Hexosamines stimulate apoptosis by altering SIRT1 action and levels in rodent pancreatic β -cells

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Abstract

The activity and levels of SIRT1, which promotes cell survival in several models, are linked to glucose concentrations and cellular energy metabolism. The present study aimed to determine whether impaired Sirt1 activity is involved in the induction of apoptosis by the nutrient-sensing hexosamine biosynthesis pathway (HBP). Pancreatic Nit-1, Rin-m5F, and Min6 β -cells were acutely treated at different doses and times with glucosamine, which enters and stimulates the HBP. *Sirt1* levels were genetically modulated by retroviral infection. Expression levels, cellular localization, and activity of apoptosis-related markers were determined by qPCR, immunoblotting, and co-immunoprecipitation. Glucosamine treatment dose- and time

dependently induced cell apoptosis in all cell lines studied. HBP stimulation time dependently modified SIRT1 protein levels, notably in the cytoplasm. This was concomitant with increased E2F1 binding to the *c-myc* promoter. In both NIT-1 and min6 β -cells, genetic knockdown of *Sirt1* expression resulted in higher susceptibility to HBP-stimulated apoptosis, whereas overexpression of *Sirt1* had the opposite impact. These findings indicate that reduction of SIRT1 levels by hexosamines contributes to β -cell apoptosis. Methods to increase SIRT1 levels or activity could thus prevent the decrease in β -cell mass, notably that observed in type 2 diabetes.

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Introduction

Hyperglycemia, occurring after a meal or during diabetes or aging, is detrimental to several systems, especially in sensitive tissues such as the brain and the pancreas, in which it has been shown that prolonged hyperglycemia leads to cell death through apoptotic signals. Pancreatic β -cells suffer from long-term high glucose levels via the induction of oxidative stress, stimulation of pro-apoptotic events, and ATP depletion from reduced glucose metabolism (Kaneto *et al.* 2001, Anello *et al.* 2004, Zachara & Hart 2004). Although apoptosis has been established as the primary factor for reduced β -cell mass in type 2 diabetes (Hanley *et al.* 2010), the molecular mechanisms linking hyperglycemia and the induction of apoptotic gene expression have not been fully determined.

Glucose metabolism through the hexosamine biosynthesis pathway (HBP) has been hypothesized to mediate many of the adverse effects of hyperglycemia and to be involved in the pathogenesis of type 2 diabetes and glucose toxicity (Brownlee *et al.* 1988, Rossetti *et al.* 1995, Hebert *et al.* 1996). Rate-limiting enzymes in the HBP are glutamine: fructose-6-phosphate amidotransferase (GFAT), which catalyzes the formation of glucosamine-6-phosphate from fructose-6-phosphate and glutamine, and O-glucosamine acetyl transferase, which adds a UDP-acetylglucosamine

group to a target protein and is overexpressed in pancreas. Adipocytes (Considine *et al.* 2000), fibroblasts, and rodents (Brownlee *et al.* 1988, Shankar *et al.* 1998, Veerababu *et al.* 2000, Einstein *et al.* 2008) exposed to glucosamine, which directly enters and stimulates the HBP, develop insulin resistance. In addition, HBP activation induces defects in insulin production and secretion by attenuating the activity of glucokinase in β -cells (Balkan & Dunning 1994, Shankar *et al.* 1998, Yoshikawa *et al.* 2002). Similar to animals exposed to glucosamine, transgenic animals overexpressing GFAT either in skeletal muscle, fat, or in liver also develop insulin resistance (Hebert *et al.* 1996, Veerababu *et al.* 2000). Less is known about the effects of HBP in humans. Although GFAT levels have been shown to correlate inversely with glucose disposal rate (Daniels *et al.* 1996) and fasting glycemia (Monauni *et al.* 2000) in humans, this issue remains controversial, as others have found that HBP has no impact on insulin sensitivity (Pouwels *et al.* 2001). In contrast, stimulation of HBP by glucosamine was demonstrated to cause apoptosis in both rodent (Konrad *et al.* 2001, Okuyama & Yachi 2001, Anello *et al.* 2004) and human (D'Alessandris *et al.* 2004) pancreatic β -cells, despite the up-regulation of cell survival factors such as MAFA (Vanderford *et al.* 2007).

