Over-expression of sterol-regulatory-element-binding protein-1c (SREBP1c) in rat pancreatic islets induces lipogenesis and decreases glucose-stimulated insulin release: modulation by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)

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Accumulation of intracellular lipid by pancreatic islet β -cells has been proposed to inhibit normal glucose-regulated insulin secretion ('glucolipotoxicity'). In the present study, we determine whether over-expression in rat islets of the lipogenic transcription factor SREBP1c (sterol-regulatory-element-binding protein-1c) affects insulin release, and whether changes in islet lipid content may be reversed by activation of AMPK (AMP-activated protein kinase). Infection with an adenovirus encoding the constitutively active nuclear fragment of SREBP1c resulted in expression of the protein in approx. 20 % of islet cell nuclei, with a preference for β -cells at the islet periphery. Real-time PCR (TaqMan[®]) analysis showed that SREBP1c up-regulated the expression of FAS (fatty acid synthase; 6-fold), acetyl-CoA carboxylase-1 (2-fold), as well as peroxisomal-proliferator-activated receptor- γ (7-fold), uncoupling protein-2 (1.4-fold) and Bcl2 (B-cell lymphocytic-leukaemia proto-oncogene 2; 1.3-fold). By contrast, levels of preproinsulin, pancreatic duodenal homeobox-1, glucokinase and

GLUT2 (glucose transporter isoform-2) mRNAs were unaltered. SREBP1c-transduced islets displayed a 3-fold increase in triacylglycerol content, decreased glucose oxidation and ATP levels, and a profound inhibition of glucose-, but not depolarisation-, induced insulin secretion. Culture of islets with the AMPK activator 5-amino-4-imidazolecarboxamide riboside decreased the expression of the endogenous SREBP1c and FAS genes, and reversed the effect of over-expressing active SREBP1c on FAS mRNA levels and cellular triacylglycerol content. We conclude that SREBP1c over-expression, even when confined to a subset of β -cells, leads to defective insulin secretion from islets and may contribute to some forms of Type II diabetes.

Key words: 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), AMP-activated protein kinase (AMPK), insulin secretion, lipotoxicity, pancreatic islet, sterol-regulatory-element-binding protein (SREBP).

INTRODUCTION

Accumulation of lipid within islet β -cells, prompted by elevated circulating concentrations of glucose and non-esterified fatty acid, has been proposed to cause 'glucolipotoxicity' and defective insulin secretion in some forms of Type II diabetes [1,2]. While the mechanisms responsible for these changes are not fully elucidated, up-regulation of SREBP1c (sterol-regulatory-element-binding protein-1c) [3–5] may be involved.

Three SREBP isoforms, each of which regulates the expression of genes involved in cholesterol and fatty acid metabolism [6,7], have been identified. Whereas SREBP1a and SREBP1c (also known as adipocyte determination and differentiation factor 1; ADD1) are the alternatively spliced products of a single gene [8], SREBP2 is encoded at a separate locus [9]. Each SREBP isoform is synthesized as an endoplasmic-reticulum-bound precursor (approx. 125 kDa) which is cleaved to liberate an active nuclear fragment (approx. 50 kDa) [10].

We [3] and others [4] have previously shown that over-expression of SREBP1c in insulinoma-derived β -cell lines leads to the up-regulation of ACC1 (acetyl-CoA carboxylase 1) and FAS (fatty acid synthase), and strongly suppresses GSIS (glucose-stimulated insulin secretion). Given the potential for alterations in lipid metabolism in transformed β -cells [3,4], and indirect effects of obesity on islet function *in vivo* [11,12], the main aim of the present work was to examine, in isolated rat islets, the impact of over-expressing a constitutively active nuclear form of SREBP1c (amino acids 1–403, SREBP CA) [13] on: (1) triacylglycerol (TG) accumulation, (2) glucose metabolism and GSIS, and (3) the expression of potential SREBP1c target genes. We demonstrate that SREBP1c activates the expression of the FAS and ACC1 genes, increases islet TG levels, and diminishes GSIS. An activator of AMPK (AMP-activated protein kinase), AICAR (5-amino-4-imidazolecarboxamide riboside), reverses the accumulation of lipid under these conditions, but does not rescue defective insulin release.

Abbreviations used: ACC1, acetyl-CoA carboxylase-1; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; Bcl2, B-cell lymphocytic-leukaemia proto-oncogene 2; DMEM, Dulbecco's modified Eagle's medium; eGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; FAS, fatty acid synthase; FCS, fetal calf serum; GK, glucokinase; GLUT2, glucose transporter isoform-2; GSIS, glucose-stimulated insulin secretion; KRBH, Krebs–Ringer bicarbonate Hepes buffer; MOI, multiplicity of infection; PDX1, pancreatic duodenal homeobox-1; PPAR γ , peroxisomal proliferator-activated receptor γ ; PPI, pre-proinsulin; RT-PCR, reverse-transcriptase PCR; SREBP1c, sterol-regulatory-element-binding protein-1c; SREBP CA, constitutively active nuclear form of SREBP1c; TG, triacylglycerol; TRITC, tetramethylrhodamine β -isothiocyanate; UCP2, uncoupling protein-2.

