Inhibition of SH2-domain containing inositol phosphatase 2 (SHIP2) in insulin producing INS1E cells improves insulin signal transduction and induces proliferation

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Abstract Inhibition of the lipid phosphatase SH2-domain containing inositol phosphatase 2 (SHIP2) in L6-C10 muscle cells, in 3T3-L1 adipocytes and in the liver of db/db mice has been shown to ameliorate insulin signal transduction and established SHIP2 as a negative regulator of insulin action. Here we show that SHIP2 inhibition in INS1E insulinoma cells increased Akt, glycogen synthase kinase 3 and extracellular signal-regulated kinases 1 and 2 phosphorylation. SHIP2 inhibition did not prevent palmitate-induced apoptosis, but increased cell proliferation. Our data raise the interesting possibility that SHIP2 inhibition exerts proliferative effects in β-cells and further support the attractiveness of a specific inhibition of SHIP2 for the treatment of type 2 diabetes.

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1. Introduction

Type 2 diabetes is characterized by insulin resistance of insulin target tissues and impaired insulin secretion from pancreatic β-cells. During progression of type 2 diabetes the initial expansion of β-cell mass partially compensates for the peripheral insulin resistance. After prolonged impaired glucose tolerance the progressive decline of pancreatic β-cell mass together with β-cell dysfunction contributes to the pathogenesis of type 2 diabetes [1]. The β-cell mass is regulated by an interplay of apoptosis and proliferation. Important β-cell survival and growth factors are insulin, insulin-like growth factor-I, hepatocyte growth factor, glucagon-like peptide 1 (GLP-1) and GIP. Several findings suggest that the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB (Akt)) pathway is important for β-cell growth and survival. Overexpression of a constitutively active form of Akt in vivo enhances cell survival and cell proliferation and thereby increases β-cell mass [2,3]. Akt phosphorylates and thereby inactivates a number of pro-apoptotic proteins like glycogen synthase kinase 3 alpha/beta (GSK3α/β), BAD, Caspase-9 or FoxO1 [4]. Therefore, it is conceivable that factors that increase endogenous Akt phosphorylation and activation enhance the proliferation of β-cells and/or inhibit β-cell apoptosis [5].

The second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), generated by PI3K, is a key component of insulin signal transduction [6]. SH2-domain containing inositol phosphatase 2 (SHIP2) is expressed in insulin target tissues and in the pancreas [7] and dephosphorylates PtdIns(3,4,5)P3 at position 5 [8,9]. A 16 bp deletion in the 3′-untranslated region of the human SHIP2 gene which leads to overexpression of SHIP2 contributes to the genetic susceptibility to type 2 diabetes in humans [10]. In agreement with these data, liver-specific overexpression of SHIP2 in db/+ mice decreased insulin-induced Akt phosphorylation and causes hepatic insulin resistance [11]. On the other hand, the liver-specific overexpression of a phosphatase defective SHIP2 mutant that acts in a dominant negative manner (dnSHIP2) in diabetic KKA-/-mice as well as in diabetic db/db-mice showed a marked reduction of blood glucose [11,12]. At the cellular level, inhibition of SHIP2 via overexpression of dnSHIP2 has been shown to increase phosphorylation of Akt in differentiated L6 myotubes, 3T3-L1 adipocytes and in C3A hepatoma cells [12–14]. In L6 myotubes and 3T3-L1 adipocytes the increased phosphorylation of Akt leads to an enhanced glycogen synthase activity with an enhanced glycogen synthesis rate [13,14].

Although it has been shown that insulin signalling plays an important role for β-cell function [15], the effects of SHIP2 inhibition in β-cells are completely unknown. It is intriguing that SHIP2 inhibition could enhance insulin signal transduction in β-cells and could thereby protect the cells from apoptosis or induce proliferation, both of which would be beneficial for type 2 diabetics. To gain insight into the effects of SHIP2 inhibition in β-cells we used adenovirus-mediated gene transfer for overexpression of a dominant negative SHIP2 mutant in the insulin-producing cell line INS1E and analysed the impact on insulin signal transduction, apoptosis and proliferation.