SIRT1 is a deacetylase enzyme mostly located in the nucleus but also found in the cytoplasm under certain

conditions (Tanno *et al.* 2007). Its NAD-dependent activity suggests that this protein may sense the metabolic state of cells and modulate gene expression accordingly. SIRT1 was shown to impact on cell survival through physical binding with p53, HSF1, Hif-2 α , FOXO, and Ku70 (Dioum *et al.* 2009, Westerheide *et al.* 2009, Gagarina *et al.* 2010) and reviewed in Yamamoto *et al.* (2007). Importantly, SIRT1 and its yeast homolog SIR2 appear to be very sensitive to changes in cellular glucose content such as those occurring upon caloric restriction (Lin *et al.* 2000, Cohen *et al.* 2004, Nisoli *et al.* 2005, Civitarese *et al.* 2007, Chen *et al.* 2008, Kanfi *et al.* 2008, Nedachi *et al.* 2008). In cultured pancreatic β -cells, overexpression of *Sirt1* was shown to protect against cytokine toxicity (Lee *et al.* 2009). Moreover, glucosamine treatment in the hepatic HepG2 cell line acutely reduces SIRT1 protein levels (Sun *et al.* 2007). In this context, we postulated the hypothesis that HBP stimulation could induce β -cell death through a reduction in SIRT1 level and/or activity.

Materials and Methods

Cell culture and treatment

Mouse Nit-1 and rat Rin-m5F cell lines were bought from ATCC (Rockville, MD, USA). Mouse Min6 cells were a kind gift from Dr André Marette (Université Laval, Canada). Nit-1 and Rin-m5F cells were cultured in Ham's F12K medium (Sigma-Aldrich) containing 5 mM glucose added with 1.5 g/l sodium bicarbonate and 10% heat-inactivated dialyzed fetal bovine serum (FBS). Min6 cells were cultured in DMEM high glucose supplemented with 15% FBS, 5 μ l/l β -mercaptoethanol, and 10 mM HEPES. Cells were cultured at 37 °C in a humidified incubator at 5% CO₂. Experiments were typically performed at 80% confluence. Following the treatments, cells were harvested, centrifuged in collection tubes for mRNA and protein extractions, and were frozen at -20 °C until subsequent analysis. Viral production and infection were performed exactly as previously described (Picard *et al.* 2004). Infected cells were kept in a medium containing puromycin throughout the study.

Measurement of cell death

Cell death and apoptosis were quantified using three complementary approaches. Cells were harvested by treatment with a cell dissociation buffer (an enzyme free Hank's based solution; Gibco). Dead cells were counted based on dye exclusion after trypan blue staining in a hemacytometer. Annexin V assays were used to measure cell death through reorganization of phosphatidylserine in cell membrane. Briefly, cells were resuspended in annexin binding buffer (10 mM HEPES, pH=7.4, 140 mM NaCl, and 5 mM CaCl₂) containing annexin V-FITC (Roche Diagnostic) and Hoechst 33258 (Sigma-Aldrich) for 10 min in the dark. Cells were washed three times with ice-cold PBS, plated on

slides, and allowed to dry. Cells were then coverslipped with MOWIOL 4-88 mounting medium (Calbiochem, Gibbstown, NJ, USA). TUNEL assays were conducted following the manufacturer's instructions (Roche Diagnostic). For TUNEL assays, floating cells were not taken into account, which at least underestimated apoptosis ratios. Slides were analyzed by microscopy (IX-81 by Olympus, Markham, ON, Canada). Pictures were taken using an Evolution QEi camera and image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

RNA analysis and real-time quantitative RT-PCR

Total RNA from cultured cells was extracted according to the standard protocol provided with RNAspin mini isolation kit (GE Healthcare, Baie d'Urfe, QC, Canada) and quantified by Experion automated electrophoresis system (Bio-Rad). A reverse transcription step was carried on 1 μ g purified RNA by using oligo dT and SuperScript II reverse transcriptase (Invitrogen) at 42 °C for 1 h. Relative quantification of cDNA amplification was performed on reactions containing 40 ng cDNA, 0.25 μ M sense and antisense primers, 2 μ M MgCl₂, and SYBR Green JumpStart Taq ReadyMix without MgCl₂ (Sigma-Aldrich) in a final volume of 20 μ l for each reaction. Amplification and detection of specific products were obtained with the Rotor-Gene RG-3000 (Corbett Research, Concord, NSW, Australia) (primer sequences and qPCR protocols available on request). Relative level of gene expression was determined via a standard curve composed of a cDNA mix from all groups. PCR data were normalized over HPRT expression (hypoxanthine-guanine phosphoribosyltransferase) used as a housekeeping gene.

