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PKM2 aggravates palmitate-induced insulin resistance in HepG2 cells via STAT3 pathway

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ABSTRACT

Studies have identified that PKM2 is related to the development of glucose intolerance and insulin resistance in rodents and humans. However, the underlying mechanism remains largely unknown. In the present study, we found that PKM2 expression was significantly elevated in insulin-resistant hepatic tissues and hepatocytes, implicating an association between PKM2 expression and hepatic insulin resistance (IR). In vitro study revealed that overexpression of PKM2 impaired the insulin signaling pathway by decreasing the phosphorylation of protein kinase B (Akt) and glycogen synthase kinase-3β (GSK3β). Furthermore, PKM2 overexpression enhanced the effects of PA on the lipid accumulation, the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) and hepatic glucose uptake. Intriguingly, PA-induced insulin resistance was suppressed following by the ablation of PKM2 in HepG2 cells. We also found that STAT3 was significantly activated by PKM2 overexpression. Moreover, we identified that PKM2 could interact directly with STAT3. Taken together, these studies demonstrate that PKM2 may promote hepatic IR via STAT3 pathway and would provide a new insight into dissecting the molecular pathogenesis of hepatic insulin resistance.

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1. Introduction

Type 2 diabetes, one of the most common chronic diseases worldwide, currently endangers the quality of life and health of human population [1]. Insulin resistance (IR) is among the earliest pathological changes in type 2 diabetes mellitus (T2DM) [2,3]. Prolonged elevated plasma free fatty acids (FFAs) plays an important role in hepatic IR [4]. However, the mechanisms linking FFAs and hepatic insulin-resistance are not fully clarified.

It is well known that FFAs-induced insulin-resistance in liver is usually accompanied by the abnormal accumulation of ROS [5]. ROS can promote PKM2 upregulation and enable its nuclear translocation, driving systemic and tissue inflammation [6,7]. These findings thus propose a link between PKM2 and hepatic IR.

PKM2, a rate-limiting glycolytic enzyme in glucose metabolism, is expressed ubiquitously in most cells [8]. Previous studies have been established that PKM2 could stimulate hypoxia-inducible factor-1α (HIF-1α) dependent transactivation of glycolytic genes in cancer cells [9]. It is reported that HIF-1α improves insulin sensitivity in the adipocytes in high-fat-diet-fed mice [10]. In colorectal cells, PKM2 contributes to the upregulation of STAT3 activation via phosphorylation of STAT3 [11]. Studies have shown that STAT3 plays a significant role in regulating hepatic insulin resistance induced by free fatty acids [12,13]. Additionally, PKM2 is elevated in adipose tissue after high-fat diet [14]. These data suggest that PKM2 is involved in FFAs-induced insulin resistance. However, the molecular mechanisms by which PKM2 directly promotes hepatic insulin-resistance remain obscure.

In order to illustrate the underlying mechanism, we first examined the level of PKM2 in liver extracts of db/db mice and in palmitate (PA) exposed HepG2 cells. We also analyzed the influence of PKM2 in insulin signaling pathway transduction through PKM2 overexpression or PKM2 depletion. Furthermore, we found that PKM2 could induce insulin resistance by activating STAT3 signaling.

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These observations demonstrate that PKM2 is a potential molecular target of IR and T2DM.

2. Research design and methods

2.1. Animal experiments

Four-week-old male db/db mice (C57BL/6J) were purchased from the Experimental Animal Center of Nantong University, and fed with normal chow diet (NCD) or high-fat diet (HFD) for 16 weeks. HFD contained 50% carbohydrate, 20% protein and 25% fat in a temperature-controlled (22–24 °C) and humidity-controlled (45–55%) environment (12:12 h light-dark cycle). All procedures were performed following the National Institutes of Health Animal Care and Use Guidelines.

2.2. Cell culture and treatments

Human hepatoma cell line HepG2 cells were obtained from the Chinese Academy of Science (Shanghai China). The HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were maintained at 37 °C with humidified air and 5% CO_2, with medium changes three times a week. In order to establish a hepatocellular model of insulin-resistance, HepG2 cells were stimulated by the indicated concentrations of PA (Sigma, 0.25 mM) for 24 h. Before harvest, HepG2 cells were subsequently treated with insulin (100 nM) for 20min.

As for cell transfection, HepG2 cells were plated in 6-well plates, and transfected with HA-tagged full-length PKM2 from GeneChem, PKM2 siRNA (GeneChem) or mock construct (control, 3.2 μg per well) using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's protocol. The siRNA target sequence is 5′-GGT GAA CTT TGC CAT GAA T-3′. 24 h after transfection, HepG2 cells were treated with or without 0.25 mM PA for 24 h and 100 nM insulin for 20 min before harvest.

