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PID1 in adipocytes modulates whole-body glucose homeostasis

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ABSTRACT

The novel obesity-associated protein **P**hosphotyrosine **I**nteraction **D**omain containing 1 (PID1) inhibits insulin-PI3K/Akt signaling pathway and insulin-stimulated glucose uptake *in vitro*. In this study, we generated fat tissue-specific aP2-PID1 transgenic (aP2-PID1^{tg}) mice and PID1 knockout (PID1$^{-/-}$) mice to explore how PID1 affects glucose metabolism *in vivo*. We observed insulin resistance and impaired insulin-PI3K/Akt signaling in aP2-PID1^{tg} mice. Consistent with these data, the PID1$^{-/-}$ mice displayed improved glucose tolerance and insulin sensitivity under chow diet, with increased Akt phosphorylation in white adipose tissue (WAT). We further demonstrated that PID1 could interact with low density lipoprotein receptor-related protein 1 (LRP1) but not the insulin receptor (IR) in adipocytes, and its overexpression could lead to decreased GLUT4 level. Our results thus indentify PID1 as a critical regulator of glucose metabolism in adipocytes.

1. Introduction

PID1 was initially identified as a novel obesity-associated gene from the abdominal subcutaneous fat tissue of obese subjects, and was gradually characterized by our laboratory [1]. PID1 protein contains a phosphotyrosine binding (PTB) domain that is capable of recognizing phosphorylated tyrosine residues. The expression of PID1 was mainly detected in adipose tissue, heart, liver, and skeletal muscle, and was augmented in the fat tissue of obese human [1]. Gain-of-function analysis revealed that PID1 promoted the proliferation of 3T3-L1 cells by regulating cell cycle without affecting adipogenic differentiation [1]. Furthermore, PID1 inhibited insulin-stimulated GLUT4 translocation and glucose uptake with impaired insulin-PI3K/Akt pathway in rodent adipocytes and skeletal muscle cells [2–5].

Intriguingly, PID1 also plays a key role in other pathological processes. The expression of PID1 was attenuated in the brains of Alzheimer disease patients [6]. In addition, PID1 may have a growth-modulating function in brain tumor formation [7]. *In vitro* experiment results further showed that PID1 significantly promoted cell death and inhibited proliferation of different brain tumor cell lines by inducing mitochondrial depolarization and affecting serum-mediated phosphorylation of Akt and ERK [7]. All these findings suggested that PID1 functions as a cell type-dependent manner in different signaling pathways to affect important metabolic and pathologic processes.

Although previous *in vitro* studies have suggested that PID1 may be a novel key candidate related to obesity-associated insulin resistance [2–5], the underlying mechanisms remain un-elucidated, and the *in vivo* data are lacking. In these studies, we generated fat tissue-specific PID1 transgenic and knockout mice models to study how PID1 affects glucose metabolism *in vivo*.

2. Research design and methods

2.1. Generation of adipose-specific aP2-PID1^{tg} mice and PID1$^{-/-}$ mice lines

The plasmid used to generate adipose tissue-specific PID1^{tg} mice was generated by subcloning mouse PID1 sequence (NM_001003948.2) into the plasmid pL253-aP2, which contained the 5.4-kb adipocyte-specific aP2 promoter [8] and a HA tag (Fig. S1), and was obtained from Model Animal Research Center of Nanjing University. We obtained several independent mouse lines with HA-PID1 overexpression in the adipose tissues, among which two lines with moderate expression level were used for subsequent experiments and hereafter referred as the aP2-PID1^{tg} mice. The PID1$^{-/-}$ embryonic stem cells in the C57BL/6N background (PID1$^{tm1a(KOMP)Wtsi}$, EPD0579_4_G03) (Fig. S2) were

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constructed by KOMP (www.KOMP.org), which inserted a vector with the L1L2_Bact_P cassette into the mouse PID1 gene between exons 2 and 3. Then the cells were injected into blastocysts of C57BL/6J mice to generate heterozygous PID1 knockout ($PID1^{+/-}$) mice.

2.2. Animal study and metabolic analysis

All animal studies were approved by the Ethics Committees at the Model Animal Research Center of Nanjing University. Mice were housed under standard conditions and allowed food and water *ad libitum*. Obese model was generated by feeding 60% high-fat diet (HFD) started at 8 weeks of age. Fat and lean mass of mice were assessed by the PIXImus dual-energy X-ray absorptiometry system (GE). All glucose tolerance tests (GTTs), insulin tolerance tests (ITTs), and glucose-stimulated insulin secretion tests were performed in fasted animals as previously described [9]. The serum insulin levels were determined by using an Ultrasensitive Mouse Insulin ELISA kit (R&D).

2.3. Glucose uptake in mature adipocytes

The 3T3-L1 preadipocytes and human preadipocytes were subjected to differentiation, and glucose uptake was measured as previously described [5,9].

2.4. Real-time quantitative PCR analysis

RNA was isolated from tissue flash-frozen in liquid nitrogen using the RNeasy Mini Kit (QIAGEN) following the manufacturer's protocol. The quality of RNA was quantified and reverse-transcribed into cDNA using the M-MLV First Strand Kit (Invitrogen). Reaction conditions were as what described in the manufacturer's instructions. Real-time PCR was performed by using SYBR Green master mix (Applied Biosystems). Expression levels were normalized to the expression level of GAPDH mRNA. All primers used are listed in Table S1.