Protein extraction and western blot analysis

Cells were scraped in lysis buffer A (10 mM HEPES, pH=7.9, 10 mM KCl, 100 μ M EDTA, 100 μ M EGTA, 1 M dithiothreitol (DTT) (1:1000), and Protease Inhibitor Cocktail (Sigma-Aldrich) (1:1000)) and combined with floating cells fraction. NP40 was added to each sample before vigorous mixing and nucleus recovery through centrifugation. Then, nuclei were resuspended in lysis buffer C (20 mM HEPES, pH=7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 M DTT (1:1000), and Protease Inhibitor Cocktail (1:1000)) and mixed. Proteins were recovered in the supernatant and dosed on a u.v. plate reader by DC Protein Assay (Bio-Rad). A total of 20 μ g of each sample were resolved by SDS-PAGE and transferred to PVDF membranes. After a 2 h transfer, membranes were blocked in a 5% milk (in BSA-1X) for 35 min. Then, membranes were incubated with different primary antibodies at 4 °C overnight against Bax (1:500) (Santa Cruz sc-526, Santa Cruz, CA, USA), c-Jun (1:700) (Santa Cruz sc-45), E2F1 (1:700) (Santa Cruz sc-193), p53 (1:700) (Santa Cruz sc-6243), p65-NFKB (1:700) (Santa Cruz sc-372), Sirt1 (1:2000) (Upstate 07-131), ubiquitin (1:700) (Santa Cruz sc-9133), and glycosylated proteins (Affinity BioReagents MA1-072). The measure of actin (1:25 000)

(Upstate MAB1501) was used as a loading control. The next day, membranes were rinsed four times in wash buffer (TBS 1× with Tween 20 and NP40) before incubation for 1 h at 4 °C with respective HRP-conjugated secondary antibodies (1:5000) (Amersham). Membranes were washed four times in wash buffer, and tagged proteins were visualized by ECL system (GE Healthcare).

Immunofluorescence

Cells were plated on coverslips and treated with 5 mM glucosamine for 1, 5, and 24 h. Cells were fixed in methanol at 4 °C for 10 min. After three washes with ice-cold PBS, non-specific binding sites were saturated with 1% BSA–PBS for 10 min at room temperature. Then, cells were incubated with Sirt1 antibody (1:25) in 1% BSA–PBS. Secondary antibodies used were FITC-conjugated affinipure goat anti-rabbit IgG (1:1000) (Jackson ImmunoResearch, West Grove, PA, USA) with nuclear staining with Hoechst 33258 (1:1000) in 1% BSA–PBS. Coverslips were mounted on slides using Vectashield mounting medium (VectorLabs, Burlington, ON, Canada) and analyzed by microscopy (IX-81 by Olympus). Pictures were acquired using an Evolution QEi camera and image Pro Plus 6.0 software (Media Cybernetics).

Chromatin immunoprecipitation

Nit-1 cells were cross-linked with 1% paraformaldehyde at room temperature after 6, 12, 18, or 24 h of 5 mM glucosamine treatment. Cells were washed three times with ice-cold PBS and scraped in ChIP lysis buffer (50 mM HEPES, pH=7.5, 140 mM NaCl, 1% (v/v) Triton X-100, and Protease Inhibitor Cocktail 1:1000). Cells were sonicated to shear DNA by repeating seven cycles of sonication: 9 s at 30% of the maximum amplitude (Sonifier Cell Disrupter 185, Branson, Danbury, CT, USA) with 1 min pauses of 10 s on dry ice followed by 50 s on ice. After taking fractions of sheared DNA for inputs, samples were pre-cleared with 50 µl of Protein A Sepharose Fast Flow (GE Healthcare) beads and 20 µg salmon sperm ssDNA (Invitrogen) for 1 h at 4 °C. Supernatants were then split to incubate with 50 µl beads, 20 µg ssDNA, and 4 µg rabbit IgG (Santa-Cruz, sc-2027) as a negative control, and with 4 µg E2F1 or 2 µg Sirt1 antibodies for ChIP assay and were rocked overnight at 4 °C. On the next day, samples were washed twice with ChIP lysis buffer, one time with ChIP lysis buffer high salt (50 mM HEPES, pH=7.5, 0.5 M NaCl, 1% (v/v) Triton X-100, and Protease Inhibitor Cocktail 1:1000), twice with wash buffer (10 mM Tris,

