Chapter 6

PRAS40 protects A14 fibroblasts against palmitate-induced insulin resistance

Emmani Nascimento, Bruno Guigas, Jan Kriek, Gerard van der Zon, Ton Maassen, Margriet Ouwens
Abstract

Obesity is associated with an increased risk for the development of insulin resistance and type 2 diabetes mellitus. The adipose tissue is able to release free fatty acids like palmitate which are known factors to induce insulin resistance. At the cellular level, signalling through protein kinase B (PKB/Akt) and the mammalian target of rapamycin complex 1 (mTORC1) are altered in the insulin resistant state. Proline-rich Akt substrate 40 kDa (PRAS40) provides a link between PKB/Akt- and mTORC1-signalling. PRAS40-Thr246 phosphorylation is PKB/Akt dependent, while phosphorylation of PRAS40-Ser183 is mTORC1 dependent. Furthermore, PRAS40 has been proposed to regulate mTORC1 activity. The current study aimed to further define the function of PRAS40 in insulin action, under normal conditions and following palmitate-induced insulin resistance. Incubation of insulin-sensitive A14 fibroblasts with palmitate reduced insulin-stimulated PKB/Akt phosphorylation and lowered IRS1 expression. Whereas neither silencing nor overexpression of PRAS40 had a significant effect on insulin signalling, the absence of PRAS40 worsened the impact of palmitate-induced insulin resistance by further reducing IRS1 expression and insulin-mediated PKB/Akt phosphorylation. Conversely, overexpression of human PRAS40 (hPRAS40) resulted in protection from palmitate-induced insulin resistance when monitored at the level of PKB/Akt phosphorylation and IRS1 protein degradation. Finally, forced nuclear accumulation of PRAS40 through expression of hPRAS40 with a mutated nuclear export sequence failed to prevent palmitate-induced inhibition of PKB/Akt phosphorylation and IRS1 protein degradation. Taken together our data indicate that PRAS40 protects against palmitate-induced insulin resistance. Furthermore, the protective effect requires a cytosolic localisation of the protein.
**Introduction**

Obesity is highly associated with increased risk for development of insulin resistance and type 2 diabetes mellitus (1). Obese patients have enlarged adipose tissue secretes fatty acids, resulting in increased plasma free fatty acids (FFAs). FFAs are known to induce insulin resistance (2-4). Insulin action is initiated by binding of insulin to the insulin receptor (IR). The activated IR phosphorylates the insulin receptor substrate (IRS) proteins, which results in activation of phosphatidyl inositol 3’-kinase (PI3K) and activation of its downstream target protein kinase B (PKB/Akt). For full activation PKB/Akt is recruited to the cell membrane to become phosphorylated on Thr308 by PDK1 and on Ser473 by mammalian target of rapamycin complex 2 (mTORC2) (5;6). PKB/Akt has numerous downstream phosphorylation targets, through which PKB/Akt is a regulator of many metabolic functions, including glucose metabolism (7). Moreover, PKB/Akt can activate the mammalian target of rapamycin complex 1 (mTORC1) signalling pathway via direct phosphorylation of tuberous sclerosis complex (TSC) proteins. The two main downstream targets of mTORC1 are p70 S6 kinase (S6K) and eukaryotic translation initiation factor 4E binding protein (4EBP1). Through these intermediates mTOR is able to regulate cell growth and protein synthesis (8). Increased activity of S6K is linked to the development of insulin resistance via the induction of inhibitory serine phosphorylation on IRS1. Serine phosphorylation of IRS1 inhibits tyrosine phosphorylation by the IR, which results in abrogation of insulin-mediated activation of PI3K and PKB/Akt (9-13).

Proline-rich Akt substrate of 40 kDa (PRAS40) provides a link between PKB/Akt and mTORC1 mediated signalling. PRAS40 has mTORC1 mediated phosphorylation sites (14-17). The mTORC1 dependent phosphorylation sites include Ser183 and Ser221, and the PKB/Akt phosphorylation site is on residue Thr246 (18). Both PRAS40-Ser183 and PRAS40-Thr246 phosphorylation are reduced in insulin resistant rats (17;19), however the precise function of PRAS40 in insulin action is unclear. Some reports mention that silencing of PRAS40 leads to increased basal S6K phosphorylation and IRS1 degradation (20-23). Accordingly, overexpression of PRAS40 lowers the basal phosphorylation of S6K and 4EBP1. These findings suggest that PRAS40 could function as a substrate inhibitor of mTORC1.