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EXPERIMENTAL

Materials

Collagenase (type V) was obtained from Sigma (Poole, Dorset, U.K.). Culture medium, FCS (fetal calf serum) and glutamine were obtained from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Histopaque solutions and antibiotics were from Sigma and AICAR was from Toronto Research Chemicals (Toronto, Canada).

Islet preparation and culture

Male Wistar rats (200–225 g) were killed by cervical dislocation. Islets were isolated by pancreatic distension, as described previously [14], and purified using either Histopaque gradient solutions (10 ml of 1.119 g/l; 6 ml of 1.083 g/l, 6 ml of 1.077 g/l) or gradients of BSA [35 %, 31 % and 27 % (w/v); see Table 3]. After centrifugation for 20 min at 1000 g, islets were removed from the top layer and washed once with DMEM (Dulbecco's modified Eagle's medium). Isolated islets were cultured in suspension for 16 h in DMEM containing 30 % (v/v) FCS, 11 mM glucose, 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin, and incubated at 37 °C with 95 % air and 5 % CO₂.

Islet dissociation

Infected islets were rinsed twice with free Ca²⁺ Krebs buffer then digested with trypsin/EDTA for 3–5 min at 37 °C until dispersed into single cells. Digestion was stopped by adding cold DMEM, and cells were collected by centrifugation (1000 g; 5 min). After further rinsing with DMEM, the cell pellet was resuspended in 150 µl of DMEM per coverslip and incubated at 37 °C, then allowed to adhere to coverslips for ≥ 6 h prior to immunocytochemical analysis.

Amplification of recombinant adenoviruses and islet infection

Adenoviruses encoding SREBP CA [3,13] or eGFP (enhanced green fluorescent protein; 'null' virus) only were amplified as described previously [15]. Virus particles were purified on CsCl gradients before infection [16] at an MOI (multiplicity of infection) of 30 viral particles/cell. Islets were infected with adenoviruses the day after isolation and cultured for 24 h in DMEM containing 11 mM glucose and supplemented as above, then for a further 24 h in DMEM containing 3 mM glucose before use, unless specified otherwise.

Immunocytochemistry

Islets were infected with adenoviruses and cultured for 48 h, then washed with PBS and fixed with 3 % (w/v) formaldehyde for 16 h at 4 °C. For sectioning, islets were fixed with Zamboni's fixative [17] overnight at 4 °C, immersed in a solution of 30 % (w/v) sucrose in PBS overnight, and then frozen in OCT compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan). Sections of islets (7 µm) were obtained using a cryostat (Bright OTF5000; Jencons, Leighton Buzzard, Bedfordshire, U.K.). Whole islets, islet slices or dissociated cells were permeabilized with 0.3 % (v/v) Triton X-100 overnight (20 min for dissociated cells), then blocked in 3 % (w/v) BSA in PBS for 15 min. Incubation with primary antibodies was performed at 4 °C overnight in a humidified chamber. Mouse monoclonal antibody (IgG-2A4), raised against amino acids 301–407 of SREBP-1a (Santa Cruz Biotechnology), was used at 1:500 dilution for whole islets and 1:50 for islet sections, and guinea-pig anti-insulin antibody at 1:500 dilution. After washing with PBS, islets were incubated

again overnight at 4 °C (1 h at 22 °C for dissociated islets) with TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated secondary antibodies against mice or guinea-pig IgG (1:500 dilution), before finally mounting in a mixture of Mowiol (Merck, Poole, Dorset, U.K.) and glycerol. Images were captured on a Leica SP2 laser-scanning confocal microscope using a × 63 oil immersion objective, with excitation at 488 nm (Ar) and 543 nm (He/Ne). Emitted light was detected at > 515 nm for eGFP (green), or > 560 nm for SREBP1 or insulin (red).

Apoptosis and islet viability

An Apoptosis Detection kit (Sigma), based on annexin V binding, was used to monitor the impact of null or SREBP-CA-encoding adenoviruses on apoptosis. Islets were incubated in DMEM containing 11 mM glucose with or without the null or SREBP CA viruses (30 MOI), and in the presence or absence of 5 µM staurosporine as a positive control. After 48 h, islets were washed twice in PBS, then with 300 µl of binding buffer (Sigma) before incubation with 50 µl of annexin V (2 µg/ml) for 1 h at 22 °C. Islets were washed twice with 250 µl of binding buffer and apoptotic cells were imaged on a Leica SP2 laser-scanning confocal microscope using a × 40 oil immersion objective, with excitation at 543 nm, > 560 nm emission for annexin V-Cy3 (red), or 488 nm, emission > 515 nm (eGFP; green). Cell viability was judged by incubation of islets with 10 µg/ml propidium iodide (Sigma) for 20 min at 37 °C, and imaging was carried out as described above.

Western (immunoblot) analysis

Total islet protein was extracted using RIPA assay buffer, comprising PBS supplemented with 1.0 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate and 0.1 % (w/v) SDS. Protein concentration was determined using BCA kit (Pierce, Rockford, IL, U.S.A.). Proteins were separated by SDS/PAGE [7.5 % (w/v) acrylamide] and transferred on to PVDF membranes using a discontinuous buffer system [18]. Blots were probed with a monoclonal antibody to SREBP1 (Santa Cruz Biotechnology; 1:500 dilution) and revealed with horseradish-peroxidase-conjugated anti-mouse IgG (1:3000) using an enhanced chemiluminescence detection system (Roche).

