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The role of HSP70 on ENPP1 expression and insulin-receptor activation

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Abstract

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Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) inhibits insulin-receptor (IR) signaling and, when over-expressed, induces insulin resistance in vitro and in vivo. Understanding the regulation of *ENPP1* expression may, thus, unravel new molecular mechanisms of insulin resistance. Recent data point to a pivotal role of the *ENPP1* 3'UTR, in modulating *ENPP1* mRNA stability and expression. We sought to identify *trans-acting* proteins binding the *ENPP1*-3'UTR and to investigate their role on ENPP1 expression and on IR signaling. By RNA electrophoresis mobility shift analysis and tandem mass spectrometry, we demonstrated the binding of heat shock protein 70 (HSP70) to *ENPP1*-3'UTR. Through this binding, HSP70 stabilizes *ENPP1* mRNA and increases *ENPP1* transcript and protein levels. This positive modulation of ENPP1 expression is paralleled by a reduced insulin-induced IR and IRS-1 phosphorylation. Taken together these data suggest that HSP70, by affecting ENPP1 expression, may be a novel mediator of altered insulin signaling.

Keywords

Inhibitors of insulin signaling; Insulin resistance; 3' untranslated regions; Tyrosine-kinase receptors

Introduction

Insulin resistance is pathogenic for type 2 diabetes and cardiovascular disease [1]. Unraveling the molecular mechanisms underlying this syndrome is urgently needed.

Ecto-enzyme nucleotide pyrophosphate phosphodiesterase 1 (ENPP1) has been proposed as a pathogenic factor for insulin resistance [2]. ENPP1 affects insulin signaling by binding to IR α -subunit and inhibiting receptor β -subunit autophosphorylation [3, 4]. ENPP1 expression is increased in tissues of insulin-resistant individuals [5, 6]. In addition, ENPP1 over-expression causes insulin resistance in rodents. [7, 8]. Finally, several data indicate that gain of function of *ENPP1* (as induced by the missense K121Q polymorphism) contribute to insulin resistance and type 2 diabetes [7–9]. Thus, determining the mechanisms whereby *ENPP1* is over-expressed may help develop strategies to counteract and possibly reverse some forms of insulin resistance. Recent data point to a pivotal role of the *ENPP1*-3'UTR [10, 11], suggesting the existence of *trans-acting* proteins that affect *ENPP1* mRNA stability. Our aim was to identify these proteins and investigate their role in the modulation of ENPP1 expression and insulin signaling.

Material and methods

Preparation of RNA probes as well as cell culture and solubilization are described in the online appendix methods

RNA electrophoresis mobility shift analysis (REMSA)

Fifty microgram of HEK293 lysates were incubated with the ^{32}P -labeled-RNA probe for 20 min at room temperature (RT) [12]. Following incubation with heparin (5 mg/ml), gel electrophoresis was carried out at RT on 5% non-denaturing PAGE and visualized by autoradiography on Typhoon 8600 (Amersham). For supershift analysis, HEK293 lysates were incubated with ^{32}P -labeled-RNA before adding either heat shock protein 70 (HSP70) specific antibody (SPA-812, Stressgen) or total IgG (Santa Cruz Biotechnology) for 30 min at RT.

Isolation of ENPP1-RNA binding protein

REMSA was carried as described above. Four gels of 15 lanes each were loaded. High molecular weight complexes, located by a 1-h exposure to X-ray film at -80°C , were excised and eluted from the gel [12]. Proteins were pooled, concentrated by acetone precipitation and resolved on 10% SDS-PAGE. The only band present in the gel with an apparent molecular weight of 70 kD was excised, and washed twice with 50% HPLC-grade acetonitrile before subsequent analysis. After proteolytic digestion, peptide composition analysis was performed at the Harvard Micro-chemistry Facility, Harvard University, (Cambridge, MA USA; <http://www.mcb.harvard.edu/microchem/>) by micro-capillary reverse-phase HPLC nano-electrospray tandem mass spectrometry ($\mu\text{LC/MS/MS}$) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer.

