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Downregulation of protein kinase B/Akt-1 mediates INS-1 insulinoma cell apoptosis induced by dominant-negative suppression of hepatocyte nuclear factor-1alpha function

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Abstract *Aims/hypothesis:* Inactivating mutations in *Tcf1*, which encodes the transcription factor hepatocyte nuclear factor (HNF)-1alpha, cause maturity-onset diabetes of the young-3. We have previously shown that a dominant-negative mutant (DN-HNF-1alpha) renders INS-1 insulinoma cells sensitive to the mitochondrial apoptosis pathway, but the underlying alterations in signal transduction remain unknown. *Materials and methods:* Using a reverse tetracycline-dependent transactivator system, DN-HNF-1alpha-induced apoptosis was assessed by immunoblotting and caspase assays. Alterations in AKT1 kinase/protein kinase B (AKT1) survival signalling during DN-HNF-1alpha-induced apoptosis were investigated by phospho-specific immunodetection and transient transfection experiments. *Results:* Induction of DN-HNF-1alpha caused significant changes in the activation-specific phosphorylation status of AKT1 that were preceded by a downregulation of *Ins1* gene transcription. Phosphorylation of AKT1 at Ser473 was dramatically reduced after 36 to 48 h of DN-HNF-1alpha induction and coincided with maximal apoptosis activation. Overexpression of a constitutively active mutant of *Akt1* rescued INS-1 cells from DN-HNF-1alpha-induced apoptosis, while ectopic expression of a dominant-negative mutant mimicked the effect of DN-HNF-1alpha on apoptosis activation. Pharmacological sup-

pression of growth factor survival signalling through administration of the phosphatidylinositol-3 kinase (PI-3K) inhibitor wortmannin accelerated the induction of apoptosis by DN-HNF-1alpha. *Conclusions/interpretation:* These data suggest that a decrease in PI-3K/AKT1 survival signalling mediates DN-HNF-1alpha-induced apoptosis in insulin-secreting cells.

Keywords AKT1 · Apoptosis · HNF-1alpha · INS-1 · MODY3 · TCF1

Abbreviations Akt1: Akt1 kinase/protein kinase B alpha · DN: dominant-negative · GFP: green fluorescent protein · HNF-1alpha: hepatocyte nuclear factor-1alpha · PDK-1: phosphoinositide-dependent kinase-1 · PI-3K: phosphatidylinositol-3 kinase · WT: wild-type

Introduction

Maturity-onset diabetes of the young, which accounts for approximately 2 to 5% of all patients with type 2 diabetes, comprises a number of single-gene disorders affecting pancreatic beta cell function [1, 2]. It is characterised by non-ketotic diabetes mellitus, usually before the age of 25 years, an autosomal mode of inheritance, and defective glucose-induced insulin secretion. MODY3 (which accounts for at least 50% of all MODY cases) results from heterozygous, loss-of-function mutations of the *Tcf1* gene encoding the transcription factor hepatocyte nuclear factor (HNF)-1alpha [3, 4]. HNF-1alpha is expressed in the liver, kidney, intestine and pancreatic islets [5, 6]. It participates in a transcription factor network that regulates pancreatic development as well as fatty acid, protein and carbohydrate metabolism [5–10]. Variations in genes involved in this transcription factor network have been associated with an increased susceptibility to glucose intolerance and type 2 diabetes [11–13].

Human autopsy and several animal studies [14–17] suggest that a decline in functional beta cell mass is an important factor for the pathogenesis of type 2 diabetes.

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Similarly to subjects with MODY3 [18], transgenic mice deficient in *Tcf1*, which encodes the transcription factor HNF-1 α , or beta cell transgenic mice carrying dominant-negative (DN) *Tcf1* mutants develop diabetes with defective glucose-stimulated insulin secretion without insulin resistance of target tissues [5, 6, 19, 20]. The beta cell mass in these transgenic mice is significantly reduced [19, 20]. Previous in vitro and in vivo studies have shown that DN mutants of HNF-1 α increase the expression of the cell cycle inhibitor p27, decrease cell proliferation, increase the basal apoptosis rate, and render cells vulnerable to activation of the mitochondrial apoptosis pathway [21, 22]. These studies suggest that decreased cell proliferation and increased apoptosis may contribute to the observed decrease in beta cell mass. However, the underlying alterations in signal transduction causing these defects remain largely unknown.

