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Research paper

CtBP2 ameliorates palmitate-induced insulin resistance in HepG2 cells through ROS mediated JNK pathway

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ABSTRACT

Oxidative stress plays a significant role in the development of hepatic insulin resistance, but the underlying molecular mechanisms remain poorly understood. In this study, we discovered that C-terminal-binding protein 2 (CtBP2) level was decreased in insulin resistance. Taking into account the relationship between CtBP family protein (ANGUSTIFOLIA) and reactive oxygen species (ROS) accumulation, we conjectured CtBP2 was involved in insulin resistance through ROS induced stress. In order to verify this hypothesis, we over-expressed CtBP2 in palmitate (PA) treated HepG2 cells. Here, we found that over-expression of CtBP2 ameliorated insulin sensitivity by increasing phosphorylation of glycogen synthase kinase 3β (GSK3β) and protein kinase B (AKT). These data suggest that CtBP2 plays a critical role in the development of insulin resistance. Moreover, CtBP2 reversed the effects of PA on ROS level, lipid accumulation, hepatic glucose uptake and gluconeogenesis. We also found that over-expression of CtBP2 could suppress PA induced c-jun NH2 terminal kinase (JNK) activation. Furthermore, JNK inhibitor SP600125 was shown to promote the effect of CtBP2 on insulin signaling. Thus, we demonstrated that CtBP2 ameliorated PA-induced insulin resistance via ROS-dependent JNK pathway.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by abnormal lipid and glucose (Cheng et al., 2009). Excess serum free fatty acids (FFAs) have been shown to participate in the development of insulin resistance in liver (Tang et al., 2015). It is increasingly apparent that insulin resistance could be caused by oxidative stress in target issues (Haberzettl et al., 2016; Keane et al., 2015; Manolopoulos et al., 2010). However, the molecular mechanisms linking oxidative stress and insulin resistance remains not fully understood.

Oxidative stress is an imbalance between oxidation and antioxidant ratio, which can lead to the generation of free radicals (Mohamed et al., 2016). It is well known that FFA-induced insulin resistance is usually accompanied by oxidative stress. ROS overload results in activating a variety of stress sensitive intracellular signal transduction pathways, such as JNK pathway (Gao et al., 2010). Importantly, JNK are mitogen activated protein kinase (MAPK) family members, have been reported to be involved in the mechanism of PA induced insulin resistance (Pal et al., 2016). JNK activation is increased in PA-treated HepG2 cells and the liver of high fat diet (HFD) fed mice (Tang et al., 2015; Zhou et al., 2015). It serves as an important negative regulator of insulin signaling pathway.

CtBP2 is a transcriptional co-repressor which has been reported to participate in various physiological and pathological processes, including tumorigenesis and cell apoptosis (Wang et al., 2015c), but its role in insulin resistance and T2DM remains unclear. Some studies have shown that CtBP2 inhibition leads to an increase in triglyceride concentration, an increase in lipid droplet accumulation, and an increase in the expression levels of the adipogenic marker genes (Wang et al., 2015a). In this study, we found that CtBP2 was down-regulated in insulin resistance. These data suggest that CtBP2 may be associated with the development of diabetes. Recently, studies have demonstrated the involvement of ANGUSTIFOLIA (a CtBP family protein) in the generation of ROS and insulin resistance (Gachomo et al., 2013; Hu et al., 2016; Isah et al., 2007). But little is known about the role of CtBP2. First, we studied the role of CtBP2 in the regulation of ROS in PA-induced insulin resistance. Because CtBP expression is closely associated

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with JNK activation (Wang et al., 2006), we further guess that CtBP2 may involve in insulin resistance via ROS mediated JNK pathway. The purpose of this study is to explain its possibility. In this study, we transfected HepG2 cells with vector expressing CtBP2 (Flag-CtBP2) and found that over-expression of CtBP2 could reverse PA-induced lipid accumulation, ROS accumulation and JNK activation. This study may be a new target for the treatment of T2DM.

2. Materials and methods

2.1. Cell culture and PA treatment

Human HepG2 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FBS, Hyclone, Logan, UT, USA) at 37 °C with 5% CO_2. The medium was changed every other day. HepG2 cells in 6-well plates were transfected with the plasmids containing Flag-CtBP2 (China) or Flag (3.2 mg/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 0.25 mM PA for 24 h and before harvest, HepG2 cells were treated with insulin (INS) (100 nM) for 20 min. PA preparation was performed as described previously (Cousin et al., 2001; Wang et al., 2015b).

