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Mitochondrial regulation of insulin production in rat pancreatic islets

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Abstract *Aims/hypothesis:* The study was designed to identify the key metabolic signals of glucose-stimulated proinsulin gene transcription and translation, focusing on the mechanism of succinate stimulation of insulin production. *Methods:* Wistar rat islets were incubated in 3.3 mmol/l glucose with and without esters of different mitochondrial metabolites or with 16.7 mmol/l glucose. Proinsulin biosynthesis was analysed by tritiated leucine incorporation into newly synthesised proinsulin. Preproinsulin gene transcription was evaluated following transduction with adenoviral vectors expressing the luciferase reporter gene under the control of the rat I preproinsulin promoter. Steady-state preproinsulin mRNA was determined using relative quantitative PCR. The mitochondrial membrane potential was measured by microspectrofluorimetry using rhodamine-123. *Results:* Succinic acid monomethyl ester, but not other mitochondrial metabolites, stimulated preproinsulin gene transcription and translation. Similarly to glucose, succinate increased specific preproinsulin gene transcription and biosynthesis. The inhibitor of succinate dehydrogenase (SDH), 3-nitropropionate, abolished glucose- and succinate-stimulated mitochondrial membrane hyperpolarisation and proinsulin biosynthesis, indicating that stimulation of proinsulin translation depends on SDH activity. Partial inhibition of SDH activity by exposure to fumaric acid monomethyl ester abolished the stimulation of preproinsulin gene transcription, but only partially inhibited the stimulation of proinsulin biosynthesis by glucose and succinate, suggesting that SDH activity is particularly important for the transcriptional response to glucose. *Conclusions/interpretation:* Succinate is a key metabolic mediator of glucose-stimulated preproinsulin gene transcription and translation. Moreover, succinate stimulation of insulin production depends on its metabolism via SDH. The differential effect of fumarate on preproinsulin gene transcription and translation suggests that these processes have different sensitivities to metabolic signals.

Keywords Insulin secretion · Islets · Mitochondria · Proinsulin biosynthesis · Preproinsulin gene transcription · Rat · Succinate · Succinate dehydrogenase

Abbreviations BCH: 2(+/−)-2-aminobicyclo-(2,2,1) heptane-2-carboxylic acid · CMV: cytomegalovirus · DCIP: 2,6-dichloroindophenol · DMM: malic acid dimethyl ester · FAM: fumaric acid monoethyl ester · FBS: fetal bovine serum · FCCP: carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone · KRBH: Krebs–Ringer–bicarbonate–HEPES · NPA: 3-nitropropionate · PDX-1: pancreatic-duodenal homeobox 1 · SAM: succinic acid monomethyl ester · SDH: succinate dehydrogenase · TCA: tricarboxylic acid

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Introduction

Glucose is the major physiological stimulus for insulin secretion and production in pancreatic beta cells. Tight coupling between secretion and production of insulin in response to nutrient stimuli is essential to maintain a pancreatic insulin reserve for normal glucose homeostasis. It is well established that glucose must be metabolised in beta cells to provide signals for both secretion and production of insulin [1–3]. Glucose is converted via glycolysis to pyruvate, which enters the mitochondria and is metabolised in the tricarboxylic acid (TCA) cycle. Pyruvate carbon metabolism leads to generation of ATP and hence to an increased cytosolic ATP : ADP ratio, which is

crucial for insulin secretion [4, 5] and biosynthesis [3]. Thus, the energetic state of the beta cell is an important component of the stimulus–secretion coupling mechanism of glucose-induced insulin production and secretion. Furthermore, efflux and refilling of TCA cycle intermediates (cataplerosis and anaplerosis) may generate additional important signals [2, 6, 7].

In normal beta cells, nutrient-induced insulin secretion is always associated with a marked increase in insulin production. However, the kinetics of insulin release and of proinsulin biosynthesis, and the metabolic signals mediating these processes, are not identical [3, 8]. In fact, the role of various glycolytic and TCA cycle intermediates in glucose-stimulated insulin secretion is controversial [7, 9–16], and even less is known about the metabolic signals for glucose-stimulated insulin production. A previous report by Alarcon et al. suggested that succinate is the key signal for glucose stimulation of preproinsulin mRNA translation [3]. In that study, inhibition of the mitochondrial conversion of succinate to fumarate via succinate dehydrogenase (SDH) stimulated proinsulin biosynthesis. Moreover, it was shown that cytosolic succinate could be converted to succinyl CoA, which led to the speculation that succinyl CoA is responsible for the rapid stimulation of proinsulin biosynthesis in response to glucose [3].

We and others have shown previously that glucose has a dual stimulatory effect on insulin production. On the one hand, it rapidly stimulates preproinsulin mRNA translation, leading to a marked increase in proinsulin biosynthesis within less than 1 h of glucose stimulation. This rapid increase in proinsulin biosynthesis is not dependent on preproinsulin gene transcription and is not mediated by the secreted insulin [17, 18]. On the other hand, glucose stimulates preproinsulin gene transcription, leading to a time-dependent increase in preproinsulin mRNA levels, which seems to be essential for the maintenance of proinsulin biosynthesis and islet insulin stores during prolonged stimulation [19]. The identity of the metabolic signals that mediate the glucose effect on preproinsulin gene transcription is not known.

In the present study we investigated the mechanism involved in succinate stimulation of insulin production. We found that, in addition to its previously reported stimulatory effect on preproinsulin mRNA translation [3], succinate is a key mediator of glucose-stimulated preproinsulin gene transcription. In contrast to previously published data, we demonstrate that succinate stimulation of insulin production is dependent on succinate metabolism via SDH, rather than being the consequence of a direct effect of succinate itself.

Materials and methods

Islet isolation and culture Male Wistar rats were obtained from Harlan (Jerusalem, Israel). Islets were prepared by