EMSAs (electrophoretic mobility shift assays)

To obtain nuclear extracts, islets were swollen for 15 min in 400 µl of ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF), then lysed by the addition of 25 µl of 10 % (v/v) Nonidet P40. Nuclear proteins were recovered by incubation for 15 min in 50 µl of ice-cold hypertonic buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF) before centrifugation for 5 min at 10 000 g, as described previously [19]. EMSAs were performed as described in [20] using 10 µg of islet nuclear extracts incubated for 45 min on ice in 20 µl of binding buffer [5 mM Hepes, pH 7.9, 60 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 4 mM spermidine, 1 mM EDTA, 10 % glycerol and 1 µg of poly(dI-dC)] and 0.1 to 0.5 ng of kinase-treated 5'-end-labelled double-stranded probe. The oligonucleotide sequence of SREBP1c binding site was derived from the FAS gene promoter (bp – 158 to – 133): 5'-CATCGGGCATCACCCACCGACGGCG-3' (SRE sequence underlined) [21]. Competition experiments were performed using a molar excess of unlabelled oligonucleotide, and for supershift assays, 2 µl of specific SREBP1 antibody

was added to the binding reaction. Samples were migrated on non-denaturing 5% (w/v) polyacrylamide gels in Tris/glycine/EDTA buffer [20], dried and subjected to autoradiography.

Semiquantitative RT (reverse-transcriptase) PCR

Total RNA was isolated by cell lysis in TRI Reagent (Sigma) according to the manufacturer's instructions. First-strand cDNA synthesis was performed as described previously [22] using 3.0 µg of RNA, oligo (15)-dT primers and reverse transcriptase (500 units, Moloney-murine-leukaemia virus; Promega) in a total volume of 50 µl. PCR reactions were performed in a total volume of 50 µl, comprising 5 µl of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer and 0.875 units of *Taq* polymerase (Expand High Fidelity; Roche). Oligonucleotide primer sequences used in PCR amplification were as follows: FAS, forward, 5'-GTT TGA TGG CTC ACA CAC T, reverse, 5'-TCA ACC ACT CGA GGC TCA G; SREBP1c, forward, 5'-GGA GCC ATG GAT TGC ACA TT, reverse, 5'-AGG AAG GCT TCC AGA GAG GA; ACC1, forward, 5'-AAC TAT GAG GTG GAT CGG AG, reverse, 5'-TCC ATA CCG CAT TAC CATG C. Oligonucleotide primers for β -actin mRNA are as described previously [23]. PCR was performed at 95 °C for 2 min, followed by 29 (ACC1), 29 (FAS), 24 (SREBP1c) or 19 cycles (β -actin) at 95 °C for 1 min, 52 °C (ACC1, FAS and SREBP1c) or 62 °C (β -actin) for 1 min, and 72 °C for 1 min. The last cycle was followed by a final extension step at 72 °C for 10 min. PCR products were separated on 1% agarose gels containing ethidium bromide (0.5 µg/ml) and quantified by digital imaging (ImageQuant; Molecular Dynamics).

Real-time quantitative RT-PCR

RNA samples were treated with DNA-free™ (Ambion, Austin, TX, U.S.A.) to remove any genomic DNA contamination, and quantified by RiboGreen assay (Molecular Probes). cDNA was synthesized from 1 µg of total RNA in 100 µl of using random hexamer primers and Moloney-murine-leukaemia virus reverse transcriptase (Promega). Real-time PCR was performed using 20 ng of reverse-transcribed total RNA with 900 nM of sense and anti-sense primers, 5.5 mM MgCl₂, 300 µM dNTP, 1.25 units of *Taq* polymerase and 1 × Taqman buffer A (Applied Biosystems) in a total volume of 25 µl in an ABI PRISM 7700 Sequence Detection System Instrument (Applied Biosystems). Standard curves were constructed by amplifying serial dilutions of untreated rat islet cDNA (50 ng–0.64 pg) and plotting cycle threshold (C_T) values as a function of starting reverse-transcribed RNA, the slope of which was used to calculate relative expression of the target gene.

TG and ATP measurements

Total lipids were extracted from 150 islets using chloroform/methanol (2:1, v/v) [24]. Extracted lipids were air-dried and 10 µl of a detergent (Thesit; Fluka, Gillingham, Dorset, U.K.) was added to the dry pellet. Samples were air-dried again and resuspended in 50 µl of water [25]. TG was measured using a commercial kit (Infinity™ Triglyceride Reagent, Sigma) and a standard curve of triolein (Sigma) treated in parallel with the samples. Islets were extracted into perchloric-acid based medium and assayed for ATP using partially purified firefly luciferase (Promega), as described previously [26].

