Prolonged insulin treatment sensitizes apoptosis pathways in pancreatic β cells

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Abstract

Insulin resistance results from impaired insulin signaling in target tissues that leads to increased levels of insulin required to control plasma glucose levels. The cycle of hyperglycemia and hyperinsulinemia eventually leads to pancreatic β cell deterioration and death by a mechanism that is yet unclear. Insulin induces ROS formation in several cell types. Furthermore, death of pancreatic β cells induced by oxidative stress could be potentiated by insulin. Here, we investigated the mechanism underlying this phenomenon. Experiments were done on pancreatic β cell lines (Min-6, RINm, INS-1), isolated mouse and human islets, and on cell lines derived from nonpancreatic sources. Insulin (100 nM) for 24 h selectively increased the production of ROS in pancreatic β cells and isolated pancreatic islets, but only slightly affected the expression of antioxidant enzymes. This was accompanied by a time- and dose-dependent decrease in cellular reducing power of pancreatic β cells induced by insulin and altered expression of several ER stress response elements including a significant increase in *Trb3* and a slight increase in *iNos*. The effect on *iNos* did not increase NO levels. Insulin also potentiated the decrease in cellular reducing power induced by H_2O_2 but not cytokines. Insulin decreased the expression of MCL-1, an antiapoptotic protein of the BCL family, and induced a modest yet significant increase in caspase 3/7 activity. In accord with these findings, inhibition of caspase activity eliminated the ability of insulin to increase cell death. We conclude that prolonged elevated levels of insulin may prime apoptosis and cell death-inducing mechanisms as a result of oxidative stress in pancreatic β cells.

Key Words

- ▶ apoptosis
- ▶ hyperinsulinemia
- ▶ oxidative stress
- ▶ β cell death

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Introduction

Loss of pancreatic β -cells is a major contributing factor to decreased pancreatic function in both type 1 (T1D) (Herold *et al.* 2013) and at a later stage in type 2 (T2D) diabetes (Allagnat *et al.* 2012). Infiltrating T and B cells and macrophages in T1D release reactive oxygen and nitrogen radicals and destroy β cells (Hou *et al.* 2008).

The low level of antioxidant enzymes in pancreatic β cells (Kajimoto & Kaneto 2004) renders them relatively more sensitive to oxidative stress than other cell types. Several studies indicate that treatment of cultured cells with insulin, or hyperinsulinemia induced in animal models, promotes the generation of reactive oxygen species (ROS)

(Mukherjee *et al.* 1978, May & de Haen 1979, Goldstein *et al.* 2005a, Barazzoni *et al.* 2012) or superoxides (Barazzoni *et al.* 2012). ROS, in turn, can increase oxidative stress and apoptosis in various cell types (Furukawa *et al.* 2004), including pancreatic β cells (Robertson 2004, Eizirik *et al.* 2008).

The dynamic changes in the pancreas during progression of β cell death raise certain questions regarding potential autocrine regulatory effects of insulin on β cells (Navarro-Tableros *et al.* 2004). In general, insulin acts as a growth factor that plays an antiapoptotic role and protects cells from death through activation of the PI3-kinase and ERK signaling pathways (Muller *et al.* 2006, Jensen & De Meyts 2009), and insulin therapy can protect β cells from deterioration (Del Parigi 2008). It has been shown, however, that hypersecretion of insulin (insulin resistance) may precede β cell dysfunction and may be an important factor in the progression to β cell failure (Fernandez *et al.* 2001, Aston-Mourney *et al.* 2008, Lu *et al.* 2010). Furthermore, in conditions of energy stress, hyperactivation of the mTORC1 complex, mediated by growth factors including insulin, may induce apoptosis (Gwinn *et al.* 2008). It was also shown that chronic insulin treatment of RINm pancreatic β cell line increases LDH release and potentiates the deleterious effects of H_2O_2 on cell survival (Sampson *et al.* 2010). Consistent with these findings, insulin has a proapoptotic effect on β cells exposed to glucose deprivation (Guillen *et al.* 2008), conditions that reduce the cellular redox potential.

Here, we have investigated in more detail the possible proapoptotic and death-promoting effects of insulin on pancreatic β cells, utilizing pancreatic β cell lines as well as isolated mouse and human pancreatic islets. The results indicate that long-term treatment (24–48 h) with high levels of insulin, under conditions of oxidative stress may jeopardize survival of β cells through apoptotic and nonapoptotic death-inducing mechanisms.

Materials and methods

Materials

Penicillin and streptomycin (Pen/Strep), Hanks' solution, DMEM, CMRL 1066 medium and trypsin–EDTA were obtained from Biological Industries (Bait Haemek, Israel). FBS was from Hyclone Laboratories (Logan, UT, USA). Protein-G Agarose beads and antiactin antibodies were from Santa Cruz Biotechnology. Insulin,

collagenase (type XI), protease inhibitor cocktail and 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM- H_2 DCFDA) were from Sigma Chemicals. Enzolyte-caspase RH110 Caspase3/7 Assay kit was from AnaSpec Ltd (San Jose, CA, USA). zVAD-fmk was from MegaPharm Ltd (Raanana, Israel). Mouse insulin ELISA kit was from Mercodia (Uppsala, Sweden). CellTiter-Blue assay kit was from Promega (purchased through Biological Industries, Beit HaEmek, Israel). Monoclonal p-Tyr (PY-20) antibodies were from BD Biosciences (San Jose, CA, USA). Anti MCL-1 antibodies (Ab 32087) were purchased from Abcom (London, UK). Cytokines IL-1 β and IFN- γ were purchased from MD Biosciences (Ness Ziona, Israel). TNF- α was provided by Prospecc-Tany Technogene (Rehovot, Israel). Cytokine mixture (1x-cytomix) consisted of 3 nM TNF- α , 3 nM IFN- γ and 1.5 nM IL-1 β (biological activity: 10 U/ng (TNF- α , IFN- γ) and 200 U/ng (IL-1 β)). Human insulin ELISA kits was purchased from Mercodia (Uppsala, Sweden).

Cell cultures

Mouse insulinoma (MIN6) cells were a gift from Dr J I Miazaki (Osaka University, Japan), and INS-1 cells were a gift from Prof. M Walker (Weizmann Institute of Science, Israel). All other cell lines were obtained from ATCC. INS-1 and RINm cells (passages 15–30) were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), 4.5 g/L glucose, 10 mM Hepes, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate and 1% Pen-Strep. MIN6 cells (passages 12–32) were grown in DMEM containing 15% FCS, 11.1 mM glucose, 2 mM L-glutamine, 1% Pen-Strep and 60 μ M β mercaptoethanol. Chinese hamster ovary (CHO) cells that overexpress the insulin receptor (CHO-T cells, passages 25–32) were grown in F-12 medium with 10% bovine calf serum (BCS). NIH fibroblasts expressing human insulin receptor (NIH-T cells, passages 20–32) were grown in DMEM with 10% BCS, human embryonic kidney cells (HEK 293, passages 10–25) were grown in Eagle's Minimum Essential Medium with 10% FCS and HepG2 cells (passages 20–30) were grown in MEM Eagle-Earle's salt base. Cultures were incubated in 5% CO_2 humidified atmosphere at 37°C. The medium was changed every 2–3 days. At 80% confluence, cells were transferred to 12-well plates and seeded at 5×10^5 cells/mL in serum- and glucose-free medium. After 24 h, cells were treated with H_2O_2 (70 μ M) and insulin (100 nM), alone or in combination, for 24–48 h.

Isolation of murine islets

Digested pancreases were filtered through 1000 and 500 μ m sieves, and islets >75 and <250 μ m were handpicked under a stereoscope. Islets were cultured in RPMI-1640 medium containing 5 mmol/L glucose, 10% (vol./vol.) FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin and 40 μ g/mL gentamycin, and were used within 48 h of isolation. Experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

Culture of human islets

Isolated human islets ($>90\%$ purity) were provided by the European Consortium for Islets Transplantation (ECIT; Islet for Basic Research Programme; <http://ecit.dri-sanraffaele.org/en/islet-transplantation/index.html>) through a Juvenile Diabetes Research Foundation award 31-2008-413. Islets were cultured at 37°C in a 5% CO_2 humidified atmosphere in CMRL 1066 medium containing 10% (vol./vol.) FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin and 40 μ g/mL gentamycin. The medium was changed every other day. Human islets were dispersed by 4 min incubation at 37°C with trypsin/EDTA. Trypsinized islets were washed with cold CMRL 1066 medium containing 10% (vol./vol.) FBS, gently pipetted and resuspended in CMRL 1066 containing 10% (vol./vol.) FBS. Human islets studies received approval from the Weizmann Institute Bioethics and Embryonic Stem Cell Research Oversight Committee.

