Protein kinase C δ regulates nuclear export of FOXO1 through phosphorylation of the chaperone 14-3-3 ζ

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Abstract

Aims/hypothesis Forkhead box protein O1 (FOXO1) is a transcription factor essential for beta cell fate. Protein kinase B-dependent phosphorylation of FOXO1 at S256 (P-FOXO1) enables its binding to 14-3-3 dimers and nuclear export. Dephosphorylated FOXO1 enters nuclei and activates pro-apoptotic genes. Since our previous observations suggest that protein kinase C delta (PKC δ) induces nuclear accumulation of FOXO1, the underlying mechanism was examined. **Methods** In human islets, genetically modified mice and INS-1E cells apoptosis was assessed by TUNEL staining. Subcellular translocation of proteins was examined by confocal microscopy and signalling pathways were analysed by western blotting and overlay assay.

Results In PKC δ -overexpressing (PKC δ -tg) mouse islet cells and INS-1E cells FOXO1 accumulated in nuclei, surprisingly,

as P-FOXO1. PKC δ -tg decelerated IGF-1-dependent stimulation of nuclear export, indicating that changes in export caused nuclear retention of P-FOXO1. Nuclear accumulation of P-FOXO1 was accompanied by increased phosphorylation of 14-3-3 ζ at S58 and reduced dimerisation of 14-3-3 ζ . Palmitic acid further augmented phosphorylation of 14-3-3 ζ and triggered nuclear accumulation of FOXO1 in both INS-1E and human islet cells. Furthermore, the overexpression of a phosphomimicking mutant of 14-3-3 ζ (S58D) enhanced nuclear FOXO1. In accordance with the nuclear accumulation of P-FOXO1, PKC δ overexpression alone did not increase apoptotic cell death. Additionally, insulin secretion and glucose homeostasis in PKC δ -overexpressing mice remained unaffected.

Conclusions/interpretation These results suggest that PKC δ -mediated phosphorylation of 14-3-3 ζ contributes to the nuclear retention of FOXO1, even when FOXO1 is phosphorylated as under non-stress conditions. P-FOXO1 does not induce pro-apoptotic genes, but may rather exert beneficial effects on beta cells.

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Abbreviations

BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
BIM	Bcl-2-like protein 11
CD	Chow diet
CHOP	CAAT/enhancer binding protein homologous transcription factor
CRM1	Chromosomal maintenance 1
ER	Endoplasmic reticulum

FOXO1	Forkhead box protein O1
HFD	High-fat diet
HRP	Horseradish peroxidase
JNK	c-Jun N-terminal kinase
PKB	Protein kinase B, Akt
PKC	Protein kinase C
PKC δ KN	Kinase-negative protein kinase C δ
PP2A	Protein phosphatase 2A
RIPA	Radioimmunoprecipitation assay
TBS	TRIS-buffered saline
TXNIP	Thioredoxin-interacting protein

Introduction

Type 2 diabetes mellitus correlates with insufficient insulin secretion and reduction of beta cell mass [1]. Numerous studies suggest that the reduced beta cell mass originates from enhanced beta cell death. Factors of the diabetogenic milieu, including NEFA, hyperglycaemia and cytokines, induce apoptosis via distinct signalling pathways: NEFA induce endoplasmic reticulum (ER) stress; hyperglycaemia triggers oxidative stress and mitochondrial dysfunction, whereas cytokines activate death receptor pathways [2–4]. The transcription factor forkhead box protein O1 (FOXO1) plays a unique role in the induction of apoptosis [5]. The inhibition or downregulation of FOXO1 counteracts beta cell apoptosis, indicating the pro-apoptotic role of FOXO1 [6, 7]. On the contrary, Accili and co-workers showed that FOXO1 deficiency in mice induces dedifferentiation, suggesting that FOXO1 is necessary for the maintenance of beta cell identity under non-stress conditions [8, 9]. Indeed, mice with beta cell specific deletion of FOXO1 display reduced insulin secretion without beta cell loss [10]. FOXO1, together with FOXO3 and FOXO4, regulates genes linked to mitochondrial oxidative phosphorylation which in turn ensures proper beta cell function [11]. In humans, a single nucleotide polymorphism of FOXO1 associates with reduced insulin secretion, impaired glucose homeostasis and type 2 diabetes [12].

Post-translational modifications of FOXO1 enable the regulation of opposing cellular processes, including proliferation, apoptosis, cell cycle progression and metabolism [13]. FOXO1 is phosphorylated by protein kinase B (PKB/Akt) at S256 (P-FOXO1) upon insulin receptor or IGF-1 receptor stimulation and this coincides with cytosolic accumulation of FOXO1. Stress conditions (i.e. hyperglycaemia, impaired IGF-1 and insulin signalling) or increased protein phosphatase 2A (PP2A) activity determine dephosphorylation of FOXO1 and its nuclear import [14, 15]. Dephosphorylated FOXO1 stimulates the expression of pro-apoptotic proteins like B cell lymphoma 2 (Bcl-2)-interacting mediator of cell death (BIM) and CAAT/enhancer binding protein homologous transcription factor (CHOP) [6, 14, 16]. Phosphorylation of FOXO1

by PKB/Akt is required for its binding to 14-3-3 ζ or 14-3-3 γ dimers and exportin-1 (chromosomal region maintenance-1 [CRM1])-dependent nuclear export [17–20]. 14-3-3 ζ dimerisation is again regulated by post-translational changes. Protein kinase C δ (PKC δ) phosphorylates 14-3-3 ζ at S58, which impairs chaperone dimerisation and binding to target proteins like Bcl-2-associated X protein (BAX) and Bcl-2-associated death promoter (BAD) [19–21]. In beta cells, 14-3-3 ζ is highly expressed and sequesters the pro-apoptotic proteins BAD and BAX in the cytosol. In accordance, reduced levels of 14-3-3 ζ induce mitochondrial-linked beta cell death [22].