RNA extraction, cDNA synthesis, and gene expression analysis

Total RNA was isolated from cells using RNeasy Quick kit (Qiagen). cDNA was generated by reverse transcription with M-MLV Reverse Transcriptase (Promega) and used as template in the subsequent analyses. Gene Expression Assay on Demand Kit Reagents (Applied Biosystems) were used to quantify relative gene expression levels of *ENPP1* and *GAPDH* on ABI-PRISM 7500 (Applied Biosystems). Expression levels of *ENPP1* were normalized against *GAPDH* using the comparative C_t method, and expressed as percentage of control.

siRNA, cell transfections

Cells were seeded in six-well plates and grown in DMEM/F12 complete medium for 48 h. To down-regulate HSP70 expression, 150 nmol/L of siRNA targeted against *HSP70* mRNA (Ambion ID number: 202680 was the only oligonucleotide used in our experiments) were either cotransfected or not with *ENPP1* cDNAs by using TransMessenger Transfection Reagent (Qiagen) according to the manufacturer's instructions.

Cell lines stably over-expressing *IR* cDNA (HEK293-IR) were generated by co-transfection of the *prk5-IR* (provided by Dr. Axel Ulrich, Martinsried Germany) and *prk5-neo* followed by geneticin selection. IR expression was evaluated by western blot (WB) as described below. HEK293-IR were transiently transfected with *prk7-ENPP1* and/or with *pCMVSPORT6-HSP70* plasmid (ATCC) by using FuGENE6 (Roche).

Western blot analysis

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were probed with following antibodies: anti-HSP70 (SPA-812, Stressgen), anti-IR β -subunit (C19, Santa Cruz Biotechnology), anti-PY (PY99 HRP, Santa Cruz Biotechnology), anti-ENPP1 (N-20, Santa Cruz Biotechnology) and anti-IRS-1 (A-19, Santa Cruz Biotechnology). Alternatively, cell lysates were immunoprecipitated with anti-PY antibody (4G10, Millipore) and analyzed by WB using IR β -subunit or IRS-1 antibodies. Immunocomplexes were detected with the ECL Western Blotting System (Amersham Pharmacia Biotech).

ENPP1 mRNA stability

ENPP1 mRNA stability was evaluated by adding Actinomycin D (5 $\mu\text{g/ml}$) 60 h after *HSP70* silencing. RNA extraction was performed at different times as described above, and *ENPP1* expressions determined as described.

Insulin stimulation

Insulin (10 nmol/L for 5' at 37°C) was added to cells and total cell lysates were either immunoprecipitated or not before SDS-PAGE. IR β -subunit and IRS-1 phosphorylation were evaluated by WB as described.

Results

Identification and characterization of an ENPP1-3'UTR protein complex

REMSA was performed by incubating HEK293 cell extracts with a 395-bp probe corresponding to the *ENPP1*-3'UTR showing a high degree of conservation between human and mouse genomes (Supplementary Figure 1). RNA-protein complex was indicated by band-shift observed in the presence of the probe (Supplementary Figure 2, lanes 1–2). SDS-PAGE of the eluted shifted-band revealed a single protein in the RNA-protein complex. This was identified by tandem mass spectrometry as the 70 kDa HSP70 encoded by the *HSPA1B* isoform (Supplementary Figure 3). Addition of increasing amount of HSP70 antibody to the RNA-cell extract mixture induced a gradual loss of the high molecular complex (Supplementary Figure 2, lanes 3–10), confirming the specific interaction between HSP70 and *ENPP1*-3'UTR.

Effect of HSP70 on ENPP1 expression

We evaluated the effect of HSP70 down-regulation on *ENPP1* mRNA stability and expression by transfecting HEK293 cells with *HSP70*-siRNA. Exposure to the siRNA decreased HSP70 by approximately 80% (Fig. 1a). After transcription inhibition by Actinomycin D, a progressive reduction in *ENPP1* mRNA levels was observed (Fig. 1b); the reduction, however, was significantly greater in HSP70 down-regulated than control cells ($p < 0.01$; Fig. 1b). We also evaluated the effect of *HSP70*-siRNA on steady state levels of *ENPP1* mRNA and protein in cells transfected with *ENPP1* cDNA. Both mRNA (Fig. 1c) and protein (Fig. 1d, lanes 1–2) contents were lower following HSP70 down-regulation. This was not observed when *ENPP1* cDNA lacked the 3'UTR (Fig. 1d, lanes 3–4). We then evaluated the effect of HSP70 over-expression (i.e. by *HSP70* cDNA transfection) on ENPP1 protein content in cells co-transfected with *ENPP1* cDNA. ENPP1 protein levels were approximately 30% higher in co-transfected cells as compared to HEK293 transfected with *ENPP1* cDNA alone (Fig. 2), thus confirming that HSP70 levels affects ENPP1 expression.