Insulin secretion

MIN6 cells or primary human islets were incubated for 60 min with 0.3 or 3.3 mmol/L glucose, respectively, in KRBH buffer (124 mmol/L NaCl, 5.6 mmol/L KCl, 2.5 mmol/L CaCl_2 and 20 mmol/L Hepes, pH 7.4). Cells were then incubated with 20.0 or 16.7 mmol/L glucose, respectively, for 60 min at 37°C . Cellular insulin content and the content of insulin secreted to the medium was determined using mouse or human insulin ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

LDH release

10^5 – 10^6 cells/mL were seeded in 24-well plates. Cells were washed, treated with insulin and H_2O_2 , and the extent

of cell death was measured by the LDH assay kit (Roche) according to manufacturer's instructions.

Preparation of cell lysates and Western blotting

Cultured β -cells were mechanically detached and centrifuged at $1500g$ for 5 min at 4°C . Cells were resuspended in RIPA buffer containing cocktails of proteases and phosphatase inhibitors for 40 min on ice, centrifuged and the supernatants were collected. Samples of 50–150 μ g protein were resolved by SDS-PAGE under reducing conditions and were transferred into nitrocellulose membrane for Western blot with the indicated antibodies. The intensity of the bands under study was quantified using the NIH Image densitometry program.

Assay of caspase-3/7 activity and cellular reducing power

Cells (15,000 cells/well) or primary human islets (10–15 islets/well) were seeded in 384-well plates in 50 μ L DMEM and treated as indicated. Caspase 3/7 activity was assayed using Enzolyte-RH110 caspase3/7 assay kit (AnaSpec, San Jose, CA, USA) according to the manufacturer's instruction. Fluorescence intensity was measured using a fluorescence microplate reader (Lumitron Electronic Instruments, Lod, Israel) at Ex/Em = 496 nm/520 nm. Assays were performed in triplicates. Cellular reducing power was assayed using the CellTiter-Blue cell (CTB) viability fluorimetric assay (Promega). Fluorescence intensity was measured using a fluorescence microplate reader (Wallac Victor 1420 multilabel counter at Ex/Em = 560 nm/590 nm).

Detection of intracellular ROS accumulation

Cells were treated with 10 μ M CM- H_2DCFDA for 30 min at 37°C in the dark as described (Dembele *et al.* 2009). Following treatment, cells were incubated with RPMI medium at 37°C for 15 min to recover, and fluorescence of the oxidized product DCEH-DA was monitored using Tecan Infinite F200 fluorescence microplate reader (Ex 485 nm, Em 520 nm). Values for each well were corrected for intracellular protein and background fluorescence. Alternatively, cells (4×10^5 /mL) were grown on cover slips in multiwell plates, treated with the CM- H_2DCFDA reagent (20 μ M), fixed, and the fluorescence intensity of DCEH-DA was monitored by fluorescence microscope (Ex 460–500 nm, Em 510–560 nm).

Quantitative real-time PCR (qRT-PCR)

RNA was extracted using the PerfectPure RNA kit (5Prime, Gaithersburg, MD, USA), and cDNA was generated by cDNA Reverse Transcription kit (Applied Biosystems). Quantitative detection of specific mRNA transcripts was carried out by real-time PCR using ABI-PRISM 7300 instrument (Applied Biosystems) using SYBR green PCR mix (Invitrogen) and the following primers: *miNos*: forward 5'-GCCCTGCTTTGTGCGAAGTG-3', reverse-5'-AGCCCTTTGTGCTGGGAGTC-3'; *mTrb3*: forward 5'-GTAGAGGGACCTTTGCCCTG-3'; reverse 5'-GCCGTGATCCTCTCTCAACC-3'; *mChop*: forward 5'-CCTTCGACCAGTCGGGTTTG-3', reverse 5'-GTCGCTGGAGAACCCATGAG-3'; *mAtf4*: forward 5'-CCTTCGACCAGTCGGGTTTG-3', reverse 5'-GTCGCTGGAGAACCCATGAG-3'; *mBip*: forward-5'-GGCACCTTCGATGTGTCTCT-3', reverse 5'-ATGACC-CGCTGATCAAAGTC-3'; *mXbp1*: (spliced isoforms): forward 5'-AACTCACGGCCTTGTTGGT-3', reverse 5'-AGGATCCAGCGTGTCCATTC-3'. *Superoxide dismutase 1 (Sod1)* (204bp) forward 5'-tgccaggcgctcattcactt-3', reverse 5'-caacatgcctctcttcatcc-3'. *Sod2* (197bp) forward 5'-cacattaacgcgcagat-3', reverse 5'-ctcaggtttgtccagaaa-3'. *Catalase (Cat)* (220bp) forward 5'-agcagtggaggagcagc-3', reverse 5'-tcaaagtatccaaagcacc-3'. *Glutathione peroxidase (Gpx)* (160bp) forward 5'-catgaccgacccaagtaca-3', reverse 5'-gggttgctaggctgcttga-3'. Data were normalized for the content of actin mRNA.

Statistical analyses

Results are presented as the mean $\pm$ S.E.M. of data obtained in at least three separate experiments. In some cases, data were normalized and expressed as per cent of control because of the variability of fluorescence readings among different repeat experiments due to differences in cell number, batch number and passage number of cultured cells, and as a consequence of differences among human sources. Data were analyzed by Student's *t*-test for unpaired samples or ANOVA.

Results

Insulin increases death of MIN6 cells

An earlier study showed that insulin (100 nM for 24 h) decreased viability of RinM cells, and increased the effect of H_2O_2 , as determined by increased LDH release (Sampson *et al.* 2010). In initial experiments on MIN6

Figure 1

High concentrations of insulin induce dose- and time-related decreases in cellular reducing power of MIN6 cells. MIN6 cells were plated at a density of $3\text{--}4 \times 10^5/\text{mL}$ in 96-well plate (0.1 mL/well) and then treated with insulin at the designated times with various concentrations. Cellular reducing power was determined as described in the 'Materials and methods' section. Each bar represents the mean $\pm$ S.E.M. of replicate (3 or more) values obtained in three experiments (* $P < 0.01$, ** $P < 0.001$ vs untreated; #not significant).

cells, we examined the effects of 1–300 nM insulin, given over a period of 4–48 h, on cell viability as measured by Cellular Reducing Power (Beck *et al.* 2011). We found that insulin caused a dose- and time-dependent decrease in cellular reducing power, with maximum effects most consistently occurring with an insulin concentration of 100 nM administered to the cells for 24 h (Fig. 1). Similarly, H_2O_2 in the range of 25–100 μ M, given for 24 h, induced a significant increase in cell death (as measured by LDH release). These concentrations of H_2O_2 are in accord with those used in other studies on pancreatic β cells (Mehmeti *et al.* 2014, Wali *et al.* 2014, Fu *et al.* 2015, Lortz *et al.* 2015). Accordingly, in subsequent studies, we examined effects of 100 nM insulin and 50–100 μ M H_2O_2 given for 24 h.

Similar to their effects on RinM cells, Fig. 2A shows that 24 h incubation of MIN6 cells with insulin (100 nM) or H_2O_2 (50 μ M) significantly increased LDH release. Insulin increased LDH release by nearly 2.7-fold, compared with control untreated cells, while 50 μ M H_2O_2 increased LDH release four-fold. Moreover, the effects of insulin and H_2O_2 in combination were greater than their individual effects and reached the levels of LDH release in the presence of the positive control Triton-X-100. We also examined the effects of insulin and H_2O_2 , alone and in combination on cell viability as assessed by the Cell Titer Blue assay. In accord with its effects on LDH release,

Figure 2

Insulin increases cell death, and augments H₂O₂-induced cell death in pancreatic β cells. Cells were plated at a density of $3-4 \times 10^5/\text{mL}$ in 96-well plate (0.1 mL/well). After 16 h incubation in serum and glucose-free medium, cells were treated with H₂O₂ (50 μM) or insulin (100 nM), alone or in combination for 24 h. (A) LDH release from MIN6 cells. (B) Viability of MIN6 cells measured by CTB reagent. (C) LDH release from RINm cells. (D) Viability of Ins1 cells measured by CTB reagent. Results are mean $\pm$ s.e.m. of replicate (3 or more) values obtained in three to four experiments (* $P < 0.01$, ** $P < 0.001$ vs untreated; *** $P < 0.01$, H₂O₂ + Ins vs H₂O₂ alone).