Gene deletion of endogenous *Tcf1* or dominant-negative suppression of HNF-1 α function have been shown to impair insulin gene transcription and glucose-stimulated insulin secretion [5, 6, 9, 19]. Insulin activates a complex signal-transduction pathway that involves receptor-mediated recruitment of insulin receptor substrates and subsequent activation of class 1a phosphoinositide-3 kinases (PI-3K). PI-3K-generated 3-phosphoinositides are recognised by a subset of pleckstrin-homology-domain-containing protein kinases, including phosphoinositide-dependent kinase-1 (PDK-1) and its downstream target AKT1/protein kinase B α (AKT1). AKT1 activation involves translocation of the enzyme to the plasma membrane, PDK-1-mediated phosphorylation within the activation loop as well as mammalian target of rapamycin kinase-mediated phosphorylation on residue Ser473 within the hydrophobic motif [23–27]. Once fully activated, AKT1 phosphorylates several downstream targets that promote proliferation and survival of cells [28–30]. In the present study we investigate the role of AKT1 in the increased sensitivity of INS-1 insulinoma cells to apoptosis that is induced by DN suppression of HNF-1 α function.

Materials and methods

Cultivation and treatment of insulinoma INS-1 cells overexpressing HNF-1 α in an inducible system

Rat INS-1 insulinoma cells overexpressing the gene encoding wild-type (WT) HNF-1 α or a DN mutant of HNF-1 α (DN-HNF-1 α) (SM6) under control of a doxycycline-dependent transcriptional activator have been described previously [9]. The SM6 mutant contains a substitution of 83 amino acids in the HNF-1 α DNA-binding domain, resulting in the formation of non-functional heterodimers with endogenous HNF-1 α [31]. The level of HNF-1 α production can be tightly controlled by culturing the cells over defined time periods with doxycycline, with maximal induction at a concentration of 500 ng/ml [9, 22]. INS-1 cells conditionally overproducing WT- or DN-HNF-1 α were cultured in

RPMI 1,640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 6 mmol/l glucose, 2 mmol/l L-glutamine, 1 mmol/l pyruvate, penicillin (100 U/ml), streptomycin (100 μ g/ml), 10% FCS (PAA, Cölbe, Germany), 10 mmol/l HEPES (pH 7.4) and 50 μ mol/l 2-mercaptoethanol. Cells were plated at a density of 5×10^4 cells/cm². After 36 h, culture medium was supplemented with 500 ng/ml doxycycline (Sigma, Munich, Germany) for 12–48 h to induce the production of HNF-1 α .

Immunoblotting

Cells were rinsed with ice-cold PBS and lysed in Tris-buffered saline containing SDS, glycerol and protease inhibitors. Protein content was determined using the Pierce (Rockford, IL, USA) BCA Micro Protein Assay kit. Samples were supplemented with 2-mercaptoethanol and denatured at 95°C for 5 min. An equal amount of protein (20–50 μ g) was separated with 5–15% SDS-PAGE and blotted to nitrocellulose membranes (Protein BA 85; Schleicher & Schuell, Dassel, Germany). The blots were blocked with 5% non-fat milk in blocking solution (15 mmol/l Tris-HCl pH 7.5, 200 mmol/l NaCl and 0.1% Tween-20) for 2 h at room temperature. Membranes were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-HNF-1 α antibody diluted 1:5,000 (kindly provided by Dr R. Cortese, IRBM, Rome, Italy), mouse monoclonal anti- α -tubulin antibody (clone DM 1A, 1:10,000; Sigma), and a rabbit monoclonal AKT antibody (diluted 1:1,000 in 5% BSA; Cell Signaling Technology, Beverly, MA, USA). Phosphorylation of AKT1 was investigated using specific antibodies raised against the active, phosphorylated form of AKT1 (Ser473) (1:1,000 in 5% BSA, clone 193H12; Cell Signaling Technology). Membranes were washed and incubated with anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate (1:1,000–1:5,000; Promega, Mannheim, Germany). Antibody-conjugated peroxidase activity was visualised using the SuperSignal chemiluminescence reagent (Pierce). Membranes were stripped in standard stripping buffer (2% SDS, 62.5 mmol/l Tris-HCl, 100 mmol/l 2-mercaptoethanol, pH 6.8) at 60°C for 30 min, washed twice and reprobed. Densitometric analysis of immunoblots was performed using Metamorph software (Molecular Devices, Downingtown, PA, USA).

