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ER stress and SREBP-1 activation are implicated in β-cell glucolipotoxicity

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Summary

The reduction in insulin secretory capacity and β-cell mass observed in type 2 diabetes is thought to be caused by glucolipotoxicity secondary to hyperglycemia and hyperlipidemia. Our aim in this study was to elucidate the underlying molecular mechanisms. We found a strong correlation between chronic high-glucose treatment and SREBP-1c activation in INS-1 cells and rat islets. Both high-glucose treatment and SREBP-1c activation in INS-1 cells resulted in lipid accumulation, impaired glucose-stimulated insulin secretion, apoptosis, and strikingly similar gene expression patterns, including upregulation of lipogenic and pro-apoptotic genes and downregulation of *IRS2*, *Bclxl* and *Pdx1*. These lipotoxic effects of high glucose were largely prevented by induction of a dominant-negative mutant of SREBP-1c, suggesting SREBP-1c is a major factor responsible for β cell glucolipotoxicity. Moreover, overexpression of another lipogenic transcription factor, ChREBP, in INS-1 cells did not cause lipotoxicity. Intriguingly, chronic high glucose treatment in INS-1 cells led to pronounced induction of the ER stress marker genes, *BIP* and *Chop10*. Treatment of rat islets with both chronic high glucose and two ER stress inducers, thapsigargin and tunicamycin, enhanced SREBP-1 binding to the human IRS2 promoter. These results suggest that SREBP-1 activation caused by ER stress is implicated in β-cell glucolipotoxicity.

Key words: ER stress, SREBP-1c, Glucolipotoxicity, β cell

Introduction

The reduction in insulin secretary capacity and β-cell mass observed in type 2 diabetes is thought to be caused at least in part by hyperglycemia and hyperlipidemia (Butler et al., 2003; Unger, 2002; Unger and Zhou, 2001; Unger et al., 1999; Weir et al., 2001). Hyperglycemia is proposed as the prerequisite for hyperlipidemia-induced β-cell lipotoxicity (Bonner-Weir et al., 1983; Harmon et al., 2001; Poitout and Robertson, 2002; Roche et al., 1998; Weir, 1982; Weir et al., 2001). Over accumulation of lipids in non-adipose tissues such as the pancreatic islets, liver and heart is often associated with type 2 diabetes and its complications (Unger, 2002; Unger and Zhou, 2001; Unger et al., 1999). However, the molecular mechanism underlying the pathogenesis of lipotoxicity in pancreatic β cells and other non-adipose tissues remain undefined. Lipogenic gene expression in adipocytes and liver are known to be regulated by several transcription factors including sterol regulatory element binding proteins (SREBPs), carbohydrate-response element binding protein (ChREBP), CCAAT-enhancer binding proteins (C/EBP), and peroxisome proliferator-activated receptors (PPAR) (Dentin et al., 2004; Iizuka et al., 2004; Ishii et al., 2004; Loftus and Lane, 1997). SREBP are transmembrane proteins of the endoplasmic reticulum (ER). In response to low sterol and other unidentified factors, SREBP cleavage-activating protein (SCAP) escorts SREBPs from the ER to the Golgi, where SREBPs are sequentially cleaved by site-1 protease (S1P) and site-2 protease (S2P). The processed mature SREBPs enter the nucleus and transactivate target genes (Brown and Goldstein, 1997). Three SREBP isoforms have been identified: SREBP-1a and -1c (alternatively known as adipocyte determination and differentiation factor-1; ADD-1) (Tontonoz et al., 1993) that are derived from the same gene through alternative splicing, and SREBP-2 that is encoded by a distinct gene (Brown and Goldstein, 1997). SREBPs are expressed ubiquitously, play an essential role in regulation of lipid homeostasis in animals and have been shown to directly activate the expression of more than 30 genes dedicated to the biosynthesis of cholesterol, fatty acids, triglycerides and phospholipids (Horton et al., 2002).

Elevated expression of SREBP-1c has been demonstrated in islets and liver of many animal models of diabetes (Kakuma et al., 2000; Lin et al., 2000; Matsuzaka et al., 2004; Riddle et al., 2001; Shimomura et al., 1999a; Shimomura et al., 1999b; Shimomura et al., 1999c; Shimomura et al., 2000; Sun et al., 2002; Takaishi et al., 2004; Tobe et al., 2001; Ueki et al., 2004; Unger and Zhou, 2001; Unger et al., 1999; Werstuck et al., 2001; Xu et al., 2004; Yahagi et al., 2002; You et al., 2002) and patients with severe lipodystrophy (Bastard et al., 2002; Petersen et al., 2002). Lipid accumulation, impaired glucose-stimulated insulin secretion, defective β-cell gene expression (insulin, *Pdx1*, glucokinase and *Glut2*), disorganized mitochondrial ultrastructure and 'lipoapoptosis' have been reported in the β cells of diabetic animals (Unger and Zhou, 2001; Unger et al., 1999). Moreover, preventing SREBP-1c overexpression and activation is common to leptin, metformin, adiponectin and PPARγ agonists. Thus there is a good

correlation between suppression of SREBP-1c function and antidiabetic effects of these agents (Kakuma et al., 2000; Lin et al., 2000; Petersen et al., 2002; Shimomura et al., 1999a; Shimomura et al., 1999b; Shimomura et al., 1999c; Tobe et al., 2001; Unger and Zhou, 2001; Unger et al., 1999; Wang, M. Y. et al., 1998; Xu et al., 2004; Zhou et al., 2001). We and others have demonstrated that overexpression of a nuclear active form of SREBP-1c (naSREBP-1c) in insulinoma (INS-1 and MIN6) cells and isolated rat islets results in β-cell lipotoxicity (Andreolas et al., 2002; Diraison et al., 2004; Wang et al., 2003; Yamashita et al., 2004). We have previously shown that long-term exposure of INS-1 cells to 30 mM glucose generated the mature nuclear form of SREBP-1c (Wang et al., 2003). Glucose also raises SREBP-1c mRNA levels in rat β cells (Flamez et al., 2002). It is well known that chronic high glucose treatment causes glucolipotoxicity in both insulinoma cells and rat islets (Efanova et al., 1998; Roche et al., 1998). We hypothesise that SREBP-1c activation could play an essential role in development of β-cell glucolipotoxicity. To further substantiate this notion we established an INS-1 stable cell line that allowed suppression of SREBP-1c function through induction of a dominant-negative mutant of SREBP-1c (DN-SREBP-1c) (Kim and Spiegelman, 1996). Strong experimental evidence demonstrated that dominant-negative suppression of SREBP-1c activity markedly prevented β-cell glucolipotoxicity. In addition, we found that ER-stress-generated SREBP-1c processing and activation could be the underlying mechanism in β-cell glucolipotoxicity.

Materials and Methods

Cell culture

The standard rat insulinoma INS-1E cells and two previously established INS-1-derived stable cell lines, ChREBP*16 (Wang and Wollheim, 2002) and naSREBP-1c*233 (Wang et al., 2003), allowing inducible expression, respectively, of ChREBP and a nuclear active form of SREBP-1c, were cultured in RPMI1640 containing 11.2 mM glucose (Asfari et al., 1992), unless otherwise indicated.