Figure 3

Incubation of insulin with trypsin (0.25%, 12–20 h) eliminates effects of insulin on IR phosphorylation (A) LDH release (B) and cellular reducing power (C) (A) Western blot showing elimination of insulin-induced phosphorylation of IR in CHO cells over expressing IR by incubation of insulin with 0.25% trypsin (Tr-Ins) for 12–20 h before addition to the cultures. Cells were plated at a density of $3-4 \times 10^5/\text{mL}$. Treatment of cells, immunoprecipitation and Western blotting were done as described in the 'Materials and methods' section. The blot is representative of results obtained in three separate experiments. (B and C) MIN6 cells were plated and treated with insulin as described in the 'Materials and methods' section. Triton $\times 100$ (Tri) was administered as a control for maximum effects on LDH release and viability. Each bar represents the mean $\pm$ s.e.m. of data obtained in three experiments (* $P < 0.01$, # P NS vs untreated).

prolonged insulin treatment of MIN6 cells decreased their cellular reducing power (viability) by 20% when compared with untreated control cells, and augmented the reduction in cellular reducing power induced by H_2O_2 (Fig. 2B). We also confirmed these effects on RINm cells (Fig. 2C) and further obtained comparable results on additional pancreatic β cell line (Ins1E cells; Fig. 2D). In the latter case, however, insulin did not potentiate the effects of H_2O_2 . Preincubation of insulin with trypsin (0.25%) for 12–20 h eliminated, as expected, insulin-induced Tyr phosphorylation of the insulin receptor in CHO cells (Fig. 3A) and abrogated the effects of insulin on LDH release and cellular reducing power in MIN6 cells (Fig. 3B and C), suggesting that it is insulin *per se* that mediates these effects. Given that insulin is secreted by MIN6 cells, we measured the ambient insulin concentration in the conditioned media of these cells. Cells were treated with 1, 10 and 100 nM insulin for 24 h. The insulin concentration in the media of control cells after 24 h was approximately 32 nM. In 24 h insulin-treated cells, the insulin concentration did not increase on exposure to 1 nM and increased to 41 nM and 135 nM when incubated with 10 and 100 nM insulin, respectively. These findings suggest that the MIN6 cells were exposed to somewhat higher ambient insulin concentrations than those that were added exogenously.

Insulin decreases cellular reducing power of isolated mouse and human islets

To further validate the physiological significance of the above findings, we examined the effects of insulin on cellular reducing power of murine and human pancreatic islets. Twenty-four hours after isolation, mouse islets were treated with insulin (100 nM) or H_2O_2 (25 μ M), alone or in combination, and cellular reducing power was measured after an additional 24 h. Figure 4A shows that insulin decreased the cellular reducing power of mouse islets to $58 \pm 2\%$ of untreated, control islets, while H_2O_2 decreased the reducing power of murine islets to 8%. Combined treatment further decreased the islets' reducing power. Thus, the effects of insulin on viability of primary mouse islets were similar to those obtained with the pancreatic β cell lines. As a further confirmation, experiments were conducted on isolated human pancreatic islets. Treatment of either intact or dispersed human islets with 10 nM insulin for 24 h decreased their reducing power by 15–25% (Fig. 4B). The lower insulin concentration suggests that human islets may be more sensitive than mouse islets to these effects of insulin.

Figure 4

Insulin decreases the reducing power and augments the effect of H_2O_2 on isolated mouse and human pancreatic islets. (A) Mouse islets. Twenty-four hours after isolation, 10 islets per well were plated in 384-well plates and were treated with insulin (100 nM) or H_2O_2 (50 μ M), alone or in combination for 24 h. Islets' reducing power was then measured using the CTB reagent. Results are mean $\pm$ S.E.M. of replicate (3 or more) values obtained in three experiments (* P < 0.01, ** P < 0.001 vs control; *** P < 0.01 H_2O_2 /Ins vs H_2O_2 alone). (B) Human pancreatic islets. Intact or dispersed human islets were incubated in CMRL1066 medium containing 10% FBS (see the 'Materials and methods' section) for 24 h, following which they were treated with insulin (10 nM) for an additional 24 h. Cellular reducing power was measured by the CTB reagent. Results are mean $\pm$ S.E.M. of replicate (3 or more) values obtained in three experiments (* P < 0.01).

Insulin promotes caspase activity in MIN6 cells and human islets

To determine whether the reduction in cellular reducing power induced by insulin might be associated with increased apoptosis, we assayed caspase 3/7 activity in untreated and insulin-treated cells. As shown in Fig. 5A, treatment of MIN6 cells with insulin for 16–24 h increased caspase activity by 70%. A more moderate, still significant, increase could be detected in intact human

Figure 5

Insulin increases caspase 3/7 activity in MIN6 cells and human pancreatic islets. MIN6 cells (A) and intact and dispersed human islets (B) were grown and treated for 16 h with insulin (MIN6 cells 100 nM; islets (10 nM), as described. At the end of incubation, apoptosis was assayed by caspase-3/7 activity. Results are mean $\pm$ S.E.M. of replicate values obtained in independent experiments (3 experiments for MIN6 cells and intact islets; 2 experiments on dispersed human islets) (* P > 0.05, ** P < 0.01).

islets (Fig. 5B) and the same trend was observed even in dispersed human islets.

We next examined whether the increase in caspase activity might be linked to insulin's effects on cellular reducing power. To accomplish this, we treated MIN6 cells with insulin (100 nM) in the presence or absence of the caspase inhibitor z-VAD-fmk (20 μ M) for 24 h. Figure 6A shows that insulin significantly increased caspase 3/7 activity compared with untreated control cells, and this

Figure 6

Z-VAD abrogates insulin-induced increase in caspase activity and the decrease in reducing power of MIN6 cells. MIN6 cells were grown as described in Fig. 1 and were treated with insulin (100 nM) or insulin and Z-VAD (25 μ M) for 24 h. Following treatment, measurements were made of caspase activity (A) and cellular reducing power (B) as described in Methods. Results are mean $\pm$ S.E.M. of replicate (4 or more) values obtained in three to four experiments (* P < 0.05; ** P < 0.01).

effect of insulin was abrogated by z-VAD-fmk. Similarly, z-VAD-fmk eliminated the 23% reduction in cellular reducing power caused by insulin treatment (Fig. 6B).

We also investigated the effects of insulin on cytokine-induced decrease in β cell viability and increase in caspase activity. MIN6 cells were treated with insulin (100 nM) or a cytokine mixture (3 nmol/L TNF α , 3 nmol/L

Figure 7

Insulin does not increase the effects of cytokines on cellular reducing power or caspase activity. Cells were plated as in Fig. 1 and treated with insulin (100 nM) or cytomix (3 nmol/L TNF α , 3 nmol/L IFN- γ and 1.5 nmol/L IL-1 β), alone and in combination for 24 h. At the end of incubation, apoptosis was assayed by caspase-3/7 activity (A) and cellular reducing power using the CTB reagent (B). Each bar represents the mean $\pm$ S.E.M. of 4–6 replicates samples in each of 4 independent experiments. (A: * P < 0.01 Ins vs Cyto; ** P < 0.01 cyto and Ins/cyto vs none; #not significant Ins/cyto vs Ins. B: ** P < 0.01, Ins and cyto vs none; #not significant cyto vs Ins/cyto; see text for further details.

IFN- γ and 1.5 nmol/L IL-1 β ; (Beck *et al.* 2013)), alone and in combination for 24 h. As shown in Fig. 7A, insulin induced a small yet significant increase in caspase activity, while cytokines induced a much bigger effect, yet the effect of the two administered in combination was not significantly different from cytokine treatment alone. Similarly, both insulin and cytokine mix each caused a significant decrease in cellular reducing power with the cytokine mix having a greater effect (Fig. 7B). Here again,

a combined treatment for 24 h with both insulin and cytokines did not further decrease the effect of cytokines on viability.

The insulin-induced increase in cell death and caspase activity is selective for β cells

We reported that insulin increased LDH release from RINm and RINm5F pancreatic β cell lines but not from nonpancreatic β cells such as PC12 (rat pheochromocytoma) or AML-12 (mouse liver) cells (Sampson *et al.* 2010). To further verify these findings, we examined the effects of insulin on cellular reducing power and LDH release from three additional non- β cell lines – HEK-293 cells, as well as CHO cells and NIH fibroblasts that overexpress human IR (CHO-T and NIH-T cells). In each of these cell lines, insulin, unlike H₂O₂, neither induced LDH release nor affected their reducing power (Fig. 8A, B, C and D). Indeed, insulin actually decreased LDH release from HEK293 cells.

The various nonpancreatic β cell lines so far studied are not classical insulin target cells; therefore, we performed additional experiments on a liver cell line (human liver carcinoma cells HepG2) that is considered a common insulin target and is used in numerous studies on IR signaling (Hah *et al.* 1992, Fawcett *et al.* 2001, Patel *et al.* 2004, Biswas *et al.* 2007). HepG2 cells were treated with insulin (100 nM) or a cytokine mixture alone or in combination for 24 h (as done for MIN6 cells) and measurements of caspase activity and cell reducing power were then made. As shown in Fig. 9A, caspase activity of HepG2 cells was slightly increased by insulin and even more so by cytokines. Moreover, the simultaneous treatment of HepG2 cells with both cytokines and insulin caused an increase in caspase activity greater than that by either substance alone. In contrast, cellular reducing power of HepG2 cells (Fig. 9B) was not significantly altered by either insulin or cytokines. Combined treatment, however, did cause a slight but significant reduction in cellular reducing power, albeit not as strongly as might have been expected from the effects on caspase activity.