Quantitative real-time PCR

Expression of *Ins1* (insulin) and *Akt1* mRNA was examined using relative quantitative real-time PCR. In relative quantification the expression of *Ins1* and *Akt1* mRNA was measured with respect to the reference gene *Actb* (β -actin), which was expressed constitutively and at the same level in all the samples analysed. INS-1 cells were harvested from culture treatments at the appropriate time-points. Total RNA was extracted using the RNeasy mini Kit

(Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed using 2 µg total RNA as template and M-MLV reverse transcriptase (Invitrogen, Paisley, UK) primed with 50 pmol random hexamers (New England Biolabs, Ipswich, MA, USA). Quantitative real-time PCR was performed using the LightCycler (Roche Diagnostics, Indianapolis, IN, USA) and the QuantiTech SYBR Green PCR kit (Qiagen) as per manufacturers' protocols and 25 pmol of primer pair concentration. In order to obtain the highest efficiency in real-time PCR using SYBR Green I, the targets were designed to be 150–200 bp in length. Specific primers for each gene analysed were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve which was used to calculate the PCR efficiency. The PCR reactions were performed in a 20-µl volume. The PCR was performed as follows: there was an activation step of HotStartTaq polymerase at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 20 s, and extension at 72°C for 20 s. After the PCR reaction, the temperature was decreased to 65°C and then gradually raised to 95°C at a rate of 0.2°C/s. The fluorescence signal was continuously monitored during this process for melting curve analysis. The results were judged positive or negative by the presence or absence of the melting temperature peak of 88°C and by the effectiveness of the quantitative score. The data were analysed using Lightcycler Software 4.0 with all samples normalised to *Actb*.

Transient transfection experiments and evaluation of apoptosis

For transfections, INS-1 cells were plated onto 24-well tissue culture plates. One day later cells were cotransfected with plasmids pEGFP-C1 (Clontech, Palo Alto, CA, USA) and plasmids encoding a constitutively active mutant of *Akt1* (CA-AKT1) (myr-AKT1; Upstate Biotechnology, Lake Placid, NY, USA), a DN form of *Akt1* (DN-AKT1) (AKT1K179M; Upstate Biotechnology), or with an empty vector (pCMV) using the F2 transfection reagent (Targeting Systems, Santee, CA, USA). Cells were transfected with pEGFP-C1 (20 ng) and a 14-fold excess of the respective kinase expression vector or the empty vector (280 ng). For transfections, 300 ng of the total plasmid DNA mix and 0.3 µl F2 reagent were diluted in 200 µl RPMI medium under serum-free conditions and preincubated at room temperature for 20 min. Cultures were incubated with the DNA-F2-transfection mixture at 37°C for 1 h. After 24 h, the culture medium was supplemented with 500 ng/ml doxycycline to induce DN-HNF-1α production. After a further 48 h, nuclei were stained live with Hoechst 33258. Apoptotic nuclei and expression of green fluorescent protein (GFP) were observed and quantified by epifluorescence microscopy as described previously [22].

Measurement of executioner caspase (DEVDase) activity

Cells were lysed in 200 µl lysis buffer (10 mmol/l HEPES, pH 7.4, 42 mmol/l KCl, 5 mmol/l MgCl₂, 1 mmol/l phenylmethylsulphonyl fluoride, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l dithiothreitol, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 5 µg/ml aprotinin, 0.5% CHAPS). Fifty microlitres of this lysate were added to 150 µl reaction buffer (25 mmol/l HEPES, 1 mmol/l EDTA, 0.1% CHAPS, 10% sucrose, 3 mmol/l dithiothreitol, pH 7.5 and 10 µmol/l of the caspase substrate Ac-DEVD-AMC). The substrate is efficiently cleaved by the apoptotic executioner caspases 3, 6 and 7 [32]. Accumulation of fluorescent AMC fluorescence was monitored over 120 min using an HTS fluorescence plate reader (Perkin Elmer, Langen, Germany) (excitation 380 nm, emission 465 nm). Fluorescence of blanks containing no cell lysate were subtracted from the values. Protein content was determined using the Pierce Coomassie Plus Protein Assay reagent. Caspase activity was expressed as change in fluorescent units per microgram protein and per hour.