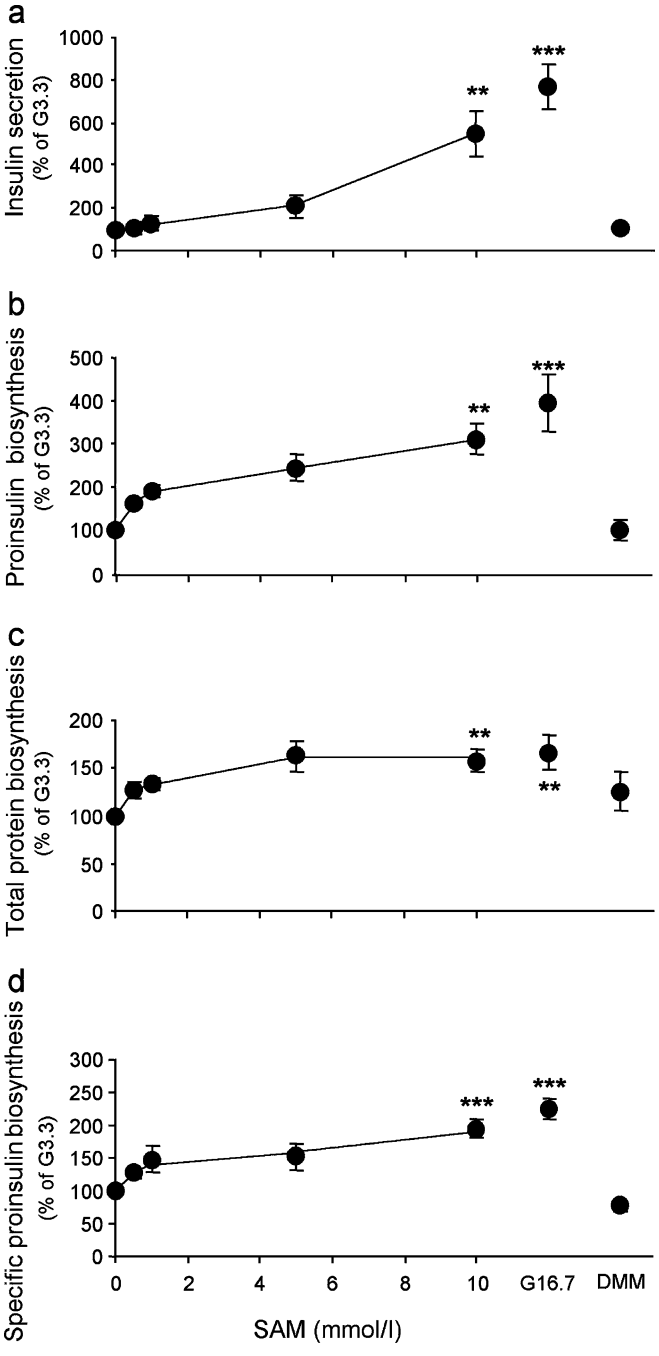

Fig. 1 Dose–response curves for the stimulation by succinate of **a** insulin secretion, **b** proinsulin biosynthesis, **c** total protein biosynthesis, and **d** specific proinsulin biosynthesis (corrected for total protein biosynthesis). Rat islets were incubated for 1 h at 37°C with 3.3 mmol/l glucose (G3.3) alone or supplemented with different concentrations of succinic acid monomethyl ester (SAM) (0.5–10 mmol/l), 10 mmol/l malic acid dimethyl ester (DMM) or with 16.7 mmol/l glucose (G16.7). Insulin secretion and tritiated leucine incorporation into proinsulin and total protein were determined as described under Materials and methods. Results are means±SEM for four or five individual experiments, each performed on islets pooled from three animals. Results are normalised to 3.3 mmol/l glucose. $^{**}p<0.01$ and $^{***}p<0.001$ relative to islets at 3.3 mmol/l glucose

collagenase digestion (Collagenase P; Roche Diagnostics, Mannheim, Germany) as described [20]. When large quantities were required, islets were hand-picked once under the stereomicroscope, followed by purification on Histopaque 1083 density gradient (Sigma, St Louis, MO, USA). The islets were used after repeated washes with Hanks' balanced salt solution, unless otherwise specified. Batches of 200–300 islets of similar size were collected and maintained at 37°C in a 5% CO_2 atmosphere in suspension in 5 ml RPMI 1640 medium (Biological Industries, Beit-Haemek, Israel), containing 3.3 mmol/l or 16.7 mmol/l glucose, 10% fetal bovine serum (FBS; Biological Industries) and different agents according to the experimental protocols. The use of animals was approved by the Institutional Animal Care and Use Committee of the Hebrew University and Hadassah Medical Organization, and principles of laboratory animal care (NIH Publication no. 85–23, revised 1985) were followed.

Experimental protocols Isolated islets were cultured in the presence of 3.3 or 16.7 mmol/l glucose with and without different concentrations of esters of the mitochondrial metabolites: succinate (succinic acid monomethyl ester, SAM), fumarate (fumaric acid monoethyl ester, FAM), glutamate (glutamic acid methyl ester), oxaloacetate (oxaloacetic acid diethyl ester) and malate (malic acid dimethyl ester, DMM). In other experiments, islets were treated with 10 mmol/l glutamine together with 10 mmol/l 2(+/−)-2-aminobicyclo-(2, 2, 1)heptane-2-carboxylic acid (BCH), which allosterically activates glutamate dehydrogenase [21], thereby stimulating mitochondrial metabolism. To study the role of SDH we used the SDH inhibitor 3-nitropropionate (NPA). Islets were treated with the different mitochondrial metabolites and/or NPA for 1 and 24 h to study their short- and long-term effects on insulin secretion and on preproinsulin gene transcription and translation. All esters of mitochondrial metabolites, glutamine, BCH and NPA were purchased from Sigma.

Proinsulin biosynthesis For the short-term experiments, islets were cultured in RPMI 1640 medium containing 3.3 mmol/l glucose for 3 h to allow recovery from the collagenase isolation, whereas for long-term experiments they were cultured for 23 h with RPMI 1640 containing 3.3 or 16.7 mmol/l glucose and test compounds, as indicated. Groups of 25 islets were collected following culture and incubated for 1 h at 37°C in modified Krebs–Ringer bicarbonate buffer containing 20 mmol/l HEPES and 0.25% BSA (KRBH-BSA), supplemented with glucose and test compounds [19]. After incubation, the islets were centrifuged and labelled in 50 μl fresh KRBH-BSA buffer containing glucose and test compounds, as above, as well as 25 μCi L-[4,5-^{3}H]leucine (150 Ci per mmol/l; Amersham, Aylesbury, UK). Leucine incorporation was terminated after 15 min of labelling at 37°C by the addition of 1 ml ice-cold glucose-free KRBH-BSA buffer and rapid centrifugation. The islet pellet was suspended in 450 μl of 0.2 mol/l glycine buffer containing 0.1% RIA-grade BSA and 0.5% NP-40, pH 8.8 (GB/NP40 buffer) and subjected to four freeze–thaw cycles in liquid nitrogen. Each sample (50 μl) was pretreated with protein A Sepharose (Sigma) prior to immunoprecipitation with anti-insulin serum (Sigma), to correct for non-specific binding. This procedure was validated by HPLC. Aliquots were used for determination of total insulin content by RIA and for measurement of total protein biosynthesis by trichloroacetic acid precipitation [22].

Insulin release and content during culture Batches of islets were incubated for 1 h in KRBH-BSA containing various glucose concentrations and test compounds. At the end of the incubation the medium was collected, centrifuged and frozen at −20°C pending insulin analysis. Islets were collected and counted for reference. Islet insulin content was determined by RIA in extracts of batches of islets subjected to repeated freeze–thaw cycles in 1.5 ml microfuge tubes containing 450 μl of 0.1% BSA in 0.1 N HCl,

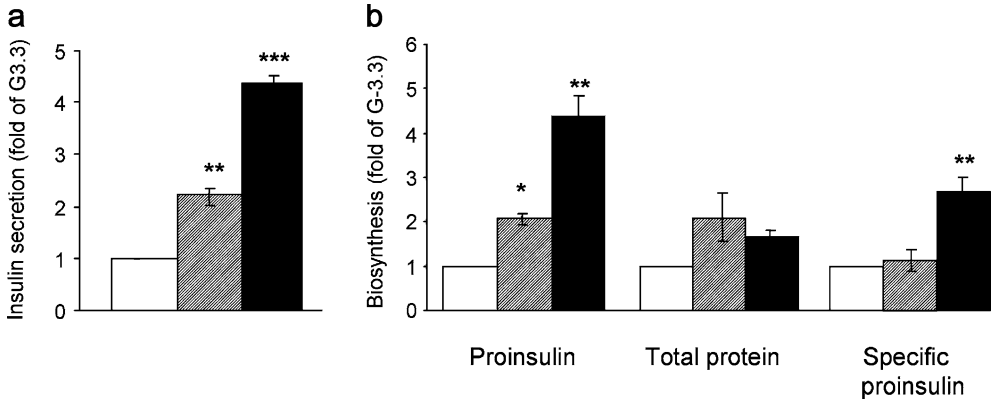

Fig. 2 Effects of glutamine plus 2(+/−)-2-aminobicyclo-(2,2,1) heptane-2-carboxylic acid (BCH) on **a** insulin secretion and **b** proinsulin, total protein and specific proinsulin biosynthesis (corrected for total protein biosynthesis). Rat islets were incubated for 1 h at 37°C with 3.3 mmol/l glucose alone (*white columns*) or supplemented with 10 mmol/l glutamine and 10 mmol/l BCH (*shaded columns*) or with 16.7 mmol/l glucose (*black columns*). Results are means±SEM for three individual experiments, each performed on islets pooled from three animals. Results are normalised to 3.3 mmol/l glucose (G3.3). *p<0.05, **p<0.01 and *** p<0.001 relative to islets at 3.3 mmol/l glucose

followed by centrifugation. Insulin RIA was performed using anti-insulin-coated tubes (ICN Pharmaceuticals, Costa Mesa, CA, USA) and ^{125}I-insulin from Linco Research (St Charles, MO, USA). Rat insulin was used as a standard. The routine intra-assay CV was 4–6% and the inter-assay CV 6–10%.