Establishment of INS-1 cells permitting inducible expression of DN-SREBP-1c

The first step stable INS-rβ (also refer to as r9) cell line, which carries the reverse tetracycline/doxycycline-dependent transactivator (Gossen et al., 1995), was described previously (Wang and Iynedjian, 1997; Wang et al., 2001). The plasmid used in the secondary stable transfection was constructed by subcloning the cDNA encoding the dominant-negative form of SREBP-1c (DN-SREBP-1c/ADD1 1-403(Y320A) [(Kim and Spiegelman, 1996) kindly supplied by B.M. Spiegelman] into the expression vector PUHD10-3 [(Gossen et al., 1995) a generous gift from H. Bujard]. The procedures for stable transfection, clone selection and screening were described previously (Wang and Iynedjian, 1997).

Immunofluorescence

Cells grown on polyornithine-treated glass coverslips were treated for 24 hours with or without 500 ng/ml doxycycline. Cells were then washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 1% BSA (PBS-BSA). The preparation was then blocked with PBS-BSA before incubating with the first antibody, anti-SREBP-1 (Santa Cruz, Basel, Switzerland; 1:100 dilution), followed by the second antibody labelling.

Nuclear protein extraction and electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from INS-1 cells were prepared according to the method of Schreiber et al. (Schreiber et al., 1988). Rat islets were isolated by collagenase digestion as described previously (Rubi et al., 2001) and their nuclear proteins were extracted as previously reported (Schreiber et al., 1988). The double-stranded oligonucleotides corresponding to the sterol regulatory element (SRE) in the human insulin receptor substrate 2 (IRS2) promoter (Ide et al., 2004), 5′cctgcgtaacgccgagtcacatgttgtt3′, was used as a probe. EMSA procedures, including conditions for probe labelling and binding reactions were performed as in Wang et al. (Wang, H. et al., 1998). Mouse monoclonal antibodies against SREBP-1 (NeoMarkers, Fremont CA, USA) and SREBP-2 (purified from the supernatant of the hybridoma cell line CRL2121, American Tissue Culture Collection, Rockville, MD, USA) were used for supershift experiments.

Staining of lipid accumulation by Oil Red O

Cells were cultured in standard (11.2 mM) or 30 mM glucose medium in the presence or absence of 500 ng/ml doxycycline for 48 hours. Cells were fixed and stained as previously reported (Kim and Spiegelman, 1996). Lipid droplets were visualized using phase-contrast microscopy (Nikon Diaphot).

Measurements of insulin secretion and cellular insulin content

Insulin secretion in INS-1E or DN-SREBP-1c#23 cells was measured in 24-well plates over a period of 30 minutes, in Krebs-Ringer-bicarbonate-Hepes buffer (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM Hepes, 0.1% BSA) containing the indicated concentrations of glucose. Cellular insulin content was determined after extraction with acid ethanol following the procedures of Wang et al. (Wang, H. et al., 1998). Insulin was detected by radio-immunoassay using rat insulin as a standard (Wang, H. et al., 1998).

Total RNA isolation and northern blotting

Total RNA was extracted and blotted to nylon membranes as described previously (Wang and Iynedjian, 1997). The membrane was prehybridized and then hybridized to ^{32}P-labelled random primer cDNA probes according to the method of Wang and Iynedijian (Wang and Iynedjian, 1997). To ensure equal RNA loading and even transfer, all membranes were stripped and re-hybridized with a ‘house-keeping gene’ probe, cyclophilin. cDNA fragments used as probes for SREBP-1c, ChREBP, glucokinase, GLUT2, L-pyruvate kinase, insulin, BIP, CHOP10 and PDX1 mRNA detection were digested from the corresponding plasmids. cDNA probes for rat mitochondrial uncoupling protein 2 (UCP2), aldolase B, fatty acid synthase, acetyl-CoA carboxylase, glycerol-phosphate acyltransferase, HMG-CoA reductase, $P21^{WAF1/CIP1}$, BAD, APO1, Bcl_{XL}, IRS2 and low density lipoprotein receptor (LDLR), were prepared by RT-PCR and confirmed by sequencing.

Cell proliferation/viability and apoptosis

Quantification of cell proliferation/viability was measured using a Quick Cell Proliferation Assay Kit (LabForce/MBL, Nunningen, Switzerland) according to manufacturer’s protocol. This assay is based on the cleavage of a tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases. Expansion in the number of viable cells results in an increase in the overall activity of the mitochondrial dehydrogenases and subsequently an augmentation in the amount of formazan dye formed. The formazan dye produced by viable cells was quantified with a multiwell spectrophotometer by measuring the

absorbance at 440 nm. Experiments for DNA fragmentation were performed using a Quick Apoptosis DNA Ladder Detection Kit (LabForce/MBL, Nunningen, Switzerland) following the manufacturer's protocol.

Statistics

Results are expressed as mean ± s.e.m. and statistical analyses were performed using Student's *t*-test.

Results

Effects of chronic high-glucose treatment on insulin secretion and β-cell gene expression in INS-1E cells

As shown in Fig. 1A, 30 mM glucose treatment of INS-1E cells for 48 hours resulted in reduced glucose- and leucine-stimulated insulin secretion. In contrast, KCl-depolarization-elicited insulin release was unaffected, suggesting that Ca^{2+}-stimulated exocytosis was unaffected. We could also confirm, as previously reported (Roche et al., 1998), that chronic high glucose treatment led to lipid accumulation and INS-1 cell apoptosis (see below). To explore the underlying mechanism, quantitative northern blotting was performed to investigate the gene expression patterns in INS-1E cells treated for 48 hours with 30 mM glucose. The mRNA levels of essential β-cell genes such as insulin, *Pdx1*, *Glut2*, glucokinase and *IRS2* were drastically suppressed by high-glucose treatment (Fig. 1B). In addition, pro-apoptotic genes, *P21*, *Bad* and *APO-1* were upregulated, whereas the anti-apoptotic gene *Bclxl* was downregulated (Fig. 1B). Furthermore, lipogenic genes, fatty acid synthase, acetyl-CoA carboxylase, glycerol-phosphate acyltransferase, HMG-CoA reductase and LDL receptor were all markedly induced by high glucose treatment (Fig. 1C). Similar alterations in all genes mentioned above have been reported in an INS-1 stable cell line expressing naSREBP-1c (Wang et al., 2003). This strikingly similar gene expression profile suggested that chronic high glucose could cause β-cell glucolipotoxicity through activation of SREBP-1c.

However, we have also reported that the transcription, nuclear translocation and DNA-binding activity of ChREBP are regulated by glucose in INS-1 cells (Wang and Wollheim, 2002). As demonstrated in Fig. 1C, mRNA levels of ChREBP, L-PK and aldolase B were markedly increased by high glucose. ChREBP is known to regulate lipogenic gene expression in the liver (Dentin et al., 2004; Iizuka et al., 2004; Ishii et al., 2004), we therefore examined whether it is also involved in β-cell glucolipotoxicity.