Detection of cytochrome-*c* release

The release of cytochrome-*c* from mitochondria into the cytosol was analysed by selective permeabilisation of the plasma membrane [22]. Briefly, cells were permeabilised with 100 µg/ml digitonin at 4°C for 10 min. The supernatant representing the cytosol and the mitochondria-containing pellet fraction were separated by centrifugation and denatured. SDS-PAGE and western blot analysis were performed as described. Cytochrome-*c* was detected with a monoclonal cytochrome-*c* antibody (7H8.2C12; Pharmingen, Franklin Lakes, NJ, USA), diluted 1:1,000.

Statistics

Data are given as means±SEM. For statistical comparison, ANOVA and subsequent Tukey's test were employed. A *p* value of *p*<0.05 was considered to be statistically significant.

Results

INS-1 cells undergoing apoptotic cell death after prolonged suppression of HNF-1α function show a marked reduction in Ser473 phosphorylation of AKT1 kinase

Using a reverse tetracycline-dependent transactivator system we have previously shown that prolonged DN suppression of HNF-1α function increases the basal apoptosis rate of rat INS-1 insulinoma cells by sensitising cells to the mitochondrial apoptosis pathway [22]. Treat-

ment with 500 ng/ml doxycycline led to a rapid production of WT-HNF-1 α and DN-HNF-1 α in INS-1 cells stably transfected with the respective reverse tetracycline-dependent transactivator systems (Fig. 1a). To investigate the relationship between production of DN-HNF-1 α , activation of apoptosis, and alterations in AKT1 survival signalling in more detail, production of WT-HNF-1 α and DN-HNF-1 α was induced in INS-1 cells by treatment with doxycycline for 24–48 h. In agreement with our previous observations [22], apoptosis activation, detected by measuring the cleavage of the caspase substrate Ac-DEVD-AMC, was mildly elevated after 24 h, and maximally activated after 36–48 h of DN-HNF-1 α induction (Fig. 1b). In contrast, induction of WT-HNF-1 α for up to 48 h did not lead to significant caspase substrate cleavage (Fig. 1b).

Because insulin is an HNF-1 α target gene [9, 10] and has been implicated in autocrine survival signalling in insulin-secreting cells [33–35], we next determined whether apoptosis activation by DN-HNF-1 α was paralleled by changes in *Ins1* gene transcription. Quantitative PCR analysis of *Ins1* mRNA expression demonstrated a rapid and prominent downregulation of *Ins1* mRNA expression after the induction of DN-HNF-1 α (Fig. 1c).

Since insulin mediates survival signalling via the PI-3K/AKT1 pathway [25], we next investigated the activation status of AKT1 kinase after induction of DN-HNF-1 α . The activation status of AKT1 kinase was detected by western blotting experiments using a Ser473-phosphorylation specific antibody. Induction of DN-HNF-1 α dramatically reduced Ser473 phosphorylation of AKT1 kinase (Fig. 2a,b). Dephosphorylation of AKT1 kinase coincided with the time-points of maximal caspase activation (36 and 48 h). Densitometric analysis of the relative expression levels of phosphorylated to total AKT1 for duplicate experiments revealed a 7% decrease in the ratio after 24 h, a 28% decrease after 36 h and a 91% decrease after 48 h. Inducible production of WT-HNF-1 α did not alter the activation status of AKT1 kinase (Fig. 2a,b).

Interestingly, the above western blot experiments also indicated a delayed reduction in total AKT1 protein levels after the induction of DN-HNF-1 α . Quantitative PCR analysis of *Akt1* mRNA expression normalised to *Actb* mRNA expression confirmed a significant reduction in *Akt1* mRNA expression 48 h after the induction of DN-HNF-1 α (Fig. 2c).

