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PCBP2 regulates hepatic insulin sensitivity via HIF-1α and STAT3 pathway in HepG2 cells

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ABSTRACT

Elevated free fatty acids (FFAs) are fundamental to the pathogenesis of hepatic insulin resistance. However, the molecular mechanisms of insulin resistance remain not completely understood. Transcriptional dysregulation, post-transcriptional modifications and protein degradation contribute to the pathogenesis of insulin resistance. Poly(C) binding proteins (PCBPs) are RNA-binding proteins that are involved in post-transcriptional control pathways. However, there are little studies about the roles of PCBPs in insulin resistance. PCBP2 is the member of the RNA-binding proteins and is thought to participate in regulating hypoxia inducible factor-1 (HIF-1α) and signal transducers and activators of transcription (STAT) pathway which are involved in regulating insulin signaling pathway. Here, we investigated the influence of PCBP2 on hepatic insulin resistance. We showed that the protein and mRNA levels of PCBP2 were down-regulated under insulin-resistant conditions. In addition, we showed that over-expression of PCBP2 ameliorates palmitate (PA)-induced insulin resistance, which was indicated by elevated phosphorylation of protein kinase B (AKT) and glycogen synthase kinase 3β (GSK3β). We also found that over-expression of PCBP2 inhibits HIF1α and STAT3 pathway. Furthermore, glucose uptake was found to display a similar tendency with the phosphorylation of Akt. The expressions of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two key gluconeogenic enzymes, were down-regulated following Over-expression of PCBP2. Accordingly, PA-induced intracellular lipid accumulation was suppressed in over-expression of PCBP2 HepG2 cells. In addition, we found that over-expression of PCBP2 inhibits HIF1α and STAT3 pathway. Our results demonstrate that PCBP2 was involved in hepatic insulin sensitivity might via HIF-1α and STAT3 pathway in HepG2 cells.

1. Introduction

The rising prevalence of type 2 diabetes mellitus (T2DM) continues to be a growing concern all over the world. Hepatic insulin resistance play a crucial role in the development of T2DM [1]. Elevated plasma free fatty acid (FFA) levels in obesity play a pathogenic role in the development of insulin resistance [2]. The abnormality in insulin signaling pathway, including insulin receptor, insulin receptor substrate, protein kinase B (AKT), glycogen synthase kinase-3β (GSK3β) and their downstream molecules [3]. However, the molecular mechanisms underlying insulin resistance and regulation of insulin signaling network in the liver are not completely understood yet. Among these, transcriptional dysregulation, post-transcriptional modifications and protein degradation contribute to the pathogenesis of T2DM [4].

PCBPs are a family of RNA-binding proteins, and characterized by high affinity and sequence-specific interactions with single-stranded RNA or DNA rich in cytosines [5]. PCBPs participate in almost every aspect of RNA metabolism, including transcriptional

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control, nuclear RNA splicing, mRNA translational control and mRNA stability [6]. However, little is known about their roles in insulin resistance and T2DM.

PCBP2 is the member of the hnRNP E family of RNA binding proteins [7]. PCBP2 was firstly identified as a component of the α-complex of the human α-globin mRNA, which greatly increases mRNA stability [8]. And it is also implicated in other important biological processes, including transcriptional regulation and translational silencing [5]. Studies have shown that PCBP2 plays pivotal roles in multifarious diseases. PCBP2 was involved in delivering iron to cytosolic nonheme iron enzymes by increasing degradation of HIF1α [9]. PCBP2 enhance cellular anti-HCV immune response by stabilizing the mRNA of STAT1 and STAT2 [7]. STAT3 functions via cellular PCBP2 to restrain EBV lytic activation in B lymphocytes [10]. These studies suggest a potential link among PCBP2, HIF-1α and STAT pathway. Besides, HIF-1α and STAT pathway are all involved in regulating insulin signaling pathway [11,12].

However, whether PCBP2 plays a role in insulin resistance through regulating above pathways remains unknown. The present study was designed to elucidate the possibility. In this study, we used biochemical assay to test the change of expression of PCBP2 in livers and hepatocytes under insulin resistance condition or normal condition. Furthermore, we analyzed the upstream and downstream pathway of PCBP2 in HepG2 cell. We found that PCBP2 was involved in regulation of hepatic insulin sensitivity in HepG2 cell. The function of PCBP2 may via HIF-1α and stat3 pathway.