The present study aimed at detailing the function of PRAS40 in insulin action, under normal conditions and under conditions of insulin resistance induced by the long-chain fatty acid palmitate in insulin-sensitive A14 fibroblasts. Palmitate was found to abrogate insulin-mediated phosphorylation of PKB/Akt and PRAS40 in this cell type (17). We examined expression of IRS1 as well as phosphorylation of PKB/Akt and S6K in A14 cells under conditions of either decreased or increased PRAS40 expression and in the presence or absence of palmitate. Our data suggest that PRAS40 protects A14 cells against palmitate-induced insulin resistance.
Materials and methods

Materials. Tissue culture media were obtained from Invitrogen (Carlsbad, CA, USA). Wortmannin and rapamycin were purchased from Calbiochem (Darmstadt, Germany). Insulin and palmitic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Palmitate was complexed to 2% fatty acid-free BSA by sonification (5 min) and heating (10 min, 55 °C). Phospho-specific antibodies against PKB/Akt-Ser473 (#4051), PKB/Akt-Thr308 (#9275), S6K-Thr389 (#9205) and total antibodies against anti-PRAS40 (#2610, #2691) were from Cell signalling technology (Danvers, MA, USA). Antibodies against Akt1 (sc-55523) and IRβ (sc-711) were from Santa Cruz biotechnology (Santa Cruz, CA, USA) and antibodies against Akt2 (07-372) were from Upstate (Millipore, Billerica, MA, USA). The polyclonal antibody against IRS1 has been described previously (24).

Cells. A14 cells are NIH3T3 fibroblasts overexpressing the human insulin receptor (25). Cells were grown on Dulbecco’s Modified Eagle’s Medium (DMEM), 10% FBS, penicillin and streptomycin.

Plasmid generation. Constructs expressing human PRAS40 (hPRAS40) were described previously (17). cDNA encoding hPRAS40 was kindly provided by Dr. R.A. Roth (Stanford University, Stanford, CA, USA) and cloned behind a CMV promoter (26). Via mutagenesis PCR, the following residues were mutated into alanine: L225 and L227. This resulted in plasmids expressing wild type hPRAS40 and mutant L225A/L227A-hPRAS40. Constructs were verified by sequence analysis. Plasmids were used for production of lentiviruses (26).

For knockdown of the PRAS40 protein, the pLKO.1 lentiviral plasmids were purified from E. coli bacteria. Two different shRNA clones were used (TRC number: 0000198644 / CCG GCG ATC GTC AGA TGA GGA GAA TCT CGA GAT TCT CAT CTG ACG ATC GTT TTT and TRC number: 0000181472 / CCG GCA ATA CCA GCG ACT TCC AGA ACT CGA GTT CTG GAA GTC GCT GGT ATT GTT TTT). As control SHC002 (CCG GCA ACA AGA TGA AGA GCA CCA ACT CGA GTT GGT GCT CTG CAT CTG GTT GTT TTT) was used from the mission library, which is pLKO.1 based vector carrying an insert that does not target human or mouse genes. Purified plasmids were used to produce lentiviruses (26).

Transduction with lentiviruses. For overexpression of wild type or mutant hPRAS40 A14 fibroblasts were transduced at 20% confluence at a MOI of 2. Three days after transduction, A14 cells were split and grown to confluence for experiments. For knockdown of the PRAS40 protein in A14 fibroblasts, cells were infected with shRNAs against PRAS40 at MOI of 4. 24 h after infection cells were split and grown to confluence for experiments.

Stimulation of cells. Prior to insulin stimulation, A14 cells were starved overnight on DMEM supplemented with 2% fatty acid-free BSA, penicillin and streptomycin) and where
indicated further supplemented with 0.75 mM palmitate. Cells were subsequently stimulated with insulin (5 min, 100 nM).

**Analysis of insulin signalling.** Cells were lysed in 100 mM Tris.HCl (pH 6.8), 3% SDS and 10% glycerol. Protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Expression and phosphorylation of proteins was studied by SDS-PAGE and Western blotting as described (19).

**Statistical analysis.** Western blots were analyzed using a two-tailed Student’s t-test in Microsoft Excel. $p<0.05$ was considered significantly different.

**Results**

**Effects of PRAS40 knockdown on palmitate-induced insulin resistance.** We started out by investigating the role of PRAS40 in palmitate-induced insulin resistance by examining the effect of PRAS40 knockdown on insulin-induced activation of insulin signalling intermediates. Insulin sensitive A14 fibroblasts were infected with a plasmid driving expression of a shRNA against PRAS40. Knockdown of PRAS40 had no significant effect on insulin-stimulated phosphorylation of PKB/Akt on Ser473 and Thr308 (Fig. 1A-1C). However, insulin-stimulated S6K-Thr389 phosphorylation was decreased after PRAS40 knockdown (Fig. 1A & 1D). In line with a previous report (14), knockdown of PRAS40 decreased total IRS1 protein expression (Fig. 1A & 1E).