2.5. Western blot analysis

Mouse tissues and cell samples were prepared in lysis buffer (20 mM Tris (pH = 8.0), 150 Mm Nacl, 10%glycerol, 20 mM beta-glycerol phosphate, 1%NP-40, 5 mM EDTA, 0.5 mM EGTA) with protease inhibitors (PMSF, NaF, leupeptin, Benzamidine, DTT, Na3VO4), quantitated, subjected to 8–10% SDS-PAGE, and transferred to 0.2-μm PVDF membranes for immunoblot analysis using specific antibodies [9]. Antibodies we used were: LRP1 (Epitomics, 2703-1), PID1 (Abcam, ab107978), Adiponectin (CST, 2789), HA (Abcam,ab9110), Phospho-AKT(thr308) (Epitomics, 2214-1), Phospho-AKT(ser473) (CST, 4060L), IRβ (Santa Cruz, sc-711),T-AKT (CST, 9272S), IRAP (CST, 9876), GLUT4 (R&D,MAB1262), β-actin (Bioworld,BS6007M) and GAPDH (Bioworld, AP0063).

2.6. Co-immunoprecipitation assay

Co-immunoprecipitation was carried out by using a previously described method [10]. Briefly, pre-cleared fat tissue and cell lysates were incubated with antibody-coupled protein G-sepharose beads overnight at 4 °C. The immunoprecipitates were dissolved in SDS sample buffer for subsequent analysis by WB. Antibodies we used were: LRP1 (Epitomics, 2703-1) and HA (Abcam, ab9110).

2.7. Immunofluorescence and confocal microscopy

Cells were fixed 20 min in 4% paraformaldehyde, blocked and then incubated with indicated primary antibodies diluted in goat serum (BOSTER, AR0009) overnight at 4 °C. The cells were subsequently stained with AlexaFluor 488-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies for 2 h at room temperature. The nuclei were stained with DAPI. The slides were mounted with 50% glycerol in PBS and photographed with a Leica TCS SP2 confocal laser scanning microscope. Primary antibodies we used were: LRP1 (Epitomics, 2703-1) (1/2000 dilution), IRβ (Santa Cruz, sc-711) (1/100 dilution), Flag (Sigma, F1804) (1/1000 dilution) and GLUT4 (Abcam, ab654) (1/100 dilution).

2.8. Clinical datasets

Firstly, we searched NCBI GEO database by using "obese/obesity" as key words, and obtained > 500 high-throughput gene expression profiles of human obesity and normal samples. To minimize confounding factors, the samples with insulin resistance, the samples from cultured human adipocytes or blood of obesity, and the samples with known medical problems such as chromosomal abnormalities, autoimmune diseases and malignant tumor were all excluded. The data from subcutaneous, omental and visceral fat tissues were all included. In our study, total 187 subjects from different datasets were used, with the ID for GSE48964, GSE29718, GSE9624, GSE5090 and GSE22070 (Table 2 and Table S2). According to different regions of fat tissues, the datasets were divided into subcutaneous and omental/visceral groups. Each gene mRNA expression was normalized to GAPDH and then used to analyze the PID1 expression level in different groups.

2.9. Statistical analysis

Statistical analysis was performed by using two-tailed, unpaired Student's *t*-tests. $p < 0.05$ was considered to be statistically significant. The results are presented as the mean ± SEM.

3. Results

3.1. aP2-$PID1^{tg}$ mice exhibited impaired glucose homeostasis and reduced insulin sensitivity under HFD challenge

To determine whether fat tissue-expressed PID1 is sufficient to induce glucose tolerance and insulin resistance, we generated a transgenic mice line that expressed HA-tagged PID1 (HA-PID1) in fat tissues by utilizing a full-length aP2 promoter/enhancer, which is specific to adipose tissue and is active during fat cell differentiation (Fig. S1A) [8]. Two founder lines with moderate levels of PID1 DNA copies were used to study the phenotype. Western blot analysis revealed that the HA-PID1 of aP2-$PID1^{tg}$ mice is only detectable in fat tissues but not others (Fig. S1B). And the expression at both mRNA and protein levels was significantly increased in epididymal, subcutaneous, and perirenal WAT and brown adipose tissues (BAT) of the aP2-$PID1^{tg}$ mice compared with WT controls (Fig. S1C–D).

Because PID1 has been implicated in insulin-stimulated glucose transport in adipocytes, we firstly sought to determine if whole-body glucose homeostasis could be altered in the aP2-$PID1^{tg}$ animals. We observed that the aP2-$PID1^{tg}$ mice showed impaired insulin tolerance but normal glucose tolerance (Fig. 1A–B) after HFD feeding for 10 weeks. When the mice were fed on HFD for 14–15 weeks, both glucose intolerance and insulin intolerance in aP2-$PID1^{tg}$ mice were further exacerbated (Fig. 1C–D). Meanwhile, GTTs and ITTs results were comparable under chow diet (CD) (Fig. 1C–D). Even at 14 months age old, the CD-fed WT mice exhibited little or no difference in glucose homeostasis compared with CD-fed aP2-$PID1^{tg}$ mice (Fig. S3A–B). Besides, we found that the glucose-induced increase in plasma insulin levels was similar between HFD-fed WT mice and HFD-fed aP2-$PID1^{tg}$ mice after glucose injection (Fig. 1E), indicating that altered insulin levels in the blood were unlikely to be responsible for the glucose intolerance phenotype of the aP2-$PID1^{tg}$ mice.

To test whether Akt activity is necessary for the regulation of glucose homeostasis, we observed that the insulin-stimulated phosphorylation of Akt was inhibited in aP2-$PID1^{tg}$ mice fat tissue (Fig. 1F–G),