Quantification of preproinsulin mRNA by RT-PCR Total islet RNA was extracted from 200–300 islets using RNAzol B (Tel-Test, Friendswood, TX, USA). For PCR analysis, total RNA was reverse-transcribed using AMV reverse transcriptase (Promega, Madison, WI, USA). The resulting cDNA was amplified by PCR using oligonucleotides complementary to sequences in the rat preproinsulin I gene: 5′-CCTGCCCAGGCTTTTGTCA-3′ and 5′-GGTGCAGCACTGATCCACAATG-3′. Primers were designed to cross an intron and amplified fragments of 208 bp of the coding sequence of the rat preproinsulin gene. 18S rRNA (QuantumRNA kit; Ambion, Austin, TX, USA) was used as an internal control. The polymerisation reaction was performed in a 25 μl reaction volume containing 2.5 μl cDNA (25 ng RNA equivalents), 80 μmol/l cold dNTPs, 2.5 μCi (alpha-^{32}P)dCTP, 100 pmol/l of appropriate oligonucleotide primers and 1.5 U of *Taq* polymerase (MBI Fermentas, Amherst, NY, USA). PCR amplification conditions were as follows: 5 min at 94°C followed by 14 cycles of 94°C, 60°C and 72°C, 30 s each step. Using these amplification conditions, the PCR reaction was linear over a wide range of RNA concentrations (10–50 ng) for both preproinsulin and 18S rRNA. The amplimers were separated on a 6% polyacrylamide gel in Tris-borate EDTA (TBE) buffer, the gel was dried and the incorporated (alpha ^{32}P)dCTP measured by PhosphorImager (FUJIX, BAS 1000; Fuji Photo Film, Tokyo, Japan). For quantification of islet preproinsulin mRNA, the ratio of preproinsulin : 18S rRNA band intensity was determined for each reaction.

Preproinsulin gene transcription A recombinant adenovirus encoding firefly luciferase under the control of the rat preproinsulin I promoter (corresponding to bases −310 to −12 relative to the transcription start site) (Ad-RIP-Luc) was kindly provided by Christopher J. Rhodes (Northwest Research Institute, Seattle, WA, USA) [23]. An adenovirus encoding firefly luciferase under the control of the cytomegalovirus (CMV) promoter (Ad-CMV-Luc) was used as a control. Batches of 200 islets were transduced overnight with 10^7 plaque-forming units/islet of Ad-RIP-Luc or Ad-CMV-Luc in RPMI 1640 containing 10% FBS and 11.1 mmol/l glucose and then in 3.3 mmol/l glucose for 6 h. Islets were then transferred to fresh RPMI 1640 containing 3.3 mmol/l or 16.7 mmol/l glucose and different treatments according to the experimental protocols, and incubated for 24 h. Luciferase activity was measured in islet extracts using the Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Preproinsulin promoter activity was normalised to the corresponding CMV promoter activity.

Mitochondrial membrane potential measurements Wistar rat islets were precultured for 5–7 days in serum-free RPMI medium containing 10 mmol/l glucose and 5 g/l bovine serum albumin. These preculture conditions preserve glucose stimulus-secretion coupling events and islet cell morphology for at least 2 weeks [24]. After culture, the islets were incubated for 1 h in Krebs buffer containing 3.3 or 16.7 mmol/l glucose and various test substances. Rhodamine-123 (10 μg/ml; Molecular Probes, Eugene, OR, USA) was added to the medium for the last 20 min of that incubation. After loading, the islets were rinsed and perifused for 30 min in the same medium without rhodamine-123, and the fluorescence of rhodamine-123

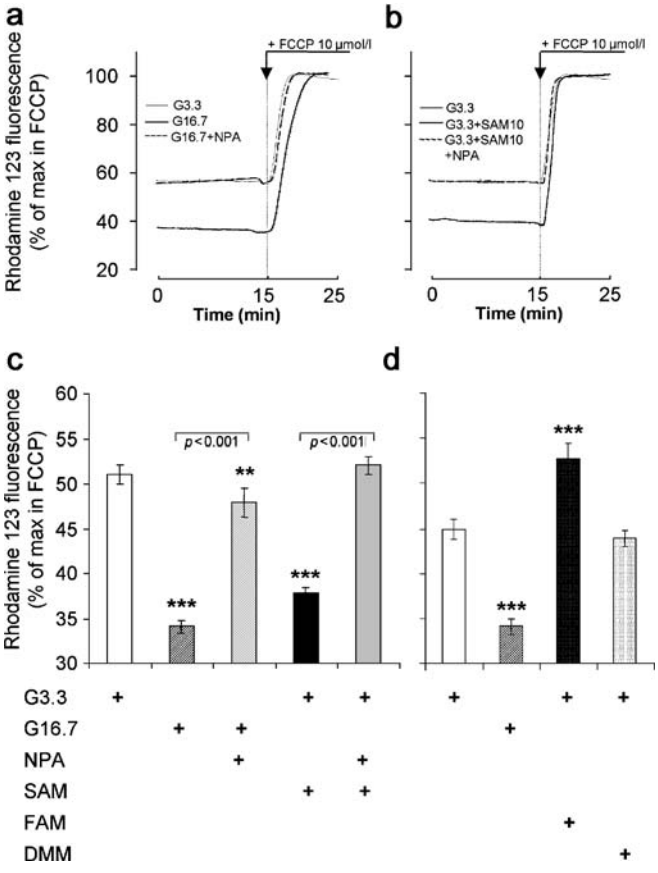

Fig. 3 Effects of various TCA cycle intermediates on islet mitochondrial membrane potential and its alteration by SDH inhibition. A decrease in rhodamine-123 fluorescence corresponds to mitochondrial membrane hyperpolarisation. Islets were incubated for 1 h in 3.3 mmol/l glucose with 10 mmol/l SAM or in 16.7 mmol/l glucose with or without 1 mmol/l 3-nitropropionate (NPA) (**a**–**c**), or for 1 h in 3.3 mmol/l glucose with 10 mmol/l malic acid dimethyl ester (DMM), fumaric acid monoethyl ester (FAM), or in 16.7 mmol/l glucose (**d**). Rhodamine-123 fluorescence was measured as described under Materials and methods and expressed as a percentage of the maximal fluorescence measured after addition of FCCP. Representative traces for six to nine islets from the same preparation tested on the same day are shown in (**a**) and (**b**). Average rhodamine-123 fluorescence over the 15 min preceding addition of FCCP is shown in (**c**) and (**d**). Results are means±SEM for 17–69 islets from three to eight isolations. $^{**}p<0.01$ and $^{***}p<0.001$ relative to islets at 3.3 mmol/l glucose

was measured by microspectrofluorimetry (excitation/emission 490/530 nm, 505 nm long-pass dichroic mirror) 15 min before and after addition of 10 μmol/l carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) [25]. After background subtraction, the rhodamine-123 fluorescence in each islet was normalised to the maximal fluorescence measured in the presence of FCCP [26], and the average relative fluorescence over the 15 min preceding FCCP addition was calculated.

Measurement of SDH activity SDH enzymatic activity was measured in isolated mitochondria according to Rosen et al. [27]. Mitochondria were isolated from rat heart and isolated islets, as described [28], and suspended in 50 mmol/l potassium phosphate buffer, pH 7.4. The mitochondrial extract was adjusted to give a protein concentration of 100 μg in 50 μl solution, which was added to a 1 ml cuvette containing 20 mmol/l sodium succinate, 144 μmol/l 2,6-dichloroindophenol (DCIP), 2.6 mmol/l phenazine metasulphate and 100 μmol/l sodium cyanide in potassium phosphate buffer and incubated for 1 min at 30°C. The decrease in absorbance at 600 nm was measured during the following 5 min in a spectrophotometer. SDH activity was determined based on the molar extinction coefficient ε=21,000. Activity was calculated as mmol DCIP reduced per min per mg protein.

Data presentation and statistical analysis Data shown are means±SEM. The statistical significance of differences between groups was determined by one-way ANOVA followed by the Newman–Keuls test using the InStat statistical program (GraphPad Software, San Diego, CA, USA). A paired-sample *t*-test was used when the difference between a reference (taken as 100%) and test was analysed. A p value of <0.05 was considered significant.