We used palmitate to induce insulin resistance. Incubation of A14 fibroblasts with palmitate was inhibited insulin-stimulated phosphorylation of PKB/Akt-Ser473 by 55%, phosphorylation of PKB/Akt-Thr308 by 54% and phosphorylation of S6K-Thr389 by 19% (Fig. 2A-2C). Furthermore, palmitate decreased IRS1 protein expression by 38% (Fig. 2D). After knockdown of PRAS40, palmitate deteriorated the inhibition of insulin-mediated phosphorylation of PKB/Akt-Ser473 to 79%, of PKB/Akt-Thr308 to 78% and of S6K-Thr389 to 25% (Fig. 2A-2C). Knockdown of PRAS40 also further decreased IRS1 protein expression to 68% (Fig. 2D) under conditions of palmitate-induced insulin resistance.
Figure 1. PRAS40 knockdown inhibits palmitate induced insulin resistance in A14 fibroblasts. A. A14 fibroblasts were infected with a plasmid driving expression of a shRNA against PRAS40 (shRNA PRAS40) or a non target shRNA (NT). Where indicated cells were incubated overnight with 0.75 mM palmitate. Cells were left untreated or were insulin stimulated for 5 min at 100 nM (+). Samples were analyzed using Western blotting techniques using phospho-specific antibodies against PKB/Akt-Ser473, PKB/Akt-Thr308, S6K-Thr389 or antibodies against PRAS40, PKB/Akt, IRS1 and IRβ. B-E. Quantification of blots obtained in panel A for pPKB/Akt-Ser473 (A), pPKB/Akt-Thr308 (B), pS6K-Thr389 (C) and IRS1 (D). White bars indicate untreated cells, black bars indicate insulin stimulated cells. Data are expressed as mean ± SEM and obtained from three independent experiments. *, p<0.05 compared to basal; $, p<0.05 compared to NT.
Figure 2. PRAS40 knockdown increases palmitate-induced inhibition of insulin signalling intermediates in A14 fibroblasts. Quantifications from Fig. 1A were recalculated to express the inhibitory effect of palmitate on pPKB/Akt-Ser473 (A), pPKB/Akt-Thr308 (B), pS6K-Thr389 (C) and IRS1 (D). Data are expressed as mean ± SEM and obtained from three independent experiments. $, p<0.05 compared to NT.

Effects of nuclear accumulation of hPRAS40 on palmitate-induced insulin resistance.
Since knockdown of PRAS40 worsened the inhibitory effects of palmitate on insulin signalling, we next studied whether ectopic expression of hPRAS40 could reverse palmitate-induced insulin resistance. A14 fibroblasts were infected with a plasmid driving expression of hPRAS40. After the infection, we examined phosphorylation of PKB/Akt-Ser473, PKB/Akt-Thr308, S6K-Thr389 and expression of total IRS1 (Fig. 3A). Control cells were infected with an empty plasmid driving expression of GFP (EV). Comparable to untransfected cells, in cells expressing only GFP, palmitate was able to inhibit insulin-stimulated phosphorylation of PKB/Akt-Ser473, PKB/Akt-Thr308 and S6K-Thr389 and to decrease the expression of IRS1 protein. Overexpression of hPRAS40 (WT) resulted in diminished palmitate-induced inhibition of PKB/Akt-Ser473 and PKB/Akt-Thr308 (Fig. 3B & 3C). Furthermore, in cells overexpressing hPRAS40, the palmitate-induced reduction of IRS1 was decreased (Fig. 3E). Finally, cells infected with a plasmid driving overexpression of mutant L225A/L227A-hPRAS40 (NES), which accumulates in the nucleus of the cell. Nuclear accumulation of mutant L225A/L227A-hPRAS40 resulted in an increase in palmitate-induced inhibition of PKB/Akt-Thr308 and S6K-Thr389 (Fig. 3B & 3C).
Figure 3. Expression of mutant L225A/L227A-hPRAS40 is not able to protect A14 fibroblasts from palmitate-induced insulin resistance. A. A14 cells were infected with plasmids driving expression of wild type hPRAS40 (WT) or mutant L225A/L227A-hPRAS40 (NES). Control cells were infected with an empty plasmid driving expression of only expressing GFP (EV). Where indicated, cells were incubated overnight with 0.75 mM palmitate. Cells were left untreated (-) or were stimulated with 100 nM insulin for 5 min (+). Samples were analyzed with Western blotting techniques using phospho-specific antibodies against PKB/Akt-Ser473, PKB/Akt-Thr308 and S6K-Thr389 or antibodies against PRAS40, PKB/Akt, IRS1, IRβ. Shown blots are representative of three independent experiments. B-E. Based on the quantifications from the blots in fig. 3A, new values were calculated to express the inhibitory impact of palmitate on pPKB/Akt-Ser473 (B), pPKB/Akt-Thr308 (C), pS6K-Thr389 (D) and IRS1 (E)). Data are expressed as mean ± SEM and obtained from three independent experiments. *, p<0.05 compared to EV.
Discussion