SREBP-1c rather than ChREBP is implicated in glucolipotoxicity in INS-1 cells

Differential gene expression patterns in INS-1 cells induced by ChREBP and naSREBP-1c are illustrated in Fig. 2A,B. Quantitative northern blotting was performed in two stable INS-1 cell lines previously established to express, respectively,

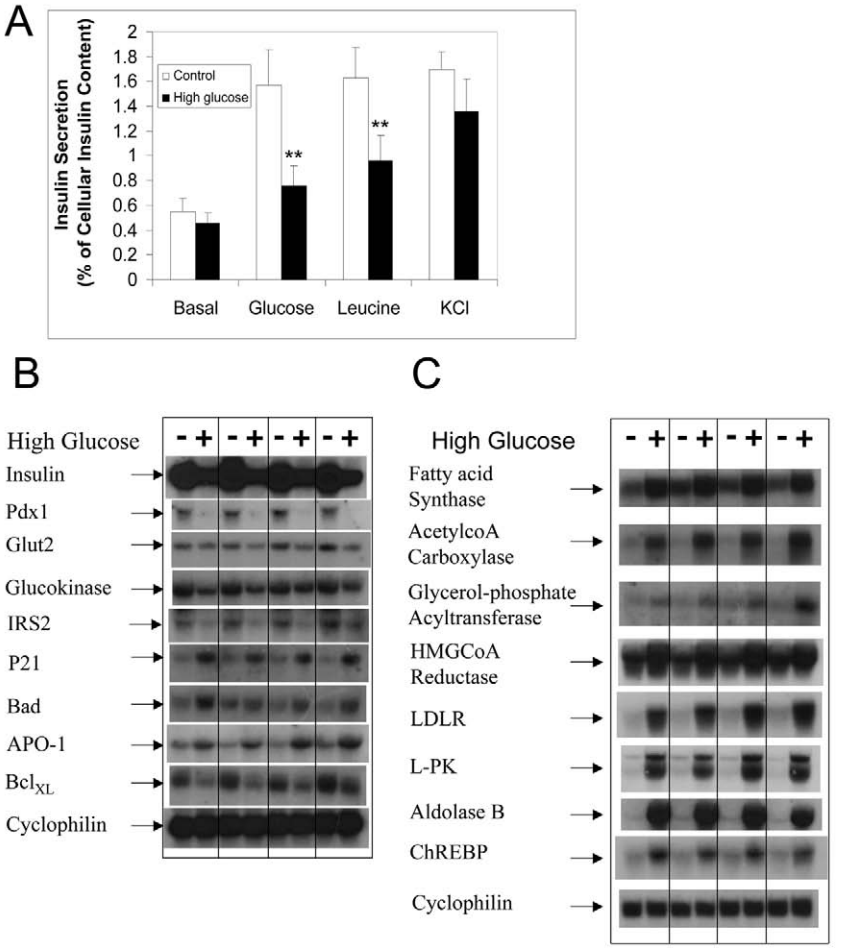

Fig. 1. Effects of chronic high glucose treatment on insulin secretion and gene expression in INS-1E cell. (A) INS-1E cells in 24-well dishes were cultured in either standard (11.2 mM) or 30 mM glucose medium for 48 hours. After 5 hours equilibration in 2.5 mM glucose medium, cells were washed twice with KRBH. Insulin release from INS-1E cells stimulated with 20 mM glucose, 20 mM leucine and 20 mM KCl in KRBH buffer containing 2.5 mM (referred to Basal) was determined by radio-immunoassay and expressed as a percentage of cellular insulin content. The equilibration period is necessary to see the glucose-responsiveness of insulin release in INS-1 cells. Cellular insulin content was decreased by 32.4±6.7% after 48 hours treatment with 30 mM glucose. Data represent mean ± s.e.m. of six independent experiments. $^{**}P<0.001$. (B,C) The gene expression patterns in INS-1E cells cultured in either standard (11.2 mM) or 30 mM glucose medium for 48 hours were quantified by northern blotting. Total RNA samples (20 μg/lane) were analysed by hybridizing with the indicated cDNA probes. Four independent experiments are shown side by side to demonstrate the consistency of the results.

