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 H2O2 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.

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 P13-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 H2O2 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 (24–48h) 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-H2DCFDA) 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-1β and IFN-γ were provided by Prospect-Tany Technogene (Rehovot, Israel). Cytokine mixture (1x-cytomix) consisted of 3nM TNF-α, 3nM IFN-γ and 1.5nM IL-1β (biological activity: 10U/ng (TNF-α, IFN-γ) and 200U/ng (IL-1β)). Human insulin ELISA kits was purchased from Merodia (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, 1mM sodium pyruvate and 1% Pen-Strep. MIN6 cells (passages 12–32) were grown in DMEM containing 15% FCS, 11.1 mM glucose, 2mM l-glutamine, 1% Pen-Strep and 60μM 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% CO2 humidified atmosphere at 37°C. The medium was changed every 2–3days. At 80% confluence, cells were transferred to 12-well plates and seeded at 5×10⁴ cells/mL in serum- and glucose-free medium. After 24h, cells were treated with H2O2 (70μM) and insulin (100nM), alone or in combination, for 24–48h.
Isolation of murine islets

Digested pancreases were filtered through 1000 and 500 μm sieves, and islets >75 and <250 μm 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 μg/mL streptomycin and 40 μg/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% CO₂ humidified atmosphere in CMRL 1066 medium containing 10% (vol./vol.) FBS, 2 mM l-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin and 40 μg/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 KRBBH buffer (124 mmol/L NaCl, 5.6 mmol/L KCl, 2.5 mmol/L CaCl₂, 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

10⁵–10⁶ cells/mL were seeded in 24-well plates. Cells were washed, treated with insulin and H₂O₂, 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 μg 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 μL 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 μM CM-H₂DCFDA 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 x 10⁵/mL) were grown on cover slips in multiwell plates, treated with the CM-H₂DCFDA reagent (20 μM), 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: mNos: forward 5′-GCCCTGCTTTGTGCGAAGTG-3′, reverse 5′-GCCGTGATCACCTCTCAACC-3′; mTib3: forward 5′-GTAGAGGGAACCTTTGCGCTG-3′, reverse 5′-GCCGTGATCCTCTCTCAACC-3′; mChop: forward 5′-CCTCGACCAGTCGGGTTTG-3′, reverse 5′-GCCGTGATCCTCTCTCAACC-3′; mAtf4: forward 5′-GCCCTGGCTTGTGCGAAGTG-3′, reverse 5′-ATGACC-CGCTGATCAAAGTC-3′; miNos: forward 5′-GCCCTGCTTTGTGCGAAGTG-3′, reverse 5′-ggggttgctaggctgcttgga-3′. Data were normalized for the content of actin mRNA.

Statistical analyses

Results are presented as the mean ± 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 H2O2, as determined by increased LDH release (Sampson et al. 2010). In initial experiments on MIN6 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, H2O2 in the range of 25–100 μM, given for 24 h, induced a significant increase in cell death (as measured by LDH release). These concentrations of H2O2 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 μM H2O2 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 H2O2 (50 μM) significantly increased LDH release. Insulin increased LDH release by nearly 2.7-fold, compared with control untreated cells, while 50 μM H2O2 increased LDH release four-fold. Moreover, the effects of insulin and H2O2 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 H2O2, 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 × 10⁵/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 ± 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 × 10⁵/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 x100 (Tri) was administered as a 
control for maximum effects on LDH release and 
viability. Each bar represents the mean ± 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 H₂O₂ (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 H₂O₂. 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 H₂O₂ (25 µM), 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 ± 2% of untreated, control islets, while H₂O₂ 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 H₂O₂ 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 H₂O₂ (50 µM), alone or in combination for 24 h. Islets’ reducing power was then measured using the CTB reagent. Results are mean ± S.E.M. of replicate (3 or more) values obtained in three experiments (*P<0.01, **P<0.001 vs control; ***P<0.01 H₂O₂/Ins vs H₂O₂ 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 ± 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
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 (100nM) in the presence or absence of the caspase inhibitor z-VAD-fmk (20μM) for 24h. Figure 6A shows that insulin significantly increased caspase 3/7 activity compared with untreated control cells, and this 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 (100nM) or a cytokine mixture (3nmol/L TNFα, 3nmol/L
IFN-γ and 1.5 nmol/L IL-1β; (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 24h 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 RI Nm 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
Insulin-induced β cell death 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 cysnteine (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.
Insulin-induced β cell death

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 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),
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Figure 11
Insulin increases ROS production in MIN6 cells. MIN6 cells were plated at a density of 2.5–3 × 10⁵/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. (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 5–6 × 10⁵/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 ± 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 enzymes. MIN6 cells were plated at a density of 2.5–3 × 10⁵/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 ± 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).

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...
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 10nM in human islets compared with 100nM 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: IRE1α, double-stranded RNA-dependent protein kinase-like kinase (PERK) and ATF6α (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 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

Figure 13
Insulin alters the expression of ER stress genes in MIN6 cells. MIN6 cells were plated at a density of 2.5–3 × 10⁶/mL in 12-well plates and, after 16 h incubation, were treated with insulin (100nM) 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 mix to elevate Trb3 expression. In all graphs, bars represent the mean ± 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).
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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 H₂O₂ 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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