2. Materials and methods

2.1. Antibodies

Antibodies anti-phospho-AKT (Ser473), anti-phospho-GSK3β, anti-phospho-Stat3 were purchased from Cell Signaling, anti-AKT, anti-GSK3β, anti-Stat3, anti-PCBP2, anti-HIF1α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH antibody was purchased from Sigma.

2.2. Fatty acid preparation

FFAs were prepared with the methods of protein adsorption: Firstly, a 100 mM stock of PA was prepared in 0.1 nM NaOH by heating to 70 °C. Secondly, PA was complexed with BSA at a 1:1 M ratio to make a 50 mM working stock via dropwise addition to 10% endotoxin/fatty acid-free BSA. The PA/BSA mixture was sterile filtered before use and kept at −20 °C.

2.3. Animals

We obtained db/db mice (C57B L/6 J) from the Experimental Animal Center of Nantong University. Mice were fed a normal diet (ND) or high fat diet (HFD) containing 50% carbohydrate, 20%protein and 25% fat for 10 weeks in a temperature-controlled (22–24 °C) and humidity-controlled (45–55%) environment. A 12 h/12 h light/dark cycle was maintained, as described previously

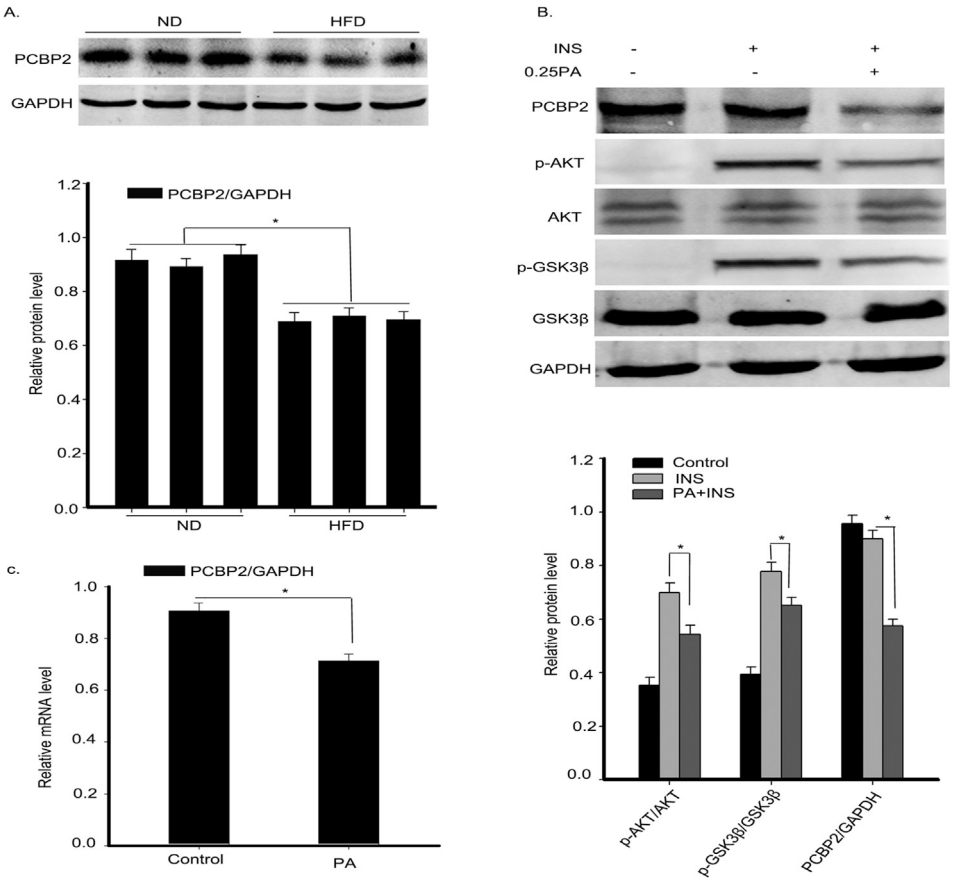

Fig. 1. PCBP2 was down-regulated in insulin-resistant livers and hepatocytes. A: The protein level of PCBP2 detected by western blotting in HFD mice livers and quantitative analysis of the intensity of protein expression relative to GAPDH in the indicated groups. B: The protein levels of PCBP2 and insulin-stimulated phosphorylation of AKT and GSK3β in PA-induced HepG2 cells and quantitative analysis of the intensity of protein expression relative to AKT and GSK3β in the indicated groups. HepG2 cells were incubated with or without 0.25 mM PA for 24 h followed by stimulation with or without 100 nM insulin for 20 min. C: PCBP2 mRNA levels were detected by qRT-PCR in HepG2 cells. HepG2 cells were incubated with or without 0.25 mM PA for 24 h. Values were mean ± SEM (n = 3, *p < 0.05.).