Abbreviations: βGal, beta-galactosidase; ERK1/2, extracellular signal-regulated kinases 1 and 2; GLP-1, glucagon-like peptide 1; GSK3, glycogen synthase kinase 3; PI3K, phosphatidylinositol 3-kinase; PKB (Akt), protein kinase B; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; SHIP2, SH2-domain containing inositol phosphatase 2

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2. Materials and methods

2.1. Reagents

The following antibodies were used: polyclonal anti-phospho (Ser\(^{198}\)-)-Akt, anti-phospho (Ser\(^{283}\)-)GSK3\(\beta\), anti-phospho (Thr\(^{202}/\)Tyr\(^{204}\))-extracellular signal-regulated kinases 1 and 2 (ERK1/2), anti-Akt, anti-GSK3\(\beta\) and anti-ERK1/2 (Cell Signaling Technology, Beverly, MA), anti-cyclin A and anti-p-actin (Santa Cruz Biotechnology, Santa Cruz, CA). All other reagents were of analytical grade and purchased from Sigma–Aldrich (Gillingham, UK) or Roche Applied Science (Mannheim, Germany).

2.2. Cell culture and infection with adenoviruses

The construction of recombinant adenoviruses expressing β-galactosidase and dominant negative SHIP2 has been described previously [12]. INS1E insulinoma cells [16,17] were maintained in RPMI 1640 containing 11 mM glucose and 2 mM glutamine (Invitrogen, Carlsbad, CA) supplemented with 50 μM β-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate and 5% fetal bovine serum (FBS) at 37 °C. Cells were infected at a multiplicity of infection (MOI) of 25 infectious units (IU)/cell. This results in >90% infection of INS1E cells as determined by adenoviral eGFP expression (data not shown). The following antibodies were used: polyclonal anti-phospho (Ser\(^{198}\)-)-Akt, anti-phospho (Ser\(^{283}\)-)GSK3\(\beta\), anti-phospho (Thr\(^{202}/\)Tyr\(^{204}\))-extracellular signal-regulated kinases 1 and 2 (ERK1/2), anti-Akt, anti-GSK3\(\beta\) and anti-ERK1/2 (Cell Signaling Technology, Beverly, MA), anti-cyclin A and anti-p-actin (Santa Cruz Biotechnology, Santa Cruz, CA). All other reagents were of analytical grade and purchased from Sigma–Aldrich (Gillingham, UK) or Roche Applied Science (Mannheim, Germany).

2.3. Expression analysis

Total RNA was extracted from INS1E cells using the RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and 100 ng RNA was transcribed into cDNA. Cyclin A mRNA levels were quantified using a TaqMan real-time quantitative PCR (qRT-PCR) chemistry and detection system (Applied Biosystems, Foster City, CA). All samples were run in triplicate. Relative mRNA levels were calculated from a standard curve and normalized to the amount of 18sRNA in the sample.

2.4. Immunoblotting

Protein concentrations of INS1E cell lysates were determined by the Bradford method. The proteins (25 μg/lane) were electrophoretically separated on 4–15% Criterion Precast Gels (BioRad, Hercules, CA) and Western blots were performed and analysed as previously described [12,18].

2.5. Determination of caspase-activity

Cells were infected with adenoviruses encoding βGal or dnSHIP2 as described. After 24 h the medium was changed to RPMI without FCS and the cells were incubated with or without 0.5 mM palmitate in the absence or presence of 100 nM insulin for 22 h. Caspase activity was assessed using the homogenous caspase assay (Roche Diagnostics, Mannheim, Germany) that detects the activity of caspases 2, 3, 6, 7, 8, 9, and 10 with different sensitivity.

2.6. Crystal violet staining

The cell number was determined by crystal violet staining. After fixation of the cells with 4% formaldehyde in 1 × PBS, the cells were incubated with crystal violet solution (0.125% crystal violet, 5% ethanol, 2% PBS) for 1 h. Then the plates were washed three times with 1 × PBS and dried for 2 h. After incubation with 5% ethanol +1% SDS for 1 h the absorbance was measured at 570 nm.