2.2. Measurement of ROS production

Intracellular ROS levels were detected with a fluorescent marker, DCFH-DA. Treated cells were incubated with 10 μmol/L DCFH-DA in serum-free medium for 30 min at 37 °C. The slides were examined under a Leica DMLS epifluorescence microscope, the images were captured with a Leica DC 100 digital camera using identical acquisition settings. The mean fluorescent intensity was calculated randomly from five visual fields per coverslip.

2.3. Antibodies and reagents

Anti-phospho-AKT (Ser473), anti-phospho-GSK3β, anti-AKT, anti-GSK3β, anti-JNK, anti-phospho-JNK antibodies were purchased from Cell Signaling, anti-GAPDH, anti-Flag, anti-CtBP2, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PA and Oil red O were purchased from Sigma. Lipofectamine 2000 was obtained from Invitrogen. ROS examination kit was purchased from Beyotime Biotechnology.

2.4. Mice

Male C57BL/6J mice were approved by the Experimental Animal Center of Nantong University. Study plans were approved by the Animal Experiment Committee of Nantong University. Mice were housed in groups at 22–24 °C on a 12 h light/dark cycle with free access to water and given a normal diet (ND) or HFD containing 50% carbohydrate, 20% protein and 25% fat for 12 weeks. Glucose tolerance test was performed as described previously (Zhao et al., 2015).

2.5. Western blot analysis

HepG2 cells were washed in cold PBS and lysed in SDS-mercaptoethanol sample buffer containing protease inhibitor mixture and phosphatase inhibitor mixture (Sigma). Whole cell extracts were boiled and quantified using the Bradford procedure. 200 μg of proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were blocked with 5% skim milk for 1 h, and incubated overnight at 4 °C with the primary antibody at the dilutions recommended by the supplier. The secondary antibodies were diluted 1:5000 in PBS and incubated for 2 h at room temperature. Image J (NIH) was used to analyze the densities of the bands.

2.6. Oil red O staining

HepG2 cells were transfected with CtBP2 (Flag-CtBP2) or control vector (Flag) under insulin-resistant conditions, and then cells were washed three times with ice-cold PBS and 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 washing with 70% ethanol. The stained cells were finally washed three times with PBS. Cells were imaged by inverted microscope.

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

Total RNA from HepG2 cells were extracted using Trizol reagent (Invitrogen, CA), according to the manufacturer's instructions. For further analysis, total RNA was reverse-transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit(Thermo Fisher Scientific, MA). Target genes were amplified by quantitative real-time PCR using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio, Otsu, Japan). The Roche LightCycler 480 System (Roche Diagnostics, Burgess Hill, UK) was used for analysis. Target gene mRNA level was normalized to GAPDH. The primer sequences for real time PCR assays were: G6pase (F) 5′-TGGTTGGGATTCTGGGCTGT-3′; and (R) 5′-TCTACACCCAGTCCCTTGAG-3′; PEPCK (F) 5′-GTTCAA TGCCAGGTTCCCAG-3′; and (R) 5′-TTGCAGGCCAG TTGTTGAC-3′; GAPDH (F) 5′-TGATGACATCA AGAAGGTGGTGAAG-3′; and (R) 5′-T CCTTGGAGGCCATGTGGGCCAT-3′.

2.8. Glucose uptake assays

We use Glucose Colorimetric/Fluorometric Assay Kit (BioVision) to test glucose uptake in HepG2 cells. Firstly, HepG2 cells were treated as described. Then stimulation with insulin (100 nM) for 30 min. Add 30 ml test samples to a 96-well plate. Adjust the volume to 50 ml/well with Glucose Assay Buffer. Mix enough reagents for the number of assays to be performed: For each well, prepare a total 50 ml Reaction Mix containing Glucose Assay Buffer 46 ml, Glucose Probe** 2 ml, Glucose Enzyme Mix 2 ml. Mix well. Add 50 ml of the Reaction Mix to each well containing the Glucose Standard and test samples. Mix well. Incubate the reaction for 30 min at 37 °C, protect from light. Measure absorbance (OD570 nm) or Fluorescence (Ex/Em = 535/590 nm) for in a microplate reader.

2.9. JNK inhibition

SP600125 (10 mM) was added to cell culture for 30 min prior to stimulation. An equivalent volume of DMSO without SP600125 was added to control cultures. The inhibitor was left in medium for the duration of experiment.

2.10. Statistical analysis

Data were expressed as the mean ± SEM 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. $P < 0.05$ was considered to be statistically significant.

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