Results

Succinate stimulates proinsulin biosynthesis Succinic acid monomethyl ester (SAM) stimulated insulin secretion and proinsulin biosynthesis in a dose-dependent manner in islets incubated for 1 h (Fig. 1a and b). Maximal proinsulin biosynthesis occurred at 10 mmol/l SAM and was similar to that induced by 16.7 mmol/l glucose. SAM also augmented total protein biosynthesis (Fig. 1c), but to a lesser extent than the stimulation of proinsulin biosynthesis (when calculated as a percentage of the value for 3.3 mmol/l glucose), indicating that succinate has a specific effect on proinsulin biosynthesis (Fig. 1d). In contrast, 10 mmol/l dimethyl ester of the TCA cycle intermediate malate (DMM) had no effect on proinsulin, total protein and specific proinsulin biosynthesis, or on insulin secretion (Fig. 1), indicating that the effect of succinate was specific and did not result from the supply of TCA cycle intermediates alone.

Since the effect of succinate on proinsulin biosynthesis correlated with its effect on insulin secretion, we performed additional studies to test the specificity of the succinate effect on proinsulin biosynthesis. In contrast to succinate, activation of mitochondrial metabolism with glutamine plus BCH had no specific effect on proinsulin biosynthesis despite stimulating insulin secretion (Fig. 2).

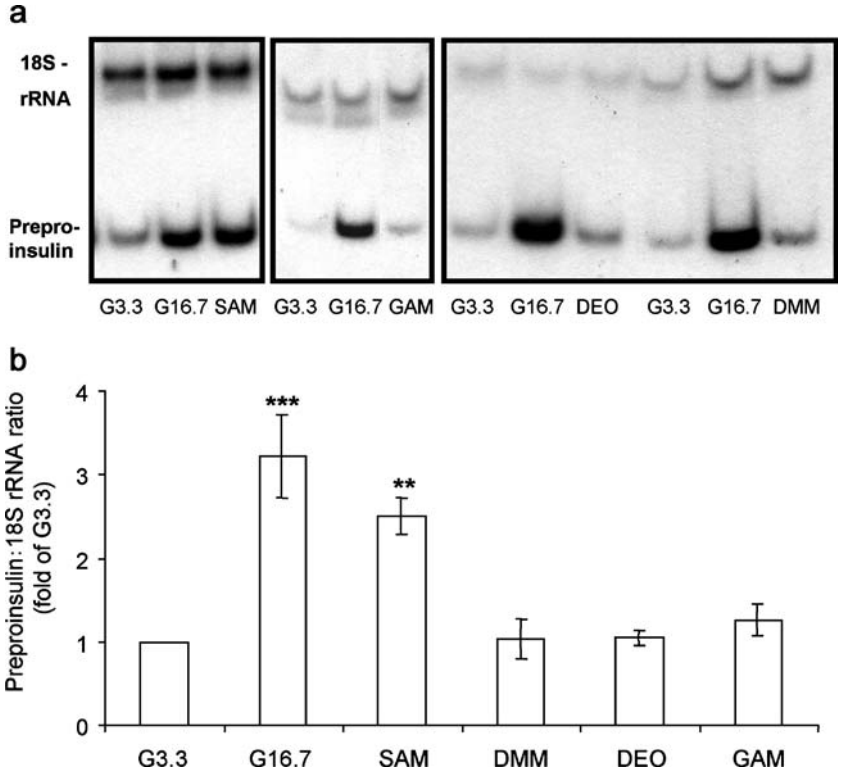

Fig. 4 Long-term effect of succinate and other mitochondrial metabolites on steady-state preproinsulin mRNA in rat islets. Islets were cultured at 3.3 mmol/l glucose alone or with 10 mmol/l succinic acid monomethyl ester (SAM), malic acid dimethyl ester (DMM), oxaloacetic acid diethyl ester (DEO), glutamic acid methyl ester (GAM) or 16.7 mmol/l glucose for 24 h. Preproinsulin gene expression, normalised to 18S rRNA, is expressed relative to the level measured in control islets (3.3 mmol/l glucose). **a** Photomicrograph of representative experiments. **b** Results of three individual experiments, shown as means±SEM. **p<0.01 and *** p<0.001 relative to islets at 3.3 mmol/l glucose

Comparison of the effects of glucose and different mitochondrial intermediates on mitochondrial membrane potential A decrease in rhodamine-123 fluorescence reflects mitochondrial membrane hyperpolarisation [24, 25]. Compared with control islets maintained in 3.3 mmol/l glucose, the mitochondrial membrane was hyperpolarised by a 1-h exposure to 16.7 glucose or 3.3 mmol/l glucose with 10 mmol/l SAM, but the effect of the latter was slightly lower than that of 16.7 mmol/l glucose (Fig. 3a, b). The specificity of the glucose and succinate effect is demonstrated by the observation that the SDH inhibitor NPA completely abolished the induction of mitochondrial membrane hyperpolarisation (Fig. 3a–c). In contrast, addition of 10 mmol/l DMM to 3.3 mmol/l glucose did not affect the mitochondrial membrane potential, whereas 10 mmol/l FAM depolarised the mitochondrial membrane (Fig. 3d).

Succinate augments preproinsulin gene expression To test whether succinate stimulates preproinsulin gene expression, islets were cultured in 3.3 mmol/l glucose with and without SAM or at 16.7 mmol/l glucose for 24 h, and preproinsulin mRNA levels were analysed by relative quantitative RT-PCR. Compared with 16.7 mmol/l glucose, 10 mmol/l SAM was slightly less effective in stimulating preproinsulin gene expression (2.5- vs 3.2-fold increase; Fig. 4). Esters of other mitochondrial metabolites, including malate, oxaloacetate and glutamate, did not stimulate preproinsulin gene expression (Fig. 4). Succinate stimulation of preproinsulin gene expression could result from increased preproinsulin gene transcription and/or stabilisation of preproinsulin mRNA. To study the stimulatory effect of succinate on preproproinsulin gene transcription, islets were transduced with adenoviral vectors expressing luciferase under the control of the rat preproinsulin I promoter (Ad-RIP-Luc) or the CMV promoter (Ad-CMV-Luc). Glucose and SAM increased CMV promoter activity ~1.4 fold (Fig. 5a). RIP-Luc was 4.9-fold and 2.7-fold higher in islets exposed to 16.7 mmol/l glucose and 10 mmol/l SAM, respectively, compared with islets at 3.3 mmol/l glucose (Fig. 5b). The RIP-Luc : CMV-Luc ratio was 3.8±0.7- and 2.0±0.3-fold higher in islets cultured at 16.7 mmol/l glucose and 3.3 mmol/l glucose supplemented with SAM relative to islets at 3.3 mmol/l glucose (Fig. 5c). Thus, both succinate and glucose stimulated preproinsulin promoter activity; however, the glucose effect was nearly twice that of succinate.