2.7. Proliferation assay

Proliferation was assessed by [\(^{3}H\) thymidine incorporation into INS1E cells in the absence or presence of insulin. For this, the cells were seeded in Cytostar™ 96-well scintillating microplates (Amer-sham Biosciences, Buckinghamshire, UK) and infected as described above. After 24 h the medium was changed to RPMI without FCS and the cells were incubated with 2μl [\(^{3}H\) thymidine (1.85 MBq/ml) per well for 22 h with different insulin concentrations. Incorporation of radioactive thymidine was measured with a TopCount NXT (Perkin–Elmer Life Sciences, Wellesley, MA).

2.8. Statistical analysis

Results are given as means ± S.E.M. For comparisons the unpaired Student’s t-test was used. Parameters with values P < 0.05 were considered to differ significantly.

3. Results

3.1. Overexpression of a dominant negative SHIP2 mutant improves phosphorylation of Akt, GSK3 and ERK1/2 in INS1E insulinoma cells

Consistent with SHIP2 expression in the human pancreas [7], expression of endogenous SHIP2 was also detected in INS1E cells (Fig. 1A). A phosphatase defective SHIP2 mutant that acts in a dominant negative fashion has been described previously [13]. A similar human SHIP2 mutant (dnSHIP2) was constructed and expressed via adenovirus mediated gene transfer in INS1E cells. As a control, an adenovirus encoding beta-galactosidase (Ad5-βGal) was used in all experiments. INS1E cells were infected and the basal and insulin-induced Akt phosphorylation was analysed by Western blot. Overexpression of dnSHIP2 was 5- to 6-fold over endogenous SHIP2 in all experiments (Fig. 1A). The overexpression of dnSHIP2 resulted in a significant increase of Akt and GSK3 phosphorylation in the absence or presence of 100 nM insulin (Fig. 1A–D). Furthermore, overexpression of dnSHIP2 significantly increased the phosphorylation of both ERK1 and ERK2 in the absence of insulin, but not in the presence of insulin (Fig. 1E/F). Therefore, functional SHIP2 inhibition increased the basal and insulin-induced Akt phosphorylation in INS1E cells.

3.2. Overexpression of dnSHIP2 does not protect INS1E cells against palmitate-induced apoptosis

To assess the impact of SHIP2 inhibition on apoptosis, INS1E cells were infected with Ad5-βGal or Ad5-dnSHIP2 as described above and incubated for 22 h with 0.5 mM palmitate in the absence or presence of 100 nM insulin. The cells were then either lysed and the activity of the caspases 2, 3, 6, 7, 8, 9, and 10 was measured or fixed for the determination of the number of living cells by crystal violet staining.

SHIP2 inhibition significantly increased the cell number independent of treatment with palmitate or insulin (Fig. 2A). Palmitate incubation decreased the cell number in both dnSHIP2- and βGal-expressing conditions to a similar extent, approximately by 30% (Fig. 2A). Therefore, dnSHIP2 expression could not rescue the cells from palmitate-induced apoptosis.

The incubation of INS1E cells with 0.5 mM palmitate resulted in a significant increase of caspase activity (Fig. 2B). However, dnSHIP2 overexpression was not able to significantly reduce the caspase activity in control cells and insulin-stimulated cells (Fig. 2B).

As no difference in either caspase activity (Fig. 2B) or the percentage of surviving cells (Fig. 2A) between the dnSHIP2 and βGal-expressing cells was observed, the data indicate that SHIP2 inhibition may impact on cell proliferation rather than anti-apoptosis.
3.3. SHIP2 inhibition induces proliferation of INS1E cells

To test the hypothesis that SHIP2 inhibition affects cell proliferation of INS1E cells we infected the cells with adenoviruses encoding βGal or dnSHIP2. Cells were incubated with or without 100 nM insulin for 10 min. (A, C, and E) Cell lysates were subjected to immunoblot analysis with anti-SHIP2, anti-Akt, anti-phospho (Ser^473)-specific Akt, anti-phospho (Ser^9/21)-specific GSK3α/β antibodies, anti-ERK1/2 antibody or anti-phospho-(Thr^202/Tyr^204)-specific ERK1/2 antibody. (B, D, and F) Densitometric analysis of (B) Akt phosphorylation, (D) GSK3 phosphorylation and (F) ERK1/2 phosphorylation of three independent experiments. Values represent means ± S.E.M. of three independent experiments. *P < 0.05 vs. βGal-expressing controls without insulin, #P < 0.05 vs. βGal-expressing controls with insulin.