We also examined the cellular content of MCL-1, a family member of the BCL-2 proteins that regulates cell survival in response to extracellular signals (Allagnat *et al.* 2011) in both MIN6 and HepG2 cells. As shown in Fig. 10A and C (black bars), MCL-1 protein levels in MIN6 cells were reduced as early as 2 h following treatment with insulin, and this effect persisted for up to 24 h. In contrast, and consistent with its effects on HepG2 cell viability, insulin

Figure 8

Insulin does not induce LDH release and does not affect the reducing power of nonpancreatic cells. Cells were plated as in Fig. 1 and, after 16 h incubation were treated with H₂O₂ (50 μ M) or insulin (100 nM), alone or in combination, for an additional 24 h. (A) LDH release from T-293 cells; (B) LDH release from CHO-T cells, cellular reducing power of CHO-T cells (C) or NIH-T cells (D). Results are mean $\pm$ S.E.M. of replicate (3 or more) values obtained in three to four experiments (* P < 0.01, ** P < 0.001 vs untreated), #not significant Ins vs con.

had no detectable effect on MCL-1 protein levels in HepG2 cells (Fig. 10B and C-gray bars). These results collectively indicate that insulin may act selectively on pancreatic β cells to prime a decrease in their viability, whereas it has an apparently minor role in nonpancreatic β cells.

Insulin increases ROS production in MIN6 cells

Among the possible mediators of insulin-induced increase in cell death are ROS and NO, which have been shown to be deleterious to β cell viability (Konishi *et al.* 1997, Nakamura *et al.* 2006, Hou *et al.* 2008, Sampson *et al.* 2010, Bedoya *et al.* 2012, Broniowska *et al.* 2014). Moreover, insulin was shown to increase ROS release in several cell types (Mahadev *et al.* 2001, Goldstein *et al.* 2005a,b, Biswas *et al.* 2007, Guichard *et al.* 2008), including RINm (rat) pancreatic β -cells (Sampson *et al.* 2010). Little appears to be known regarding the effects of insulin on NO release. To address this possibility, we studied the effects of insulin on *iNos* transcription and NO production. As shown in Fig. 11A, exposure of MIN6 cells to insulin for 24–48 h induced only a slight 1.5–2.0-fold increase in *iNos* expression, but not in NO production (not shown). In contrast, we could show that the treatment of MIN6 cells with 100 nM insulin for 18 h resulted in generation of ROS, as indicated by a significant accumulation of the fluorescent oxidized product (DCEH-DA, see the 'Materials and methods' section) (Fig. 11B). Quantification of the cellular fluorescent intensity revealed that insulin induced a 50% increase in ROS production, when compared with

control untreated cells; H₂O₂ induced an approximately two-fold increase, while a combined treatment with insulin and H₂O₂ significantly increased ROS production approximately three-fold (Fig. 11C). In contrast, insulin did not increase ROS levels in CHO-T cells, either when added alone or in combination with H₂O₂ (Fig. 11D). We were also able to confirm earlier findings (Sampson *et al.* 2010) that insulin-induced ROS generation is abrogated by N-acetyl cysteine (not shown).

Insulin does not appear to directly affect antioxidative enzyme expression

Our results indicate that ROS generated by pancreatic β cells in response to high concentrations of insulin may contribute to the deleterious effect of insulin on β cell viability. The generation of ROS might be expected to increase the relatively low expression of antioxidant enzymes. Alternatively, it is possible that insulin might directly alter the expression of these enzymes. Accordingly, we examined MIN6 cells for the effects of 100 nM insulin given for 24 and 48 h on expression of *Sod1*, *Sod2*, *Cat* and *Gpx*, which are the main antioxidant enzymes in pancreatic β cells (Laybutt *et al.* 2002, Chetboun *et al.* 2012, Bhakkiyalakshmi *et al.* 2014, Liu *et al.* 2015). As shown in Fig. 12, the expressions of *Sod1* and *Sod2* were not significantly altered after either 24 or 48 h insulin exposure. In contrast, *Cat* and *Gpx* expressions were increased after both 24 and 48 h insulin treatment.

Figure 9

Insulin increases caspase 3/7 activity but only slightly affects cellular reducing power of HepG2 cells. HepG2 cells were grown in 96-well plates as described in the 'Materials and methods' section and treated with insulin (100 nM) or cytomix (3 nmol/L TNF α , 3 nmol/L IFN- γ and 1.5 nmol/L IL-1 β), alone and in combination for 24 h. At the end of incubation, apoptosis was assayed by caspase-3/7 activity (A) and cellular reducing power using the CTB reagent (B). Each bar represents the mean $\pm$ s.e.m. of four to six replicate samples in each of three independent experiments. (A: * P < 0.001 Ins vs none; ** P < 0.01 Ins/cyto vs Ins. B: #not significant none vs Ins and none vs cyto; * P < 0.01 Ins/cyto vs Ins). See text for further details.

Insulin induces changes in expression of ER stress genes

Excess accumulation of ROS in pancreatic β cells induces both oxidative and endoplasmic reticulum (ER) stress, which triggers the unfolded protein response (UPR) (Vanderford 2010) which may lead, if sustained, to β -cell dysfunction and apoptosis (Kitiphongspattana *et al.* 2007, Lin *et al.* 2012). To examine whether insulin might alter

Figure 10

Insulin downregulates Mcl-1 protein in MIN6 but not HepG2 cells. MIN6 and HepG2 cells were grown and treated with insulin for the designated times. (A and B) Western blots showing levels of Mcl-1 in MIN6 and HepG2 cells. (C) Graph of densitometry measurements of Mcl-1 relative to actin levels following treatment of MIN6 cells with insulin for the designated times (black bars – MIN6; gray bars – HepG2). Each bar represents the mean $\pm$ s.e.m. of measurements made of Western blots in six independent experiments. In two of these experiments, measurements were made after 12 h. These values were pooled with the 24 h data. (* P < 0.05; #NS).

expression of UPR-associated genes, we determined the mRNA levels of *Bip*, *Xbp1*, *Atf4* and *Chop* (Walter & Ron 2011) in MIN6 cells treated with insulin for 24–48 h. As shown in Fig. 13A, insulin significantly reduced the expression of *Bip* and *Xbp1* (25 and 33%, respectively), while it did not significantly affect the mRNA levels of *Atf4* in MIN6 cells. In contrast, the expression levels of *Chop* were significantly increased (30%) in the presence of insulin. Thus, insulin affected the expression of at least three UPR-associated proteins, which could be accounted for by its effects on ROS production in pancreatic β cells. Hence, insulin alone or in combination with potentially deleterious factors may induce effects consistent with ER stress.

Among the candidate ER stress proteins that might be involved in insulin effects is *Trb3*, a downstream target of *Atf4*, which has been found to be increased by cytokines (Kanki *et al.* 2009, Humphrey *et al.* 2010, Kanwar 2010),

Figure 11

Insulin increases ROS production in MIN6 cells. MIN6 cells were plated at a density of $2.5\text{--}3 \times 10^5/\text{mL}$ in 12-well plates and, after 16 h incubation, were treated with insulin (100 nM) for 24 h. (A) mRNA levels of iNOS were determined by qRT-PCR as described in the 'Materials and methods' section, and were quantified following normalization to actin mRNA levels. (B) Representative photomicroscope images of DCF-DA accumulation in untreated and insulin-treated MIN6 cells. Accumulation was measured by an increase in fluorescence at 530 nm when the sample was excited at 485 nm. (calibration line = 20 microns). (C and D) Fluorescence measurements of CM-H₂DCFDA (ROS release) in MIN6 (C) and CHO-T (D) cells. Cells were plated at a density of $5\text{--}6 \times 10^5/\text{mL}$ in 24-well plates (0.5 mL/well). After overnight incubation, CM-H₂DCFDA (10 μM) reagent was added to the cells as described in the 'Materials and methods' section. Two hours later, MIN6 and CHO-T cells were treated with insulin (100 nM) or H₂O₂ (75 μM), alone or in combination, as indicated. Fluorescence was measured after treatment of 18 h. Results are mean $\pm$ S.E.M. of measurements obtained in three experiments (* $P < 0.01$, ** $P < 0.001$ vs untreated control cells; *** $P < 0.001$, H₂O₂ alone vs H₂O₂ + Ins; #not significant Ins/H₂O₂ vs H₂O₂).

