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Short communication

Effects of small interference RNA against PTP1B and TCPTP on insulin signaling pathway in mouse liver: Evidence for non-synergetic cooperation

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Abstract

Metabolic deregulation accompanying type II diabetes is characterized by insulin resistance in peripheral tissues (liver, muscle, and adipose), mediated by impairments in insulin receptor (IR) signaling. Two closely-related protein tyrosine phosphatases, PTP1B and TCPTP both showed abilities to negatively regulate insulin receptor signaling. In order to test whether these two phosphatases can act synergistically, hydrodynamic injection was applied to deliver small interfering RNA (siRNA) of PTP1B and/or TCPTP to mouse liver. By measuring insulin-sensitive reporter gene expression and plasma glucose of diabetic mice, we found siRNA of PTP1B or TCPTP alone can sensitize insulin signal transduction, but combined treatment of both siRNAs had no better effects than siRNA of PTP1B. These results suggested siRNA of PTP1B and TCPTP can strengthen insulin signaling, but their effects do not appear to be synergistic in mouse liver.

Keywords: PTP1B; TCPTP; siRNA; Fatty acid synthase promoter; Diabetic mouse model

1. Introduction

A major characteristic of type II diabetes, also named non-insulin dependent diabetes, is insulin resistance in peripheral tissues including liver, fat, and skeletal muscle. Although the molecular mechanism underlying insulin resistance is not clear, it is believed to involve the impairment of insulin receptor (IR) signal transduction (Chakraborty, 2006; Kruszynska and Olefsky, 1996).

The protein tyrosine phosphatase PTP1B can dephosphorylate activated IR and insulin receptor substrate to function as a negative regulator of insulin signaling. Mice lacking PTP1B showed increased insulin sensitivity with resistance to weight gain on a high-fat diet but no significant defects (Elchebly et al., 1999). Furthermore, PTP1B antisense treatment can enhance insulin sensitivity and normalize blood glucose in genetic diabetic mouse models (Rondinone et al., 2002; Zinker et al., 2002).

TCPTP is the PTP most closely-related to PTP1B, with 72% sequence identity and 86% similarity in the catalytic domain (residues 43–288). Some studies have shown that the closely-related tyrosine phosphatase TCPTP might also contribute to the regulation of insulin receptor (IR) signaling in vivo (Galic et al., 2003).

In order to investigate the functions of PTP1B and TCPTP on insulin signaling, endonuclease-hydrolyzed PTP1B (esiMP) and TCPTP siRNA (esiTP) were prepared. PTP1B-EGFP and TCPTP-EGFP fusion constructs were used to estimate the inhibitory effects of esiMP and esiTP. The ability of esiRNAs to up-regulate insulin signaling and normalize plasma glucose was verified by reporter system with insulin controlled fatty acid synthase promoter, and alloxan induced diabetic mouse model. But the combination of esiMP and esiTP did not show better effects than esiMP alone.

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2. Materials and methods

2.1. Plasmid constructs

The plasmids p1B-EGFP, pET2p-1B and the reporter plasmid pFP-IHBS were constructed according to Xu et al. (2005).

Mouse Igκ-chain leader sequence was amplified from pSecTag2A (Invitrogen, USA) with sense primer GGAATTCCACCATGGAGACAGACACAC and antisense primer GAATCCTGATGTTGTGCTCTCGTCACCAGTGGAA CCTGGAAC. DNA sequence coding for HBV s-antigen was amplified from pUC-HBV (a gift from Dr. Wei Lixin at SMMU, China) with sense primers CCATATCGTCAATCTTCTCG and antisense primer GGCCCGGGTC AAATGTATACCCAAAGAC. Igκ-chain leader sequence and HBV s-antigen sequence were linked by overlap PCR, and replaced EGFP sequence of pEGFP_N2 (BD Clontech, USA) between *Eco*RI and *Sma*I restriction sites to obtain pCMV-IHBS. The secretory HBS expression was verified in Hepa1-6 cell (a gift from Dr. Wei at SMMU, China) before subsequent construction. An 812 bp long insulin responsive element (from −678 to 134 in mRNA sequence of GenBank NM007988) of mouse fatty acid synthase promoter (mFP) was amplified from mouse total DNA by PCR. Primers used are sense CCCATTAATTGTTCCCTATCCTGCCTACTG and antisense GGGCTAGCAGGCAGACGACAAGCGAGGA. The fragment was digested with *Ase*I/*Nhe*I and replaced CMV promoter sequence in pCMV-IHBS to obtain plasmid pFP-IHBS.

Full length of the mouse *TCPTP* gene was amplified from mouse cDNA using primers G*GAATTC*ATGTCGGCAACCATCGAGCG and G*GGATCC*G GGTGTCTGTCAATCTTGGCC and inserted into pEGFP_N2 at *Eco*RI and *Bam*HI sites to obtain fusion protein expression plasmid pTCPTP-EGFP.