[U-¹⁴C]Glucose oxidation

Islets were pre-incubated for 30 min at 37 °C in KRBH (Krebs–Ringer bicarbonate Hepes buffer: 130 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgSO₄, 0.5 mM KH₂PO₄, 2.0 mM NaHCO₃ and 10 mM Hepes) supplemented with 3 mM glucose/

Figure 1 Adenovirus-mediated expression of SREBP CA in pancreatic islets

Isolated islets were infected with null (N)- or SREBP CA (S)-encoding adenoviruses, and cultured for a further 24 h at 16 mM glucose, 24 h at 3 mM glucose and then 17 mM glucose for 8 h, prior to protein extraction, nuclear extraction and gel shift (A) or immunoblot analysis (B) as described in the Experimental section. (A) EMSAs were performed using either no added protein (lane 1) or 10 µg of nuclear extracts (lanes 2–5) from islets infected with either null or SREBP CA adenoviruses. (B) Nuclear extracts (30 µg of protein) from islets infected with either null or SREBP CA adenoviruses were subjected to immunoblot analysis, and SREBP1c was revealed with a monoclonal anti-SREBP1 antibody (Ab SREBP; see the Experimental section). MW, molecular mass.

0.5% (w/v) BSA. Triplicate groups of 100 islets were then placed in 250 µl of KRBH containing 3 or 17 mM unlabelled glucose, 1.7 µCi [U-¹⁴C]glucose and 0.5% BSA (w/v) in a 24-well plate. A rubber gasket the size of the 24-well plate containing 0.5 cm holes was aligned over the 24-well plate. A UniFilter-24 GF/B Plate (Packard Instrument) was sealed with an adhesive sheet and 100 µl of 40% (w/v) KOH was pipetted on to each filter. The filter plate was inverted and aligned over the rubber gasket to form a small CO₂ capture chamber. Finally, the chamber was sealed with a 6-mm glass plate, a 6-mm metal plate and a lead weight to ensure an airtight seal. The apparatus was incubated for 2 h at 37 °C. Filters were removed and captured ¹⁴CO₂ was measured by scintillation counting.

Insulin secretion

After adenoviral infection (48 h), islets were incubated for 60 min in a shaking water bath at 37 °C in 1 ml of KRBH supplemented with 3 mM glucose/0.1% (w/v) BSA, equilibrated with a mixture of 95% O₂/5% CO₂, pH 7.4. Then the islets were separated into six groups of five islets per condition, and incubated for 30 min in 1.0 ml of KRBH as above, containing either 3 or 17 mM glucose. Total insulin extracted from the islets in acidified ethanol was carried out as described previously [16]. Insulin was measured by radio-immunoassay (Linco Research, St. Charles, MO, U.S.A.).

Oil Red O staining

Islet sections were washed twice with PBS, and immersed briefly in 60% (v/v) propan-2-ol, then incubated at 22 °C for 1 h in Oil

Figure 2 Immunostaining of whole or islets section with anti-SREBP1 (A) or anti-insulin (B) antibodies

Islets were isolated, cultured at 11 mM glucose, then infected with appropriate adenovirus for 48 h before fixation (see the Experimental section). Whole (a, b, e, f, i, j, m and n) or cryostat islet sections (c, d, g, h, k, l, o and p) were permeabilized at 4 °C (16 h) in the presence of 0.3 % (v/v) Triton X-100 in PBS. Sections or whole islets were probed with mouse monoclonal antibody raised against amino acids 301–407 of SREBP-1a (A), or with guinea-pig anti-insulin (B) antibody, and were then revealed with TRITC conjugated to appropriate secondary antibodies. Immunolabelled islets were imaged using a Leica SP2 laser-scanning confocal microscope using a $\times 63$ oil immersion objective with excitation at 488 nm (Ar) and 543 nm (He-Ne) laser for excitation. Emission was detected at > 515 nm (green, eGFP) and > 560 nm (red, SREBP and insulin). Bright-field images are shown in panels a, c, e, g, i, k, m and o. Scale bar, 40 μ m.

Red O solution (Sigma). After destaining for 1 h in 60 % propan-2-ol, islets were rinsed and examined using a Leica confocal microscope in transmission mode.

Statistical analysis

Results are expressed as the means $\pm$ S.E.M. Statistical significance was assessed using ANOVA or Student's *t* test, as appropriate.

RESULTS

Adenoviral transduction of islets results in the expression of SREBP CA

Infection with adenovirus encoding SREBP CA led to the efficient expression of the SREBP1c(1–403) fragment and its trafficking to the nucleus, as shown by Western blotting (Figure 1B). EMSA (Figure 1A) revealed binding to an oligonucleotide probe containing the FAS gene SRE1 sequence, confirming the DNA-binding

Figure 3 Impact of null- or SREBP-CA-encoding adenoviruses on apoptosis

Islets were isolated and cultured at 11 mM glucose, as described in the Experimental section, before incubation with (e–j) or without (a–d) the adenoviruses indicated, followed by permeabilization at 4 °C (16 h) in the presence of 0.3 % (v/v) Triton X-100 in PBS. Uninfected islets (a–d), or islets infected with either null or SREBP CA adenoviruses, as indicated (e–j), were incubated either with no further additions or in the presence of 5 μ M staurosporine (stauro/positive control; c and d) as shown. After 48 h, incubations were continued in the presence of 2 μ g/ml annexin V conjugated to Cy3 for 1 h. Confocal imaging was then performed as described above with excitation at 543 nm, > 560 nm emission for annexin V–Cy3 (red) or 488 nm, emission > 515 nm (eGFP; green). Note the abundance of annexin V-stained cells in islets treated with staurosporine (positive control; c). Bright-field images are shown in panels b, d, g and j. Scale bar, 40 μ m.