Figure 1 Glucosamine treatment dose- and time dependently triggers β -cell death. Cells were treated with or without glucosamine at the indicated time and doses. Bright-field pictures (A and B), nucleus staining (Hoechst) (C and D), and immunofluorescent annexin V staining (E and F) of Nit-1 cells taken 24 h after glucosamine treatment (24 h). Loss of β -cells was then scored by cell count of trypan blue (G) or annexin staining (H) after correction for total cell number. (I) Time course of positive annexin staining in Nit-1 cells after 5 mM glucosamine treatment. (J) Dose–response of cell death in Rin-m5F cells treated with glucosamine for 24 h. (K) Dose–response of cell death in Nit-1 cells treated with glucosamine for 48 h. TUNEL assays (representative left panels) were done at the end of the incubation period. Data shown were obtained from at least three independent experiments done in duplicate. Bars are mean $\pm$ s.e.m. * indicates a significant effect ($P < 0.05$) compared to the control group (white bars). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0243>.

Figure 2 Glucosamine triggers an apoptotic transcriptional program in Nit-1 cells after 24 h of treatment. Quantitative real-time PCR results were corrected for mRNA levels of GAPDH as a housekeeping gene. Data shown were obtained from three independent experiments done in duplicate. Bars represent fold change compared to the control condition (dotted line). Asterisks indicate a statistical difference from the control group (* $P<0.05$; ** $P\leq 0.01$; *** $P\leq 0.0001$). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0243>.

pH=8.0, 250 mM LiCl, 0.5% (v/v) NP40, and 1 mM EDTA), and twice with TE (10 mM Tris, pH=8.0, and 10 mM EDTA). Beads were eluted (10 mM Tris, pH=8.0, 1% (v/v) SDS, and 10 mM EDTA) with two rounds of heating at 65 °C separated by vortexing. Combined supernatants and inputs were heated at 65 °C overnight. The next day, DNA was purified using PCR Purification Kit (Qiagen) followed by PCR on the c-myc promoter with specific primers (forward, 5'-GCGGGGCTCCTAGAT-AACTC-3'; reverse, 5'-CGGCTCCGGGTGTAAAC-3') or on the Hsp70 promoter (forward, 5'-GAACCCACA-ACTCCGATTACTCA-3'; reverse, 5'-TCTTGTCTTC-GCTTGTCTCTGGAT-3'). Amplification results were obtained by migration on 1% EtBr agarose gel.

Statistical analysis

Data are presented as means $\pm$ S.E.M. The main and interactive effects were analyzed by ANOVA. When justified by ANOVA, differences between individual group means were analyzed by Fisher's PLSD test. Differences were considered statistically significant at $P<0.05$.

Results

HBP stimulation induces apoptosis in β -cells

HBP stimulation by glucosamine treatment induced a large increase in the number of floating cells resulting from disorganization of β -cell colonies (Fig. 1A and B). Cells treated with glucosamine lost almost every ultrastructural feature such as beta granules and well-developed rough

Figure 3 Effects of glucosamine treatment on the protein cellular location and levels in Nit-1 cells harvested after 24 h of glucosamine treatment (A). Actin levels are shown as loading controls. Each lane from both groups represents an independent experiment. (B) Quantification of the protein levels shown in (A) relative to those of actin. * $P<0.05$ compared to the vehicle treatment.

endoplasmic reticulum. Cell death was also apparent following nucleus staining (Fig. 1C and D). These findings were confirmed by annexin V staining (Fig. 1E–H). The impact of glucosamine treatment was not due to osmotic or pH modification, as control solutions with mannitol or pH comparable to that of glucosamine did not result in any change in cell death (data not shown). Statistical analysis of cell death ratio indicated that 5 mM glucosamine stimulated cell death within 18–24 h of treatment (Fig. 1I). The significant impact of glucosamine was also observed in rat Rin-m5F β -cells (Fig. 1J), indicating that the effect of HBP stimulation on cell death is not species-specific. Further tests using highly sensitive TUNEL assays indicated that a 48 h treatment with a lower dose of 1 mM glucosamine also increased cell death (Fig. 1K). These findings demonstrate that HBP stimulation even by a low dose of glucosamine induces β -cell apoptosis.