[13]. All animal procedures were followed the National Institutes of Health Animal Care and Use Guidelines.

2.4. Cell culture and treatments

HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were maintained at 37 °C with humidified air and 5% CO_2, with medium changes three times a week. For protein and mRNA analysis, HepG2 cells were cultured for 24 h in DMEM in the presence or absence of PA (0.25 mM). Before harvest, HepG2 cells were treated with insulin (100 nM) for 20 min.

For transfection: HepG2 cells in 6-well plates were transfected with the plasmids containing HA-PCBP2 (a gift from Professor Zhengfan Jiang, PEKING University, China) or HA (3.2 μg/well), at the indicated final concentrations in the culture medium using Lipofectamine 2000 (Invitrogen, Shanghai, China). After transfection for 24 h, cells were subsequently stimulated with or without 25 mM PA for 24 h and before harvest, HepG2 cells were treated with insulin (100 nM) for 20 min.

2.5. Western blot analysis

Cell lysates were separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore Bedford, MA, USA), blocked with 5% nonfat dry milk for 2 h at room temperature, and the membranes were immunoblotted with primary antibodies at 4 °C overnight followed by incubation with a horseradish peroxidase secondary antibody (1:5000; Southern-Biotech) for 1–2 h. And then, they were visualized using an enhanced chemiluminescence system (ECL; Pierce Company, Woburn, MA, USA). ImageJ (NIH) was used to analyze densities of the bands.

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) and was reverse-transcribed. Quantitative real-time PCR (qRT-PCR) was performed with the Roche Light Cycler 480 System (Roche Diagnostics, Burgess Hill, UK). The target gene mRNA level was normalized to that of GAPDH in the same sample. The primer sequences for real time PCR assays were: PCBP2 (Human) 5′-ATACGAGAGAGTACAGGGGC-3′ and 5′-GACCACCTGCAAAGATGACC-3′; GAPDH (Human) 5′-ATGG TTTACATGTTCCAATAT-3′ and 5′-ATGAGGTCCACCACCCTGGTTG-3′; G6pase (Human) 5′-TGGTTGG GATTCTGGGCTGT-3′ and 5′-TCTACACCCAGTCCCTTGAG-3′; PEPCK (Human) 5′-GTTCAATGCCAGGTTCCCAG-3′ and 5′-TTGCAGGCCA GTTGTTGAC-3′.

2.7. Glucose uptake assay

Glucose uptake in HepG2 cells was measured by Glucose Colorimetric/Fluorometric Assay Kit (BioVision). HepG2 cells were transfected with HA-PCBP2 or HA and 24 h later incubated with 0.25 mM palmitate for 24 h, and then stimulation with insulin (100 nM) for 20 min. A quantity of 30ul of medium was sampled for measurement of glucose concentration using a glucose assay kit (Milpitas Blvd., Milpitas, CA 95035 USA) in accordance with the manufacturer's protocol.