Effect of HSP70 down-regulation on insulin signaling

When HSP70 expression was down-regulated in HEK293-IR cells, insulin-stimulated IR autophosphorylation was 2.5- to threefold higher than in control cells (Fig. 3a: lanes 2 vs. lane 1; Fig. 3b: second bar vs. first bar). In contrast, in cells transfected with *ENPP1* cDNA, insulin-induced IR autophosphorylation was markedly inhibited (Fig. 3a, lanes 3 vs. lane 1; Fig. 3b: third bar vs. first bar). This inhibition was partially abolished by HSP70 down-regulation (Fig. 3a lane 4 vs. lane 3; Fig. 3b: fourth bar vs. third bar), a rescue which was paralleled by a reduction of ENPP1 over-expression (Fig. 3a lanes 5–6 and Fig. 3b sixth bar vs. fifth bar) nor the reduced ENPP1 expression (Fig. 3a lanes 5–6) was observed when cells were transfected with *ENPP1* cDNA lacking the 3'UTR. In human liver HepG2 cells, *HSP70* down-regulation increased insulin-induced IR and IRS-1 phosphorylation (Fig. 3c lane 3 vs. lane 2). As compared to control cells, insulin-induced IR β -subunit autophosphorylation and IRS-1 phosphorylation were higher in cells treated with *HSP70* siRNA (153% and 274% increase, respectively, mean of two different experiments).

Discussion

The two major messages of our study are as follows 1) by binding *ENPP1*-3'UTR, HSP70 stabilizes *ENPP1* mRNA and eventually increases *ENPP1* transcript and protein levels; 2) through the modulation of ENPP1 expression, HSP70 affects insulin-induced IR and IRS-1 phosphorylation, thus becoming a new potential modulator of insulin resistance. Similar data were observed in two different cell lines, thus suggesting this phenomenon occurs in several insulin target tissues. To the best of our knowledge, this is the first report elucidating a molecular mechanism which modulates ENPP1 expression and, consequently, affects IR signaling.

Most of the molecular mechanisms of insulin resistance so far described are located at the downstream 'post-receptor level'. However, recent functional, metabolic [5, 13, 14] and genetic studies [9–11, 15–23] have suggested that an additional mechanism of insulin resistance resides at the 'receptor level' and is mediated by ENPP1[2]. In this context, we propose HSP70 as a new potential contributor to insulin resistance through up-regulation of ENPP1 expression and subsequent inhibition of IR-autophosphorylation. Of note, HSP70 is known to bind and modulate other 3'UTRs of yeast and human mRNAs [24, 25]. Very recently an opposite role of HSP70 on insulin signaling has been reported in transgenic animal models [26]. The proposed mechanism is mediated by HSP70-inhibition of JNK activation, a negative modulator of IRS-1 signaling. These discrepancies are difficult to reconcile and might be due to relevant differences intrinsic to the study models utilized (i.e. in vivo obese rodents vs. in vitro human cells) or variable tissue-specific expression. Further studies are clearly needed to acquire deeper insights on the potential role of HSP70 in both cellular and human insulin resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