Overexpression of a constitutively active *Akt1* rescues INS-1 cells from DN-HNF-1 α -induced apoptosis

We determined whether overexpression of *Akt1* was able to reverse DN-HNF-1 α -induced apoptosis. INS-1 cells were transiently cotransfected with an EGFP expression vector and plasmids encoding either a constitutively active AKT1 mutant targeted to the plasma membrane by myristylation (CA-AKT1), or a kinase-dead AKT1 mutant that functions as a DN inhibitor (DN-AKT1), or an empty

Fig. 1 DN-HNF-1 α -induced apoptosis in INS-1 cells. **a** Time course of induction of WT-HNF-1 α and DN-HNF-1 α in response to 500 ng/ml doxycycline. Immunodetection was performed using an anti-HNF-1 α and anti- α -tubulin antibody. Location of molecular weight marker band (in kDa) is provided on the right side of the figure. Note that the SM6 mutation contains an 86 amino acid substitution and migrates slightly faster. **b** Time-course of caspase-3-like protease activity in cytosolic protein extracts. INS-1 cells were induced to overproduce WT-HNF-1 α or DN-HNF-1 α for 0, 24, 36 and 48 h. Caspase-protease activity was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC (10 μ M). Activities are represented as increase in AMC fluorescence (in arbitrary fluorescence units, AU) over 1 h/ μ g protein. Data are means $\pm$ SEM from $n=6$ cultures. Experiments were repeated twice with similar results. * $p<0.05$, difference from non-induced controls. **c** Quantification of *Ins1* gene expression. INS-1 cells were treated with 500 ng/ml doxycycline (Doxy) from 0 to 48 h. Following treatment *Ins1* mRNA expression was examined using real-time quantitative PCR. Data shown are the mean of $n=3$ separate treatments. The experiment was repeated three times with similar results. * $p<0.05$, difference from non-induced controls

Fig. 2 DN-HNF-1alpha-induced AKT1-Ser473-dephosphorylation coincides with caspase-3 activation. INS-1 cells were induced to overproduce WT-HNF-1alpha (**a**) or DN-HNF-1alpha (**b**) for 0, 12, 24, 36 and 48 h. The activation status of AKT1 kinase was detected by western blotting using a Ser473-phosphorylation-specific antibody. As proteolytic cleavage of AKT1 by activated caspases has been described previously [50], INS-1 cells also received 100 μ mol/l of the broad spectrum caspase inhibitor zVAD-fmk. A duplicate experiment yielded comparable results. **c** Quantification of *Akt1* gene expression. INS-1 cells were treated with 500 ng/ml doxycycline (*Doxy*) from 0 to 48 h. Following treatment mRNA expression of *Akt1* was examined using real-time quantitative PCR. Data shown are the mean of $n=3$ separate treatments. * $p<0.05$, difference from non-induced controls

control vector (pCMV). Twenty-four hours after the transfection, production of DN-HNF-1alpha was induced for 24 and 48 h. Cells were assessed for apoptosis by live counterstaining with Hoechst 33258. EGFP-positive cells with condensed and fragmented nuclei were considered apoptotic and quantified. After 24 h, a pronounced increase in apoptotic nuclear morphology was detected in DN-HNF-1alpha-induced cells transfected with the DN mutant of *Akt1*, while empty vector-transfected, induced cells showed only mild apoptosis activation at this time-point (Fig. 3c). After 48 h, production of the DN mutant of *Akt1* did not further potentiate DN-HNF-1alpha-induced apoptosis compared with empty vector-transfected controls (Fig. 3b), suggesting that the effects of DN-HNF-1alpha and DN-AKT1 were not additive. After 48 h of induction, DN-HNF-1alpha-induced apoptosis was significantly in-

Fig. 3 Expression of a constitutively active mutant of *Akt1* rescues INS-1 cells from DN-HNF-1alpha-induced apoptosis. Plasmids encoding CA-AKT1, DN-AKT1 or empty vector (CMV) were transiently transfected into INS-1 cells by lipofection. After 24 h, production of DN-HNF-1alpha was induced for 24 h (**a**) or 48 h (**b**). Live cells were counterstained with Hoechst 33258 and analysed by epifluorescence and phase-contrast microscopy. All GFP-positive cells per culture (approximately 100 cells) were assessed for apoptotic nuclear morphology. Data are means $\pm$ SEM from $n=5-6$ cultures in three separate transfection experiments. * $p<0.05$, difference from respective non-induced controls; # $p<0.05$, difference between cultures

hibited by overexpression of the constitutively active mutant of *Akt1* (Fig. 3b).