To obtain double strand RNA in *Escherichia coli*, a pET2p plasmid was constructed with two promoters in opposite direction to express double strand RNA. A fragment containing T7 promoter and tac promoter flanking pET22b multi-clone site region was amplified with primers 2p5 (GGAAGATCTGGC CCACCCGTGAAGGTGAGCC CGATCCCGCGAAATTAATACGACT) and 2p3 (CCGCTCGAGTTGACAATTAATCATCGGCTCGTATAATGTGCGGC CGCAAGCTTGTC) and replaced the sequence of pET22b between *Bgl*II and *Xba*I restriction sites to get pET2p. The mouse *TCPTP* gene was then inserted into pET2p at *Eco*RI and *Bam*HI site to obtain pET2p-TCPTP.

The constitutively active Akt mutant (AktE40K) is a gift from Dr. Sha at the University of Alabama, Birmingham, USA.

2.2. esiRNAs preparation from E. coli expressed long dsRNA

The esiRNA preparation procedures were described in previous paper (Qian et al., 2005). Briefly, the dsRNA expressing vectors were transformed into *E. coli* and then cultured and induced for dsRNA expression. dsRNAs were purified from the induced bacteria cell lysate with Whatman® fibrous cellulose CF-11 (Whatman, USA). The obtained dsRNA were digested with recombinant *E. coli* RNase III in a buffer containing manganese ions. esiRNAs about 21 bp in length were purified by DEAE ion exchange chromatography and size exclusion chromatography (Xuan et al., 2005).

2.3. Cell culture and transfection

Hepa1-6 mouse hepatoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Each well was seeded with 1×10^5 cells in 0.5 ml of the medium and cultured overnight or until 90% confluency in 24-well plate. For p1B-EGFP and pTCPTP-EGFP transfection, cells were co-transfected with 0.4 μg p1B-EGFP or pTCPTP-EGFP and 0.4 μg esiMP, esiTP or esiAp and continued growing in 24-well plate. Transfection was performed with Lipofectamine 2000 (Invitrogen) following manufacturer's instruction. At 48 h post-transfection, cells were subjected to flow cytometry assay.

2.4. Animal care and treatment

About 20-g weight KM mice (provided by SMMU, China) were fasted 12 h before hydrodynamic injection, and were refed immediately after injection. Ten microgram of plasmids and 3 μg siRNAs in 2 ml Ringers' buffer were injected intravenously within 5 s. A dose of 2.5 g/kg glucose was injected introperitoneally to elicit insulin stimulation. Mouse blood was obtained at various time points after injection and the titer of secretary HBV s-antigen in the sera was done with ELISA Kit.

KM mice were rendered diabetic by intravenous injection of 75 mg/kg body weight alloxan (Sigma; freshly dissolved in 10 mM citrate buffer pH 3.0) twice in continual two days. All animals entering the study exhibited nonfasting blood glucose levels above 15 mM. siRNAs and plasmid were transferred into mouse liver by hydrodynamic injection. Blood was obtained from the tail vein for the glucose assays (Shanghai Kehua biotechnology company, China) for three days after injection.

3. Results

3.1. Reduction of PTP1B and TCPTP expression by RNAi in Hepa1-6 cells

We first constructed p1B-EGFP and pTP-EGFP in which enhanced green fluorescence protein was fused C-terminal to the PTP1B protein or TCPTP protein, and the degradation of resultant fusion protein would be easily seen if RNAi targeted against mouse PTP1b or TCPTP works. siRNAs were tested for their inhibitory effect on PTP1B-EGFP or TCPTP-EGFP expression in Hepa1-6 cell. As seen with flow cytometry assay, cells transfected with esiMP and esiTP showed specific decreases of fluorescence intensities to ∼6%, indicating the expression of PTP1B-EGFP and TCPTP-EGFP was specifically and dramatically inhibited by RNAi (Fig. 1A).

3.2. Reduction of PTP1B and TCPTP increased insulin signaling in mouse liver

In order to investigate the effect of siRNAs of PTP1B and TCPTP on insulin signaling, we measured the activity of fatty acid synthase promoter in mouse liver since fatty acid synthase gene is responsive to insulin signaling. The reporter plasmid was injected with esiMP or with both esiMP and esiTP. If TCPTP can coordinately regulate insulin signaling with PTP1B in liver, injection of both siRNAs will promote the reporter gene expression. Surprisingly, injection of both siRNAs exhibited worse effects than injection of esiMP alone (Fig. 1B).

3.3. Reduction of PTP1B and TCPTP decreased plasma glucose in diabetic model mice

Alloxan induced diabetic mouse model was used to determine the anti-diabetic effects of siRNAs. Besides siRNAs, a plasmid expressing constituent active Akt was also introduced as a positive control because phosphorylation and activation of Akt is a signature of activated insulin pathway. Although injection of Ringers' buffer also decreased plasma glucose in the second day, injection of siRNA of PTP1B, TCPTP or both can decrease plasma glucose to a lower level. Among which, the ability to decrease plasma glucose of esiMP was stronger than those of esiTP and both. And in coincidence with the results of insulin signaling promotion (Fig. 1C),