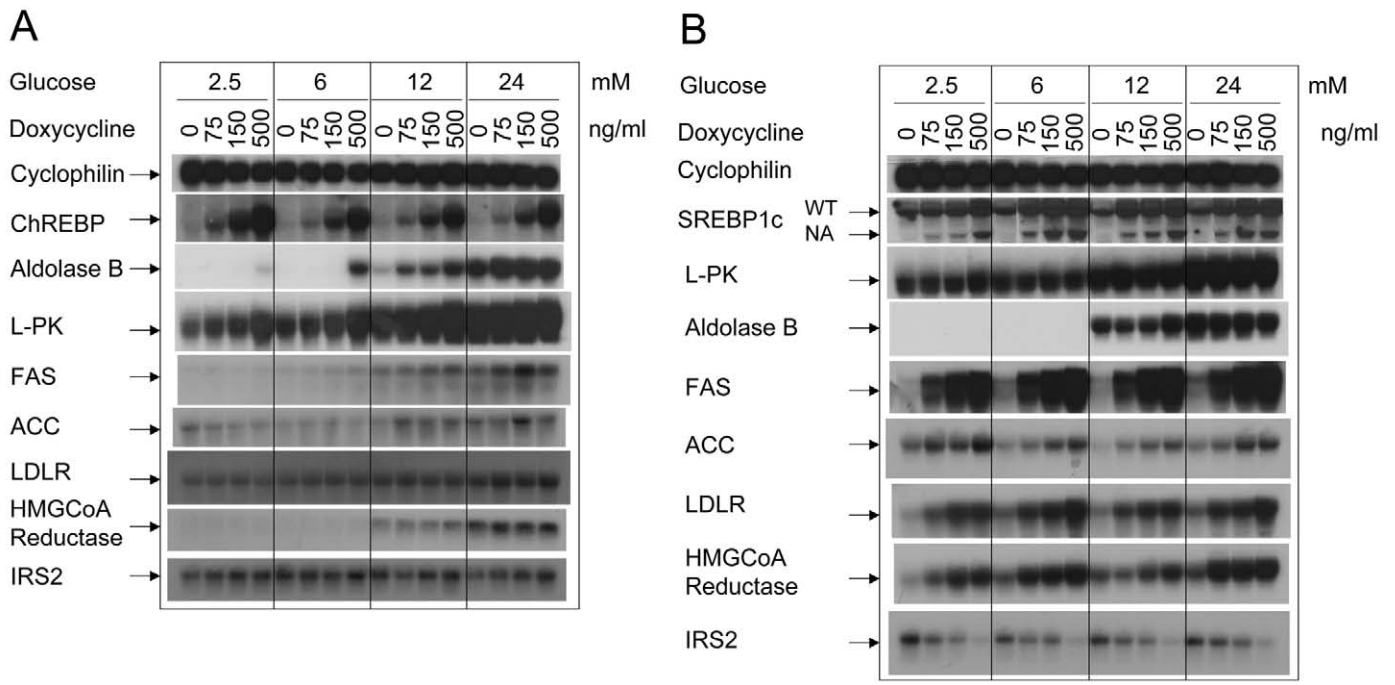

Fig. 2. Comparison of gene expression patterns in INS-1 cells allowing inducible expression, respectively, of ChREBP and the nuclear active form (na) of SREBP-1c. (A) ChREBP*16 cells were cultured in standard medium (11.2 mM glucose) with 0, 75, 150 or 500 ng/ml doxycycline for 24 hours. The culture was continued in 2.5 mmol/l glucose medium with the indicated concentrations of doxycycline for 16 hours, followed by an additional 8 hours in culture medium with 2.5, 6, 12 and 24 mM glucose and with or without doxycycline. The equilibration period is necessary to see the glucose-responsiveness of gene expression. (B) SREBP-1c*233 cells were cultured in 2.5 mM glucose medium with the indicated concentrations of doxycycline for 16 hours, followed by an additional 8 hours in culture medium with 2.5, 6, 12 and 24 mM glucose and with or without doxycycline. SREBP-1c was induced for a total of 24 hours to avoid its apoptotic effects. Gene expression patterns in these cells were evaluated by quantitative northern blotting. 20 μg total RNA samples were analysed by hybridising with indicated cDNA probes. WT, the endogenous wild-type SREBP-1c; NA, the induced nuclear active form of SRENP-1c. The experiments were repeated two times with similar results.

ChREBP (Wang and Wollheim, 2002) and naSREBP-1c (Wang et al., 2003) in a doxycycline-dependent manner. The results displayed in Fig. 2A indicate that ChREBP predominantly targeted L-PK and aldolaseB, although induction of ChREBP also slightly enhanced the expression of fatty acid synthase and acetyl-CoA carboxylase. In contrast, induction of SREBP-1c drastically increased the lipogenic gene expression for proteins such as, fatty acid synthase, acetyl-CoA carboxylase, HMG-CoA reductase and LDL receptor, whereas it did not affect the mRNA levels of L-PK and aldolase B (Fig. 2B). It is noteworthy that the function of SREBP-1c may also overlap with the function of SREBP-2 on target gene expression in INS-1 cells since HMG-CoA reductase is regulated by naSREBP-1c. In addition, the expression of IRS2 was suppressed by naSREBP-1c but unaffected by ChREBP. It has been reported recently that SREBP-1c binds to the human IRS2 promoter and suppresses its activity in rat and mouse hepatocytes (Ide et al., 2004).

Induction of ChREBP in INS-1 cells did not affect glucose-stimulated insulin secretion or cell growth. In contrast to the effect of naSREBP-1c, induction of ChREBP with 500 ng/ml doxycycline for 48 hours did not alter glucose-stimulated insulin secretion or cellular insulin content. To assess the impact of ChREBP on INS-1 cell growth and viability, we performed the WST-1 assay (Fig. 3B). This assay is based on the cleavage of a tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases. Expansion in the number of viable cells results in an increase in the overall activity of the mitochondrial dehydrogenases and subsequently an augmentation in the amount of formazan dye formed. Unlike overexpression of naSREBP-1c (Wang et al., 2003), maximum induction of ChREBP for 48 hours did not cause INS-1 cell growth arrest (Fig. 3B) or apoptosis as assessed by DNA fragmentation experiments (data not shown). We have previously shown that similar induction of SREBP-1c caused apoptosis in INS-1 cells (Wang et al., 2003).

Establishment of INS-1 stable cell line permitting inducible expression of DN-SREBP-1c

To further substantiate the implication of SREBP-1c in chronic high-glucose-induced glucolipotoxicity, we generated an INS-1 stable cell line expressing DN-SREBP-1c, called DN-SREBP-1c*23 cells. This mutant protein contains an intact dimerization domain but lacks DNA-binding activity (Kim and Spiegelman, 1996). DN-SREBP-1c therefore exerts its dominant-negative function by forming non-functional heterodimers with endogenous SREBPs (Kim and Spiegelman, 1996). Immunofluorescence staining with an antibody against the N terminus of SREBP-1c demonstrated that DN-SREBP-1c protein was induced by doxycycline in an all-or-none manner (Fig. 4A).

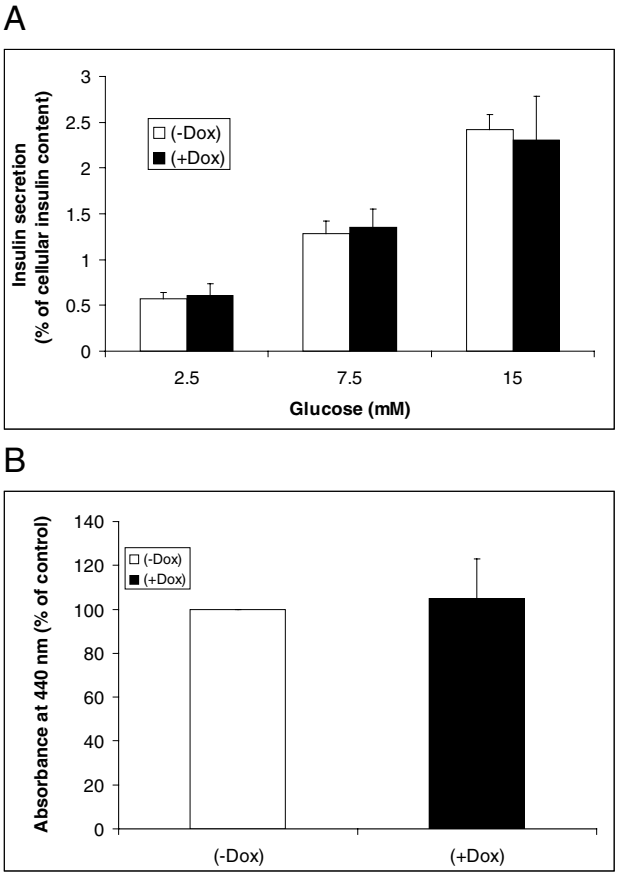

Fig. 3. Overexpression of ChREBP did not affect glucose-stimulated insulin secretion or cell growth. (A) ChREBP*16 cells were cultured with (+Dox) or without (–Dox) 500 ng/ml doxycycline in standard medium (11.2 mM glucose) for 24 hours and then equilibrated in 2.5 mM glucose medium for a further 24 hours. Insulin release from ChREBP*16 cells in KRBH buffer containing the indicated concentrations of glucose was determined by radio-immunoassay and expressed as a percentage of cellular insulin content. Data represent six independent experiments. (B) A WST-1 assay, presented as optical density at 440 nm, showed that ChREBP*16 cell growth/survival was not affected by 48 hours treatment with 500 ng/ml doxycycline.

Effects of DN-SREBP-1c on chronic high-glucose-induced glucolipotoxicity in INS-1 cells

Induction of DN-SREBP-1c abolished chronic high-glucose-induced SREBP-1 binding to the IRS2 promoter. DN-SREBP-1c*23 cells were cultured in either standard (11.2 mM) or 30 mM glucose medium for 48 hours, in the presence (+Dox) or absence (–Dox) of 500 ng/ml doxycycline. EMSA with the SRE element of the human IRS2 promoter (Ide et al., 2004) showed that SREBP protein binding was increased 10-fold by 48 hours treatment with 30 mM glucose, which was diminished by 80% after induction of DN-SREBP-1c (Fig. 4B). The specificity of SREBP-1 binding complexes was confirmed by the observed supershift with a monoclonal anti-SREBP-1 antibody (Fig. 4B). However, we could not exclude binding activity of SREBP-2 to the IRS2-SRE-element, since it would also be inhibited by DN-SREBP-1c (Rishi et al., 2004). Indeed, monoclonal anti-SREBP-2 antibody also caused a supershift of the retarded complex but to a much lesser extent than the SREBP-1-specific antibody (Fig. 4C). These results suggest that SREBP-1 accounts for the majority of the glucose-induced binding activity to the IRS2 promoter.