capacity of the expressed fragment. The specificity of the DNA binding observed with nuclear extracts was confirmed by competition with non-radioactive probe and by supershift experiments with anti-SREBP1 antibody (Figure 1A). Neither the level of expression of the SREBP CA fragment, nor the apparent DNA binding activity, was affected by the culture of islets at 17 compared with 3 mM glucose ($n = 3$ separate trials; results not shown).

We explored next the extent of adenoviral infection and SREBP1c expression by immunocytochemical analysis of the surface of whole islets (Figure 2A, a, b, e and f; Figure 2B, i, j, m and n) or islet sections (Figure 2A, c, d, g and h; Figure 2B, k, l, o and p). Both eGFP (Figure 2A, a–h) and SREBP1 (Figure 2A, e–h) were detected in the majority of cells in the first one–two layers of SREBP CA infected islets, but neither was present in the islet core (Figure 2A, c, d, g and h). The majority (> 60 %) of cells infected with the SREBP CA adenovirus (i.e. positive for eGFP) also displayed SREBP1 immunoreactivity (Figure 2A, f and h) and corresponded to β -cells (co-localization with insulin, 86.5 ± 1.0 %; results from three separate experiments; Figure 2B).

The above analysis of islet sections suggested that over-expressed SREBP1c was present in 15–30 % of all islet cells. However, this value could not be calculated more precisely due to large variations in islet diameter and uncertainty as to the position of any given section within the islet examined. We therefore counted the number of eGFP- and SREBP1-positive cells after

islet dissociation (see the Experimental section). Of the cells examined, 762 of 2529 (30.1 %) were positive for eGFP and 511 of 2529 (20.2 %) positive for SREBP1, which agrees with the above estimates in intact islets.

Effect of over-expressing active SREBP1c on islet cell viability and apoptosis

We next determined whether infection of islets affected cell viability or function. Infection with either null- or SREBP-CA-encoding adenoviruses at 30 MOI had no apparent effect on the frequency of apoptosis (Figure 3) or cell viability assessed with the carboxyfluorescein or calcein acetoxymethyl ester (results not shown). By contrast, culture of islets for 3 days at higher viral titres (> 100 MOI) resulted in progressive loss of viability and necrosis, as well as dissociation into single cells at the highest viral concentrations tested (results not shown). Neither infection with the null adenovirus, nor changes in FCS concentrations (10–30 %), had any apparent effect on either basal or glucose-stimulated insulin release (Figure 4A).

Effect of over-expressing active SREBP1c on islet TG content and insulin release

Infection of islets with the adenovirus encoding SREBP CA increased TG levels by approx. 3-fold relative to null-virus-infected

Figure 4 Effect of null- or SREBP-CA-encoding adenoviruses on TG content and insulin secretion

(A) Islets were isolated and subsequently maintained at the indicated FCS concentrations, prior to assay of insulin secretion. After infection with the indicated adenovirus at 30 MOI, and culture for 24 h at 11 mM glucose, islets were incubated for a further 24 h at 3 mM glucose. Six groups of five islets per condition were then selected and pre-incubated for 1 h in KRHB buffer. Insulin secretion was measured during a subsequent 30 min static incubation at either 3 or 17 mM glucose. Released and total insulin were quantified by radio-immunoassay. The results are expressed as the means $\pm$ S.E.M. for three independent experiments. *** $P < 0.001$ for the effects of 17 compared with 3 mM glucose. (B) Islet TG content was measured after infection with null or SREBP CA adenoviruses (150 islets per condition). Islets were cultured for 24 h in 11 mM glucose after infection and then incubated for a further 24 h at 17 mM glucose. Total lipids were extracted from 150 islets per condition. The results are expressed as the means $\pm$ S.E.M. for three independent experiments. *** $P < 0.001$ for the effect of SREBP CA compared with null adenovirus. (C) Islets were infected with (i) null or (ii and iii) SREBP CA adenoviruses before sectioning, staining with Oil Red O and confocal imaging analysis (see the Experimental section). Note the presence in SREBP-CA-infected islets of lipid droplets both in peripherally located cells (ii, box, and iii) and in the islet core (ii, arrows). Scale bars, 20 μ m (i and ii) or 7.5 μ m (iii).

islets (Figure 4B). In these islets, lipid droplets were revealed using Oil Red O, not only in cells at the islet periphery which were positive for eGFP (and presumably also SREBP1), but also in apparently uninfected (eGFP-negative) cells in the islet core (Figure 4C). SREBP1 infection caused a marked (approx. 70%) decrease in glucose oxidation at elevated concentrations of the sugar (Figure 5A), and a decrease in ATP content at both 3 and 17 mM glucose (Table 1). These changes were associated with an essentially complete suppression of glucose-stimulated insulin

Figure 5 Effect of SREBP CA adenovirus on glucose metabolism and insulin secretion from islets

(A) Oxidation of [U-¹⁴C]glucose was measured as described in the Experimental section. The results are expressed as the means $\pm$ S.E.M. for three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ for the effects of 17 mM glucose. (B and C) Insulin secretion was measured as described in Figure 4 for islets infected with the indicated adenoviruses and incubated for 30 min with the indicated glucose and KCl concentrations.