2.3. Antibodies and reagents

Anti-phospho-Akt (Ser473), anti-phospho-GSK3β (Ser9), anti-phopho-STAT3 (Thr705) were purchased from Cell Signaling. Anti-Akt, anti-GSK3β, anti-PKM2, anti-STAT3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Anti-GAPDH, anti-HA were purchased from Sigma.

FFAs were prepared with the methods of protein adsorption: First of all, 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.

2.4. Westen blot and immunoprecipitation (IP)

After cells harvest, the protein concentration was performed by Quantity One software (Bio-Rad, Hercules, CA, USA). Cell lysates were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA, USA), and the membranes were blocked with 5% w/v skim milk for 2 h at room temperature (RT). The membranes were incubated overnight at 4 °C with antibodies against PKM2, STAT3, Thr705-phosphorylated STAT3, Akt, Ser473-phosphorylated Akt, GSK-3β, Ser9-phosphorylated GSK3β and GAPDH. The immune complexes were detected using a horseradish peroxidase-conjugated secondary antibody (1:5000, SouthernBiotech) for 2 h at RT. Finally, they were visualized using an enhanced chemiluminescence system (ECL; Pierce Company Woburn, MA, USA). ImageJ (NIH) was used to analyze the densities of the bands. Each blot shown in the figures is representative of at least three experiments.

The protein extracts were pre-cleared with protein A/G plus beads (Santa Cruz Biotechnology). A total of 500 μg of protein from each sample was incubated with the indicated antibodies overnight at 4 °C at a dilution of 1:100. And the protein samples were further incubated with 15 μl of pre-cleared protein A/G plus beads for 2 h at 4 °C, followed by 3 washes with non-denaturing lysis buffer. The prepared samples were then detected with Western blot assays, as described above.

2.5. Glucose uptake assay

Insulin-induced glucose uptake in HepG2 cells was measured by Glucose Colorimetric/Fluorometric Assay kit (BioVision, Mountain View, CA). As described previously, HepG2 cells were incubated with Insulin for 20 min. And then we added 30 μl test samples to a 96 well plate and adjusted the volume to 50 μl/well with glucose assay buffer. Mix enough reagents for the number of assays to be performed: For each well, prepare a total 50 μl reaction system containing glucose assay buffer 46 μl, glucose probe 2 μl, glucose enzyme mix 2 μl and mix well. Add 50 μl reaction mix to each well including glucose standard and test samples. Mix well. Incubate the reaction for 30 min at 37 °C in the darkness. Mersure absorbance (OD 570 nm) or fluorescence (Ex/Em = 535/590 nm) using a microplate reader.

2.6. Immunofluorescence (IF)

The cells seeded in the 24-well plates were fixed by 4% paraformaldehyde, followed by permeabilization of 1% triton. Then the cells were incubated overnight at 4 °C with anti-PKM2 antibodies and the following day, they were detected with Alexa Fluor 555 goat anti-mouse IgG antibody. Hoechst (Sigma) was used to stain the nuclei. Immunofluorescence images were observed on a Zeiss LSM 510 META Confocal Microscope.

2.7. Oil Red O staining

HepG2 cells were transfected with HA-PKM2 or HA and then 24 h later treated with 0.25 mM PA for 24 h. Confluent cells were fixed in phosphate-buffered formalin (10%) for 1 h at RT. After removal of formalin, cells were rinsed with cold 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 RT. And then excess stain was removed by 70% ethanol and finally the cells were washed with PBS. The cells were obtained by inwerted microscope.

2.8. RNA isolation and quantitative real time PCR

Total RNA from liver tissue was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and was reverse-transcribed. Quantitative real-time PCR (qRT-PCR) analysis was performed with the Roche Light Cycler 480 System (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer's guidance. And the gene expression level was normalized to GAPDH for each sample. The qRT-PCR primers used were as follows: GAPDH, 5′-ATG GTT TAC ATG TTC CAA TAT-3′(forward) and 5′-ATG AGG TCC ACC ACC CTG GTT G-3′(reverse); G6Pase, 5′-TGG TTG GGA TTC TGG GCT CT-3′ (forward) and 5′-TCT ACA CCC AGT CCC TTG AG-3′(reverse); PEPCK, 5′-GTT CAA TGC CAG GTT CCC AG-3′ (forward) and 5′-TTG CAG GCC AGT TGT TGA C-3′(reverse).

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