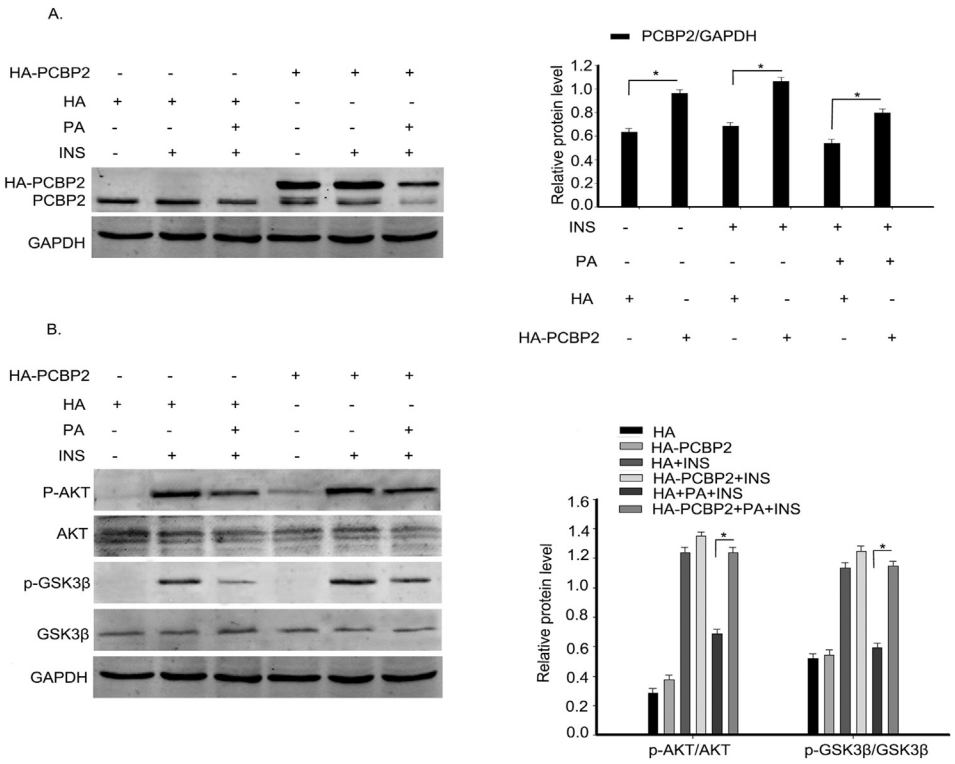

Fig. 2. Over-expression of PCBP2 ameliorated PA-induced insulin resistance in HepG2 cells. HepG2 cells were exposed to HA-PCBP2 or HA for 24 h and then incubated with or without 0.25 mM PA for 24 h, and then the cells were stimulated with or without 100 nM insulin for 20 min. A. The protein levels of PCBP2 in above cells were detected by western blotting and quantitative analysis of the intensity of protein expression relative to GAPDH in the indicated groups. B: The protein levels of phosphorylation of AKT and GSK3β were detected by western blotting and quantitative analysis of the intensity of protein expression relative to AKT and GSK3β in the indicated groups. Values were mean ± SEM (n = 3, *p < 0.05, significantly different from respective controls).

2.8. Oil red O staining

HepG2 cells were transfected with HA-PCBP2 or HA and then 24 h later incubated with 0.25 mmol/L palmitate for 24 h. Confluent cells were washed twice with ice-cold PBS and were fixed in phosphate-buffered formalin (10%) for 1 h at room temperature. After removal of formalin, cells were washed with PBS followed by 70% ethanol, and stained with Oil Red O solution (3 parts of saturated Oil Red O dye in isopropyl alcohol 2 parts of water) for 30 min at room temperature. Excess stain was removed by 70% ethanol, and then cells were finally washed with PBS. The cell was observed by inverted microscope.

2.9. Statistical analysis

Statistics Data are expressed as means ± SD of at least three independent experiments. The differences between experimental groups were performed using a one-way analysis of variance (ANOVA), followed by individual post hoc comparisons.

3. Results

3.1. PCBP2 is down-regulated under insulin-resistant conditions

To track with whether PCBP2 is involved in insulin resistance, we first examined the expression of PCBP2 in control and insulin-resistant liver tissues. We found the protein expression of PCBP2 was higher in insulin-resistant liver tissues than control (Fig. 1A). The insulin resistance mice were induced by high-fat dietary as described previously.

To further determine the role of PCBP2, we then analyzed the expression of PCBP2 in insulin-resistant hepatocytes. Human HepG2 cells were treated with PA for 24 h to induce insulin resistance [14]. Before harvest, HepG2 cells were treated with insulin (100 nM) for 20 min. As shown in Fig. 1B, insulin-stimulated phosphorylation of AKT and GSK3β, the commonly used marker of insulin signaling, was evaluated. PA suppressed insulin-induced phosphorylation of AKT and GSK3β. Consistently, the protein expression of PCBP2 was down-regulated in insulin-resistant HepG2 (Fig. 1B). We also observed significant decrease of PCBP2 mRNA level in HepG2 cells after PA exposure (Fig. 1C). All the above suggests that PCBP2 may be a link between FFA and insulin resistance.