Induction of DN-SREBP-1c diminished chronic high-glucose-induced lipid accumulation, apoptosis and impaired insulin secretion in INS-1 cells. As shown by Oil-Red-O staining (Fig. 5A), 30 mM glucose treatment of DN-SREBP-1c*23 cells for 48 hours resulted in massive lipid accumulation, which was largely prevented by induction of DN-SREBP-1c. Similarly, treatment of DN-SREBP-1c*23 cells for 48 and 72 hours with 30 mM glucose caused typical DNA fragmentation (Fig. 5B), a characteristic hallmark of cells undergoing apoptosis. DN-SREBP-1c largely protected INS-1 cells from apoptosis (Fig. 5B).

In agreement with the results obtained in INS-1E (Fig. 1A), treatment of DN-SREBP-1c*23 cells with 30 mM glucose for 48 hours also led to reduced glucose-stimulated insulin secretion (Fig. 5C). The glucose-stimulated insulin secretion was partially restored by expression of DN-SREBP-1c (Fig. 5C). Unexpectedly, induction of DN-SREBP-1c slightly increased basal (2.5 mM) insulin release in control cells (Fig. 5C).

DN-SREBP-1c corrected defective gene expression in INS-1 cells treated with chronic high glucose

As demonstrated in Fig. 6, treatment of DN-SREBP-1c*23 cells with 30 mM glucose for 48 hours increased the mRNA levels of L-PK, aldolase B and ChREBP, which were not affected by induction of DN-SREBP-1c. These results suggest that high glucose may promote the expression of L-PK and aldolase B through a SREBP-1c-independent but ChREBP-dependent pathway. In contrast, the protective effects of DNSREBP-1c in high-glucose-treated DN-SREBP-1c*23 cells revealed the SREBP-1c-dependent gene expression patterns, including upregulation of fatty acid synthase, HMG-CoA reductase, LDLR and P21, as well as downregulation of GLUT2, glucokinase, PDX1, IRS2 and BCL_{XL} (Fig. 6). These data further substantiate the notion that SREBP-1c-target genes are implicated in chronic high-glucose-mediated β-cell glucolipotoxicity. *Ucp2* is also a downstream target gene of SREBP-1c in INS-1 cells (Wang et al., 2003; Yamashita et al., 2004). Interestingly, induction of DN-SREBP-1c suppressed UCP2, which may account for the increased basal insulin secretion observed (Fig. 5C).

ER-stress and SREBP-1 activation could be the molecular mechanisms underlying β-cell glucolipotoxicity

Increased expression of GADD153/CHOP10 and BIP are characteristics of ER stress response in many cell types. We found that treatment of INS-1E cells for 48 hours with 30 mM glucose markedly induced these ER stress marker genes (Fig. 7A). Therefore, chronic high-glucose-induced ER stress could be the underlying mechanism involved in SREBP-1 activation and subsequently INS-1 cell glucolipotoxicity, since it has been well documented that ER stress causes SREBP processing through S1P and S2P (Werstuck et al., 2001; Ye et al., 2000).

A similar mechanism may also apply to islet β cells. The

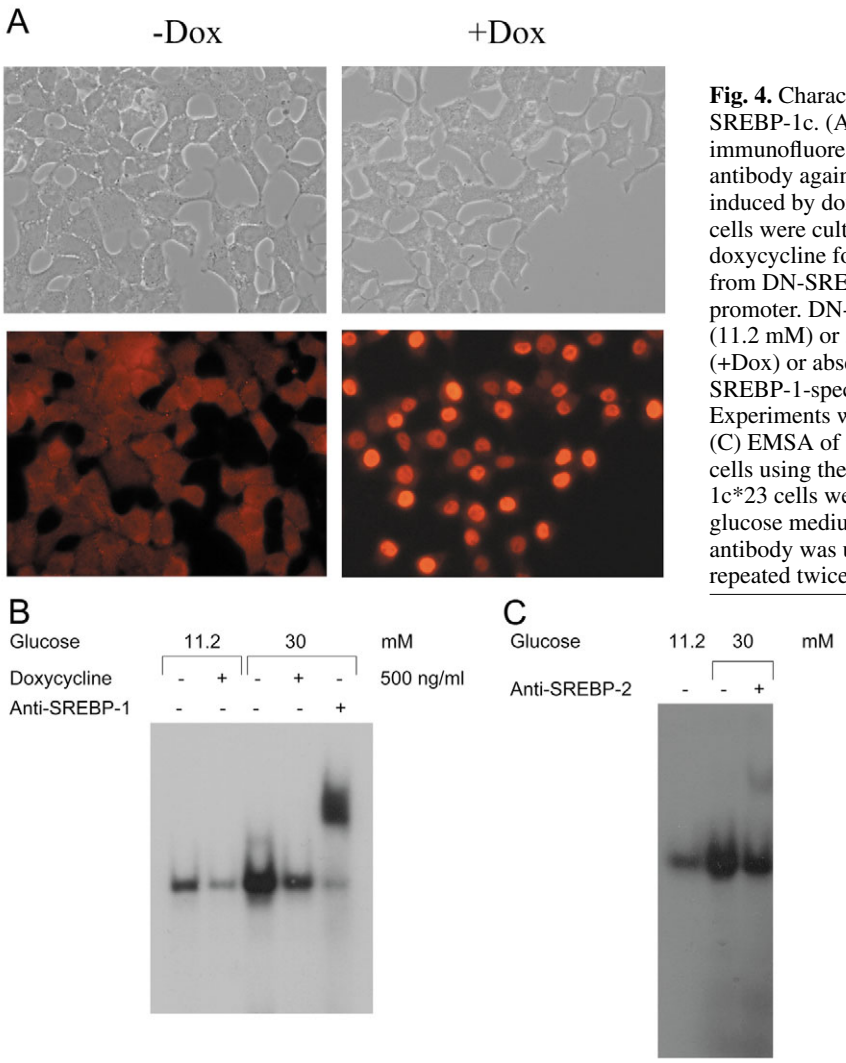

Fig. 4. Characteristics of the INS-1 stable cell line expressing DN-SREBP-1c. (A) Phase-contrast images (top) and immunofluorescence staining (bottom) with a rabbit polyclonal antibody against SREBP-1 showed that DN-SREBP-1c protein was induced by doxycycline in an all-or-none manner. DN-SREBP-1c*23 cells were cultured with (+Dox) or without (–Dox) 500 ng/ml doxycycline for 48 hours. (B) Gel-shift assay of nuclear extracts from DN-SREBP-1c*23 cells using the SRE sequence of the IRS2 promoter. DN-SREBP-1c*23 cells were cultured in either standard (11.2 mM) or 30 mM glucose medium for 48 hours, in the presence (+Dox) or absence (–Dox) of 500 ng/ml doxycycline. Monoclonal SREBP-1-specific antibody was used for the supershift experiment. Experiments were repeated two to three times with similar results. (C) EMSA of nuclear extracts from non-induced DN-SREBP-1c*23 cells using the SRE sequence of the IRS2 promoter. DN-SREBP-1c*23 cells were cultured in either standard (11.2 mM) or 30 mM glucose medium for 48 hours. Monoclonal SREBP-2-specific antibody was used for the supershift experiment. Experiments were repeated twice with similar results.