HSP70 down-regulation and ENPP1 expression. **a** *HSP70* siRNA. HEK293 cells were either transfected with *HSP70* siRNA (lane 1) or with scrambled siRNA (lane 2). HSP70 expression was evaluated by western blot by using HSP70 SPA-812 specific antibody. **b** *ENPP1* mRNA stability. *ENPP1* mRNA content was determined by real time PCR (after transcription inhibition by 5 μ g/ml Actinomycin-D) in HEK293 cells either treated (square) or not (circle) with *HSP70* siRNA. A significantly greater reduction of mRNA content over time ($p=0.04$, by two-way ANOVA) was observed in HSP70 down-regulated cells as compared to control cells. Expression levels of *ENPP1* were normalized against GAPDH using the comparative C_t method, expressed as percentage of untreated cells at time 0 (Endogenous levels of *ENPP1* expression were detectable with C_t values being 26.4 ± 1.3 , as compared to those of GAPDH being 15.7 ± 1.9). Data are mean $\pm$ SD ($n=4$ experiments in each group). **c** *ENPP1* levels in transfected HEK293 cells. *ENPP1* mRNA content was determined by real-time PCR in HEK293 cells transfected with *ENPP1* cDNA and treated (black bar) or not (white bar) with *HSP70* siRNA. The grey bar represents *ENPP1* levels in untransfected cells. HSP70 down-regulation caused 80% significant reduction ($p=0.016$ by paired sample t -test) of *ENPP1* transcript. Expression levels of *ENPP1* were normalized against GAPDH using the comparative C_t method, expressed as percentage of untreated cells (white bar). Data are means $\pm$ SD ($n=3$ experiments in each group). **d** *ENPP1* protein content. HEK293 cells transfected with either *ENPP1* cDNA or with *ENPP1* cDNA lacking 3'UTR (*ENPP1* $\Delta 3'$ UTR lanes 3–4) were treated (+) or not (–) with *HSP70* siRNA. Total cell lysates were then used to measure HSP70, (by using HSP70 SPA-812 specific antibody) and ENPP1 (by using ENPP1 N-20 specific antibody) protein content. HSP70 down-regulation had no effect on ENPP1 protein content when *ENPP1* $\Delta 3'$ UTR cDNA was used

Fig. 2.

HSP70 over-expression and ENPP1 protein content. HEK293 cells were either transfected with *ENPP1* cDNA (*lane 2*) or with both *ENPP1* and *HSP70* cDNAs (*lane 3*). Total cell lysates were then used to measure HSP70 (*bottom panel*) and ENPP1 (*upper panel*) protein content, by western blot analysis. HSP70 up-regulation (*bottom panel, lane 3*) increased by approximately 30% ENPP1 protein content (*lane 3 vs. lane 2*). A representative experiment is shown

Fig. 3.

HSP70 and insulin signaling. **a** HEK293-IR cells were either transfected (*lanes 3–6*) or not (*lanes 1–2*) with either *ENPP1* cDNA (*lanes 3–4*) or with *ENPP1* cDNA lacking the 3'UTR (Δ 3'UTR, *lanes 5–6*) and then treated (*lanes 2, 4 and 6*) or not (*lanes 1, 3 and 5*) with *HSP70* siRNA. Sixty hrs after silencing, cells were stimulated with 10 nmol/L insulin for 5 min at 37°C and IR β -subunit autophosphorylation evaluated. IR β -subunit autophosphorylation was assessed with anti-PY antibody. Blot was then stripped and reprobed with anti IR β subunit. HSP70 protein content was evaluated with specific SPA-812 antibody. IR and HSP70 bands are from the same blot, but were cropped because of their different motilities. A representative experiment is shown. **(b)** IR β -subunit autophosphorylation in each condition was calculated as percentage of that of control untransfected cells (first bar, from the left). IR β -subunit autophosphorylation was almost 3 times higher in cells treated with *HSP70* siRNA (second bar) and 70% lower in cells transfected with *ENPP1* cDNA (third bar). As compared to that of cells transfected with *ENPP1* cDNA (third bar), IR β -subunit autophosphorylation was doubled in those co-transfected, with *HSP70* siRNA (fourth bar), so that an almost complete rescue of IR β -subunit autophosphorylation was observed. This rescue was not observed when HSP70 expression was down-regulated in cells transfected with *ENPP1* cDNA Δ 3'UTR (*lanes 5–6*). Data shown are mean $\pm$ SEM of 3 experiments. * $p < 0.04$ vs. control cells. **(c)** HepG2 cells were either treated (lane 3 or not lane 2) with *HSP70* siRNA. Sixty hrs after silencing, cells were stimulated with 10 nmol/L insulin for 10 min at 37°C and cell lysates were immunoprecipitated with anti-PY antibody. Blots were probed with both IRS-1 and IR β -subunit antibodies, respectively. IR β -subunit and IRS-1 bands are from the same blot, but were cropped because of their different motilities. A representative experiment is shown.