HBP stimulation modulates the levels and cellular location of pro-apoptotic factors

Previous studies suggested that glucosamine treatment of rat β -cells results in reduced insulin synthesis (Andreozzi *et al.* 2004), and in the inactivation of the IRS1/PI3K pathway, leading to increased c-Jun/JNK and a down-regulation of FOXO1 activity (Andreozzi *et al.* 2004, D'Alessandris *et al.* 2004). In the present study, further analysis of apoptotic

pathways by real-time quantitative PCR revealed that glucosamine treatment dose dependently stimulated the mRNA expression of Myc, Bax, Jun, and the p65 unit of NFkB (Fig. 2). In contrast, fatty acid synthase (FAS) expression levels were significantly down-regulated by glucosamine treatment. However, no change in glucokinase mRNA levels was found. Insulin, *Apaf-1*, and *Sirt1* mRNA levels also remained unchanged after incubation with glucosamine (Fig. 2).

Determination of protein cellular location and expression levels confirmed the increase in BAX levels upon glucosamine treatment, which was mainly observed in the nucleus (Fig. 3). Overall, ubiquitination of proteins did not change upon treatment (Fig. 3). Cytoplasmic protein levels of p65-NFkB and p53 were reduced (Fig. 3). Interestingly, E2F1 levels were slightly diminished in the nucleus 24 h after glucosamine treatment (Fig. 3).

HBP affects SIRT1 level and activity

Consistent with previous findings in hepatic HepG2 cells (Sun *et al.* 2007), western blotting revealed a robust decline in the cytoplasmic fraction of SIRT1 24 h after incubation with glucosamine (Fig. 3). Given the possibility that SIRT1 serves as a node interacting with and inhibiting pro-apoptotic factors such as E2F1 and p53 while

Figure 4 Stimulation of HBP by glucosamine alters SIRT1 protein levels and location. (A) Western blot of SIRT1 protein at 0–24 h after glucosamine exposure in Nit-1 cells. (B) Quantitative analysis of the changes in SIRT1 protein levels upon glucosamine treatment ($n=3$). Signals for SIRT1 were divided by those for actin to account for loading. * indicates $P<0.0001$. (C) Time course showing localization of SIRT1 (green) using immunofluorescence in Nit-1 cells treated with 5 mM glucosamine. Cell nuclei are shown in blue (Hoechst). Data shown are representative of three independent experiments done in duplicate. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0243>.

Figure 5 Time-dependent transactivation of Hsp70 and Myc promoters by HBP stimulation. (A) ChIP assay on Sirt1 and IgG (control)-immunoprecipitated DNA from Nit-1 cells treated with 5 mM glucosamine for up to 5 h. A sequence containing the Hsp70 promoter was amplified by specific primers. (B) ChIP assay on E2F1 and IgG (control)-immunoprecipitated DNA from Nit-1 cells treated with 5 mM glucosamine for 6–24 h. A sequence containing E2F1 response elements from the Myc promoter was amplified by specific primers. For both A and B, quantification of the binding signals is shown on the right panels. Data shown are representative of three independent experiments. * $P < 0.05$ compared to 0 h of treatment.

stimulating anti-apoptotic factors such as HSF1, a time-course study was performed to document the decrease in SIRT1 protein level upon glucosamine treatment. The reduction (58%, $P < 0.0001$) was noticeable only 24 h after the beginning of the incubation period (Fig. 4A and B). At that time point, most of SIRT1 protein was observed in the nucleus (Fig. 4C). This diminution in SIRT1 protein expression while mRNA levels remained constant suggests that SIRT1 protein degradation might be increased by HBP stimulation.