3.2. Over-expression of PCBP2 ameliorates PA-induced insulin resistance

To further explore whether PCBP2 plays a functional role in PA-induced insulin resistance in HepG2 cells, we examined the effect of PCBP2 over-expression on phosphorylation of AKT (ser473) and GSK3β, by transfecting HepG2 cells with vector expressing PCBP2 (HA-PCBP2) or control vector (HA). As shown in Fig. 2, transfection of HA-PCBP2 significantly elevated the level of PCBP2 in the cells. We observed that PA-induced down-regulation of p-AKT and

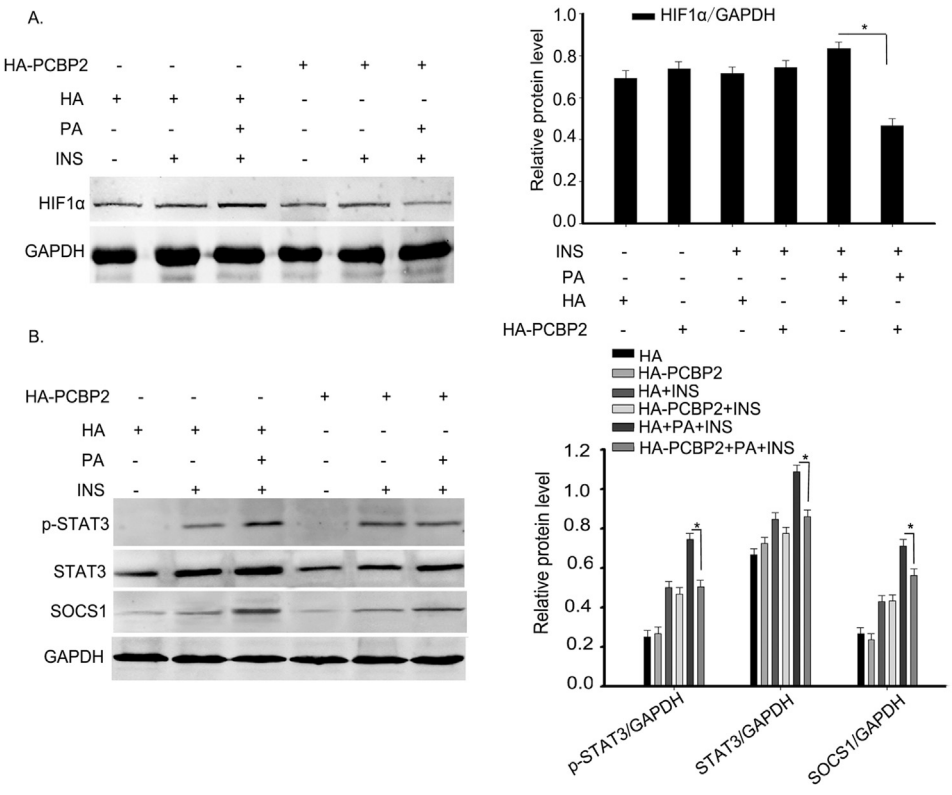

Fig. 3. Over-expression of PCBP2 inhibits HIF1α and STAT3 pathway. HepG2 cells were exposed to HA-PCBP2 or HA for 24 h and then incubated with or without 0.25 mM PA for 24 h, and then the cells were stimulated with or without 100 nM insulin for 20 min. A: The protein levels of HIF1α in above cells were detected by western blotting and quantitative analysis of the intensity of protein expression relative to GAPDH in the indicated groups. B: The protein levels of phosphorylation of STAT3 and SOCS1 were detected by western blotting and quantitative analysis of the intensity of protein expression relative to STAT3 and GAPDH in the indicated groups. Values were mean ± SEM ($n = 3$, $^*p < 0.05$, significantly different from respective controls).

p-GSK3β was enhanced after PCBP2 over-expression. These data indicated that endogenous PCBP2 exerted a critical regulatory role in the insulin sensitivity in vitro.

3.3. Over-expression of PCBP2 inhibits HIF1α and STAT3 pathway

Previous studies have proposed HIF1α as a downstream target of PCBP2 [9], and HIF1α has also been implicated in regulating hepatic insulin responses [15], suggesting the possibility that PCBP2 may mediate the effect of HIF1α on insulin sensitivity. To test this possibility, we examined the effect of PCBP2 over-expression on HIF1α, by transfecting HepG2 cells with vector expressing PCBP2 (HA-PCBP2) or control vector (HA). As shown in Fig. 3A, transfection of HA-PCBP2 significantly reversed the effect of PA on increasing HIF1α.