PKC δ is activated by cytokines and metabolites, including saturated NEFA [23–26]. Obesity is associated with higher PKC δ levels in humans and high-fat feeding upregulates hepatic PKC δ expression in mice [27, 28]. Furthermore, hepatic overexpression of PKC δ impairs insulin sensitivity and triggers nuclear accumulation of FOXO1, while PKC δ deficiency protects against diet-induced insulin resistance [29]. Our previous study suggested that overexpression of a kinase-negative PKC δ (PKC δ KN) in beta cells reduces nuclear accumulation of FOXO1, while overexpression of wild-type PKC δ increases nuclear FOXO1 [26]. The aim of the present study was to understand the modulatory role of PKC δ in FOXO1 trafficking and function.

Methods

The sources of materials are listed in electronic supplementary materials (ESM) [Methods](#).

Generation of β PKC δ WT transgenic mice C57BL/6 mice with beta cell specific overexpression of PKC δ (B6-Tg^{RIP-Prkcd}Fbos) were generated as previously described [26, 30]. All animal experiments were carried out in accordance with the accepted standard of animal care and were approved by the local authorities.

GTT Male littermates were used for in vivo experiments. Mice (4 weeks old) were fed with regular chow diet (CD) or high-fat diet (HFD) for 8 weeks. GTTs were performed after the mice had been fasted overnight and given an intraperitoneal injection of glucose (2 g/kg body weight). Blood glucose was measured with a glucometer. Plasma insulin concentrations were assessed by RIA. The selection of animals that received high-fat diet (HFD) or chow diet (CD) was determined randomly. HFD feeding and GTT were blinded. Furthermore, GTT was performed using simple randomisation (random selection of animals per feeding and genetically differentiated group).

INS-1E cell transfection INS-1E cells (kindly provided by C. B. Wollheim, Geneva, Switzerland) were cultured in RPMI 1640 supplemented with (in mmol/l): 10 HEPES, 1 sodium pyruvate, 2 L-glutamine, 0.05 2-mercaptoethanol and 10% (vol./vol.) FCS. Only glucose-responsive and mycoplasma free cells were used (regularly tested by insulin secretion analysis and PCR/DNA staining, respectively). The cells were stably transfected with a retrovirus driven by LTR (long terminal repeats) promoter, encoding PKC δ or 14-3-3 ζ wild type and mutants (S58A and S58D) [31].

Insulin secretion For determining insulin secretion, cells were incubated in a buffer containing (in mmol/l): 135 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.3 CaCl₂, 1.2 KH₂PO₄, 5 NaHCO₃, 10 HEPES (pH 7.4), 5 g/l BSA and glucose, as indicated. Secreted insulin and insulin content were measured by RIA.

Primary islet and cell culture Human islets were cultured in CMRL 1066 supplemented with 2 mmol/l L-glutamine, 10 mmol/l HEPES and 10% (vol./vol.) FCS. All experiments were approved by the ethics committee of the Faculty of Medicine of the University of Tübingen (533/2010BO2). Mouse islets were isolated with collagenase (1 mg/ml) [32] and further digested with trypsin (40 U/ml). Dispersed cells were seeded onto L-poly-ornithine (0.001% [vol./vol.])-coated coverslips and cultured in INS-1E cell medium without addition of 2-mercaptoethanol. Palmitic acid, from a stock solution of 100 mmol/l in dimethyl sulfoxide, was dissolved in FCS at a concentration of 6 mmol/l [26].

Immunocytochemistry and TUNEL assay Islets, INS-1E cells and human pancreatic tissue were fixed with 4% formaldehyde in PBS, permeabilised with 0.2% Triton X-100 and blocked in 10% (vol./vol.) FCS-PBS. The cells were incubated overnight with primary antibodies against CHOP (1:800), PKC δ (1:400), 14-3-3 ζ (1:50), FOXO1 (1:100), P-S256-FOXO1 (1:50), insulin (1:100) and glucagon (1:150), washed with 10% (vol./vol.) FCS-PBS and incubated for 1 h with the secondary antibodies (1:400). Nuclei were stained with 1 μ mol/l TOPRO-3. The TUNEL assay was performed according to the protocol provided with the kit. Samples were examined using confocal laser scanning microscopy (Leica, Wetzlar, Germany).

In-cell crosslinking The in-cell crosslinking of INS-1E cells was performed using 0.5 mmol/l dithiobis(succinimidyl) propionate in PBS for 3 h on ice [33]. After removal of the cross-linker, the cells were incubated 15 min with 20 mmol/l Tris-HCl, pH 7.5, and thereafter lysed in radioimmunoprecipitation assay (RIPA) buffer (in mmol/l): 25 Tris-HCl pH 7.5, 150 NaCl, 2 EDTA, 10 NaF, 1 Na₃VO₄, 10% (vol./vol.) glycerol, 1% (vol./vol.) Nonidet-P40, 0.1% (wt./vol.) SDS, 0.1% (wt./vol.) C₂₄H₃₉NaO₄ and protease

inhibitors. Cell homogenates were boiled in non-reducing Laemmli buffer. To cleave the cross-linked disulfide bonds, aliquots of the lysates were incubated with 50 mmol/l dithiothreitol for 15 min at 37°C prior to boiling.

Immunoprecipitation and overlay assay INS-1E cells were lysed in RIPA buffer and 500 μ g protein were incubated with FOXO1 antibody (1:100) and 20 μ l of protein-A agarose beads (50% (vol./vol.) slurry) for 3 h at 4°C. The immunoprecipitates were washed with detergent-free RIPA buffer, subjected to SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with 5% milk-TBS-Tween and incubated overnight with FOXO1 antibody (1:1,000 in 5% (wt/vol.) BSA-TBS-Tween) followed by incubation with horseradish peroxidase (HRP)–14-3-3 ζ (1:1,000 in 5% (wt/vol.) milk-TBS-Tween) for 1 h.