Inhibition of PI-3K-mediated survival signalling by wortmannin accelerates DN-HNF-1alpha-induced apoptosis

Finally, we determined whether suppression of growth factor survival signalling at the level of PI-3K accelerated DN-HNF-1alpha-induced caspase activation. Treatment of non-induced INS-1 cells with the selective PI-3K inhibitor wortmannin alone led to a moderate activation of executioner caspases, detected by measuring the cleavage of the fluorogenic caspase substrate Ac-DEVD-AMC (Fig. 4a).

Fig. 4 Inhibition of PI-3K activity by wortmannin accelerates DN-HNF-1alpha-induced apoptosis. **a** Caspase-3-like protease activity in cytosolic protein extracts. INS-1 cells were induced to produce DN-HNF-1alpha for 24 h followed by a treatment with wortmannin (50 and 100 nmol/l) for 2 h. Executioner caspase protease activity was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC (10 μmol/l). Activities are represented as increase in AMC fluorescence (in arbitrary fluorescence units, AU) over 1 h/μg protein. Data are means±SEM from $n=4$ cultures. Non-induced cells served as controls. Experiments were repeated three times with

similar results. * $p<0.05$, difference from non-induced controls; # $p<0.05$, difference between untreated and treated DN-HNF-1alpha-producing cells. **b** INS-1 cells were induced to produce DN-HNF-1alpha for 24 h whereas non-induced cells served as controls. After DN-HNF-1alpha induction, cells were treated with the PI-3K inhibitor wortmannin (100 nmol/l) for 2 h and were then subjected to digitonin permeabilisation of the plasma membrane. Soluble cytochrome-c was detected in the supernatant by Western blot analysis. Membrane was stripped and reprobed with an anti-alpha-tubulin antibody to prove equal loading of samples

Treatment with the PI-3K inhibitor wortmannin greatly accelerated caspase activation in cells induced to produce DN-HNF-1alpha for 24 h (Fig. 4a). Likewise, treatment with wortmannin accelerated the release of the caspase-activating factor cytochrome-c from mitochondria (Fig. 4b), suggesting that the potentiation of apoptotic caspase activation by wortmannin occurred via the mitochondrial apoptosis pathway.

Discussion

The present study demonstrates that suppression of endogenous HNF-1alpha function renders insulin-secreting INS-1 cells sensitive to the mitochondrial apoptosis pathway via reduced PI-3K/AKT1 kinase survival signalling. Previous studies in *Drosophila* as well as in transgenic mice have demonstrated that the PI-3K/AKT kinase signalling pathway is centrally involved in mitogen-regulated control of cell growth and cell survival [36–38]. Dominant-negative suppression of HNF-1alpha has been shown to reduce the expression of insulin and insulin-like growth factor-I in insulin-secreting cells [9, 10], as HNF-1alpha acts as a transcriptional transactivator of both genes [39, 40]. Our data demonstrated a rapid and substantial downregulation of *Ins1* gene expression during suppression of endogenous HNF-1alpha function that was followed by substantial caspase and apoptosis activation in

the cultures. Gene deletion of *Tcf1* or DN suppression of HNF-1alpha function has also been shown to reduce the release of insulin from insulin-secreting cells. This is due to defects in nutrient-secretion coupling and ATP production [6, 9, 10]. Insulin release serves as an important autocrine survival signal for the pancreatic beta cells [33, 35]. It also activates *Ins1* gene transcription via the insulin receptor/PI-3K/p70 S6 kinase pathway [34]. We also observed a partial reduction in *Akt1* mRNA expression and AKT1 protein production in response to DN-HNF-1alpha. Hence combined defects in *Ins1* mRNA expression, insulin secretion and *Akt1* mRNA expression induced by a lack of HNF-1alpha function may initiate a vicious cycle, leading to a severe reduction in beta-cell function and beta-cell survival signalling. It is likely that this dysfunction is sensed internally by the beta cell and eventually leads to the activation of an apoptotic cell death programme.