SDH is required for glucose- and succinate-stimulated proinsulin biosynthesis The SDH inhibitor NPA was used to study the role of SDH in mediating the stimulatory effects of glucose and succinate on insulin production. NPA inhibited glucose-stimulated insulin secretion, and total and specific proinsulin biosynthesis, in a dose-dependent manner (Fig. 6). The median inhibitory dose of NPA on glucose-stimulated insulin secretion and proinsulin biosynthesis was ~1 mmol/l. (Fig. 6d). One mmol/l

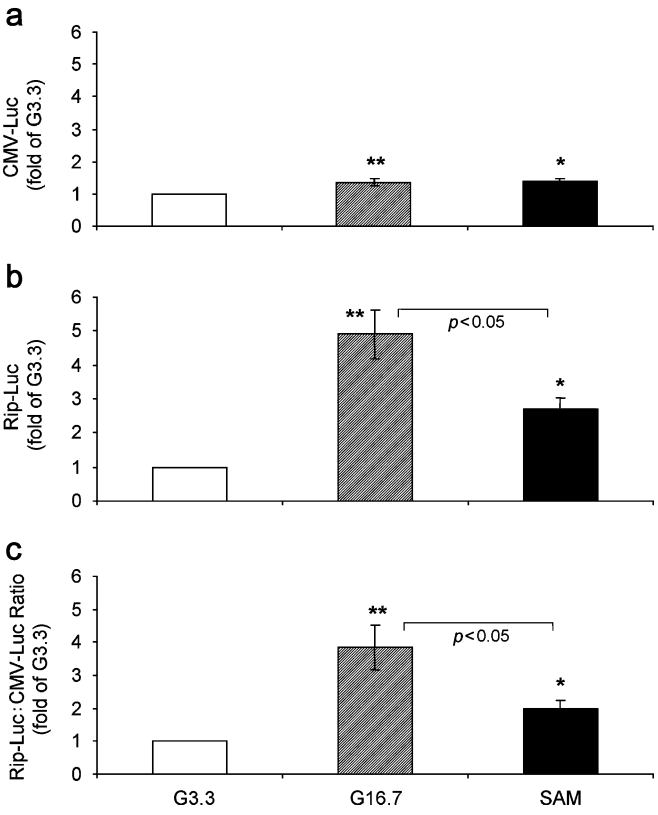

Fig. 5 Effect of succinate on preproinsulin promoter activity. Isolated rat islets were transduced with adenoviral vectors expressing luciferase under the control of the rat preproinsulin I (Ad Rip-Luc) or CMV promoter (Ad CMV-Luc). Luciferase activity was determined in islets following 24 h of incubation in 3.3 mmol/l glucose without and with 10 mmol/l succinic acid monomethyl ester (SAM) or 16.7 mmol/l glucose. **a** CMV-Luc activity. **b** Rip-Luc activity. **c** Ratio of Rip-Luc to CMV-Luc (specific preproinsulin promoter activity). Results are expressed relative to the value measured in control islets (3.3 mmol/l glucose) and shown as means± SEM for six to nine individual experiments. *$p<0.05$ and **$p<0.01$ relative to islets at 3.3 mmol/l glucose

NPA resulted in an average inhibition of 50, 62 and 49% of succinate-induced insulin secretion, total and specific proinsulin biosynthesis, respectively (Fig. 6).

Effect of fumarate on SDH activity SDH is a bidirectional enzyme that can catalyse the conversion of succinate to fumarate or the inverse reaction, fumarate to succinate, i.e. fumarate reductase. To further study the role of SDH, we tried to modulate the activity of the enzyme by treating the islets with fumarate. The depolarisation of the mitochondrial membrane by the addition of 10 mmol/l FAM to 3.3 mmol/l glucose (Fig. 3d) suggests reversal of the enzymatic reaction. Indeed, both FAM and fumaric acid inhibited SDH activity in isolated mitochondria in a dose-dependent manner (Fig. 7); as expected, a lower inhibitory effect was observed with the ester form of the mitochondrial metabolite. In addition, fumarate exhibited a weaker inhibitory activity compared with NPA.

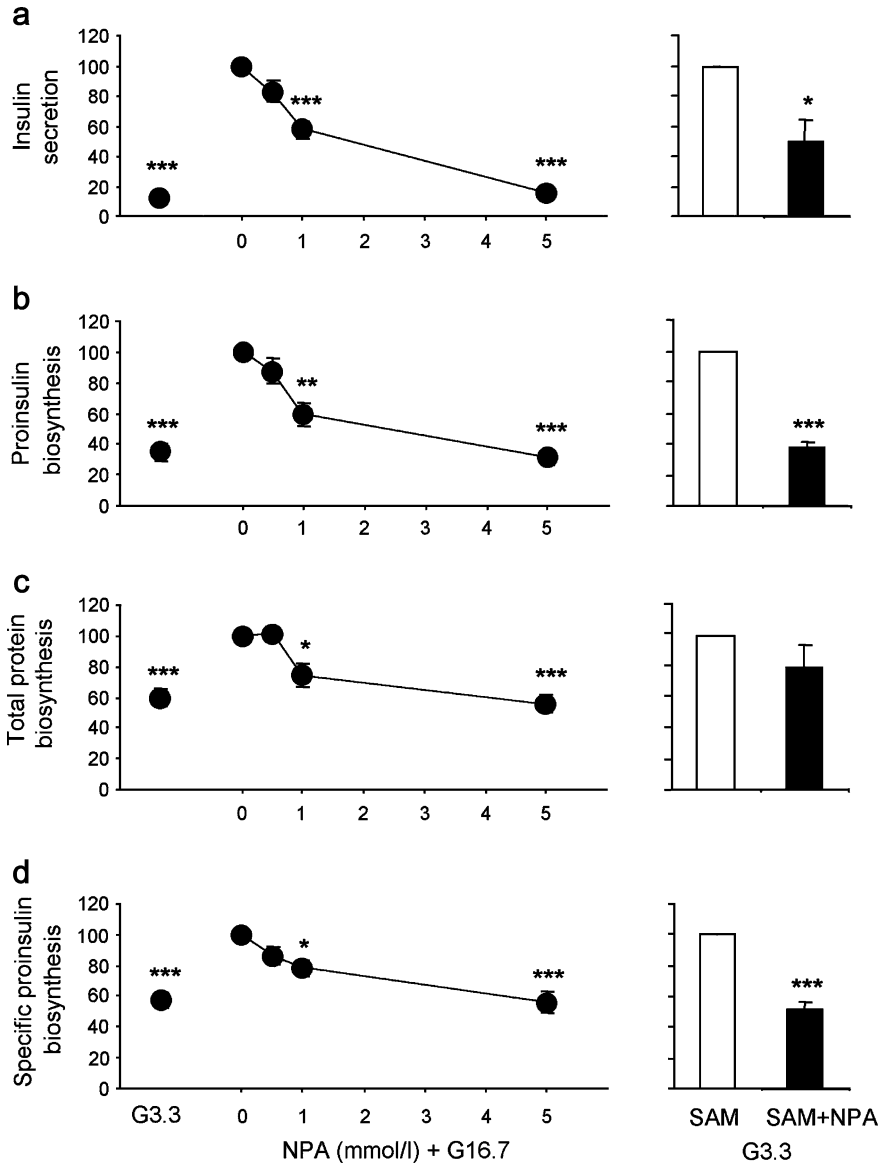

Fig. 6 Effect of SDH inhibition on glucose- and succinate-stimulated **a** insulin secretion, **b** proinsulin biosynthesis, **c** total protein biosynthesis and **d** specific proinsulin biosynthesis. Islets were incubated for 1 h in 3.3 mmol/l glucose, in 16.7 mmol/l glucose with increasing concentrations of 3-nitropropionate (NPA) or in 3.3 mmol/l glucose with 10 mmol/l SAM with and without 1 mmol/l NPA. Results are shown as means±SEM of five individual experiments, each performed on a pooled islet sample from three animals. Data are normalised to the maximal stimulation in 16.7 mmol/l glucose or 3.3 mmol/l glucose+SAM. *$p<0.05$, ** $p<0.01$ and ***$p<0.001$ relative to maximal stimulation

Effect of fumarate on glucose- and succinate-stimulated preproinsulin gene transcription and translation Short-term exposure to 10 but not to 2 mmol/l FAM partially reversed glucose- and succinate-induced hyperpolarisation of the mitochondrial membrane (Fig. 8a) but did not affect glucose- and succinate-stimulated insulin secretion (not shown), or proinsulin biosynthesis (Fig. 8b). There was a tendency for increased total protein biosynthesis in response FAM at 16.7 mmol/l glucose (Fig. 8c), resulting in a modest decrease of specific proinsulin biosynthesis (Fig. 8d).

Long-term exposure (24 h) of islets to 10 mmol/l, but not 2 mmol/l, FAM induced cell death (data not shown); therefore, we used 2 mmol/l FAM in long-term culture experiments. Incubation of islets with 2 mmol/l FAM for 24 h reduced somewhat the glucose-induced hyperpolarisation of the mitochondrial membrane, but completely abolished the hyperpolarising effect of succinate (Fig. 8a). Specific glucose- and succinate-stimulated proinsulin biosynthesis was inhibited by 18 and 43%, respectively, after 24-h exposure to 2 mmol/l FAM (Fig. 8d). Thus, modulation of specific proinsulin biosynthesis by glucose and succinate correlated with changes in the mitochondrial membrane potential. In contrast to these modest effects on proinsulin biosynthesis, 2 mmol/l FAM completely abolished the stimulatory effect of glucose and succinate on preproinsulin gene transcription (Fig. 9a, b), suggesting different sensitivities of the insulin transcriptional and translational machineries to these metabolic signals.