Western blotting After lysis in RIPA buffer, fresh homogenates were immediately subjected to western blotting to preserve protein phosphorylation [26]. SDS-PAGE was performed as described above. Secondary HRP-coupled antibody (1:2,000 in 5% (wt/vol.) milk-TBS-Tween) was added for 1 h. Specificity of FOXO1 antibodies is shown in ESM Fig. 1.

Real-time PCR INS-1E cells were cultured as indicated, lysed and total RNA was isolated using a commercial kit. RNA (1 μ g) was transcribed into cDNA using reverse-transcriptase M-MuLV. Aliquots of cDNA were subjected to PCR using the light cycler system LC-480 (Roche Diagnostics, Basel, Switzerland). Normalised gene expression levels were calculated as a ratio of the target vs housekeeping gene (*Rps13*) transcripts ($2^{-\Delta C_t}$). The primer sequences are listed in ESM Table 1.

Statistical analysis The criterion for exclusion of animals was a >20% deviation from the mean weight of the other animals of the same group. The criterion for the exclusion of any data point was a distance $> \pm 2.5 \times \text{SD}$ from the mean of the other data from the same group.

Data are expressed as means $\pm$ SEM; $p < 0.05$ (unpaired Student's *t* test or two-way ANOVA followed by Bonferroni post test where applicable) was considered significant.

Results

PKC δ overexpression promotes nuclear accumulation of phosphorylated FOXO1 PKC δ and FOXO1 proteins are expressed in isolated human islets (Fig. 1a–d). More significantly, palmitate augmented the amount of cleaved caspase-3 and cleaved PKC δ , reduced FOXO1 phosphorylation at S256 already within 1 h and increased nuclear accumulation of

Fig. 1 Overexpression of PKC δ induces nuclear accumulation of P-FOXO1. Human islets (**a–e**), INS-1E (Ctrl, white bars) and PKC δ -tg INS-1E cells (black bars) (**f–l**) were cultured under control condition or in the presence of 600 μ mol/l palmitate for 1 h (P1h, Pal) or 24 h (P24h). (**a**) Cytosolic and nuclear staining of PKC δ . (**b, c**) Western blot detection of PKC δ at 77 kDa and cleaved PKC δ at 38 kDa (**b**) and P-FOXO1 and FOXO1 at 80 kDa (**c**). (**d, e**) Cytosolic and nuclear staining of FOXO1, scale bar 100 μ m (**d**) and percentage of FOXO1-positive nuclei of five independent human islet cell preparations (**e**). (**f**) Quantification of PKC δ on western blots. (**g**) Evaluation of FOXO1-positive nuclei by confocal microscopy. (**h–l**) Western blot analyses of P-PKB, PKB and P-FOXO1. Representative western blots of whole islets and cell lysates and means $\pm$ SEM of three independent experiments are shown. * p <0.05 effect of palmitate; † p <0.05 effect of PKC δ -tg

FOXO1 (Fig. 1a–e and ESM Fig. 2a, b). These results strongly suggest that our previous findings with rodent cells may translate to humans [27]. To gain insight into the molecular

mechanism of PKC δ -dependent nuclear accumulation of FOXO1, an INS-1E cell clone with more than fivefold higher expression of wild-type PKC δ (PKC δ -tg) was used (Fig. 1f).

In PKC δ -tg INS-1E cells nuclei stained positive for FOXO1 already under control culture condition (Fig. 1g and [26]).

Since the nucleocytoplasmic distribution of FOXO1 depends on PKB/Akt activity, the phosphorylation of PKB/Akt at S473 (P-PKB) and of FOXO1 at S256 (P-FOXO1) was analysed next (Fig. 1h–l). Under control culture condition P-PKB was significantly higher in PKC δ -tg INS-1E cells than in parental INS-1E cells (Fig. 1h, i). Palmitate (1 h) reduced phosphorylation of PKB, an effect that became significant in PKC δ -tg INS-1E cells, while the protein levels of PKB remained unchanged (Fig. 1h–j). In parallel with the inhibition of PKB phosphorylation, palmitate also reduced FOXO1 phosphorylation in parental INS-1E cells (Fig. 1k, l). To our surprise, in PKC δ -tg INS-1E cells, the level of P-FOXO1 was extremely high and remained elevated after palmitate treatment.

To clarify the discrepancy between nuclear accumulation of FOXO1 and the high level of phosphorylation of FOXO1 in PKC δ -tg cells, the nucleocytoplasmic distribution of FOXO1 and P-FOXO1 was determined after subcellular fractionation (Fig. 2). The results confirmed our previous observation that palmitate (1 h) augmented the amount of nuclear FOXO1 in parental INS-1E cells, while increased FOXO1 levels were

detectable in nuclei of PKC δ -tg cells already under control condition (Fig. 2a, c and [26]). The level of cytosolic FOXO1 remained largely unchanged (Fig. 2e). Unexpectedly, both nuclear and cytosolic P-FOXO1 were three- to fivefold higher in PKC δ -tg cells compared with parental INS-1E cells and remained high after palmitate exposure (Fig. 2b, d, f, h). The correlation of nuclear P-FOXO1 with nuclear FOXO1 revealed a significant reduction of phosphorylation by palmitate (Fig. 2g). Concomitantly, nuclear PKB phosphorylation was transiently reduced in INS-1E and PKC δ -tg cells, while PKB expression remained unchanged (ESM Fig. 3a). Compatible with nuclear accumulation of P-FOXO1, palmitate did not induce *Bcl2l1* (gene encoding *BIM*) expression, while inhibition of PKB/Akt-dependent phosphorylation of FOXO1 by Akti-1/2 significantly augmented *Bcl2l1* mRNA levels in INS-1E cells (ESM Fig. 3b). The stress kinase c-Jun N-terminal kinase (JNK) was reported to induce nuclear translocation of FOXO1 [7, 34]. However, neither the level of P-T183/P-Y185-JNK nor of its downstream target P-S73-cJun were higher in PKC δ -tg cells compared with parental INS-1E cells, indicating that increased JNK activity does not account for nuclear accumulation of P-FOXO1 (ESM Fig. 3c–f). Thus, neither stimulation of JNK nor inhibition of PKB/Akt seems