In the present study, we have examined the role of PRAS40 in palmitate-induced insulin resistance. Knockdown of PRAS40 further enhanced palmitate-induced insulin resistance at the level of PKB/Akt-Ser473, PKB/Akt-Thr308 and IRS1. Overexpression of hPRAS40 protected cells from palmitate-induced insulin resistance at the level of PKB/Akt-Ser473 and IRS1. Interestingly expression of mutant nuclear accumulating L225A/L227A-hPRAS40 was not able to protect cells from palmitate-induced insulin resistance. Palmitate induced insulin resistance was even enhanced at the level of PKB/Akt-Thr308 and S6K-Thr389 in cells expressing mutant L225A/L227A-hPRAS40. This indicates that normal cytosolic localisation of PRAS40 is required for the protective effects of PRAS40 against palmitate-induced insulin resistance.

From in vitro and in vivo studies it is known that long-chain fatty acids such as palmitate can induce insulin resistance. However the degree of insulin resistance depends on the concentration of the fatty acid, the measured output (eg. phosphorylation of PKB/Akt-Ser473 or GLUT4 translocation) and the studied cell type. Downstream of PKB/Akt, PRAS40 phosphorylation was first affected by palmitate-induced insulin resistance at a concentration of 0.38 mM (17). Another study L6 myotubes has shown insulin resistance in GLUT4 translocation at a concentration of 0.15 mM palmitate, however in this case signalling remained intact. Only at higher concentrations, insulin resistance was evident when examining phosphorylation of IRS1, PKB/Akt-Ser473 and PKB/Akt-Thr308 in L6 myotubes (27). Therefore we choose to use higher concentration of palmitate to examine the effect of PRAS40 on signalling. Next to insulin resistance, palmitate can induce reactive oxygen species (ROS), which also affect insulin signalling (28). In the present study we have not examined the effect of PRAS40 on ROS production for technical reasons. Since expression of hPRAS40 increased insulin sensitivity at the level of PKB/Akt-Ser473 and IRS1 (Fig. 3), we cannot exclude that PRAS40 ameliorates palmitate-mediated ROS production in vitro.

Looking upstream in the insulin signalling pathway at total IRS1 protein expression, we observed a decrease in IRS1 protein expression after palmitate incubation or PRAS40 knockdown (Fig. 1). In a recent article, knockdown of PRAS40 resulted in inactivation of IRS1 (12). In line with these findings, we observed that overexpression of hPRAS40 resulted in IRS1 upregulation and increased phosphorylation of PKB/Akt-Ser473 and PKB/Akt-Thr308. We did not measure IRS1 phosphorylation, either stimulatory Ser phosphorylation or inhibitory Tyr phosphorylation. This information could tell us whether IRS1 protein activity is affected under conditions of PRAS40 knockdown. According to literature palmitate-induced insulin resistance is mediated via IRS1 independent mechanisms (27). In our cell system, phosphorylation/activity of IRS1 needs to be
determined first to draw a conclusion towards the role of IRS1 in palmitate-induced insulin resistance.

From literature it is known, that knockdown of PRAS40 increases basal S6K-Thr389 phosphorylation (14). However in the present study, knockdown of PRAS40 did not increase basal S6K-Thr389 phosphorylation (Fig. 1A & 1D). On the other hand, overexpression of hPRAS40 was able to decrease basal S6K phosphorylation in our cell system. These findings do match observations from others (14;29-31). It remains unclear why a decrease in S6K phosphorylation after PRAS40 knockdown was not detected in our cell system. Cell line specificity may be involved. More information could be acquired by looking at phosphorylation of S6K at different residues (eg. Thr42/Tyr424 or Ser), however phosphorylation of S6K-Thr389 is mostly closely related to S6K activity in vivo (32). The next step would be to examine activity/phosphorylation of mTORC1 itself or other mTORC1 substrates.

The differences observed between wild type hPRAS40 and mutant nuclear accumulating L225A/L227A-hPRAS40 in the protection against palmitate-induced insulin resistance, indicates the importance of the nuclear export signal present in the PRAS40 protein (Fig. 3). Within the nuclear export signal, the mTORC1 dependent PRAS40-Ser221 phosphorylation site is present (30). It would be interesting to establish the impact of this phosphorylation site on palmitate-induced insulin resistance.

In the present study, PRAS40 overexpression is able to protect A14 fibroblasts from palmitate-induced insulin resistance. Cytosolic localisation of the protein is of importance for this process. Further studies are needed to unravel the involvement of phosphorylation sites within the PRAS40 protein in palmitate-induced insulin resistance. And whether thus alterations in PRAS40 phosphorylation might already reverse palmitate-induced insulin resistance.

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PRAS40 and palmitate-induced insulin resistance