Table 1 Effect of over-expression of SREBP CA on total islet ATP content

Islets were infected with the indicated adenovirus for 24 h at 11 mM glucose, then incubated for 24 h at 3 mM glucose. ATP content was determined as described in the Experimental section. The results are expressed as the means $\pm$ S.E.M. for four separate experiments. † $P < 0.01$ for the effect of SREBP-CA-over-expressing compared with null adenovirus.

Adenovirus	[Glucose] (mM)	ATP content (pmol/islet)
Null	3.0	5.26 $\pm$ 0.55
Null	17.0	6.97 $\pm$ 0.89
SREBP CA	3.0	3.11 $\pm$ 0.29†
SREBP CA	17.0	4.08 $\pm$ 0.31†

secretion (Figure 5B), but had no effect on depolarization-induced secretion (Figure 5C). Total insulin content was similar in null-virus-infected and SREBP-CA-expressing islets (241.2 $\pm$ 32.2 compared with 247.9 $\pm$ 10.9 ng insulin/islet respectively; $n = 3$; not significant).

Effect of elevated glucose concentrations and over-expression of SREBP CA on lipogenic gene expression

To investigate the mechanisms which may mediate the above effects of SREBP1c over-expression on glucose-stimulated insulin

Figure 6 Effects of glucose and over-expression of SREBP CA on FAS and ACC1 genes expression in rat islets

Islets were infected with the indicated adenovirus for 24 h at 11 mM glucose, and then incubated for 24 h at 3 mM glucose before incubation at the given glucose concentrations for 8 h. FAS, ACC1 and SREBP1c mRNA levels were estimated by semi-quantitative RT-PCR (see the Experimental section). The results of a typical experiment (upper panels) and combined results from three separate experiments (histograms) are shown (means $\pm$ S.E.M.). * $P < 0.05$, effect of 17 compared with 3 mM glucose; # $P < 0.05$, effect of SREBP CA over-expression compared with null adenovirus.

secretion, we used either semi-quantitative (Figure 6) or real-time RT-PCR analysis (Tables 2 and 3) to monitor changes in lipogenic gene expression at high and low glucose concentrations.

Table 2 Primer and probe sequences used in quantitative RT-PCR analysis

All primer sequences were designed using Primer Express™ (Applied Biosystems) software and were from Biosource International. Probes were labelled with 6-carboxyfluorescein (FAM) and 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA).

Gene (accession number)	Forward primer (5'–3')	Reverse primer (5'–3')	Probe (5'–3')
PPAR γ (AB011365)	TGCGAGCCCTGGCAAA	GCCCTCGCCTTGGCTTT	FAM-ATTGTATGACTCATACATAAAGTCCTTCCCGCTG-TAMRA
18 S (M10098)	CGCCGCTAGAGGTGAAATCT	CATTCTGGCAATGCTTTTCG	FAM-ACCGGCGCAAGACGAGCAGA-TAMRA
UCP-2 (AB006613)	ACCAGAGCACTGTGGAAGCC	AGGTCCCTTTCAGAGGCC	FAM-CAAGACCATTCACGAGAGGAAGGAT-TAMRA
Cyclophilin (NM_017101)	TATCTGCACTGCCAAGACTGA	CCACAATGCTCATGCCTTCTTCA	FAM-CCAAAGACCACATGCTTGCATCCA-TAMRA
Insulin (NM_019129)	GGGATCTTCAGACCTTGGCA	GCAGCACTGATCCACAATGC	FAM-TGGAGGTTGCCCGGCAAGC-TAMRA
GLUT2 (J03145)	GAAGAGTGGTTCCGCCCC	TCACACAGTCTCTGATGACCC	FAM-CCACGCAAGCCACTGTACAAATGGAA-TAMRA
GK (NM_012565)	CAGATCTGGCAGAGTCCAG	CGGTCCATCTCTTCTGCAAT	FAM-CCGTCTCATCCTTCTTCAGGTCTCC-TAMRA
PDX-1 (NM_022852)	TGAAATCCACCAAGCTCAGC	TGGAAAAGCCAGTGGGAGGAGG	FAM-TCCGGTTCTGCTGCTATG-TAMRA
Bcl2 (NM_016993)	CTGCACCTGACGCCCTTC	CCACAATCTCCCCAGTTCACCC	FAM-ACACACATGACCCACCGA-TAMRA

Table 3 Effects of SREBP CA and glucose on rat islet gene expression as determined by real-time RT-PCR