Fig. 2 Nuclear FOXO1 is phosphorylated at S256. INS-1E (Ctrl, white bars) and PKC δ -tg INS-1E cells (black bars) were cultured under control conditions or in the presence of 600 μ mol/l palmitate for 1 h (P1h, Pal). Representative western blots and means $\pm$ SEM of three independent experiments are shown for nuclear FOXO1 (a, c) and P-FOXO1 (b, d, g) and cytosolic FOXO1 (e) and P-FOXO1 (f, h). * p <0.05 effect of palmitate; † p <0.05 effect of PKC δ -tg; ‡ p <0.05 effect of palmitate in PKC δ -tg cells

to be the cause of nuclear accumulation of FOXO1 in PKC δ -tg cells.

PKC δ delays nuclear export causing nuclear retention of P-FOXO1 The subcellular distribution of FOXO1 depends on the relative rates of nuclear export and import. Since dephosphorylation of FOXO1 correlates with an increased import rate and phosphorylation of FOXO1 with nuclear export, we examined whether the inhibition of export induces nuclear accumulation of P-FOXO1. In human islet and parental INS-

1E cells, the inhibition of CRM1-dependent nuclear export by leptomycin B (20 ng/ml, 2 h) led to a strong nuclear accumulation of FOXO1 (Fig. 3a, b). Moreover, in the presence of leptomycin B, FOXO1 and P-FOXO1 staining remained nuclear even after stimulation with IGF-1 (20 ng/ml, 15 min), which, by phosphorylating FOXO1, promotes nuclear export of FOXO1 in the absence of leptomycin B (Fig. 3a–c). These observations suggest that P-FOXO1 accumulates in the nuclei when the export is inhibited.

Fig. 3 Inhibition of nuclear export leads to nuclear accumulation of FOXO1. Human islet cells (a) and INS-1E cells (b, c) were treated in cell culture as indicated and the subcellular distribution of FOXO1 (a, b) (green) and P-FOXO1 (c) (green) was visualised by confocal microscopy. Nuclei are stained in red. Representative pictures of three independent experiments are shown. Concentrations and incubation times: IGF-1, 20 ng/ml, 15 min; leptomycin B, 20 ng/ml, 2 h; Akti1/2, 10 μ mol/l, 3 h. Scale bar, 50 μ m (a) or 100 μ m (b, c). White arrows indicate P-FOXO1 in nuclei

To assess whether the rate of nuclear export differs between control and PKC δ -tg INS-1E cells, nuclear accumulation of FOXO1 was first triggered with palmitate (1 h) and thereafter the export of FOXO1 was stimulated with IGF-1 (20 ng/ml) and the cellular distribution of FOXO1 was examined over time. In parental INS-1E cells palmitate-induced nuclear accumulation of FOXO1 was counteracted by IGF-1 already after 5 min and FOXO1 staining was mainly cytosolic 10 and 15 min after addition of IGF-1 (Fig. 4a). In PKC δ -tg INS-1E cells nuclear FOXO1 accumulation was still high 5 and 10 min after exposure to IGF-1 (Fig. 4b). These results indicate that PKC δ , by decelerating the nuclear export, causes nuclear retention of P-FOXO1 in PKC δ -tg INS-1E cells. Notably, nuclear P-FOXO1 is also detectable in islets of human pancreatic resections, indicating that this is not a cell culture phenomenon (ESM Fig. 2c).

PKC δ phosphorylates 14-3-3 ζ at S58 and impairs its dimerisation The chaperone 14-3-3 ζ is highly expressed in beta cells and its dimerisation is impaired by phosphorylation at S58, which is also a phosphorylation site of PKC δ [22, 35, 36]. Since nuclear export of FOXO1 requires binding of P-FOXO1 to 14-3-3 dimers [19], we used an overlay assay to examine the interaction between the immunoprecipitated

FOXO1 and HRP-labelled 14-3-3 ζ (Fig. 5a, b). HRP-14-3-3 ζ bound to FOXO1 and the binding was significantly reduced after cell exposure to palmitate (1 h) and increased after cell stimulation with IGF-1 (1 h). Thus, the degree of 14-3-3 ζ binding to FOXO1 correlated with the degree of FOXO1 phosphorylation (Fig. 5a, b and ESM Fig. 3g).

Next, we examined whether PKC δ and palmitate increase phosphorylation of 14-3-3 ζ at S58 (P-14-3-3 ζ). In human islets, incubation with palmitate for 1 and 24 h increased levels of P-14-3-3 ζ and immunostaining of human islet cells suggested that 14-3-3 ζ protein is not only distributed throughout the cytosol but also in nuclei (Fig. 5c, d). In the nuclear fraction of PKC δ -tg INS-1E cells, levels of P-14-3-3 ζ were significantly higher than in those of parental INS-1E cells and P-14-3-3 ζ was further increased by palmitate (24 h) (Fig. 5e, f). The cytosolic levels of P-14-3-3 ζ remained unchanged (ESM Fig. 3i, j). To examine whether phosphorylation of 14-3-3 ζ at S58 affects its dimerisation, the amount of 14-3-3 ζ dimers was determined on western blots after in-cell crosslinking. Both palmitate and PKC δ -tg significantly reduced 14-3-3 ζ dimerisation (Fig. 5g, h). These observations suggest that palmitate- and PKC δ -dependent phosphorylation of 14-3-3 ζ may hinder chaperone dimerisation and its interaction with P-FOXO1, which, consequently, delays nuclear

Fig. 4 PKC δ impedes nuclear export of FOXO1. INS-1E (Ctrl) (a) and PKC δ -tg INS-1E cells (b) were treated as indicated and the subcellular distribution of FOXO1 (green) was visualised by confocal microscopy. Nuclei are stained in red. Representative pictures of three independent experiments are shown. Concentrations used: palmitate (P1h), 600 μ mol/l; IGF-1, 20 ng/ml. Scale bar, 50 μ m

export of P-FOXO1. In agreement, the overexpression of a phosphomimicking mutant of 14-3-3 ζ (S58D) increased nuclear staining of FOXO1, whereas the overexpression of wild type or a phosphorylation-deficient mutant (S58A) of 14-3-3 ζ did not affect the subcellular distribution of FOXO1 (Fig. 5i and ESM Fig. 3h).