To test the effect of this phenomenon on Sirt1 transcriptional activity, ChIP assays were first performed on the promoter of the heat shock protein 70 (Hsp70), which has a protective, anti-apoptotic effect against stress (Daugaard *et al.* 2007). SIRT1 binding to the Hsp70 promoter was induced within 1 h by glucosamine treatment and remained high 5 h after HBP stimulation (Fig. 5A). Then, as SIRT1 protein levels are decreasing as a function of glucosamine treatment (Fig. 4), possibly because of progressing changes in protein conformation, we postulated that its inhibitory activity on E2F1 would also diminish, thus allowing E2F1 to stimulate pro-apoptotic gene transcription. Consistent with this concept, binding of E2F1 on the Myc promoter, as evidenced by ChIP, was increased from 6 to 18 h of HBP stimulation, but was greatly reduced at 24 h (Fig. 5B).

Genetic manipulation of SIRT1 levels modulates apoptotic susceptibility to HBP

To confirm that reduced SIRT1 contributes to HBP-induced apoptosis, virus-mediated, specific Sirt1 knockdown (T1 KD) was performed in Nit-1 cells, which resulted in a 60% decrease in its protein level (Fig. 6A). In normal conditions, death ratios were similar between control and T1 KD cells (Fig. 6B). In contrast, compared to control cells, T1 KD cells were significantly more susceptible to apoptosis when incubated with glucosamine (Fig. 6B). These findings indicate that reduction of SIRT1 exacerbates HBP-induced cell death in Nit-1 β -cells.

To further validate this observation, levels of the Sirt1 gene were increased (pBabe-Sirt1) or reduced (pSuper-Sirt1-RNAi) in mouse Min6 cells by retroviral infection. On the one hand, overexpression of Sirt1 resulted in a protection against glucosamine-induced cell death as quantified by trypan blue (Fig. 7A) and annexin (Fig. 7B) staining. On the other hand, cells with down-regulation of Sirt1 levels showed increased basal and glucosamine-stimulated apoptosis (Fig. 7A and B). These results confirmed that modulation of SIRT1 levels impacts on HBP-induced apoptosis.

Figure 6 Reduction in Sirt1 levels exacerbates HBP-induced apoptosis in Nit-1 β -cells. (A) SIRT1 protein levels in Nit-1 cells infected with viruses produced through an empty (pSuper) plasmid, or with a sequence specifically targeting Sirt1 (T1 KD) (Picard *et al.* 2004). (B) Cell death in infected cells incubated or not with 5 mM glucosamine for 12 h. Results are mean $\pm$ S.E.M. of three independent experiments done in triplicate. * indicates significant effect of glucosamine; † indicates significant difference with empty virus.

Figure 7 Overexpression of *Sirt1* protects against HBP-induced apoptosis in Min6 β -cells. Overexpression (pBabe-Sirt1) or down-regulation (pSuper-Sirt1-RNAi) of *Sirt1* was achieved by retroviral infection. After at least 10 days of antibiotic selection, Min6 cells were incubated with glucosamine. Trypan blue- (A) and annexin- (B) positive cells were quantified 48 h after. * indicates a significant effect of glucosamine ($P < 0.05$). † indicates a significant effect of the virus compared to the control pSuper group ($P < 0.05$). Results are mean $\pm$ S.E.M. of two independent experiments done in triplicate. pS-Sirt1-scr represents the same plasmid as pS-Sirt1-RNAi except that the RNAi sequence was scrambled as a negative control.

Discussion

It has previously been shown that stimulation of the HBP results in apoptosis in several models of pancreatic β -cells. The findings of the present study indicate that this pathway can modify SIRT1 protein levels and location, resulting in reduced activity and a concomitant transactivation of pro-apoptotic target genes such as *Myc*.

The present study confirms in three different cell lines that HBP stimulation by glucosamine triggers β -cell apoptosis and subsequent cell death at a dose even lower than that used in previous reports (Konrad *et al.* 2001, Okuyama & Yachi 2001, Anello *et al.* 2004, D'Alessandris *et al.* 2004). On the one hand, consistent with strong annexin staining, *Myc* and *NF κ B* transactivation, morphologic differences observed after glucosamine exposure as well as modified expression patterns

of genes such as *Fas* showed that cell metabolism was robustly altered, likely in a time-dependent manner. On the other hand, the mRNA expression of some, but not other, pro-apoptotic factors was not significantly changed upon 5 mM glucosamine treatment, suggesting activation of specific pathways implicated in apoptosis. These could be linked to altered mitochondrial function (Balestrieri *et al.* 2008) and production of reactive oxygen species, as SIRT1 controls the expression of UCP2 in pancreatic β -cells (Bordone *et al.* 2005, Moynihan *et al.* 2005) and consequently insulin secretion.