Discussion

The present study is an extension of a previous study by Alarcon et al. on the mitochondrial regulation of proinsulin biosynthesis in pancreatic islets [3]. Our data confirm that

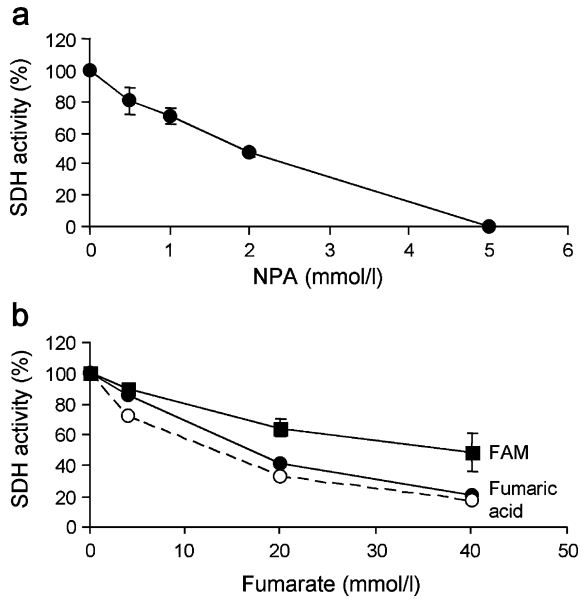

Fig. 7 Dose–response curves of NPA (**a**) and fumarate (**b**) effects on SDH activity. SDH activity was measured in isolated rat heart mitochondria, as described (see Materials and methods). The enzyme activity assay was performed at 30°C in the presence of 20 mmol/l succinic acid and different concentrations of NPA (**a**), FAM and fumaric acid (**b**). The decrease in absorbance at 600 nm was measured during 5 min of incubation in a spectrophotometer. SDH activity was determined based on the molar extinction coefficient ε=21,000. Activity was calculated as mmol DCIP reduced per min per mg protein and expressed as the percentage of the controls. Results are means±SEM for NPA and FAM (n=3). The effect of fumaric acid in two separate experiments is shown

succinate rapidly stimulates proinsulin biosynthesis at the level of preproinsulin mRNA translation [3]. Most importantly, we show that succinate also mimics the glucose effect on preproinsulin gene transcription. Like glucose, succinate increased the general transcriptional activity and total protein biosynthesis in pancreatic islets; however, the specific stimulation of preproinsulin gene transcription and proinsulin biosynthesis was two- to three-fold higher. Since exposure to high glucose increases islet succinate levels [3, 29], this metabolite may indeed serve as an important mediator between glucose metabolism and preproinsulin gene transcription and translation. It has to be stressed that other mitochondrial metabolites had no effect on insulin production; thus the succinate effect seems to be specific.

Insulin production stimulated by succinate correlated with the effect of succinate on insulin secretion. This is important for the global function of the beta cell in preserving islet insulin content. The same metabolic signals can be important for insulin secretion, proinsulin and total protein biosynthesis. Therefore, it is difficult to completely dissociate these processes and distinguish exclusive mechanisms. The fact that mitochondrial activation via glutamate dehydrogenase does not increase specific proinsulin biosynthesis contrasts with the strong specific effect of succinate, emphasising its unique role in signalling proinsulin biosynthesis. Allosteric activation of glutamate dehydrogenase by L-leucine or its non-metabolisable analogue BCH increases glutamine oxidation and insulin secretion [21, 30]. The conversion of glutamate to alpha-ketoglutarate eventually increases mitochondrial succinate levels. However, this metabolic pathway provides additional signals that stimulate general protein biosynthesis [31], resulting in dilution of the specific effect of succinate on proinsulin biosynthesis.

How does succinate stimulate insulin production? Alarcon et al. found that inhibition of the enzymatic conversion of succinate to fumarate by malonic acid methyl ester augmented proinsulin biosynthesis [3]. This observation was the basis for their suggestion that succinate itself or its cytosolic metabolite succinyl CoA may serve as a coupling factor for glucose-stimulated proinsulin biosynthesis. However, they observed only a modest augmentation of proinsulin biosynthesis over a narrow range of malonate concentrations. In contrast, we show here that the potent SDH inhibitor NPA abolished glucose- as well as succinate-induced proinsulin biosynthesis, emphasising the importance of this enzyme for the physiological regulation of preproinsulin mRNA translation, while rendering a direct effect of succinate less likely. NPA abolished glucose- and succinate-stimulated hyperpolarisation of the mitochondrial membrane, indicating that this compound indeed inhibited the islet activity of SDH, which is part of complex II of the mitochondrial respiratory chain. We cannot exclude the possibility that succinate and/or succinyl CoA may have an intrinsic stimulatory effect on proinsulin biosynthesis; yet, based on our data, we believe it to be marginal compared with the role of SDH. NPA also reduced glucose- and succinate-stimulated insulin secretion, thus pointing to a role for SDH in beta cell stimulus–secretion coupling. It is unlikely that the secreted insulin is involved in the regulation of proinsulin biosynthesis in our experimental system, since complete inhibition of glucose-stimulated insulin secretion with diazoxide had no effect on preproinsulin gene expression and biosynthesis [19]. In addition, Alarcon et al. have shown that the inhibition of insulin secretion by somatostatin or calcium removal did not inhibit succinate- and glucose-stimulated proinsulin biosynthesis [3].

We suggest that SDH activity stimulates proinsulin biosynthesis by modulating TCA cycle flux and ATP production. SDH is directly linked to the electron transport chain in the inner membrane of the mitochondria and therefore plays an important role in oxidative phosphorylation. The electron transport chain promotes proton pumping across the inner mitochondrial membrane, thereby hyperpolarising the membrane. In isolated rat islets there is an excellent parallelism between glucose-induced changes in mitochondrial membrane potential and modification of islet ATP : ADP ratio [24]. Therefore, mitochondrial membrane hyperpolarisation is a good indicator of mitochondrial ATP production. Indeed, glucose and succinate stimulation of proinsulin biosynthesis was associated with hyperpolarisation of the mitochondrial membrane; prevention of the glucose and succinate effects on mitochondrial membrane potential by the SDH inhibitors