Effect of PKC δ on regulation of FOXO1 target genes Although it is generally accepted that dephosphorylated FOXO1 contributes to glucolipotoxicity, it is unclear whether P-FOXO1 affects gene expression [37]. One of the putative FOXO1 target genes that strongly associates with palmitate-dependent beta cell death is *DDIT3*, encoding the ER stress

Fig. 5 PKC δ and palmitate increase phosphorylation of 14-3-3 ζ at S58 and impair dimerisation of 14-3-3 ζ . INS-1E cells (Ctrl, white bars) and PKC δ -tg INS-1E cells (black bars) (**a**, **b**, **e**–**i**) and human islets (**c**, **d**) were cultured under control condition or treated with 600 μ mol/l palmitate for 1 h or 24 h (Pal, P1h, P24h) or with IGF-1 (50 ng/ml, 1 h), as indicated. (**a**, **b**) Representative in vitro overlay assay of HRP-labelled 14-3-3 ζ with immunoprecipitated FOXO1 (**a**) and relative binding of HRP-14-3-3 ζ to FOXO1 (**b**). (**c**, **d**) Representative western blots of P-S58-14-3-3 ζ and 14-3-3 ζ in human islets (**c**) and subcellular distribution of 14-3-3 ζ (green) in human islet cells (**d**). (**e**–**h**) Representative western blots and relative amounts of nuclear P-S58-14-3-3 ζ and 14-3-3 ζ (**e**, **f**), and of whole-cell 14-3-3 ζ dimers and monomers (**g**, **h**) without ($\emptyset$) and after (+) in-cell crosslinking. (**i**) FOXO1 immunostaining in INS-1E cells (Ctrl) and after transfection with wild-type (WT), S58A- or S58D-mutated 14-3-3 ζ . Scale bar, 50 μ m. Data are expressed as means $\pm$ SEM of three independent experiments; * p <0.05 vs Ctrl

marker CHOP. Chronic treatment with palmitate increased mRNA levels of *Ddit3* and induced the expression of CHOP protein in INS-1E cells (Fig. 6a, b). PKC δ -tg had no significant effect on *Ddit3* mRNA levels and the effect of palmitate was not significantly altered (Fig. 6a, b).

In contrast, the mRNA and protein levels of the proapoptotic thioredoxin-interacting protein (TXNIP), which were downregulated by palmitate, were strongly reduced in PKC δ -tg cells already under control culture conditions (Fig. 6c, d). Neither the basal rate of apoptosis nor palmitate-induced apoptosis were increased in PKC δ -tg cells compared with parental INS-1E cells (Fig. 6e). In human islets, palmitate induced apoptosis and changed mRNA levels, i.e. downregulated *TXNIP*, upregulated *DDIT3* but did not change *BCL2L1* mRNA levels, effects that are comparable with our observations with rodent cells (Fig. 6f–h). These findings suggest that P-FOXO1, as opposed to FOXO1, may support transcriptional activity beneficial for beta cells.

Nuclear accumulation of FOXO1 without beta cell failure: the unexpected role of PKC δ

To examine whether PKC δ impairs beta cell function in vivo, mice specifically

overexpressing PKC δ in beta cells (β PKC δ -tg) were generated. An increased amount of PKC δ protein was detectable in isolated islets of β PKC δ -tg mice, while the expression in other tissues remained unchanged (Fig. 7a, b). As expected, significantly more nuclei of β PKC δ -tg islet cells stained positive for FOXO1 when compared with wild-type cells, already under control culture conditions (Fig. 7c, d). However, PKC δ overexpression neither increased basal apoptosis nor accentuated palmitate cytotoxicity in cultured islet cells (Fig. 7e). In addition, glucose tolerance and plasma insulin levels after CD and HFD feeding did not significantly differ between β PKC δ -tg and wild-type mice (ESM Fig. 4a–d), nor was the islet area significantly altered (ESM Fig. 4e, f). In accordance PKC δ -tg INS-1E cells displayed normal insulin content and glucose-induced insulin secretion (ESM Fig. 4g). Thus, increased levels of PKC δ did not impair beta cell function.

In conclusion, our study proposes PKC δ as an inhibitor of nuclear export of P-FOXO1 and provides evidence that PKC δ can modulate the expression of FOXO1 target genes by increasing nuclear accumulation of P-FOXO1, which is beneficial for beta cells (Fig. 8).

Fig. 6 Nuclear P-FOXO1 modulates gene expression without triggering cell death. INS-1E (Ctrl, white bars) and PKC δ -tg INS-1E cells (black bars) (a–e), human islets (f, g) and human islet cells (h) were cultured under control culture condition or in the presence of 600 μ mol/l palmitate for 24 h (Pal, P24h) as indicated. (a, c, f, g) Relative mRNA levels. (b) CHOP immunostaining (green); nuclei are stained in red. Scale bar, 50 μ m. (d) Western blot for TXNIP protein. (e, h) Percentage of TUNEL-positive nuclei. Data are expressed as means+SEM of three or four independent experiments. * p <0.05, ** p <0.01 palmitate vs respective control and ††† p <0.001 effect of PKC δ -tg vs Ctrl. Concentrations used: 600 μ mol/l palmitate (a–g); 100, 300, 600 μ mol/l palmitate (h), as indicated

