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Role of somatomedin-B-like domains on ENPP1 inhibition of insulin signaling

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ABSTRACT

The exact mechanism by which ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) inhibits insulin signaling is not known. ENPP1 contains two somatomedin-B-like domains (i.e. SMB 1 and 2) involved in ENPP1 dimerization in animal cells. The aim of the present study was to investigate if these domains modulate ENPP1 inhibitory activity on insulin signaling in human insulin target cells (HepG2). ENPP1 (ENPP1–3′ myc), ENPP1 deleted of SMB 1 (ENPP1-ΔI-3′myc) or of SMB 2 (ENPP1-ΔII-3′myc) domain were cloned in frame with myc tag in mammalian expression vector pRK5. Plasmids were transiently transfected in human liver HepG2 cells. ENPP1 inhibitory activity on insulin signaling, dimerization and protein–protein interaction with insulin receptor (IR), reported to mediate the modulation of ENPP1 inhibitory activity, were studied. As compared to untransfected cells, a progressive increase of ENPP1 inhibitory activity on insulin-induced IR β-subunit autophosphorylation and on Akt-S^{473} phosphorylation was observed in ENPP1–3′ myc, ENPP1-ΔI-3′myc and ENPP1-ΔII-3′myc cells. Under non reducing conditions a 260 kDa homodimer, indicating ENPP1 dimerization, was observed. The ratio of non reduced (260 kDa) to reduced (130 kDa) ENPP1 was significantly decreased by two thirds in ENPP1-ΔII-3′myc vs. ENPP1–3′myc but not in ENPP1-ΔI-3′myc. A similar ENPP1/IR interaction was detectable by co-immunoprecipitation in ENPP1–3′myc, ENPP1-ΔI-3′myc and ENPP1-ΔII-3′myc cells. In conclusion, SMB 1 and SMB 2 are negative modulators of ENPP1 inhibitory activity on insulin signaling. For SMB 2 such effect might be mediated by a positive role on protein dimerization.

1. Introduction

Insulin resistance is a major contributor to type 2 diabetes and cardiovascular disease and thus to morbidity and mortality in developed countries [1].

The molecular mechanisms of insulin resistance are mostly unknown [2]. A candidate molecule is the class II transmembrane glycoprotein ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1), which binds to the insulin receptor (IR) β-subunit and inhibits IR β-subunit autophosphorylation and downstream signaling [3–6]. As a matter of fact, in both cultured cells [3,7–11] and rodents [12,13], ENPP1 overexpression affects insulin signaling and action. In addition, increased ENPP1 expression has been observed in several tissues of insulin-resistant subjects [5,14–17]. Further support to the notion that ENPP1 may play a role on human insulin resistance derives from the ENPP1 K121Q polymorphism (rs1805101) [18], with Q121 being a gain of function aminoacid substitution with a stronger inhibitory activity on IR signaling [9,19] and a genetic determinant of insulin resistance-related abnormalities in several [20–22], although not all [23], studies.

The exact mechanism by which ENPP1 inhibits IR β-subunit autophosphorylation and downstream signaling is not yet understood.

ENPP1 is a homodimer of about 130 kDa [24,25]. The monomers consist of a short intracellular N-terminal domain (10–80 residues) involved in the targeting to plasma membrane [26], a single transmembrane domain (~20 residues) and a large extracellular part (~800 residues) comprising several domains. Among these, are two consecutive somatomedin-B-like (i.e. SMB 1 and 2) domains which have been reported to take part of mouse ENPP1 dimerization in monkey cells

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[27]. Whether these two domains modulate ENPP1 inhibitory activity on insulin signaling is not known. The aim of the present study was to address this issue in human insulin target cells.

2. Materials and methods

2.1. Cell lines

HepG2 cells (ATCC, Manassas, USA) were maintained at 37 °C and 5% CO_2 in DMEM/F12 containing 10% FBS.

2.2. Plasmids

Full-length cDNA of human ENPP1 was kindly provided by Dr. I.D. Goldfine (San Francisco, University of California, USA). ENPP1 open reading frame was cloned in frame with myc tag in mammalian expression vector pRK5 (ENPP1–3′myc, expected molecular weight (MW) 119.6 kDa); ENPP1 lacking amino acids from 107 to 144 which represent SMB 1 domain (ENPP1-ΔI-3′myc, expected MW 115.5 kDa) and ENPP1 lacking amino acids from 145 to 186, which represent the SMB 2 domain (ENPP1-ΔII-3′myc, expected MW 110.9 kDa) were generated by mutagenesis of pRK5 ENPP1–3′myc by using ExSite PCR-Based Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene), by using the following primers:

- ENPP1-ΔI-3′myc:
  5′-TATATGGACTTGCAACAAATTCAGGTGTGGTGAG-3′
  5′-AACTTCTTTGGCACAGCTTGGTTTCAACCC-3′;
- ENPP1-ΔII-3′myc:
  5′-AGTTGGGTAGAAGAACCATGTGAGAGCATTAATG-3′
  5′-ATGTTCTGGTTCTATGCACGTCTCCTGGTAATCTAAAC-3′.