Figure 12

Insulin affects the expression of certain antioxidative enzyme. MIN6 cells were plated at a density of $2.5\text{--}3 \times 10^5/\text{mL}$ in 12-well plates and, after 16 h incubation, were treated with insulin (100 nM) for 24 and 48 h. mRNA levels of the indicated genes were determined by qRT-PCR as described in the 'Materials and methods' section, and were quantified following normalization to actin mRNA levels. In all graphs, bars represent the mean $\pm$ S.E.M. of duplicate determinations obtained in four separate experiments. Sod1 and Sod2, superoxide dismutase 1 and 2; Cat, catalase; Gpx, glutathione peroxidase. (* $P < 0.05$; ** $P < 0.01$. All other differences were not significant at $P < 0.05$).

and by insulin in Fao rat hepatoma and in 3T3-L1 adipocytes (Du & Ding 2009). Accordingly, we examined the effect of insulin on the expression of *Trb3* in MIN6 cells. Exposure of MIN6 cells to insulin for 24–48 h induced a three-fold increase in the expression of *Trb3* (Fig. 13B). Given that insulin increases the effect of cytokines on caspase 3/7 activity in HepG2 cells (Fig. 9), we thought it of interest also to examine potential interaction between insulin and cytokines on *Trb3* expression in MIN6 cells. In these experiments (Fig. 13C), we used a low concentration (0.1 to 0.25 $\times$) of cytokine mix, which did not elicit a significant increase in *Trb3* expression. When, however, cells were treated with low cytokine concentration together with insulin for 24–48 h, *Trb3* expression was approximately doubled. These data demonstrate that insulin is able to boost the effects of low cytokine concentrations on *Trb3*.

Discussion

Insulin resistance is a state in which target cells fail to respond to ordinary levels of insulin resulting in the need for increased levels of insulin to control plasma glucose levels. The cycle of hyperglycemia and hyperinsulinemia

Figure 13

Insulin alters the expression of ER stress genes in MIN6 cells. MIN6 cells were plated at a density of $2.5\text{--}3 \times 10^5/\text{mL}$ in 12-well plates and, after 16 h incubation, were treated with insulin (100 nM) for 24 h. mRNA levels of the indicated genes were determined by qRT-PCR as described in the 'Materials and methods' section, and were quantified following normalization to actin mRNA levels. (A) Insulin decreased the expression of *Bip* and *Xbp1*, and increased the expression of *Chop*. (B) Insulin increased *Trb3* expression and (C) augmented the effect of cytokine to elevate *Trb3* expression. In all graphs, bars represent the mean $\pm$ s.e.m. of data obtained in three to four separate experiments (A and B $*P < 0.01$, $**P < 0.05$ insulin vs untreated; #not significant. C – #not significant, $*P < 0.01$, $**P < 0.05$ insulin + cytokine mix vs cytokine mix alone).

eventually leads to pancreatic β cell deterioration and death. In this study, we provide evidence that elevated levels of insulin itself might contribute an important component to the increased demise of β cells, possibly attributable to insulin-induced production of ROS in

β cells, induction of ER stress-related genes and subsequent stimulation of apoptotic processes. Apoptosis may be an important mechanism of insulin-induced β cell death, because insulin-induced changes in caspase 3/7 activity and cell viability were completely abrogated by z-VAD-fmk, a selective inhibitor of caspases. Hence, prolonged elevated levels of insulin may prime β cell apoptosis and thereby exacerbate β -cell deterioration in response to other factors.

Several lines of evidence support this conclusion. First, we could show that prolonged (24 h) insulin treatment increased caspase activity, decreased the reducing power and promoted the release of LDH from three pancreatic β cell lines (MIN6, RINm, INS-1) as well as isolated mouse and human islets. Human islets appeared to be more sensitive to the effects of insulin, as the decrease in cell viability and increase in caspase 3/7 activity were readily detectable at an insulin concentration of 10 nM in human islets compared with 100 nM in mouse islets. This difference might be explained by the fragile state of human islets, as conditions for isolation and transportation were considerably more severe than those for mouse islets (see the 'Materials and methods' section). Insulin not only promoted death of pancreatic β cells, but sustained insulin treatment also inhibited β -cell survival, as evidenced by the significant decrease in cellular content of MCL-1, a family member of the BCL antiapoptotic proteins, in β cells subjected to prolonged insulin treatment. The insulin-induced increase in β cell death was associated with elevated production of ROS that could result in increased ER stress. This idea is supported by our finding of a decrease in the expression of *Bip*, which triggers an unfolded protein response (UPR) (Walter & Ron 2011). UPR involves the activation of three linked signal transduction pathways emanating from three principle ER stress sensors: IRE1 α , double-stranded RNA-dependent protein kinase-like kinase (PERK) and ATF6 α (Ron & Walter 2007). Failure of the UPR to reduce the ER stress and resume homeostasis results in the initiation of an UPR-triggered apoptotic process (Walter & Ron 2011). Indeed, we could show that insulin triggers induction of the UPR. We found an insulin-dependent reduced expression of *Xbp1*, presumably due to *Xbp1* splicing (He et al. 2010), which contributes to a sustained UPR that leads to apoptosis (Walter & Ron 2011). Sustained insulin treatment also increased the expression of *Trb3* and its downstream target *Chop*, a key mediator of UPR-induced apoptotic process in β cells (Vanderford 2010). The effect of insulin to increase *Trb3* expression is in accord with a recent study in Fao hepatoma and 3T3 L1-adipocytes; indeed, insulin promoted the expression of several

stress-related genes in these cells similar to our findings in this study (Du & Ding 2009). It still remains unclear why insulin did not affect the transcription of *Atf4*, the upstream regulator of *Trb3*. Our results are thus in accord with a recent study showing that the balance between *Xbp1*-mediated adaptive and *Chop*-dependent apoptotic UPR is critically important for β -cell survival during ER stress (Chan *et al.* 2015).

Along with the generation of ROS, relatively long-term exposure of MIN6 cells to insulin did appear to increase significantly the expressions of the antioxidant enzymes *Cat* and *Gpx*, as occurs with some other inducers of cellular stress (Tiedge *et al.* 1997, Laybutt *et al.* 2002, Palsamy & Subramanian 2010, Chetboun *et al.* 2012, Bhakkiyalakshmi *et al.* 2014, Tersey *et al.* 2014, Vasu *et al.* 2014, An *et al.* 2015). In contrast, the expressions of *Sod1* and *Sod2* were only slightly and not significantly increased by 24 and 48 h insulin exposure. Furthermore, we could not detect any significant interaction between cytokines and insulin on antioxidant enzymes expression (not shown). We would, therefore, propose that the effects on *Cat* and *Glx* were secondary to the generation of ROS, and that insulin itself has no direct effect on expression of these enzymes.

In addition to its effects as a mild inducer of β cell death, insulin seems to increase β cell death triggered by other stress inducers. Here, as well as in an earlier study (Sampson *et al.* 2010), we could show that insulin significantly increased caspase activity and LDH release, apparently triggered by H_2O_2 , an inducer of oxidative stress. In addition, we found that insulin not only increased *Trb3* expression but also could increase the effect of subthreshold cytokine levels on *Trb3* expression. Hence, prolonged insulin treatment potentiates detrimental effects of stress inducers on β cell function. We suggest that these effects of insulin are most likely due in part to its own effects on ROS production and the UPR.

In contrast to its positive interaction with H_2O_2 , insulin did not significantly influence the effects of cytokines on cell viability and caspase activity. One possible explanation might be related to the report that NO plays a major role in mediation of the antiproliferative effects of proinflammatory cytokines on cultured β cells (Quintana-Lopez *et al.* 2013), and that we found only a trivial and not significant effect of insulin on *iNos* expression and NO activity. The lack of a detectable positive interaction might also be due to the very strong effect of the cytokine mixture that could mask any additional effect of insulin. Moreover, certain cytokines induce production of ROS (Prause *et al.* 2016), and this effect may not be further

increased by insulin. Additional studies are necessary in order to explore any further possible effects of insulin on cytokine-induced cell death.

We could also show that insulin effects are relatively selective for pancreatic β cells, because cells from different origins, such as CHO, HEK293 and NIH fibroblast cells (as well as AML12 liver and pheochromocytoma cells (Sampson *et al.* 2010), did not demonstrate insulin-induced LDH release or decreased cellular reducing power. Interestingly, we found that HepG2 liver cells responded to prolonged insulin exposure with a slight increase in caspase 3/7 activity, an indication that apoptosis mechanisms might be slightly activated. Yet, neither cytokine mix nor insulin had a significant effect on cellular reducing power despite their effects on caspase activity, suggesting that the minor increase in caspase activity in HepG2 cells is not sufficient to affect their viability.