Fig. 7 Overexpression of PKC δ in mouse beta cells augments nuclear accumulation of FOXO1 without increasing cell death. **(a, b)** Representative western blot for PKC δ in isolated islets **(a)** and brain, liver, muscle and white adipose tissue (WAT) **(b)** of wild-type (WT) and β PKC δ -tg (tg) mice. **(c)** FOXO1 immunostaining (red) in isolated islet cells cultured under standard conditions or in the presence of palmitate for 1 h (P1h). Nuclei are stained in blue. **(d, e)** Percentage of FOXO1-positive nuclei **(d)** and TUNEL-positive cells **(e)** of WT (white bars) and β PKC δ -tg (black bars) mouse islet cells. Data are expressed as means $\pm$ SEM of three independent experiments; * p <0.05 vs respective control; † p <0.05 vs control WT cells

Discussion

This study reveals PKC δ to be an important regulator of nucleocytoplasmic distribution of FOXO1 and proposes that PKC δ decelerates CRM1-dependent nuclear export of P-FOXO1 by impairing 14-3-3 ζ dimerisation.

PKC δ is expressed in human and rodent insulin-secreting cells. However, it is controversially discussed as a kinase which positively and negatively regulates proliferation and apoptosis [38, 39]. Our previous observations indicate that PKC δ KN counteracts nuclear accumulation of FOXO1 and reduces palmitate-mediated islet cell apoptosis [26]. In accordance, FOXO1-deficient mice are protected against HFD-impaired glucose tolerance [10]. Therefore, it was unexpected that overexpression of PKC δ in insulin-secreting cells, despite nuclear accumulation of FOXO1, neither impaired in vivo glucose homeostasis of mice nor accentuated palmitate cytotoxicity in vitro. The increased S256-phosphorylation of nuclear FOXO1 in PKC δ -tg INS-1E cells could explain why nuclear FOXO1 was not pro-apoptotic. Indeed, inhibition of PKB/Akt and FOXO1 dephosphorylation are necessary for induction of the pro-apoptotic genes *Bax* and *Bcl2/11*

[6, 16, 40]. In PKC δ -tg cells nuclear FOXO1 was highly phosphorylated and *Bcl2/11* mRNA levels were not significantly increased (data not shown). Palmitate-induced nuclear accumulation of FOXO1 was also not sufficient to augment *Bcl2/11* expression, while pharmacological inhibition of PKB/Akt triggered nuclear accumulation of dephosphorylated FOXO1 and augmented *Bcl2/11* mRNA levels [16]. More significantly, PKC δ -tg reduced the mRNA and protein levels of the pro-apoptotic protein TXNIP. The expression of TXNIP was shown to be repressed by FOXO1 and palmitate, but upregulated by hyperglycaemia [41, 42]. The present study suggests that PKC δ -dependent nuclear accumulation of P-FOXO1 may be involved in the inhibition of TXNIP expression.

The phosphorylation of nuclear FOXO1 could result from stimulation of a kinase (i.e. PKB) or inhibition of a phosphatase (i.e. PP2A) [15, 19, 43]. PKC δ was found to stimulate insulin-IGF-1 signalling through phosphorylation of IRS1 at S318 in muscle cells [44]. Indeed, the levels of P-PKB were slightly but significantly higher in PKC δ -tg INS-1E cells (Fig. 1h–k). The role of other kinases that target FOXO1, like serum- and glucocorticoid-inducible kinase, AMP-activated protein kinase or Raf-1, remains to be defined [45–47].

Fig. 8 Nuclear export of P-FOXO1 is inhibited by PKC δ through phosphorylation of 14-3-3 ζ . Subcellular distribution of FOXO1 depends on the relative rates of nuclear import and export. Inhibition of PKB/Akt or stimulation of phosphatase PP2A reduces phosphorylation of the cytosolic P-FOXO1 and triggers nuclear import. In nuclei, FOXO1 induces pro-apoptotic genes (*BIM*) leading to apoptosis (1). Upon PKB-mediated phosphorylation (2) nuclear P-FOXO1 binds to 14-3-3 ζ dimers and CRM1 (3), steps necessary for nuclear export (4). Phosphorylation of nuclear 14-3-3 ζ at S58 by PKC δ (5) impairs chaperone dimerisation (6) and its binding to P-FOXO1, events which decelerate the export of P-FOXO1 (7). We propose that nuclear P-FOXO1, as opposed to FOXO1, exerts anti-apoptotic effects (8)

FOXO1 activity is further regulated by additional post-translational changes like acetylation [48]. In PKC δ -tg INS-1E cells, acetylation levels of nuclear FOXO1 were unaltered (data not shown). Furthermore, JNK activation is essential for palmitate-induced beta cell death [7]. Our results suggest that JNK phosphorylation was not decisive for the nuclear accumulation of P-FOXO1. Nevertheless, JNK may interfere with nuclear FOXO1 at a distal step and convert the anti-apoptotic signal into a pro-apoptotic signal. Previous observations showed that JNK phosphorylates 14-3-3 ζ at S184 and triggers the release of the pro-apoptotic FOXO3 and BAD [49].

PKC δ overexpression decreased the export rate of P-FOXO1, suggesting that the regulation of export rather than import dictates nuclear accumulation of FOXO1. PKC δ overrides the effect of PKB, enabling nuclear accumulation of P-FOXO1. This role of PKC δ might explain the diverging transcriptional activity of FOXO1 and its opposing effects on beta cell fate. Our results suggest that the nuclear export is decelerated due to PKC δ -dependent phosphorylation and impaired dimerisation of 14-3-3 ζ . Of the seven 14-3-3 isoforms expressed in beta cells, 14-3-3 ζ is a target of PKC δ [22, 36]. Indeed, nuclear P-S58-14-3-3 ζ was increased in PKC δ -tg INS-1E cells and its levels were further augmented by palmitate. Previously, cleaved PKC δ (i.e. by caspase-3)

was shown to be responsible for phosphorylation of 14-3-3 ζ [36]. In agreement, in human islets palmitate increased the levels of cleaved caspase-3 and cleaved PKC δ (i.e. a 38 kDa protein which bound the PKC δ antibody).