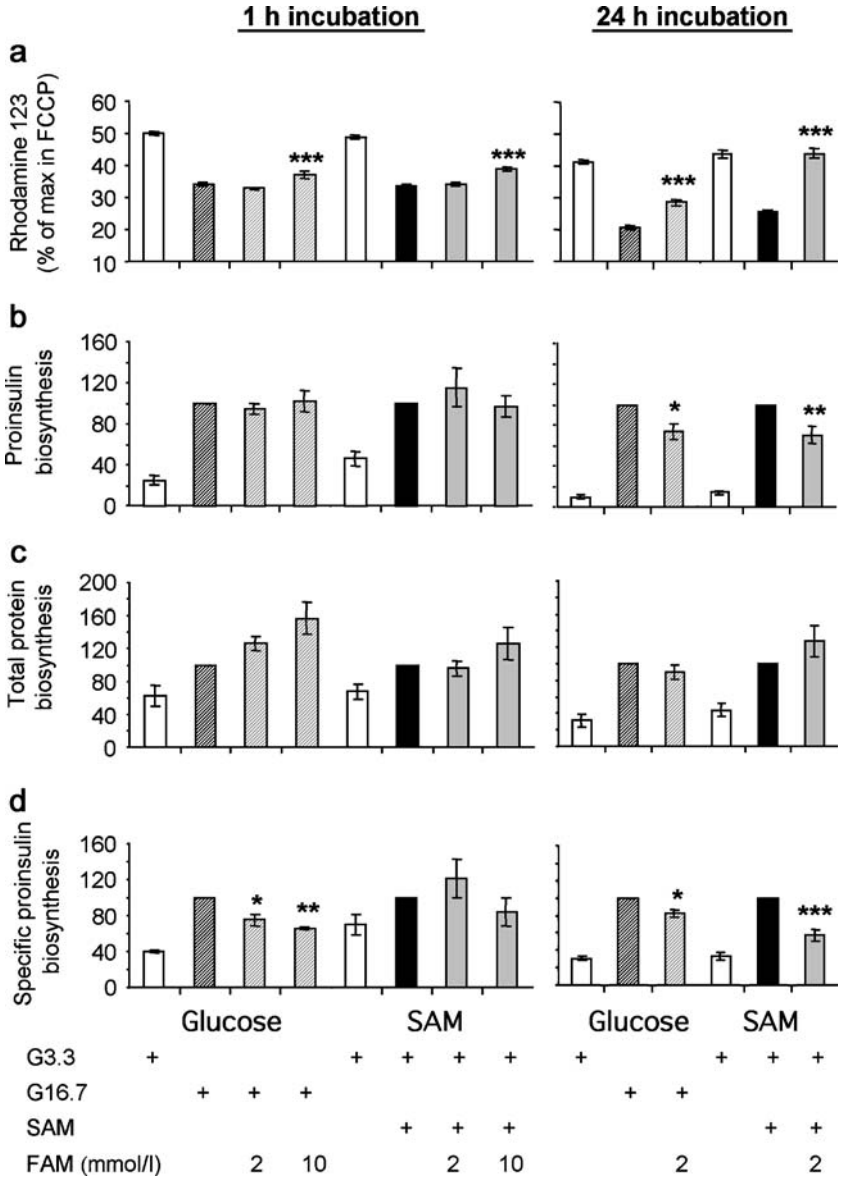

Fig. 8 Short- and long-term effects of fumarate on **a** glucose- and succinate-induced mitochondrial membrane hyperpolarisation, **b** proinsulin biosynthesis, **c** total protein biosynthesis and **d** specific proinsulin biosynthesis. Results (**a**) are means±SEM for 17–51 islets from two to six isolations. Isolated rat islets were incubated with 16.7 mmol/l glucose or 10 mmol/l SAM with and without 2 or 10 mmol/l fumaric acid monoethyl ester (FAM) for 1 h or 24 h. Results (**b–d**) are means±SEM of three to seven independent experiments, each performed on pooled islets from three rats. Data are normalised to the maximal stimulation in 16.7 mmol/l glucose or 3.3 mmol/l glucose+SAM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ relative to maximal stimulation

NPA and fumarate abolished proinsulin biosynthesis. Furthermore, TCA cycle intermediates that failed to hyperpolarise the mitochondrial membrane showed no stimulatory effect on proinsulin biosynthesis. All these findings support our suggestion that SDH activity stimulates proinsulin biosynthesis by augmenting mitochondrial ATP production.

It was previously shown that inhibitors of the electron transport chain and uncouplers of the mitochondrial inner membrane provoked a similar inhibition of islet ATP, total protein biosynthesis and glucose-induced proinsulin biosynthesis, suggesting that electron transport and ATP are not specific coupling factors for glucose-stimulated proinsulin biosynthesis [3]. Our findings do not support this interpretation, since inhibition of SDH activity reduced specific proinsulin biosynthesis (Fig. 6). We therefore suggest that SDH catalytic activity is a specific, key coupling-factor for glucose-stimulated proinsulin biosynthesis.

The couple FAD–FADH2, which is the prosthetic group of succinate dehydrogenase, has a lower impact on mitochondrial proton gradient and ATP production than NAD–NADH (2 ATP molecules produced for each FADH2 molecule entering the electron transport chain vs 3 ATP molecules for the couple NAD–NADH). However, malate (Fig. 3) and citrate esters (data not shown) did not hyperpolarise the mitochondrial membrane despite the fact that malate dehydrogenase and isocitrate dehydrogenase use NAD–NADH as cofactor. Our results may therefore suggest that FAD–FADH2 is a key metabolic mediator for insulin production.

In contrast to exposure to NPA, short-term exposure to FAM, a less potent inhibitor of SDH (Fig. 7), did not affect the mitochondrial membrane potential, suggesting that FAM was not an efficient inhibitor of SDH at this time point. Indeed, this resulted in only a modest effect on glucose-stimulated proinsulin biosynthesis and no effect

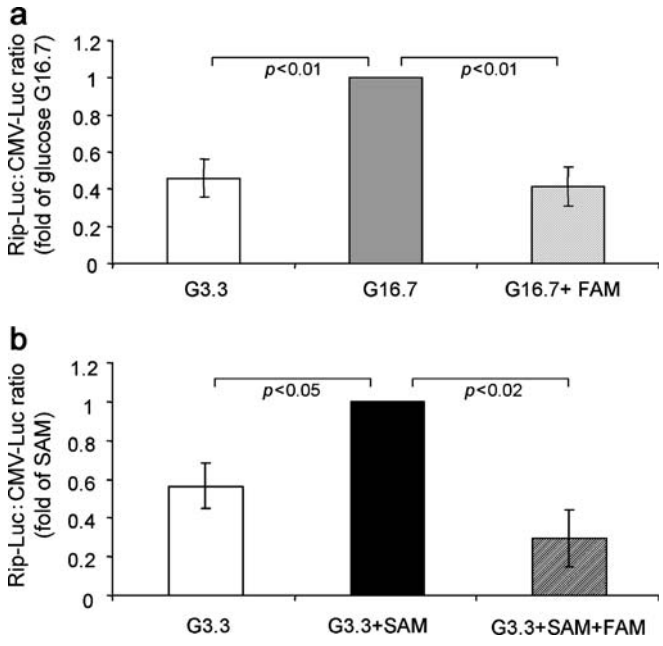

Fig. 9 Inhibitory effect of fumarate on glucose- and succinate-stimulated preproinsulin gene transcription. Isolated rat islets were transduced as in Fig. 5, then incubated for 24 h with **a** 16.7 mmol/l glucose or **b** 10 mmol/l SAM with and without 2 mmol/l FAM. Specific preproinsulin promoter activity is shown as the ratio of Rip-Luc : CMV-Luc normalised to the ratio in islets at **a** 16.7 mmol/l glucose or **b** 3.3 mmol/l glucose+10 mmol/l SAM. Results are mean±SEM for three or four individual experiments, each performed on islets pooled from six animals

on succinate-stimulated proinsulin biosynthesis at the level of translation. In contrast, long-term (24 h) exposure to 2 mmol/l FAM completely reversed succinate-induced mitochondrial hyperpolarisation. It is likely that the concentration of fumarate in the islet increases time-dependently as FAM is hydrolysed to the active product fumaric acid. This could explain the lag in the effect of FAM on the mitochondrial membrane potential. The possibility that prolonged exposure to 2 mmol/l FAM induced a small amount of cell death cannot be completely excluded; however, propidium iodide staining showed no increase in the mortality of cells treated with low-dose FAM relative to control islets in 16.7 mmol/l glucose (not shown). Moreover, this treatment did not impair insulin secretion and cellular protein biosynthesis. FAM inhibition of succinate-induced hyperpolarisation was higher than that of glucose, resulting in greater inhibition of specific succinate-induced proinsulin biosynthesis. Whereas fumarate had only a modest effect on proinsulin biosynthesis, it completely abolished glucose- and succinate-stimulated preproinsulin gene transcription, indicating greater sensitivity of the transcriptional machinery to reduction in SDH activity. Glucose-stimulated preproinsulin gene transcription is required for maintaining proinsulin biosynthesis during prolonged exposure to a high concentration of glucose [19]. As an example, complete inhibition of preproinsulin gene transcription with actinomycin D resulted in a 50% decrease in proinsulin biosynthesis following 24 h of exposure to 16.7 mmol/l glucose [19]. In line with this observation, fumarate inhibition of glucose- and succinate-stimulated preproinsulin gene transcription was associated with a 20–40% decrease in proinsulin biosynthesis over time.