EMSA shown in Fig. 7B indicates that treatment of isolated rat islets with either chronic high glucose or two ER stress inducers, thapsigargin and tunicamycin, drastically enhanced SREBP-1 binding to the SRE sequence of the IRS2 promoter. The specificity of retarded SREBP-1 complexes was confirmed by the supershift in the presence of the monoclonal antibody against SREBP-1 (Fig. 7B).

Discussion

It is well known that chronic high-glucose treatment in vitro causes β-cell glucolipotoxicity, including lipid accumulation, impaired glucose-stimulated insulin secretion and apoptosis (Efanova et al., 1998; Roche et al., 1998). These characteristics resemble the description of β-cell lipotoxicity in *ob/ob* mice and Zucker Diabetic Fatty (ZDF) rats (Unger, 2002; Unger and Zhou, 2001). Overexpression or overactivation of SREBP-1c in the islets of these animals has been proposed as the causal factor (Unger, 2002; Unger and Zhou, 2001). Consistently, overexpression of a nuclear active form of SREBP-1c in insulinoma cells (INS-1 and MIN6) and isolated rat islets results in typical characteristics of β-cell glucolipotoxicity (Andreolas et al., 2002; Diraison et al., 2004; Wang et al., 2003; Yamashita et al., 2004). Some investigators refer to lipotoxicity as the consequence of increased circulating free fatty acids and cellular lipid accumulation (Unger, 2002; Unger and Zhou, 2001; Unger et al., 1999), whereas others suggest that glucotoxicity is the prerequisite for lipotoxicity, at least in β cells (Bonner-Weir et al., 1983; Harmon et al., 2001; Poitout and Robertson, 2002; Roche et al., 1998; Weir, 1982; Weir et al., 2001). Therefore, glucolipotoxicity is a more proper term to describe β cell dysfunction in type 2 diabetes.

To elucidate the molecular mechanisms underlying β-cell glucolipotoxicity, we first investigated the quantitative gene expression patterns in INS-1 cells treated chronically with high-glucose. The drastically reduced expression of essential β-cell genes such as *IRS2,* insulin, *Pdx1, Glut2,* glucokinase and *Bclxl*, and markedly increased mRNA levels of lipogenic and proapoptotic genes should contribute to the glucolipotoxicity. Interestingly, this gene expression profile is strikingly similar to what we have reported in the INS-1 cell line expressing naSREBP-1c (Wang et al., 2003). In contrast, the characteristics of these gene expression patterns as well as typical glucolipotoxicity were not observed in the INS-1 cell line overexpressing another lipogenic transcription factor ChREBP. Similar negative results were obtained in INS-1 cells expressing, PPARγ or C/EBPβ (H.W. and C.B.W., unpublished data), suggesting that SREBP-1c is the predominant transcription factor responsible for β-cell glucolipotoxicity.

To further substantiate this hypothesis, we established an INS-1 stable cell line with inducible expression of DN-SREBP-1c. Induction of DN-SREBP-1c diminished the chronic high-glucose-induced SREBP-1 binding to the SRE fragment of the IRS2 promoter. Consequently, the inhibitory effects of chronic high glucose on IRS2 expression were largely attenuated by DN-SREBP-1c. It has been reported that