Cytosolic 14-3-3 ζ has been found to bind to BAD and BAX, inhibiting their pro-apoptotic action [22]. Our data suggest that nuclear 14-3-3 ζ binds to P-FOXO1, thereby promoting nuclear extrusion of the transcription factor. This additional role underlines the importance of 14-3-3 ζ for beta cell function and explains why INS-1E cells do not survive after small interfering RNA-mediated downregulation of 14-3-3 ζ (own observations and [22]).

Long-term exposure to palmitate increased *Ddit3* mRNA and protein levels of CHOP in INS-1E cells. Previous studies demonstrated that overexpression of inactive FOXO1 inhibits palmitate-mediated induction of CHOP [7]. In control cultured PKC δ -tg cells CHOP protein levels were undetectable, suggesting that P-FOXO1 does not upregulate CHOP. This finding, however, does not exclude a regulatory role for dephosphorylated FOXO1.

Our observations in human islets, which mirror the results obtained with rodent cells, and the overexpression of PKC δ in a defined cell model enabled us to identify the nuclear export machinery as a regulatory site of PKC δ -dependent nuclear

accumulation of FOXO1. The proposed mechanism, namely PKC δ -mediated phosphorylation of 14-3-3 ζ impairs chaperone dimerisation, its binding to P-FOXO1 and consequently CRM1-dependent nuclear export of P-FOXO1, helps to understand the recent observations showing that FOXO1 modulates beta cell fate in opposing ways (Fig. 8).

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Contribution statement FG contributed to the design of the study, performed experiments and data analysis, prepared the figures, wrote the manuscript and revised and approved the final version of the manuscript. GK, MP, RL, TS, AMH, FM, AP and FB performed experiments and data analysis and revised and approved the final version of the manuscript. HUH contributed to the study design and the drafting, writing and revision of the manuscript and approved the final version. SU designed the study, performed data analysis, prepared the figures, wrote the manuscript and revised and approved the final version of the manuscript. FG and SU are the guarantors of this work and, as such, had full access to all the data of the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

- Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102–110
- Donath MY, Boni-Schnetzler M, Ellingsgaard H, Ehses JA (2009) Islet inflammation impairs the pancreatic β -cell in type 2 diabetes. *Physiology* 24:325–331
- Maechler P, Li N, Casimir M, Vetterli L, Frigerio F, Brun T (2010) Role of mitochondria in β -cell function and dysfunction. *Adv Exp Med Biol* 654:193–216
- Cunha DA, Hekerman P, Ladiere L et al (2008) Initiation and execution of lipotoxic ER stress in pancreatic β -cells. *J Cell Sci* 121:2308–2318
- Buteau J, Accili D (2007) Regulation of pancreatic β -cell function by the forkhead protein FoxO1. *Diabetes Obes Metab* 9:140–146
- Ren D, Sun J, Mao L, Ye H, Polonsky KS (2014) BH3-only molecule Bim mediates β -cell death in IRS2 deficiency. *Diabetes* 63:3378–3387
- Martinez SC, Tanabe K, Cras-Méneur C, Abumrad NA, Bernal-Mizrachi E, Permutt MA (2008) Inhibition of Foxo1 protects pancreatic islet β -cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. *Diabetes* 57:846–859
- Talchai C, Xuan S, Lin HV, Sussel L, Accili D (2012) Pancreatic β cell dedifferentiation as a mechanism of diabetic β cell failure. *Cell* 150:1223–1234
- Talchai SC, Accili D (2015) Legacy effect of FoxO1 in pancreatic endocrine progenitors on adult β -cell mass and function. *Diabetes* 64:1–12
- Kobayashi M, Kikuchi O, Sasaki T et al (2012) FoxO1 as a double-edged sword in the pancreas: analysis of pancreas- and β -cell-specific FoxO1 knockout mice. *Am J Physiol Endocrinol Metab* 302:E603–E613
- Kim-Muller JY, Zhao S, Srivastava S et al (2014) Metabolic inflexibility impairs insulin secretion and results in MODY-like diabetes in triple FoxO-deficient mice. *Cell Metab* 20:593–602
- Mussig K, Staiger H, Machicao F et al (2009) Association of common genetic variation in the FOXO1 gene with β -cell dysfunction, impaired glucose tolerance, and type 2 diabetes. *J Clin Endocrinol Metab* 94:1353–1360
- Eijkelenboom A, Burgering BM (2013) FOXOs: signalling integrators for homeostasis maintenance. *Nat Rev Mol Cell Biol* 14:83–97
- Martinez SC, Cras-Méneur C, Bernal-Mizrachi E, Permutt MA (2006) Glucose regulates Foxo1 through insulin receptor signaling in the pancreatic islet β -cell. *Diabetes* 55:1581–1591
- Yan L, Lavin VA, Moser LR, Cui Q, Kanies C, Yang E (2008) PP2A regulates the pro-apoptotic activity of FOXO1. *J Biol Chem* 283:7411–7420
- Kaiser G, Gerst F, Michael D et al (2013) Regulation of forkhead box O1 (FOXO1) by protein kinase B and glucocorticoids: different mechanisms of induction of beta cell death in vitro. *Diabetologia* 56:1587–1595
- Brunet A, Kanai F, Stehn J et al (2002) 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J Cell Biol* 156:817–828
- Rena G, Prescott AR, Guo S, Cohen P, Unterman TG (2001) Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targeting. *Biochem J* 354:605–612
- Brunet A, Bonni A, Zigmond MJ et al (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96:857–868
- Zhao X, Gan L, Pan H et al (2004) Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. *Biochem J* 378:839–849
- Park JK, Kang MY, Kim YH et al (2008) PKC δ in preeclamptic placentas promotes Bax dissociation from 14-3-3 ζ through 14-3-3 ζ phosphorylation. *Placenta* 29:584–592
- Lim GE, Piske M, Johnson JD (2013) 14-3-3 proteins are essential signalling hubs for beta cell survival. *Diabetologia* 56:825–837
- Eitel K, Staiger H, Rieger J et al (2003) Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* 52:991–997
- Carpenter L, Cordery D, Biden TJ (2002) Inhibition of protein kinase C δ protects rat INS-1 cells against interleukin-1 β and streptozotocin-induced apoptosis. *Diabetes* 51:317–324
- Cantley J, Boslem E, Laybutt DR et al (2011) Deletion of protein kinase C δ in mice modulates stability of inflammatory genes and protects against cytokine-stimulated beta cell death in vitro and in vivo. *Diabetologia* 54:380–389
- Hennige AM, Ranta F, Heinzlmann I et al (2010) Overexpression of kinase-negative protein kinase C δ in pancreatic β -cells protects mice from diet-induced glucose intolerance and β -cell dysfunction. *Diabetes* 59:119–127

