated with TUDCA compared with HepG2 cells treated with only CP21. G: GSK2606414 was used to inhibit the PERK pathway, and the increased expression of FGF21 induced by CP21 was not decreased after the treatment; H: STF-083010 was used to inhibit the IRE1α pathway, and the expression of IRE1α protein was decreased; I: the increased expression of FGF21 induced by CP21 was significantly decreased after the use of STF-083010. ***P < 0.001. CHOP, C/EBP homologous protein; ATF6, activating transcription factor 6; FGF21, fibroblast growth factor 21; TRB3, tribble 3; TUDCA, tauroursodeoxycholic acid; ER, endoplasmic reticulum. PERK, PKR-like endoplasmic reticulum kinase; XBP1, X-box binding protein 1; XBP1s, spliced X-box binding protein 1; IRE1, inositol requiring enzyme 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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activated. In the present study, the increased expression of FGF21 induced by CP21 was not decreased after the treatment of GSK2606414, an inhibitor of the PERK pathway. STF-083010 was used to inhibit the IRE1α pathway successfully, and the increased expression of FGF21 induced by CP21 was significantly decreased in HepG2 cells treated with STF-083010. Therefore, it seemed that the activation of the Wnt/β-catenin signaling resulted in insulin resistance and lipogenesis via regulation of the IRE1α pathway of UPR. IRE1α pathway might be the main branch of ER stress to cause insulin resistance in hepatocytes, and further investigations need to do to explore it.

In summary, the Wnt/β-catenin signaling was activated by CP21 in HepG2 cells, resulting in insulin resistance and abnormal lipogenesis. The ER stress and UPR signaling was activated after the activation of the Wnt/β-catenin signaling, and inhibition of the ER stress and UPR signaling could improve insulin resistance and abnormal lipid metabolism caused by the activation of Wnt signaling. It seemed that the activation of Wnt signaling induced insulin resistance and lipogenesis in HepG2 cells via regulation of the IRE1 pathway of the ER stress, providing new targets for the treatment of glucose and lipid metabolic disorders.

Declaration of competing interest

The author reports no conflicts of interest in this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81830113, No. 81803912, No. 31671520), the Science and Technology Project of Guangdong Province (No. 2017B050504005), the Science and Technology Planning Project of Guangzhou City (No. 201803010069), the Scientific Research Project of the Administration of Traditional Chinese Medicine of Guangdong Province (No. 20182079), the Characteristic Innovation Project (Natural Science) of the Education Department of Guangdong Province and the “Innovation Strong School Project” of Guangdong Pharmaceutical University (No. 2017KTSCX102), and the Science and Technology Project of Yue-Xiu District of Guangzhou (No. 2018-WS-011).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.03.147.

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Please cite this article as: Z. Lei et al., Activation of Wnt/β-catenin pathway causes insulin resistance and increases lipogenesis in HepG2 cells via regulation of endoplasmic reticulum stress, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.03.147