While hyperinsulinemia is associated with pancreatic β cell demise (Wang & Jin 2009), insulin itself has not been considered to be a contributor to β cell deterioration. Most studies attribute any deleterious effects of this association to overproduction of insulin by the β cells that eventually results in a classical induction of ER stress, UPR and eventual death of the β cell (Fonseca *et al.* 2011). Although the concentrations used in this study are unlikely to be attained in the blood during hyperinsulinemia, they are in accord with those used in other studies regarding insulin effects on its target tissues (Biswas *et al.* 2007, Guillen *et al.* 2008, Du & Ding 2009). Furthermore, some studies report levels as high as 700–800 pM (Movassat *et al.* 1997, Remington *et al.* 2015, Yang *et al.* 2015). Moreover, it should be noted that the concentration of insulin in the vicinity of the islets may be considerably higher than that in the serum. Interestingly, it was shown that exogenous insulin completely prevented apoptosis induced by serum withdrawal when given at picomolar or low nanomolar concentrations, but not at higher concentrations (Johnson *et al.* 2006). We would suggest, therefore, that the current study highlights another potential aspect of high nanomolar concentrations of insulin, namely that the secreted insulin *per se*, when present in the vicinity of the β cells at high enough concentrations and for prolonged periods of time, might act as a proapoptotic adjuvant agent that promotes β cell death. This notion is consistent with previous findings that have shown that insulin can increase ROS levels in target cells (Goldstein *et al.* 2005b), probably via the NOX family of NADPH oxidases (Guichard *et al.* 2008), several members of which

are expressed in pancreatic islets (Guichard *et al.* 2008). Insulin was also reported to induce apoptosis in glucose-deprived β cells (Guillen *et al.* 2008) and potentiate the deleterious effects of H_2O_2 on β cell survival (Sampson *et al.* 2010). Of special interest in this regard is our finding that insulin increased the expression of *iNos* and *Trb3*, factors whose expression is known to be increased by cytokines in pancreatic β cells (Kanki *et al.* 2009, Humphrey *et al.* 2010, Kanwar 2010, Quintana-Lopez *et al.* 2013). *Trb3* was recently shown to mediate FFA-induced apoptosis in pancreatic β cells (Qin *et al.* 2014).

Insulin is generally considered to be a factor that positively affects cell proliferation and promotes β cell survival (Wang & Jin 2009). As shown in numerous studies by us and others (Gurevitch *et al.* 2010, Tseng *et al.* 2002), insulin mainly plays antiapoptotic roles and exerts its trophic effects on β -cells through an IRS-2-mediated signaling pathway. Still, growth factors, such as insulin, might also induce ROS production and proapoptotic signals under certain circumstances (Porras *et al.* 2003, Bryson & White 2012, Graham *et al.* 2012). Production of ROS for short periods is associated with an increase in glucose-stimulated insulin secretion, but excessive or sustained production of ROS is negatively correlated with the insulin secretory process and leads to β cell death (Newsholme *et al.* 2007). This might be due to the relatively low levels of free radical detoxifying and redox-regulating enzymes, such as superoxide dismutase, glutathione peroxidase, catalase and thioredoxin in β cells. The consequence of limited scavenging systems is that upon stimulation of the NADPH oxidase systems, ROS concentrations in β -cells may increase rapidly and thereby easily reach damaging levels (Newsholme *et al.* 2007). Support to this notion is provided by the fact that inhibition of Akt, which can mediate ROS production, abrogates the effects of insulin. Another possible mechanism for apoptosis-inducing effects could be related to effects of prolonged insulin signal stimulation on cell viability. Thus, it was reported that decreased IRS-2 protein expression in β -cells, mediated via chronic activation of mTOR induced by chronic exposure to glucose, correlated with decreased PKB phosphorylation activation; this, in turn, was associated with increased caspase-9 activation, a marker of β -cell apoptosis (Briaud *et al.* 2005, Shigeyama *et al.* 2008). In support of this possibility is the report that β cell apoptosis and markers of oxidative stress in response to a high-fat diet were decreased in mice expressing a mutated IR compared with wild-type mice (Tachibana *et al.* 2015).

Clearly, the mechanisms involved in pancreatic β cell deterioration and death are many and varied and likely involve the myriad of steps and factors controlling β cell proliferation (see review by Kulkarni (Kulkarni *et al.* 2012)). The results in this work support the concept (Aston-Mourney *et al.* 2008) that increased and sustained insulin levels that promote NADPH oxidase activity and ROS production may be detrimental to pancreatic β cell survival. Hence, induction of increased insulin secretion in patients with obesity and insulin resistance that are already hyperinsulinemic may actually accelerate the deterioration of pancreatic β cells, a consideration supported by the results presented in this report.

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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References

- Allagnat F, Cunha D, Moore F, Vanderwinden JM, Eizirik DL & Cardozo AK 2011 Mcl-1 downregulation by pro-inflammatory cytokines and palmitate is an early event contributing to beta-cell apoptosis. *Cell Death and Differentiation* **18** 328–337. (doi:10.1038/cdd.2010.105)
- Allagnat F, Fukaya M, Nogueira TC, Delaroché D, Welsh N, Marselli L, Marchetti P, Haefliger JA, Eizirik DL & Cardozo AK 2012 C/EBP homologous protein contributes to cytokine-induced pro-inflammatory responses and apoptosis in beta-cells. *Cell Death and Differentiation* **19** 1836–1846. (doi:10.1038/cdd.2012.67)
- An L, Wang Y, Wang C, Fan M, Han X, Xu G, Yuan G, Li H, Sheng Y, Wang M, *et al.* 2015 Protective effect of Schisandra chinensis oil on pancreatic beta-cells in diabetic rats. *Endocrine* **48** 818–825. (doi:10.1007/s12020-014-0375-y)
- Aston-Mourney K, Proietto J, Morahan G & Andrikopoulos S 2008 Too much of a good thing: why it is bad to stimulate the beta cell to secrete insulin. *Diabetologia* **51** 540–545. (doi:10.1007/s00125-008-0930-2)
- Barazzoni R, Zanetti M, Gortan Cappellari G, Semolic A, Boschelle M, Codarin E, Pirulli A, Cattin L & Guarnieri G 2012 Fatty acids acutely enhance insulin-induced oxidative stress and cause insulin resistance by increasing mitochondrial reactive oxygen species (ROS) generation and nuclear factor-kappaB inhibitor (IkappaB)-nuclear factor-kappaB (NFkappaB) activation in rat muscle, in the absence of mitochondrial dysfunction. *Diabetologia* **55** 773–782. (doi:10.1007/s00125-011-2396-x)
- Beck A, Isaac R, Lavelin I, Hart Y, Volberg T, Shatz-Azoulay H, Geiger B & Zick Y 2011 An siRNA screen identifies transmembrane 7 superfamily member 3 (TM7SF3), a seven transmembrane orphan receptor, as an inhibitor of cytokine-induced death of pancreatic beta cells. *Diabetologia* **54** 2845–2855. (doi:10.1007/s00125-011-2277-3)