27. Bezy O, Tran TT, Pihlajamäki J et al (2011) PKC δ regulates hepatic insulin sensitivity and hepatosteatosis in mice and humans. *J Clin Invest* 121:2504–2517
28. Greene MW, Burrington CM, Lynch DT et al (2014) Lipid metabolism, oxidative stress and cell death are regulated by PKC δ in a dietary model of nonalcoholic steatohepatitis. *PLoS One* 9, e85848
29. Greene MW, Burrington CM, Luo Y, Ruhoff MS, Lynch DT, Chaithongdi N (2014) PKC δ is activated in the liver of obese Zucker rats and mediates diet-induced whole body insulin resistance and hepatocyte cellular insulin resistance. *J Nutr Biochem* 25:281–288
30. George M, Ayuso E, Casellas A, Costa C, Devedjian JC, Bosch F (2002) β cell expression of IGF-I leads to recovery from type 1 diabetes. *J Clin Invest* 109:1153–1163
31. Li W, Yu JC, Shin DY, Pierce JH (1995) Characterization of a protein kinase C- δ (PKC- δ) ATP binding mutant. An inactive enzyme that competitively inhibits wild type PKC- δ enzymatic activity. *J Biol Chem* 270:8311–8318
32. Ullrich S, Berchtold S, Ranta F et al (2005) Serum- and glucocorticoid-inducible kinase 1 (SGK1) mediates glucocorticoid-induced inhibition of insulin secretion. *Diabetes* 54:1090–1099
33. Smith AL, Friedman DB, Yu H, Carnahan RH, Reynolds AB (2011) ReCLIP (reversible cross-link immuno-precipitation): an efficient method for interrogation of labile protein complexes. *PLoS One* 6, e16206
34. Kawamori D, Kaneto H, Nakatani Y et al (2006) The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. *J Biol Chem* 281:1091–1098
35. Woodcock JM, Murphy J, Stomski FC, Berndt MC, Lopez AF (2003) The dimeric versus monomeric status of 14-3-3 ζ is controlled by phosphorylation of Ser58 at the dimer interface. *J Biol Chem* 278:36323–36327
36. Kim YS, Choi MY, Kim YHJ et al (2010) Protein kinase Cdelta is associated with 14-3-3 phosphorylation in seizure-induced neuronal death. *Epilepsy Res* 92:30–40
37. Zhang X, Gan L, Pan H et al (2002) Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J Biol Chem* 277:45276–45284
38. Basu A, Pal D (2010) Two faces of protein kinase C δ : the contrasting roles of PKC δ in cell survival and cell death. *Sci World J* 10: 2272–2284
39. Jackson DN, Foster DA (2004) The enigmatic protein kinase C δ : complex roles in cell proliferation and survival. *FASEB J* 18:627–636
40. McLoughlin TJ, Smith SM, DeLong AD, Wang H, Unterman TG, Esser KA (2009) FoxO1 induces apoptosis in skeletal myotubes in a DNA-binding-dependent manner. *Am J Physiol Cell Physiol* 297:C548–C555
41. Shalev A (2014) Minireview: thioredoxin-interacting protein: regulation and function in the pancreatic β -cell. *Mol Endocrinol* 28:1211–1220
42. Chen J, Saxena G, Mungrue IN, Lusi AJ, Shalev A (2008) Thioredoxin-interacting protein: a critical link between glucose toxicity and β -cell apoptosis. *Diabetes* 57:938–944
43. Guo S, Dunn SL, White MF (2006) The reciprocal stability of FOXO1 and IRS2 creates a regulatory circuit that controls insulin signaling. *Mol Endocrinol* 20:3389–3399
44. Weigert C, Hennige AM, Lehmann R et al (2006) Direct cross-talk of interleukin-6 and insulin signal transduction via insulin receptor substrate-1 in skeletal muscle cells. *J Biol Chem* 281:7060–7067
45. Di Pietro N, Panel V, Hayes S et al (2010) Serum- and glucocorticoid-inducible kinase 1 (SGK1) regulates adipocyte differentiation via forkhead box O1. *Mol Endocrinol* 24:370–380
46. Yun H, Park S, Kim MJ et al (2014) AMP-activated protein kinase mediates the antioxidant effects of resveratrol through regulation of the transcription factor FoxO1. *FEBS J* 281:4421–4438
47. Alejandro EU, Lim GE, Mehran AE et al (2011) Pancreatic β -cell Raf-1 is required for glucose tolerance, insulin secretion, and insulin 2 transcription. *FASEB J* 25:3884–3895
48. Qiang L, Banks AS, Accili D (2010) Uncoupling of acetylation from phosphorylation regulates FoxO1 function independent of its subcellular localization. *J Biol Chem* 285:27396–27401
49. Sunayama J, Tsuruta F, Masuyama N, Gotoh Y (2005) JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. *J Cell Biol* 170:295–304