Primary rat pancreatic islets were treated for 36 h with SREBP CA or null adenovirus (+ or – respectively) in culture medium containing 11 mM glucose, then incubated for 16 h at 3 mM glucose, and finally for 8 h at either 3 or 17 mM glucose as indicated. Levels of mRNA encoding the indicated genes were determined by quantitative real-time RT-PCR and were normalized compared with cyclophilin mRNA. Results are expressed as the fold change over control (null, 3 mM glucose) and presented as the means $\pm$ S.E.M. Statistical significance: ** $P < 0.01$ and *** $P < 0.001$ for the effect of SREBP CA; † $P < 0.05$ for the effect of 17 mM compared with 3 mM glucose.

mRNA measured	SREBP CA virus	[Glucose] (mM)	Relative expression level
PPI	–	3	1.00 $\pm$ 0.05
	–	17	0.96 $\pm$ 0.10
	+	3	1.07 $\pm$ 0.09
	+	17	1.20 $\pm$ 0.12
PDX1	–	3	1.00 $\pm$ 0.05
	–	17	0.96 $\pm$ 0.09
	+	3	1.23 $\pm$ 0.12
	+	17	1.07 $\pm$ 0.09
Glut2	–	3	1.00 $\pm$ 0.08
	–	17	1.27 $\pm$ 0.11†
	+	3	0.86 $\pm$ 0.06
	+	17	1.30 $\pm$ 0.15†
GK	–	3	1.00 $\pm$ 0.05
	–	17	0.99 $\pm$ 0.08
	+	3	1.08 $\pm$ 0.12
	+	17	1.13 $\pm$ 0.11
UCP2	–	3	1.00 $\pm$ 0.09
	–	17	0.86 $\pm$ 0.09
	+	3	1.40 $\pm$ 0.08***
	+	17	1.43 $\pm$ 0.07***
Bcl2	–	3	1.00 $\pm$ 0.08
	–	17	1.14 $\pm$ 0.1
	+	3	1.30 $\pm$ 0.02**
	+	17	1.36 $\pm$ 0.06**
PPAR γ	–	3	1.00 $\pm$ 0.09
	–	17	0.73 $\pm$ 0.12
	+	3	7.45 $\pm$ 0.53***
	+	17	8.77 $\pm$ 1.05***

Whereas elevated glucose concentrations (17 compared with 3 mM) increased the expression of SREBP1c, ACC1 (Figure 6) and GLUT2 (glucose transporter isoform-2; Table 3) mRNAs in null adenovirus-infected islets, as previously reported in MIN6 cells [3], high glucose levels had no significant effect on FAS

Figure 7 Effect of AICAR on SREBP1 and FAS gene expression in islets infected with null adenovirus or SREBP CA

(A–C) After 16 h incubation at 11 mM glucose, islets were incubated for a further 24 h at 17 mM glucose, plus AICAR at the indicated concentrations. (D–F) Islets were infected with the indicated adenovirus and incubated for 24 h at 17 mM glucose with or without AICAR. SREBP1, FAS and ACC1 mRNA levels were estimated by semi-quantitative RT-PCR. The results of a typical experiment (upper panels) and the combined results (lower panels) for three separate experiments are shown (means $\pm$ S.E.M.). * $P < 0.05$ and ** $P < 0.01$ for the effects of AICAR. # $P < 0.05$ for the effect of SREBP CA over-expression compared with null adenovirus (analysed by ANOVA).

(Figure 6), PPI (pre-proinsulin), PDX-1 (pancreatic duodenal homeobox-1), GK (glucokinase), UCP2 (uncoupling protein-2), Bcl2 (B-cell lymphocytic-leukaemia proto-oncogene 2) or PPAR γ (peroxisomal proliferator-activated receptor γ) (Table 3) mRNA levels. Infection with SREBP CA augmented FAS, PPAR γ , UCP2 and Bcl2 gene expression in islets incubated at either 3 or 17 mM glucose (Table 3 and Figure 6), but increased ACC1 mRNA levels significantly only at 3 mM glucose (Figure 6). By contrast, SREBP CA over-expression had no significant effect on PPI, PDX1, GLUT2 or GK mRNA levels (Table 3).

Effect of AICAR on SREBP1c, FAS and ACC1 gene expression in islets

Since activation of AMPK has previously been found to diminish FAS gene expression in isolated hepatocytes [27], we reasoned that AICAR may reverse the effects on islet lipid content and insulin secretion of over-expressing active SREBP1c. Correspondingly, culture of rat islets for 24 h in the presence of AICAR (2–4 mM) dose-dependently decreased expression of the endogenous SREBP1c and FAS genes, but was without effect on

Figure 8 Effect of AICAR on SREBP-CA-induced TG accumulation in islets

Isolated islets were infected with SREBP-CA-expressing adenovirus for 24 h at 11 mM glucose as described in Figure 4(B), before culture at 17 mM glucose for 24 h in the presence or absence of 4 mM AICAR as shown. TG content was measured as described in the Experimental section. The results are expressed as the means $\pm$ S.E.M. for three separate experiments.