Studies have shown that STAT1 and STAT2 as downstream targets of PCBP2 [7]. STAT3, another member of family of STAT, plays an important role in regulating hepatic insulin resistance induced by free fatty acids (FFA) [16]. To study whether STAT3 is also a target of PCBP2, we measured the protein change of STAT3 after transfecting HA-PCBP2) or HA vector. We found that over-expression of PCBP2 attenuated the protein level of STAT3 induced by PA (Fig. 3B). In addition, over-expression of PCBP2 significantly decreased phosphorylation of STAT3 and the expression of SOCS-1 compared with control cells under insulin resistance condition (Fig. 3B). Taken together, these results imply that PCBP2 functions in the regulation of insulin sensitivity under insulin-resistant condition may through the modulation of HIF1α and STAT3 pathway.

3.4. Over-expression of PCBP2 ameliorates PA-induced intracellular lipid accumulation, gluconeogenesis, and suppression of glucose uptake

Lipid accumulation in liver plays a causal role in hepatic insulin resistance [14], and SOCS1 is involved in regulating lipid metabolism [17]. So we determine whether PCBP2 have a role in regulation lipid metabolism. We examined intracellular lipid accumulation by Oil red O staining. As shown in Fig. 4A, Over-expression of PCBP2 significantly decrease accumulation of lipid droplets.

In addition, we also evaluated the effect of PCBP2 on glucose uptake by measuring insulin-stimulated glucose uptake into the HepG2 cells. Compared with controls, insulin-induced glucose uptake was suppressed by about 35% after PA treatment and over-expression of PCBP2 can reverse decreased cellular glucose uptake by PA (Fig. 4B). Exposure of HepG2 cells to palmitate enhanced gluconeogenesis, and over-expression of PCBP2 can alleviate the increase of gluconeogenesis induced by PA (Fig. 4C).

4. Discussion

PCBP2 are present in many cells and tissues [18–20]. Although numerous biological functions of PCBP2 have been identified