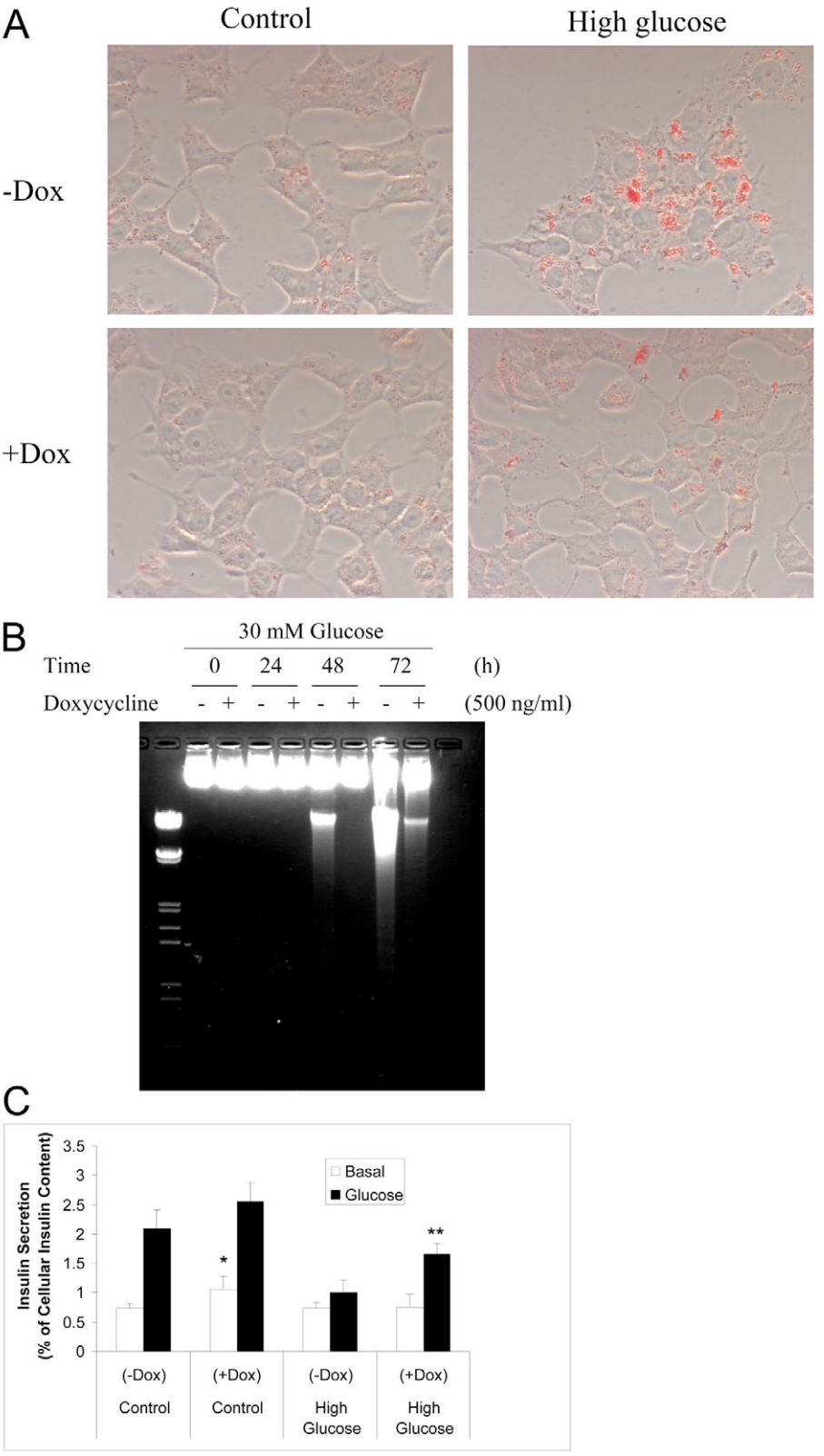

Fig. 5. Induction of DN-SREBP-1c largely prevented chronic high-glucose-induced glucolipotoxicity in INS-1 cells. (A) DN-SREBP-1c diminished lipid droplets accumulation, as seen by staining with Oil Red-O. DN-SREBP-1c*23 cells were cultured in either standard (11.2 mM; Control) or 30 mM glucose (High glucose) medium for 48 hours, in the presence (+Dox) or absence (–Dox) of 500 ng/ml doxycycline. (B) DN-SREBP-1c prevented apoptosis. DNA fragmentation was assessed in DN-SREBP-1c*23 cells cultured in 30 mM glucose medium for 0, 24, 48, and 72 hours, in the presence (+Dox) or absence (–Dox) of 500 ng/ml doxycycline. (C) DN-SREBP-1c partially restored glucose-stimulated insulin secretion. DN-SREBP-1c*23 cells in 24-well dishes were cultured in either standard (11.2 mM; Control) or 30 mM glucose (High Glucose) medium for 48 hours. After 5 hours equilibration in 2.5 mM glucose medium, cells were washed twice with KRBH. Insulin release from DN-SREBP-1c*23 cells stimulated with either 2.5 (Basal) or 20 mM glucose was determined by radio-immunoassay and expressed as a percentage of cellular insulin content. Data represent mean ± s.e.m. of six independent experiments. *P<0.01; **P<0.001.

SREBP-1c binds to the IRS2 promoter and suppresses IRS2 expression in rodent hepatocytes (Ide et al., 2004). Our results suggest a similar mechanism in pancreatic β cells. Deletion of IRS2 in mouse hypothalamus and β cells has recently been shown to cause a type 2 diabetes-like syndrome (Kubota et al., 2004; Lin et al., 2004). IRS2 is also known to promote β cell growth/survival (Withers et al., 1998). We therefore propose that suppression of IRS2 expression by SREBP-1c should contribute, at least in part, to β-cell glucolipotoxicity.

We also demonstrated that dominant-negative suppression of SREBP-1c activation largely prevented glucolipotoxicity in INS-1 cells, including lipid accumulation, impaired glucose-stimulated insulin secretion and apoptosis. The protective effects of DN-SREBP-1c could be explained by nomalized expression of fatty acid synthase, HMG-CoA reductase, LDLR, P21, GLUT2, glucokinase, PDX1, IRS2 and BCL_{XL}. In addition, DN-SREBP-1c also suppressed the expression of UCP2. In contrast, DN-SREBP-1c did not affect the expression of L-PK and aldolase B, which were targeted by ChREBP. Glucokinase is the rate-limiting enzyme for glycolysis and serves as the β-cell glucose sensor (Wang and Iynedjian, 1997). PDX1 is required for maintaining β-cell-specific gene expression and the β-cell phenotype (Ahlgren et al., 1998; Wang et al., 2001). Mouse islets deficient in LDLR

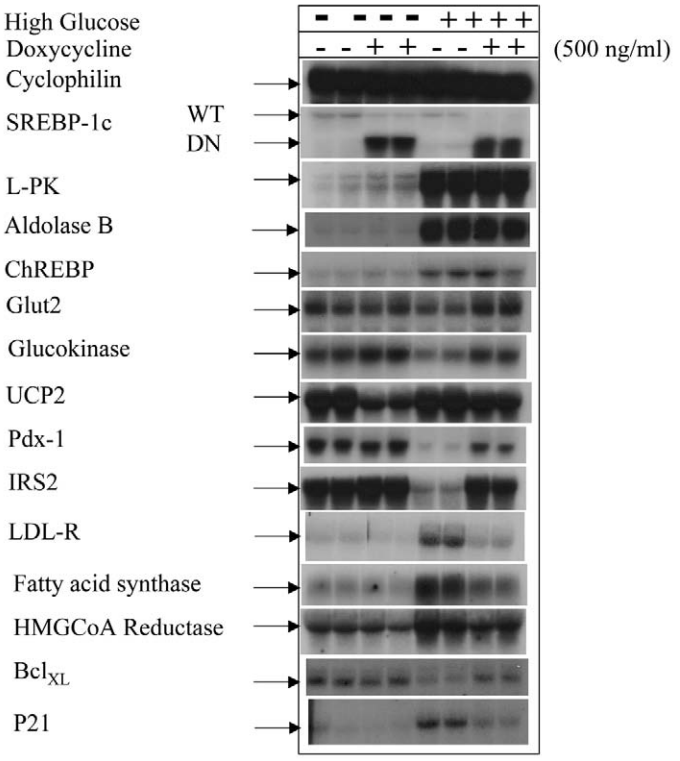

Fig. 6. Effects of DN-SREBP-1c on gene expression patterns induced by chronic high-glucose in INS-1 cells. DN-SREBP-1c*23 cells were cultured in either standard (11.2 mM; –) or 30 mM (+) glucose medium for 48 hours, in the presence (+Dox) or absence (–Dox) of 500 ng/ml doxycycline. The gene expression pattern in these cells was quantitatively evaluated by northern blotting. 20 μg total RNA samples were analysed by hybridising with the indicated cDNA probes. The experiments were repeated twice with similar results.

have been shown to be resistant to LDL-induced apoptosis (Roehrich et al., 2003). Deletion of *Ucp2* restores β-cell function in *ob/ob* mice and in animals fed a high-fat diet (Chan et al., 2004; Joseph et al., 2002; Zhang et al., 2001). In addition, mouse islets deficient in UCP2 are protected from fatty acid-induced lipotoxicity (Joseph et al., 2004), whereas its overexpression leads to impaired glucose-stimulated insulin secretion (Lameloise et al., 2001; Yamashita et al., 2004). Therefore, chronic high glucose may act through SREBP-1 to target multiple genes, causing β-cell glucolipotoxicity.

Most intriguingly, we found that chronic high glucose treatment caused ER stress response in INS-1 cells, evidenced by elevated expression of two characteristic genes, *CHOP* and *BIP*. It has been reported that glucose stimulates protein synthesis in pancreatic β cells through dephosphorylation of the eukaryotic elongation factor (eEF2) (Yan et al., 2003) and initiation factor (eIF2α) (Gomez et al., 2004) and activation of the guanine nucleotide exchange factor eIF2B (Gilligan et al., 1996). We, therefore, postulate that chronic high glucose could induce ER stress through overloading protein synthesis. Our data suggest that chronic high-glucose-induced ER stress could be the underlying mechanism in SREBP-1 processing/activation. Indeed, we found that treatment of isolated rat islets with chronic high glucose and two ER stress inducers, thapsigargin and tunicamycin, markedly increased the SREBP-1 binding activity to the IRS2 promoter. It has been well established that ER stress causes SREBP processing through S1P and S2P (Werstuck et al., 2001; Ye et al., 2000). SREBP-1c activation induced by ER stress has also been implicated in hyperhomocysteinemia-induced liver steatosis (Werstuck et al., 2001). Increased ER stress has been incriminated in *ob/ob* mice and in mice fed on a high-fat diet and proposed as the cause of insulin resistance (Ozcan et al., 2004). Likewise, response to ER stress has been reported in rat β cells treated with fatty acids (Kharroubi et al., 2004). Therefore, prolonged ER stress and SREBP-1c activation could be a common mechanism underlying both β-cell dysfunction and insulin resistance in type 2 diabetes.