ACC1 mRNA levels (Figure 7, A–C). Infection with adenovirus encoding SREBP CA (Figure 7, D–F) strongly augmented FAS expression (FAS: β -actin mRNA levels, 3.98 ± 1.32 compared with 1.0; $n = 3$; $P < 0.05$ for SREBP-CA- and null-virus-infected islets respectively, Figure 7E), but not ACC1 gene expression (Figure 7F). AICAR (4 mM) completely reversed the induction of FAS mRNA by SREBP CA (FAS: β -actin mRNA levels, 3.98 ± 1.32 compared with 0.76 ± 0.29 in control and AICAR-treated islets respectively; $n = 3$; $P < 0.05$; Figure 7E), but had no significant effect on the level of mRNA encoding the over-expressed SREBP1c fragment (Figure 7D).

We next determined whether the decrease in lipogenic gene expression caused by AICAR was reflected in a change in islet lipid accumulation. As shown in Figure 8, incubation with maximal concentrations of AICAR (4 mM) caused a partial reversal of the increase in islet TG content resulting from SREBP CA expression, but did not rescue the loss of glucose-induced insulin secretion (results not shown).

DISCUSSION

We show in the present study that over-expression of the active nuclear fragment of SREBP1c leads to a marked accumulation of TG in primary rat islets, and a profound decrease in glucose oxidation and glucose-stimulated insulin secretion (Figures 5A and 5B). Unexpectedly, these effects were associated with the infection of only a fraction of β -cells within the islet (Figure 3). There may be a number of reasons for this finding. Firstly, by targeting the islet periphery, adenoviral infection may affect those β -cells which are normally the most responsive to glucose, given the poor diffusion of the sugar into the core of cultured islets, and the possible effects of central islet necrosis [28]. Secondly, since functional coupling between individual β -cells is important for normal glucose-stimulated insulin release [29], it is possible that the inhibited function of even a minority of β -cells may impact on the whole islet syncytium. Thirdly, over-expression of SREBP1c may lead to the release of unknown inhibitory products (e.g. fatty acids or derivatives, nitric oxide [30], etc.) which might affect the secretory activity of neighbouring β -cells [31]. Alternatively, it is possible that even low levels of SREBP1c protein, undetectable by immunofluorescence, are present in a proportion of the β -cells in the core of SREBP-CA-infected islets. Whether this phenomenon, or the uptake and re-esterification of fatty acids from neighbouring infected cells, is responsible for the appearance of lipid droplets in the core cells (Figure 4C), is presently unclear. Finally, while altered secretion of glucagon, somatostatin etc. is also conceivable, it should be emphasized that

infection of β -cells was favoured over α , δ , etc. cells, even at the islet surface.

The present results are thus broadly consistent with our findings in MIN6 β -cells [3] and other studies in INS-1 cells [4], and are also compatible with the observation that adenoviral transduction with diacylglycerol acyltransferase 1 [32] leads to diminished GSIS from isolated rat islets. What mechanisms may explain the impact of these changes on insulin secretion? Firstly, infection with the SREBP CA caused a marked inhibition of glucose oxidation at elevated concentrations of the sugar (Figure 5A), and is thus likely to prevent the generation of signalling molecules which trigger insulin secretion (ATP etc.). Moreover, up-regulation of the mitochondrial uncoupling protein UCP2, as reported in the present study, has been shown to cause a decrease in intracellular ATP levels and defective insulin secretion from islets [33,34]. Thus elevated UCP-2 levels are likely to contribute to the observed decrease (approx. 40%) in total islet ATP content at both 3 and 17 mM glucose (Table 1). Furthermore, increases in PPAR γ expression (Table 3), and hence 'transdifferentiation' of islet β -cells towards an adipocyte phenotype, might potentially play a role.

In the present study, over-expression of active SREBP1c had no effect on the expression of PPI or PDX1 mRNAs, nor on the expression of the glucose sensors GK and GLUT2/Slc2a2 [35,36]. In this respect, the response to SREBP CA over-expression in primary islets differs from that in INS-1 cells, where the level of each of the above genes (insulin, PDX-1, GK and GLUT2) was reduced after the induction of SREBP CA [4]. This difference is unlikely to be due to more efficient lipid accumulation in the INS-1 cell system, since this was actually much lower (39%) than that observed in islets (> 300%; Figure 4B). Rather, the absence of detectable changes in the levels of PDX-1, PPI, GK and GLUT2 mRNAs reported in the present study seems most likely to be the result of the fact that SREBP1c was only over-expressed at high levels in a subset of islet cells (Figure 2).

We and others have previously shown that AMPK is expressed in clonal β -cells and islets, and is activated by low glucose concentrations [37–40]. The forced activation of AMPK, as achieved here using AICAR, is expected to decrease TG accumulation both by accelerating the β -oxidation of fatty acids and by decreasing the expression of lipogenic genes [39]. Although a clear decrease in islet TG content in response to AICAR treatment was indeed observed here (Figure 8), it was, however, not associated with a restoration of glucose-stimulated insulin secretion. This observation probably reflects the inhibition by active AMPK of β -cell glucose metabolism [40], as well as other effects of AMPK on more distal events in insulin trafficking and release [41]. Nevertheless, the present findings do suggest that agents which reduce lipid accumulation by β -cells without affecting the latter parameters may provide a useful means to enhance insulin release in some forms of Type II diabetes.

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