Our data indicate that a reduction in AKT1 signalling plays a crucial role in this self-destructive process. Ectopic expression of a constitutively active *Akt1* mutant was sufficient to protect INS-1 cells against DN-HNF-1alpha-induced apoptosis. We also demonstrated that in INS-1 cells induced to produce DN-HNF-1alpha for 48 h and hence lacking detectable Ser473-AKT1 phosphorylation, expression of the DN mutant of *Akt1* did not further potentiate DN-HNF-1alpha-induced apoptosis. These data suggest that the pro-apoptotic effects of DN-HNF-1alpha were largely mediated via AKT1 inactivation.

How is a reduction in PI-3K/AKT1 kinase signalling able to trigger the activation of pro-apoptotic pathways? AKT1 kinase has been reported to stimulate the expression of the anti-apoptotic protein BCL-xL via activation of cyclic adenosine monophosphate response element binding protein and the nuclear factor kappa B pathway [41, 42]. BCL-xL acts primarily at the level of mitochondria and functions to inhibit the release of pro-apoptotic, caspase-activating factors from the mitochondrial intermembrane space into the cytosol [43]. These factors include cytochrome-*c* and Smac/DIABLO, which trigger and accelerate the formation of a caspase-activating complex, the apoptosome. In previous work we demonstrated that suppression of endogenous HNF-1 α specifically sensitised INS-1 cells to this mitochondrial apoptosis pathway [22]. We also demonstrated that DN-HNF-1 α -induced apoptosis was associated with a significant decline in BCL-xL protein levels, and that DN-HNF-1 α -induced apoptosis could be rescued by ectopic expression of BCL-xL [22]. AKT1 kinase is also able to phosphorylate and inactivate other pro-apoptotic proteins. These include caspase-9, which is essential for apoptosome formation, as well as the BH3-only protein BAD, which neutralises the anti-apoptotic activity of BCL-xL [29, 30]. In addition, AKT1 promotes cell survival through phosphorylation and inactivation of Forkhead transcription factors, thereby inhibiting the transcription of the pro-apoptotic BH-3 only protein BIM [44, 45]. Similarly to BAD, BIM is able to neutralise the pro-apoptotic activity of BCL-xL. BIM is also able to directly activate BAX and BAK, two proteins required for the mitochondrial release of cytochrome-*c* and Smac/DIABLO [46]. Hence inhibition of AKT1 signalling may be sufficient to promote apoptosis in the INS-1 cells, as demonstrated in the present study by the pro-apoptotic effects of DN-*Akt1* overexpression.

Despite a clear reduction in insulin gene expression 24 h after the induction of DN-HNF-1 α , significant Ser473 phosphorylation of AKT1 was still detectable at this time-point. Apart from insulin and insulin-like growth factor-I, other beta-cell survival factors have been identified in recent years. These include GLP-1, GIP and hepatocyte growth factor [47–49]. Interestingly, evidence has been provided that the cytoprotective activity of these signalling peptides and growth factors are also largely mediated via an activation of the PI-3K/AKT1 pathway [47–49]. It is possible that signalling through alternative survival factors can transiently maintain AKT1 Ser473 phosphorylation. However, a reduction in *Akt1* mRNA expression in response to DN-HNF-1 α may eventually also impair these alternative survival pathways. The potentiation of apoptosis by DN-*Akt1* overexpression or PI-3K inhibition in cells induced to produce DN-HNF-1 α for 24 h may be associated with a suppression of survival signalling through alternative, insulin-independent pathways.

Decreased PI-3K/AKT1 kinase signalling in response to a suppression of HNF-1 α function will also affect cell proliferation. Forkhead transcription factors also down-regulate the cell cycle inhibitor p27^{kip1} in a PI-3K-

dependent manner [44]. Increased protein levels of p27^{kip1} have been observed in DN-HNF-1 α -producing INS-1 cells as well as in DN-*Tcf1* transgenic mice [21, 22], and could lead to cell cycle arrest and diminished beta cell proliferation. A progressive decline in beta cell mass due to increased cell death and diminished cell proliferation as a consequence of insufficient PI-3K/AKT1 kinase signalling may therefore be implicated in the early manifestation of glucose intolerance in subjects with mutations in the *Tcf1* gene predisposing them to development of MODY3.

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Duality of interest The authors declare no competing financial interests.

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