It is noteworthy that the genetic studies have associated SREBP-1c polymorphisms with obesity, insulin resistance and type 2 diabetes (Eberle et al., 2004; Laudes et al., 2004). SREBP-1c is the upstream suppressor of *IRS2* (Ide et al., 2004), and the transactivator of *Ucp2, Ldlr,* stearoyl-CoA desaturase 1 and

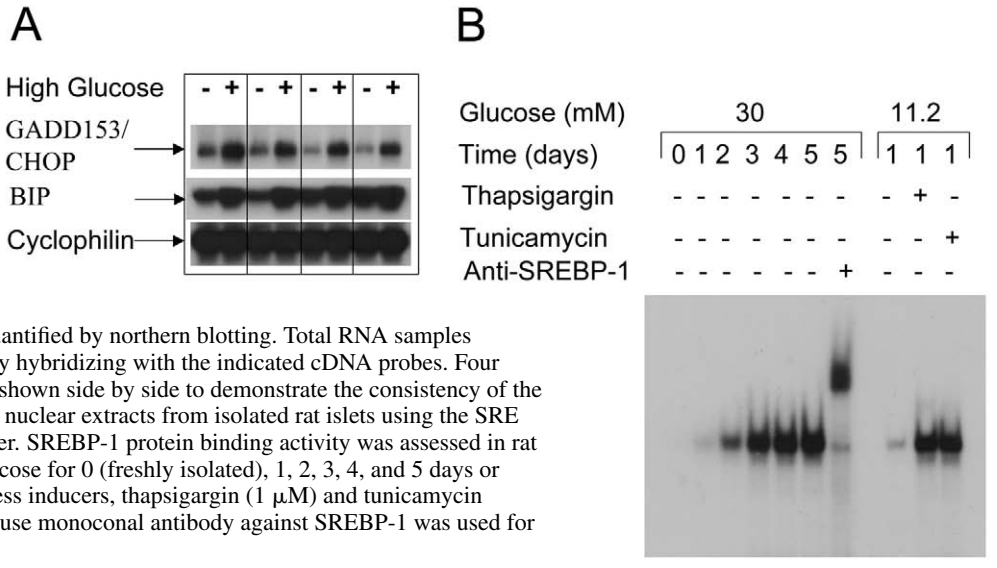

Fig. 7. ER stress and SREBP-1 activation are implicated in β-cell glucolipotoxicity. (A) The gene expression patterns in INS-1E cells cultured in either standard (11.2 mM; –) or 30 mM (+) glucose medium for 48 hours were quantified by northern blotting. Total RNA samples (20 μg/lane) were analysed by hybridizing with the indicated cDNA probes. Four independent experiments are shown side by side to demonstrate the consistency of the results. (B) Gel-shift assay of nuclear extracts from isolated rat islets using the SRE sequence of the IRS2 promoter. SREBP-1 protein binding activity was assessed in rat islets treated with 30 mM glucose for 0 (freshly isolated), 1, 2, 3, 4, and 5 days or alternatively with two ER stress inducers, thapsigargin (1 μM) and tunicamycin (10 μg/ml) for 1 day. The mouse monoconal antibody against SREBP-1 was used for supershift experiments.

many lipogenic genes (Bene et al., 2001; Horton et al., 2003; Medvedev et al., 2002; Wang et al., 2003; Yahagi et al., 1999). In addition to the aforementioned genes (*IRS2*, *Ucp2* and *Ldlr*), stearoyl-CoA desaturase 1 (SCD1) deficiency has been shown to increase insulin sensitivity, fatty acid oxidation and energy expenditure, as well as promoting resistance to diet-induced obesity (Dobrzyn et al., 2004). We, therefore, propose that SREBP-1c is one of the causative genes for development of both β-cell dysfunction and insulin resistance in type 2 diabetes. In fact, SREBP-1c over-activation has been associated with liver steatosis, lipodystrophy, diabetic renal disease and/or β-cell lipotoxicity in many animal models of type 2 diabetes, such as *ob/ob*, *db/db*, IRS2$^{-/-}$ and ap2-nSREBP-1c mice, streptozotocin-induced diabetic rats, Zucker diabetic fatty rats, and mice with liver overexpression of Socs-1/-3 (Kakuma et al., 2000; Lin et al., 2000; Matsuzaka et al., 2004; Riddle et al., 2001; Shimomura et al., 1999a; Shimomura et al., 1999b; Shimomura et al., 1999c; Shimomura et al., 2000; Sun et al., 2002; Takaishi et al., 2004; Tobe et al., 2001; Ueki et al., 2004; Unger and Zhou, 2001; Unger et al., 1999; Werstuck et al., 2001; Xu et al., 2004; Yahagi et al., 2002; You et al., 2002), as well as in patients with severe lipodystrophy (Bastard et al., 2002; Petersen et al., 2002). Treatment with some antidiabetic drugs or hormones including metformin, troglitazone, leptin and adiponectin could prevent the diabetes-associated lipotoxicity in these animals and patients through inhibition of SREBP-1c activation (Lin et al., 2000; Petersen et al., 2002; Shimomura et al., 1999c; Xu et al., 2004). Genetic manipulations to suppress SREBP-1c function are shown to be equally effective (Engelking et al., 2004; Takaishi et al., 2004; Ueki et al., 2004).

In summary, we found that chronic high glucose treatment alone could cause typical glucolipotoxicity and a gene expression profile resembling that caused by naSREBP-1c expression in INS-1 cells (Wang et al., 2003). This gene expression profile (Wang et al., 2003) has also been most recently confirmed in mice with β-cell-specific overexpression of SREBP-1c (Takahashi et al., 2005), suggesting that the results in our INS-1 cell model correlate well with native β cells in vivo. However, it remains to be determined whether naSREBP-1c-induced apoptosis and impaired glucose-stimulated insulin secretion occurs via lipid accumulation or through an independent mechanism. Most importantly, we found that dominant-negative suppression of SREBP-1c function could prevent glucolipotoxicity in INS-1 cells. We have further implicated ERnstress in the activation of SREBP-1c, leading to β-cell glucolipotoxicity. Therefore, the present study should help to clarify the molecular mechanisms underlying development of β-cell glucolipotoxicity, a process thought to cause the loss of both β-cell mass and insulin secretory response to glucose in type 2 diabetes. Prevention of excessive ER stress and SREBP-1c action should be considered for therapy aimed at protection of β-cell function in type 2 diabetes.

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The authors declare no conflict of interest.

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