- Beck A, Vinik Y, Shatz-Azoulay H, Isaac R, Streim S, Jona G, Boura-Halfon S & Zick Y 2013 Otubain 2 is a novel promoter of beta cell survival as revealed by siRNA high-throughput screens of human pancreatic islets. *Diabetologia* **56** 1317–1326. (doi:10.1007/s00125-013-2889-x)
- Bedoya FJ, Salguero-Aranda C, Cahuana GM, Tapia-Limonchi R, Soria B & Tejedo JR 2012 Regulation of pancreatic beta-cell survival by nitric oxide: clinical relevance. *Islets* **4** 108–118. (doi:10.4161/isl.19822)
- Bhakiyalakshmi E, Shalini D, Sekar TV, Rajaguru P, Paulmurugan R & Ramkumar KM 2014 Therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis mediated through Nrf2. *British Journal of Pharmacology* **171** 1747–1757. (doi:10.1111/bph.12577)
- Biswas S, Gupta MK, Chattopadhyay D & Mukhopadhyay CK 2007 Insulin-induced activation of hypoxia-inducible factor-1 requires generation of reactive oxygen species by NADPH oxidase. *American Journal of Physiology: Heart and Circulatory Physiology* **292** H758–H766. (doi:10.1152/ajpheart.00718.2006)
- Briaud I, Dickson LM, Lingohr MK, McCuaig JF, Lawrence JC & Rhodes CJ 2005 Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in beta-cells. *Journal of Biological Chemistry* **280** 2282–2293. (doi:10.1074/jbc.M412179200)
- Broniowska KA, Oleson BJ & Corbett JA 2014 beta-Cell responses to nitric oxide. *Vitamins and Hormones* **95** 299–322. (doi:10.1016/B978-0-12-800174-5.00012-0)
- Bryson BD & White FM 2012 Signaling for death: tyrosine phosphorylation in the response to glucose deprivation. *Molecular Systems Biology* **8** 591. (doi:10.1038/msb.2012.27)
- Chan JY, Luzuriaga J, Maxwell EL, West PK, Bensellam M & Laybutt DR 2015 The balance between adaptive and apoptotic unfolded protein responses regulates beta-cell death under ER stress conditions through XBP1, CHOP and JNK. *Molecular and Cellular Endocrinology* **413** 189–201. (doi:10.1016/j.mce.2015.06.025)
- Chetboun M, Abitbol G, Rozenberg K, Rozenfeld H, Deutsch A, Sampson SR & Rosenzweig T 2012 Maintenance of redox state and pancreatic beta-cell function: role of leptin and adiponectin. *Journal of Cellular Biochemistry* **113** 1966–1976. (doi:10.1002/jcb.24065)
- Del Parigi A 2008 Insulin may have a role to play in protecting beta cells from deterioration in diabetes. *Diabetologia* **51** 1340. (doi:10.1007/s00125-008-1010-3)
- Dembele K, Nguyen KH, Hernandez TA & Nyomba BL 2009 Effects of ethanol on pancreatic beta-cell death: interaction with glucose and fatty acids. *Cell Biology and Toxicology* **25** 141–152. (doi:10.1007/s10565-008-9067-9)
- Du K & Ding J 2009 Insulin regulates TRB3 and other stress-responsive gene expression through induction of C/EBP β . *Molecular Endocrinology* **23** 475–485. (doi:10.1210/me.2008-0284)
- Eizirik DL, Cardozo AK & Cnop M 2008 The role for endoplasmic reticulum stress in diabetes mellitus. *Endocrine Reviews* **29** 42–61. (doi:10.1210/er.2007-0015)
- Fawcett J, Hamel FG, Bennett RG, Vajo Z & Duckworth WC 2001 Insulin and analogue effects on protein degradation in different cell types. Dissociation between binding and activity. *Journal of Biological Chemistry* **276** 11552–11558. (doi:10.1074/jbc.M007988200)
- Fernandez AM, Kim JK, Yakar S, Dupont J, Hernandez-Sanchez C, Castle AL, Filmore J, Shulman GI & Le Roith D 2001 Functional inactivation of the IGF-I and insulin receptors in skeletal muscle causes type 2 diabetes. *Genes and Development* **15** 1926–1934. (doi:10.1101/gad.908001)
- Fonseca SG, Gromada J & Urano F 2011 Endoplasmic reticulum stress and pancreatic beta-cell death. *Trends in Endocrinology and Metabolism* **22** 266–274. (doi:10.1016/j.tem.2011.02.008)
- Fu J, Zheng H, Wang H, Yang B, Zhao R, Lu C, Liu Z, Hou Y, Xu Y, Zhang Q, et al. 2015 Protective role of nuclear factor E2-related factor 2 against acute oxidative stress-induced pancreatic beta-cell damage. *Oxidative Medicine and Cellular Longevity* **2015** 1–12. (doi:10.1155/2015/639191)
- Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M & Shimomura I 2004 Increased oxidative stress in obesity and its impact on metabolic syndrome. *Journal of Clinical Investigation* **114** 1752–1761. (doi:10.1172/JCI21625)
- Goldstein BJ, Mahadev K & Wu X 2005a Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets. *Diabetes* **54** 311–321. (doi:10.2337/diabetes.54.2.311)
- Goldstein BJ, Mahadev K, Wu X, Zhu L & Motoshima H 2005b Role of insulin-induced reactive oxygen species in the insulin signaling pathway. *Antioxidants and Redox Signaling* **7** 1021–1031. (doi:10.1089/ars.2005.7.1021)
- Graham NA, Tahmasian M, Kohli B, Komisopoulou E, Zhu M, Vivanco I, Teitell MA, Wu H, Ribas A, Lo RS, et al. 2012 Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death. *Molecular Systems Biology* **8** 589. (doi:10.1038/msb.2012.20)
- Guichard C, Moreau R, Pessayre D, Epperson TK & Krause KH 2008 NOX family NADPH oxidases in liver and in pancreatic islets: a role in the metabolic syndrome and diabetes? *Biochemical Society Transactions* **36** 920–929. (doi:10.1042/BST0360920)
- Guillen C, Bartolome A, Nevado C & Benito M 2008 Biphasic effect of insulin on beta cell apoptosis depending on glucose deprivation. *FEBS Letters* **582** 3855–3860. (doi:10.1016/j.febslet.2008.10.020)
- Gurevitch D, Boura-Halfon S, Isaac R, Shahaf G, Alberstein M, Ronen D, Lewis EC & Zick Y 2010 Elimination of negative feedback control mechanisms along the insulin signaling pathway improves beta-cell function under stress. *Diabetes* **59** 2188–2197. (doi:10.2337/db09-0890)
- Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE & Shaw RJ 2008 AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular Cell* **30** 214–226. (doi:10.1016/j.molcel.2008.03.003)
- Hah J, Jo I, Chakrabarti R & Jung CY 1992 Demonstration of an insulin-insensitive storage pool of glucose transporters in rat hepatocytes and HepG2 cells. *Journal of Cellular Physiology* **152** 56–63. (doi:10.1002/jcp.1041520108)
- He Y, Sun S, Sha H, Liu Z, Yang L, Xue Z, Chen H & Qi L 2010 Emerging roles for XBP1, a sUPeR transcription factor. *Gene Expression* **15** 13–25. (doi:10.3727/105221610X12819686555051)
- Herold KC, Vignali DA, Cooke A & Bluestone JA 2013 Type 1 diabetes: translating mechanistic observations into effective clinical outcomes. *Nature Reviews Immunology* **13** 243–256. (doi:10.1038/nri3422)
- Hou N, Torii S, Saito N, Hosaka M & Takeuchi T 2008 Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases. *Endocrinology* **149** 1654–1665. (doi:10.1210/en.2007-0988)
- Humphrey RK, Newcomb CJ, Yu SM, Hao E, Yu D, Krajewski S, Du K & Jhala US 2010 Mixed lineage kinase-3 stabilizes and functionally cooperates with TRIBBL-3 to compromise mitochondrial integrity in cytokine-induced death of pancreatic beta cells. *Journal of Biological Chemistry* **285** 22426–22436. (doi:10.1074/jbc.M110.123786)
- Jensen M & De Meyts P 2009 Molecular mechanisms of differential intracellular signaling from the insulin receptor. *Vitamins and Hormones* **80** 51–75. (doi:10.1016/S0083-6729(08)00603-1)
- Johnson JD, Bernal-Mizrachi E, Alejandro EU, Han Z, Kalynyak TB, Li H, Beith JL, Gross J, Warnock GL, Townsend RR, et al. 2006 Insulin protects islets from apoptosis via Pdx1 and specific changes in the human islet proteome. *PNAS* **103** 19575–19580. (doi:10.1073/pnas.0604208103)