4. Discussion

Despite many studies illustrating the relevance of the insulin/PI3K/Akt signalling pathway for β-cell growth and survival, little is known about the negative regulation of this pathway in β-cells. The phosphatidylinositol D3-phosphatase PTEN, which acts as a negative regulator of the PI3K pathway, inhibits the compensatory growth of β-cells in IRS2−/− mice [20]. Here we identified the phosphatidylinositol D5-phosphatase SHIP2 as another negative regulator of the PI3K signal transduction pathway in INS1E cells. We observed enhanced Akt phosphorylation in INS1E cells following SHIP2 inhibition with a similar magnitude as compared to other cell types, like 3T3-L1 adipocytes, L6 myotubes or C3A hepatoma cells [12–14]. To study the effect of SHIP2 inhibition downstream of Akt activation we measured apoptosis and proliferation rates of INS1E cells.
recent study describes a very similar mechanism in mouse epidermal C141 cells. In these cells treatment with the protein-tyrosine phosphatase inhibitor, sodium vanadate, dose-dependently increased the phosphorylation of Akt and GSK3, enhanced the expression of cyclin A and increased the number of cells in S-phase [21], which is consistent with our observations.

In agreement, strong activation of Akt by overexpression of constitutively active Akt1 increased β-cell mass in mice in part by increased proliferation [2,3,22]. But more importantly, pancreatic β-cell specific deletion of PTEN in mice which causes a moderate increase in Akt phosphorylation also increased β-cell proliferation [23].

The observed increase in basal thymidine incorporation is most probably caused by multiple signalling pathways including the P13K/Akt pathway and the Ras/Raf/MEK/ERK-pathway. However, the contribution of the Ras/Raf/MEK/ERK-pathway to the increased insulin-stimulated thymidine incorporation remains unclear, because we did not observe an increase in insulin-stimulated ERK1/2 phosphorylation after dnSHIP2 overexpression.

In our study, we could not detect an attenuation of palmitate-induced apoptosis after inhibition of SHIP2. Most reports that provide evidence for an anti-apoptotic role of Akt in INS1 cells overexpressed a constitutively active Akt1 [24,25]. However, SHIP2 inhibition with dnSHIP2 does only cause transient 1.5- to 2-fold activation of endogenous Akt, which is not comparable with effects caused by overexpression of a constitutively active Akt that causes 10- to 100-fold activation of the pathway. Furthermore, the relative contribution of the Akt isoforms is unknown. In 3T3-L1 cells it is established that SHIP2 inhibition improves the phosphorylation of Akt2 rather than Akt1 [26]. In β-cells, all three isoforms of Akt are ex-
pressed [27], but whether SHIP2 interacts with a specific Akt isoform in INS1E cells is unknown and was beyond the scope of this study.

Although, we observed an increased phosphorylation of GSK3 we could not detect inhibition of apoptosis an event that is associated with GSK3 inhibition [28]. A recent study shows that small molecule inhibitors of GSK3 can partially rescue INS1E cells from glucolipotoxicity [28]. In this study, a significant anti-apoptotic effect in INS1E cells (reduction of DNA-fragmentation of 15–20%) was achieved with two structurally different GSK3 inhibitors (1-azakenapollone and CHIR99021) starting at concentrations of 5 μM. However, a significant increase in proliferation (BrdU incorporation) of INS1E cells was already achieved at 15-fold lower concentrations (0.3 μM) of both compounds. The maximum effect in the proliferation assay was reached with both compounds at 2.5 μM. This strongly suggests that full GSK3 inhibition is needed to inhibit apoptosis, but partial GSK3 inhibition is sufficient to increase proliferation. We believe that the modest increase in GSK3 phosphorylation in our study leads to a moderate inhibition of GSK3, and therefore an anti-apoptotic effect can not be expected.

Taken together, we show here for the first time that inhibition of SHIP2 improves insulin signal transduction in INS1E insulinoma cells and thereby induces cell proliferation. Our data raise the interesting possibility that SHIP2 inhibition exerts important proliferative effects in β-cells. Therefore, these data further support the attractiveness of a specific inhibition of SHIP2 for the prevention and/or treatment of type 2 diabetes.

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