Our findings demonstrate that modulation of SIRT1 levels robustly interferes with HBP-induced β -cell apoptosis. The glucosamine-stimulated reduction of SIRT1 protein would likely affect many pathways involved in cell cycle and apoptosis. Our data suggest that SIRT1 can initially protect against HBP-induced metabolic stress by activating the Hsp70 promoter (Fig. 5A), but that, in the longer term, loss of SIRT1 function enables pro-apoptotic factors. Previous data indicate that SIRT1 can inhibit E2F1 activity through direct physical interaction (Wang *et al.* 2006). From our present findings, it could be postulated that reduced SIRT1 levels could diminish its docking to E2F1, which could then stimulate the binding of E2F1 to the MYC promoter (Fig. 5B), triggering transactivation of the gene and induction of apoptosis. Reduced E2F1 DNA binding at 24 h might be due to a slightly reduced protein level (Fig. 3) or to the fact that other pro-apoptotic mechanisms take place at this time point. However, to date, attempts to pinpoint one exclusive specific mechanism (e.g. via altered SIRT1 docking to E2F1, FOXO, or HSF1) have not been conclusive, leading to the speculation that SIRT1 may act through multiple networks.

Several studies have reported increased SIRT1 protein levels (Balestrieri *et al.* 2008, Kanfi *et al.* 2008, Nedachi *et al.* 2008) and nuclear accumulation (Nedachi *et al.* 2008) under conditions of low glucose concentrations. In line with this, a report showed that glucosamine reduced SIRT1 protein expression in HepG2 cells (Sun *et al.* 2007). These observations suggest that stimulation of HBP by glucosamine affects SIRT1 stability, a concept supported by our findings that glucosamine reduces SIRT1 protein but not mRNA levels (Figs 2–4). Phosphorylation of SIRT1 has been hypothesized to play a significant role in its deacetylase activity (Beausoleil *et al.* 2004), and glycosylation was shown to affect the same protein motifs as phosphorylation (Zachara & Hart 2004). Transcription factors and other protein components of the nucleus can become glycosylated, which triggers rapid changes in affinity, specificity, and stability of components in protein complexes. For example, this applies in β -cells to PDX-1 (Gao *et al.* 2003, Akimoto *et al.* 2007) and H6451sp60, which detaches from BAX upon HBP stimulation and triggers BAX translocation to the mitochondria (Kim *et al.* 2006). The balance between phosphorylation and glycosylation, in association with the reported sumoylation modification of SIRT1 (Yang *et al.* 2007), could thus represent a fine-tuning regulatory mechanism of SIRT1 activity. Moreover, prolonged glycosylation was previously

shown to modulate protein ubiquitination (Guinez *et al.* 2008), a mechanism that could have contributed, in the present study, to the decrease in SIRT1 observed after 24 h of glucosamine treatment. Given the proposed links between SIRT1 and longevity (Bordone *et al.* 2007, Guarente 2008) and the increase in HBP activity shown in aged rats (Einstein *et al.* 2008), more studies are required to better analyze how HBP stimulation controls SIRT1 protein status.

While confirming that stimulation of the HBP triggers β -cell apoptosis, our present findings reveal that this effect is associated with robust changes in SIRT1 protein levels, which leads to impaired activity, allowing transactivation of pro-apoptotic genes. The relevance of this novel mechanism remains, however, to be demonstrated *in vivo* or using isolated islets. In addition, compared to that of other established HBP-induced pro-apoptotic pathways, the relative contribution of this molecular mechanism to overall cell death is still to be clarified. Finally, the findings described herein suggest that post-translational modifications of SIRT1 might play an important role in the responses of β -cell to metabolic stresses.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M L-L designed the experiments, performed experiments shown in Figs 1, 3, 4A and B, 5, 6, and 7, analyzed the data, and wrote the manuscript. G D performed the experiments shown in Figs 1, 4C, and 7, and analyzed the data. F P initiated the study, designed the experiments, analyzed the data, and wrote the manuscript.

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