Our results thus indicate that succinate and its metabolism via SDH are critical mediators of glucose-stimulated preproinsulin gene transcription and translation, being required both for the rapid increase of insulin production in response to glucose and for its maintenance during prolonged stimulation. We have previously shown that glucose-stimulated preproinsulin gene transcription is mediated by augmentation of pancreatic-duodenal homeobox 1 (PDX-1) binding to specific DNA elements of the preproinsulin gene upstream region [32–34]. It is likely that succinate also stimulates preproinsulin gene transcription by augmenting the binding activity of PDX-1 and possibly other transcription factors. This hypothesis will be addressed in future studies.

Type 2 diabetes is characterised by defects in glucose-stimulated insulin secretion, preproinsulin gene transcription and proinsulin biosynthesis [19, 34]. Deranged succinate production and/or responsiveness could contribute to the failure of pancreatic beta cells to increase insulin production in the face of increased secretory demand. Studies in models of type 2 diabetes are required to clarify the potential role of succinate production and metabolism via SDH in the insulin deficiency of this pathological condition.

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References

1. Maechler P, Wollheim CB (2001) Mitochondrial function in normal and diabetic beta-cells. Nature 414:807–812
2. Maechler P (2002) Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic beta-cells. Cell Mol Life Sci 59:1803–1818
3. Alarcon C, Wicksteed B, Prentki M, Corkey BE, Rhodes CJ (2002) Succinate is a preferential metabolic stimulus-coupling signal for glucose-induced proinsulin biosynthesis translation. Diabetes 51:2496–2504
4. Cook DL, Hales CN (1984) Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. Nature 311:271–273
5. Sweet IR, Cook DL, DeJulio E et al (2004) Regulation of ATP/ADP in pancreatic islets. Diabetes 53:401–409
6. MacDonald MJ (2003) The export of metabolites from mitochondria and anaplerosis in insulin secretion. Biochim Biophys Acta 1619:77–88
7. Flamez D, Berger V, Kruhoffer M, Orntoft T, Pipeleers D, Schuit FC (2002) Critical role for cataplerosis via citrate in glucose-regulated insulin release. Diabetes 51:2018–2024

8. Skelly RH, Bollheimer LC, Wicksteed BL, Corkey BE, Rhodes CJ (1998) A distinct difference in the metabolic stimulus-response coupling pathways for regulating proinsulin biosynthesis and insulin secretion that lies at the level of a requirement for fatty acyl moieties. Biochem J 331:553–561
9. Maechler P, Wollheim CB (1999) Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature 402:685–689
10. MacDonald MJ, Fahien LA (2000) Glutamate is not a messenger in insulin secretion. J Biol Chem 275:34025–34027
11. MacDonald MJ, Fahien LA, Buss JD, Hasan NM, Fallon MJ, Kendrick MA (2003) Citrate oscillates in liver and pancreatic beta cell mitochondria and in INS-1 insulinoma cells. J Biol Chem 278:51894–51900
12. Farfari S, Schulz V, Corkey B, Prentki M (2000) Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. Diabetes 49:718–726
13. Newgard CB, Lu D, Jensen MV et al (2002) Stimulus/secretion coupling factors in glucose-stimulated insulin secretion: insights gained from a multidisciplinary approach. Diabetes 51 [Suppl 3]:S389–S393
14. Mulder H, Lu D, Finley J et al (2001) Overexpression of a modified human malonyl-CoA decarboxylase blocks the glucose-induced increase in malonyl-CoA level but has no impact on insulin secretion in INS-1-derived (832/13) beta-cells. J Biol Chem 276:6479–6484
15. Bertrand G, Ishiyama N, Nenquin M, Ravier MA, Henquin JC (2002) The elevation of glutamate content and the amplification of insulin secretion in glucose-stimulated pancreatic islets are not causally related. J Biol Chem 277:32883–32891
16. Roduit R, Nolan C, Alarcon C et al (2004) A role for the malonyl-CoA/long-chain acyl-CoA pathway of lipid signaling in the regulation of insulin secretion in response to both fuel and nonfuel stimuli. Diabetes 53:1007–1019
17. Leibowitz G, Oprescu AI, Uckaya G, Gross DJ, Cerasi E, Kaiser N (2003) Insulin does not mediate glucose stimulation of proinsulin biosynthesis. Diabetes 52:998–1003
18. Wicksteed B, Alarcon C, Briaud I, Lingohr MK, Rhodes CJ (2003) Glucose-induced translational control of proinsulin biosynthesis is proportional to preproinsulin mRNA levels in islet beta-cells but not regulated via a positive feedback of secreted insulin. J Biol Chem 278:42080–42090
19. Leibowitz G, Uckaya G, Oprescu AI, Cerasi E, Gross DJ, Kaiser N (2002) Glucose-regulated proinsulin gene expression is required for adequate insulin production during chronic glucose exposure. Endocrinology 143:3214–3220
20. Gadot M, Leibowitz G, Shafrir E, Cerasi E, Gross D, Kaiser N (1994) Hyperproinsulinemia and insulin deficiency in the diabetic *Psammomys obesus*. Endocrinology 135:610–616
21. Sener A, Malaisse-Lagae F, Malaisse WJ (1981) Stimulation of pancreatic islet metabolism and insulin release by a nonmetabolizable amino acid. Proc Natl Acad Sci U S A 78:5460–5464
22. Alarcon C, Lincoln B, Rhodes CJ (1993) The biosynthesis of the subtilisin-related proprotein convertase PC3, but not that of the PC2 convertase, is regulated by glucose in parallel to proinsulin biosynthesis in rat pancreatic islets. J Biol Chem 268:4276–4280
23. Kelpe CL, Moore PC, Parazzoli SD, Wicksteed B, Rhodes CJ, Poitout V (2003) Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. J Biol Chem 278:30015–30021
24. Khaldi MZ, Guiot Y, Gilon P, Henquin JC, Jonas JC (2004) Increased glucose sensitivity of both triggering and amplifying pathways of insulin secretion in rat islets cultured for 1 week in high glucose. Am J Physiol Endocrinol Metab 287:E207–E217
25. Scaduto RC Jr, Grotyohann LW (1999) Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 76:469–477
26. Antinozzi PA, Ishihara H, Newgard CB, Wollheim CB (2002) Mitochondrial metabolism sets the maximal limit of fuel-stimulated insulin secretion in a model pancreatic beta cell: a survey of four fuel secretagogues. J Biol Chem 277:11746–11755
27. Rosen H, Rakita RM, Waltersdorph AM, Klebanoff SJ (1987) Myeloperoxidase-mediated damage to the succinate oxidase system of *Escherichia coli*. Evidence for selective inactivation of the dehydrogenase component. J Biol Chem 262:15004–15010
28. Holmuhamedov EL, Ozcan C, Jahangir A, Terzic A (2001) Restoration of Ca^{2+}-inhibited oxidative phosphorylation in cardiac mitochondria by mitochondrial Ca^{2+} unloading. Mol Cell Biochem 220:135–140
29. MacDonald MJ (2002) Differences between mouse and rat pancreatic islets: succinate responsiveness, malic enzyme, and anaplerosis. Am J Physiol Endocrinol Metab 283:E302–E310
30. Li C, Najafi H, Daikhin Y et al (2003) Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets. J Biol Chem 278:2853–2858
31. McDaniel ML, Marshall CA, Pappan KL, Kwon G (2002) Metabolic and autocrine regulation of the mammalian target of rapamycin by pancreatic beta-cells. Diabetes 51:2877–2885
32. Melloul D, Ben-Neriah Y, Cerasi E (1993) Glucose modulates the binding of an islet-specific factor to a conserved sequence within the rat I and the human insulin promoters. Proc Natl Acad Sci U S A 90:3865–3869
33. Marshak S, Totary H, Cerasi E, Melloul D (1996) Purification of the beta-cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. Proc Natl Acad Sci U S A 93:15057–15062
34. Leibowitz G, Ferber S, Apelqvist A et al (2001) IPF1/PDX1 deficiency and beta-cell dysfunction in *Psammomys obesus*, an animal with type 2 diabetes. Diabetes 50:1799–1806