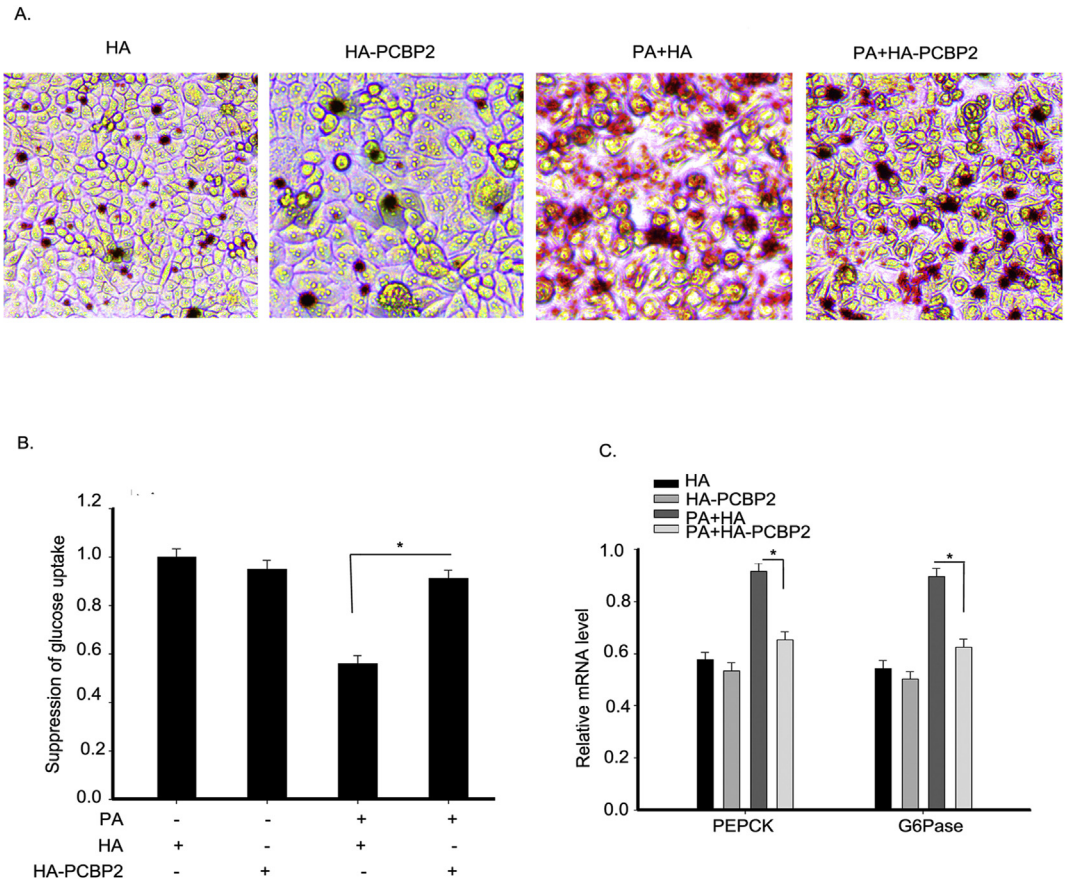

Fig. 4. Over-expression of PCBP2 ameliorates PA-induced intracellular lipid accumulation, gluconeogenesis, and suppression of glucose uptake. HepG2 cells were exposed to HA-PCBP2 or HA for 24 h and then incubated with or without 0.25 mM PA for 24 h. A. Lipid accumulation was determined by oil red staining. B. Glucose uptake was determined by glucose fluorometric assay kit. C. Quantitative real-time PCR analysis of mRNA levels of G6Pase and PEPCK. Values were mean ± SEM (n = 3, *p < 0.05, significantly different from respective controls). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[21,22], an effect of PCBP2 on insulin sensitivity and T2DM has not previously been described. In current study, we delved into the role of PCBP2 in hepatic insulin resistance revealing that PCBP2 played an essential role in insulin sensitivity in HepG2 cells. This conclusion was based on following results: PCBP2 was down-regulated in livers of HFD mice and HepG2 cells of insulin resistance induced by PA. Over-expression of PCBP2 could improve hepatic insulin signal transduction and inhibit HIF1α and STAT3/SOCS1 pathway, accompanying with inhibiting the expressions of gluconeogenic enzymes and increasing glucose uptake induced by PA. Over-expression of PCBP2 ameliorates PA-induced intracellular lipid accumulation.

PCBPs regulate protein expression at several levels, including transcription, mRNA processing, mRNA stabilization, and translation. PCBP2 is the member of the family of PCBPs and participated in expressions of various proteins. For instance, Knockdown of PCBP2 enhanced the expression of FHL3 by stabilizing its mRNA and inhibited glioma growth [23]. PCBP2 enhances the antiviral activity of IFN-α against HCV by increasing protein expression of STAT1 and STAT2 [7]. Depletion of PCBP1 and PCBP2 decreased the mRNA levels of Fatty acid desaturase 1 (FADS1) and HIF1α in K562 cells [24]. However, depletion of PCBP1 and PCBP2 increased the mRNA levels of HIF1α in Huh7 cells [9]. In addition, studies also showed that PCBP2 may be regulated by STAT3. So HIF-1α was regulated by PCBP2 in complicated manners and PCBP2 was linked to STAT pathway. HIF-1α and STAT pathway play crucial roles in insulin resistance. Increasing HIF-1α can cause inflammation and insulin resistance in obesity [25]. The activation of STAT3/SOCS1 signaling pathway participates in induction of hepatic insulin resistance [26]. So PCBP2 may be involved in hepatic insulin resistance via above pathways. Consistent with this hypothesis, we found that the decrease of PCBP2 played an essential role in HepG2 cells of insulin resistance induced by PA and the effect of PCBP2 may through HIF-1α and STAT3/SOCS1 signaling pathway.

PCBP2 participated in regulating of insulin sensitivity may by other signaling pathways. Studies have shown that PCBP2 was involved in post-transcriptional control of p21 in K562 cells [24]. P21closely links to hepatic insulin resistance [27]. PCBP2 also is a constituent of stress granules and processing bodies. Various stresses, such as oxidative stress and endoplasmic reticulum, play essential roles in insulin resistance [28,29]. So PCBP2 regulates insulin sensitivity may by p21 and stress signaling pathways. Further studies should be performed to definitely clarify the precise role of PCBP2 in the regulation of insulin resistance and diabetes.

In conclusion, our date showed that PCBP2 played a protective role in FFA-induced hepatic insulin resistance. The effect of TPCBP2 on insulin signaling was probably at least in part by inhibition of HIF1α and STAT3/SOCS1 pathway. Because PCBP2 is expressed in all tissues, the potential role of PCBP2 in the regulation of insulin sensitivity in other cells and tissues will be investigated in the future.

Conflict of interest

The authors declare no conflict of interest.

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Transparency document

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