- Kajimoto Y & Kaneto H 2004 Role of oxidative stress in pancreatic beta-cell dysfunction. *Annals of the New York Academy of Sciences* **1011** 168–176. (doi:10.1196/annals.1293.017)
- Kanki K, Kawamura T & Watanabe Y 2009 Control of ER stress by a chemical chaperone counteracts apoptotic signals in IFN-gamma-treated murine hepatocytes. *Apoptosis* **14** 309–319. (doi:10.1007/s10495-009-0318-x)
- Kanwar YS 2010 TRB3: an oxidant stress-induced pseudokinase with a potential to negatively modulate MCP-1 cytokine in diabetic nephropathy. *American Journal of Physiology: Renal Physiology* **299** F963–F964. (doi:10.1152/ajprenal.00479.2010)
- Kitiphongpattana K, Khan TA, Ishii-Schrade K, Roe MW, Philipson LH & Gaskins HR 2007 Protective role for nitric oxide during the endoplasmic reticulum stress response in pancreatic beta-cells. *American Journal of Physiology: Endocrinology and Metabolism* **292** E1543–E1554. (doi:10.1152/ajpendo.00620.2006)
- Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U & Nishizuka Y 1997 Activation of protein kinase C by tyrosine phosphorylation in response to H₂O₂. *PNAS* **94** 11233–11237. (doi:10.1073/pnas.94.21.11233)
- Kulkarni RN, Mizrahi EB, Ocana AG & Stewart AF 2012 Human beta-cell proliferation and intracellular signaling: driving in the dark without a road map. *Diabetes* **61** 2205–2213. (doi:10.2337/db12-0018)
- Laybutt DR, Kaneto H, Hasenkamp W, Grey S, Jonas JC, Sgroi DC, Groff A, Ferran C, Bonner-Weir S, Sharma A, et al. 2002 Increased expression of antioxidant and antiapoptotic genes in islets that may contribute to beta-cell survival during chronic hyperglycemia. *Diabetes* **51** 413–423. (doi:10.2337/diabetes.51.2.413)
- Lin N, Chen H, Zhang H, Wan X & Su Q 2012 Mitochondrial reactive oxygen species (ROS) inhibition ameliorates palmitate-induced INS-1 beta cell death. *Endocrine* **42** 107–117. (doi:10.1007/s12020-012-9633-z)
- Liu J, Wang X, Peng Z, Zhang T, Wu H, Yu W, Kong D, Liu Y, Bai H, Liu R, et al. 2015 The effects of insulin pre-administration in mice exposed to ethanol: alleviating hepatic oxidative injury through anti-oxidative anti-apoptotic activities and deteriorating hepatic steatosis through SRBEP-1c activation. *International Journal of Biological Sciences* **11** 569–586. (doi:10.7150/ijbs.11039)
- Lortz S, Lenzen S & Mehmeti I 2015 Impact of scavenging hydrogen peroxide in the endoplasmic reticulum for beta cell function. *Journal of Molecular Endocrinology* **55** 21–29. (doi:10.1530/JME-15-0132)
- Lu H, Koshkin V, Allister EM, Gyulhandanyan AV & Wheeler MB 2010 Molecular and metabolic evidence for mitochondrial defects associated with beta-cell dysfunction in a mouse model of type 2 diabetes. *Diabetes* **59** 448–459. (doi:10.2337/db09-0129)
- Mahadev K, Wu X, Zilbering A, Zhu L, Lawrence JT & Goldstein BJ 2001 Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. *Journal of Biological Chemistry* **276** 48662–48669. (doi:10.1074/jbc.M105061200)
- May JM & de Haen C 1979 Insulin-stimulated intracellular hydrogen peroxide production in rat epididymal fat cells. *Journal of Biological Chemistry* **254** 2214–2220.
- Mehmeti I, Lortz S, Elsner M & Lenzen S 2014 Peroxiredoxin 4 improves insulin biosynthesis and glucose-induced insulin secretion in insulin-secreting INS-1E cells. *Journal of Biological Chemistry* **289** 26904–26913. (doi:10.1074/jbc.M114.568329)
- Movassat J, Saulnier C, Serradas P & Portha B 1997 Impaired development of pancreatic beta-cell mass is a primary event during the progression to diabetes in the GK rat. *Diabetologia* **40** 916–925. (doi:10.1007/s001250050768)
- Mukherjee SP, Lane RH & Lynn WS 1978 Endogenous hydrogen peroxide and peroxidative metabolism in adipocytes in response to insulin and sulfhydryl reagents. *Biochemical Pharmacology* **27** 2589–2594. (doi:10.1016/0006-2952(78)90332-5)
- Muller D, Jones PM & Persaud SJ 2006 Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic beta-cells. *FEBS Letters* **580** 6977–6980. (doi:10.1016/j.febslet.2006.11.066)
- Nakamura U, Iwase M, Uchizono Y, Sonoki K, Sasaki N, Imoto H, Goto D & Iida M 2006 Rapid intracellular acidification and cell death by H₂O₂ and alloxan in pancreatic beta cells. *Free Radical Biology and Medicine* **40** 2047–2055. (doi:10.1016/j.freeradbiomed.2006.01.038)
- Navarro-Tableros V, Sanchez-Soto MC, Garcia S & Hiriart M 2004 Autocrine regulation of single pancreatic beta-cell survival. *Diabetes* **53** 2018–2023. (doi:10.2337/diabetes.53.8.2018)
- Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR & Curi R 2007 Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. *Journal of Physiology* **583** 9–24. (doi:10.1113/jphysiol.2007.135871)
- Palsamy P & Subramanian S 2010 Ameliorative potential of resveratrol on proinflammatory cytokines, hyperglycemia mediated oxidative stress, and pancreatic beta-cell dysfunction in streptozotocin-nicotinamide-induced diabetic rats. *Journal of Cellular Physiology* **224** 423–432. (doi:10.1002/jcp.22138)
- Patel NA, Apostolatos HS, Mebert K, Chalfant CE, Watson JE, Pillay TS, Sparks J & Cooper DR 2004 Insulin regulates protein kinase CbetaII alternative splicing in multiple target tissues: development of a hormonally responsive heterologous minigene. *Molecular Endocrinology* **18** 899–911. (doi:10.1210/me.2003-0391)
- Porras A, Zuluaga S, Valladares A, Alvarez AM, Herrera B, Fabregat I & Benito M 2003 Long-term treatment with insulin induces apoptosis in brown adipocytes: role of oxidative stress. *Endocrinology* **144** 5390–5401. (doi:10.1210/en.2003-0622)
- Prause M, Berchtold LA, Urizar AI, Hyldgaard Trauelsen M, Billestrup N, Mandrup-Poulsen T & Storling J 2016 TRAF2 mediates JNK and STAT3 activation in response to IL-1beta and IFNgamma and facilitates apoptotic death of insulin-producing beta-cells. *Molecular and Cellular Endocrinology* **420** 24–36. (doi:10.1016/j.mce.2015.11.021)
- Qin J, Fang N, Lou J, Zhang W, Xu S, Liu H, Fang Q, Wang Z, Liu J, Men X, et al. 2014 TRB3 is involved in free fatty acid-induced INS-1-derived cell apoptosis via the protein kinase C delta pathway. *PLoS ONE* **9** e96089. (doi:10.1371/journal.pone.0096089)
- Quintana-Lopez L, Blandino-Rosano M, Perez-Arana G, Cebada-Aleu A, Lechuga-Sancho A, Aguilar-Diosdado M & Segundo C 2013 Nitric oxide is a mediator of antiproliferative effects induced by proinflammatory cytokines on pancreatic beta cells. *Mediators of Inflammation* **2013** 1–10. (doi:10.1155/2013/905175)
- Remington GJ, Teo C, Wilson V, Chintoh A, Guenette M, Ahsan Z, Giacca A & Hahn MK 2015 Metformin attenuates olanzapine-induced hepatic, but not peripheral insulin resistance. *Journal of Endocrinology* **227** 71–81. (doi:10.1530/JOE-15-0074)
- Robertson RP 2004 Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *Journal of Biological Chemistry* **279** 42351–42354. (doi:10.1074/jbc.R400019200)
- Ron D & Walter P 2007 Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology* **8** 519–529. (doi:10.1038/nrm2199)
- Sampson SR, Bucris E, Horovitz-Fried M, Parnas A, Kahana S, Abitbol G, Chetboun M, Rosenzweig T, Brodie C & Frankel S 2010 Insulin increases H₂O₂ (2)-induced pancreatic beta cell death. *Apoptosis* **15** 1165–1176. (doi:10.1007/s10495-010-0517-5)
- Shigeyama Y, Kobayashi T, Kido Y, Hashimoto N, Asahara S, Matsuda T, Takeda A, Inoue T, Shibutani Y, Koyanagi M, et al. 2008 Biphasic response of pancreatic beta-cell mass to ablation of tuberous sclerosis complex 2 in mice. *Molecular and Cellular Biology* **28** 2971–2979. (doi:10.1128/MCB.01695-07)
- Tachibana K, Sakurai K, Yokoh H, Ishibashi T, Ishikawa K, Shirasawa T & Yokote K 2015 Mutation in insulin receptor attenuates oxidative stress and apoptosis in pancreatic beta-cells induced by nutrition

- excess: reduced insulin signaling and ROS. *Hormone and Metabolic Research* **47** 176–183. (doi:10.1055/s-0034-1389990)
- Tersey SA, Maier B, Nishiki Y, Maganti AV, Nadler JL & Mirmira RG 2014 12-lipoxygenase promotes obesity-induced oxidative stress in pancreatic islets. *Molecular and Cellular Biology* **34** 3735–3745. (doi:10.1128/MCB.00157-14)
- Tiedge M, Lortz S, Drinkgern J & Lenzen S 1997 Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* **46** 1733–1742. (doi:10.2337/diab.46.11.1733)
- Tseng YH, Ueki K, Kriauciunas KM & Kahn CR 2002 Differential roles of insulin receptor substrates in the anti-apoptotic function of insulin-like growth factor-1 and insulin. *Journal of Biological Chemistry* **277** 31601–31611. (doi:10.1074/jbc.M202932200)
- Vanderford NL 2010 Defining the regulation of IL-1 β - and CHOP-mediated beta-cell apoptosis. *Islets* **2** 334–336. (doi:10.4161/isl.2.5.13095)
- Vasu S, McClenaghan NH, McCluskey JT & Flatt PR 2014 Mechanisms of toxicity by proinflammatory cytokines in a novel human pancreatic beta cell line 1.1B4. *Biochimica et Biophysica Acta* **1840** 136–145. (doi:10.1016/j.bbagen.2013.08.022)
- Wali JA, Gurzov EN, Fynch S, Elkerbout L, Kay TW, Masters SL & Thomas HE 2014 Activation of the NLRP3 inflammasome complex is not required for stress-induced death of pancreatic islets. *PLoS ONE* **9** e113128. (doi:10.1371/journal.pone.0113128)
- Walter P & Ron D 2011 The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334** 1081–1086. (doi:10.1126/science.1209038)
- Wang Q & Jin T 2009 The role of insulin signaling in the development of beta-cell dysfunction and diabetes. *Islets* **1** 95–101. (doi:10.4161/isl.1.2.9263)
- Yang ZH, Inoue S, Taniguchi Y, Miyahara H, Iwasaki Y, Takeo J, Sakaue H & Nakaya Y 2015 Long-term dietary supplementation with saury oil attenuates metabolic abnormalities in mice fed a high-fat diet: combined beneficial effect of omega-3 fatty acids and long-chain monounsaturated fatty acids. *Lipids in Health and Disease* **14** 155. (doi:10.1186/s12944-015-0161-8)

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