2.3. Transfections

HepG2 cells were transiently transfected with ENPP1–3′myc, ENPP1-ΔI-3′myc, ENPP1-ΔII-3′myc and mock cDNAs as appropriate by using TransIT reagent according to the manufacturer's instructions (Mirus, Italy), and then starved overnight in DMEM/F12 containing 0.5% FBS before experiments. ENPP1 protein expression in each condition was evaluated by western blot analysis. Transfection efficiency was evaluated by immunofluorescence after co-transfecting pcDNA3–EGFP with either ENPP1–3′myc or ENPP1-ΔI-3′myc or ENPP1-ΔII-3′myc, respectively. Fluorescent cells were always >55% across all experimental conditions.

2.4. Western blot

Cells lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were probed with specific antibodies and the chemiluminescent substrate (Super Signaling West Pico Thermo Scientific, Pierce or ECL by Amersham, GE Healthcare) was used for detection following HRP-conjugated antibodies (Santa Cruz Biotechnology, CA) incubation. Gel images were acquired by using Molecular Imager ChemiDoc XRS (Biorad) and analyzed by using Kodak Molecular Imaging Software 4.0 or IMAGEJ 1.40 g (Wayne Rasband, NIH).

2.5. IR β-subunit autophosphorylation

Following human insulin stimulation (10^{-6} M for 5 min at 37 °C) and cell lysis, equal amount of proteins was immunoprecipitated with anti-PY antibody (4G10 Platinum, Millipore, Italy) and analyzed by western blot with anti IR β-subunit antibody (C19, Santa Cruz Biotechnology, CA). IR β-subunit autophosphorylation was calculated as percentage of that in stimulated untransfected cells (i.e. IR band intensities of insulin-stimulated transfected cells/IR band intensities of insulin-stimulated untransfected cells × 100) and expressed as mean ± SEM. When data were analyzed after adjusting for ENPP1 protein expression levels, the above described values were further divided by ENPP1 band intensities. Both the upper and the lower bands were considered for quantification of ENPP1–3′myc and ENPP1-ΔI-3′myc expression.

2.6. Insulin downstream signaling

Following insulin stimulation (10^{-6} M for 5 min at 37 °C) and cell lysis, equal amount of protein was analyzed by western blot with the specific anti-phospho-Akt-S^{473} antibody (Cell Signaling, MA). The blot was then stripped and re-probed with anti-Akt antibody for normalization (Cell Signaling, MA). Data were calculated as percentage of that in stimulated untransfected cells (i.e. Akt-Ser^{473} band intensities of insulin-stimulated transfected cells/Akt-Ser^{473} band intensities of insulin-stimulated untransfected cells × 100). When data were analyzed after adjusting for ENPP1 protein expression levels the above described values were further divided by ENPP1 band intensities. Both the upper and the lower bands were considered for quantification of ENPP1–3′myc and ENPP1-ΔI-3′myc expression.

2.7. ENPP1 dimerization

Equal amount of proteins lysate was either supplemented with SDS-electrophoresis buffer in the absence of reducing agent (i.e. non-reduced proteins) or supplemented with SDS-electrophoresis buffer in the presence of reducing agent (i.e. reduced proteins) boiled and analyzed by western blot with anti c-myc antibody (9E10, Roche, Italy). Data were calculated as native to denatured sample ratio and expressed as mean ± SEM.

2.8. ENPP1/IR interaction

ENPP1/IR interaction was performed as previously described [19]. Briefly, following human insulin stimulation (10^{-6} M for 5 min at 37 °C) and cell lysis, proteins (i.e. 1 mg) were immunoprecipitated with the 83–14 anti-IR α-subunit antibody (Invitrogen, Italy) and analyzed by western blot analysis by using anti c-myc antibody (9E10, Roche, Italy). IR interaction was corrected for IR and ENPP1 expression levels and expressed as mean ± SEM.

2.9. Statistical analysis

Differences between mean values were evaluated by unpaired or paired Student's *t* test, as appropriate.

3. Results

3.1. ENPP1 constructs

To elucidate the role of SMB domains of ENPP1 on IR activation, we generated two deletion mutants (i.e. ENPP1-ΔI-3′myc, ENPP1-ΔII-3′myc) whose salient features are described in Methods (Fig. 1).

According to previous report [28], two bands appeared for myc-tagged ENPP1, whose apparent molecular mass is 135–145 kDa. This was also the case for ENPP1-ΔI-3′myc (apparent molecular mass of 133–143 kDa) but not for ENPP1-ΔII-3′myc for which only a single band was observed (apparent molecular mass of 134 kDa). Accordingly to what reported for ENPP1 [28], the doublet observed for ENPP1-ΔI-3′myc, might be the consequence of post-translational modification; therefore, distinct protein modification processes between ENPP1-ΔI-3′myc and ENPP1-ΔII-3′myc may account for their different aspect at western blot analysis.