

The dicarboxylate carrier plays a role in mitochondrial malate transport and in the regulation of glucose-stimulated insulin secretion from rat pancreatic beta cells

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Abstract

Aims/hypothesis We have previously described a strong correlation between pyruvate cycling and insulin secretion. We have also demonstrated a particularly important role for a pyruvate–isocitrate cycling pathway involving the mitochondrial citrate/isocitrate carrier (CIC) and cytosolic NADP-dependent isocitrate dehydrogenase. CIC requires cytosolic malate as a counter-substrate during citrate and isocitrate export. Thus, considering that the mitochondrial dicarboxylate carrier (DIC) provides an important source of cytosolic malate, we investigated the potential role of DIC in control of glucose-stimulated insulin secretion (GSIS).

Methods We used pharmacological and small interfering RNA (siRNA) tools to assess the role of DIC in insulin release in clonal insulin-secreting 832/13 cells and isolated rat islets.

Results Butylmalonate, an inhibitor of malate transport, reduced cytosolic malate and citrate levels, and inhibited GSIS in a dose-dependent manner in 832/13 cells. Suppression of DIC expression resulted in inhibition of GSIS by 5% to 69%, the extent of inhibition of insulin

secretion being proportional to the level of *Dic* (also known as *Slc25a10*) gene knockdown. The most effective siRNA duplex against *Dic* did not affect glucose utilisation, glucose oxidation or ATP/ADP ratio, but did suppress glucose-induced increments of the NADPH/NADP⁺ ratio. Confirmation of our results in primary cultures of isolated rat islets showed that butylmalonate and an adenovirus expressing an siRNA against *Dic*-inhibited GSIS.

Conclusions/interpretation Malate transport by DIC may play an important role in GSIS, possibly by providing cytosolic malate as a counter-substrate for citrate and/or isocitrate export by CIC. These studies also suggest that malate transport by DIC is (1) a critical component of NADPH production mediated by pyruvate-cycling and (2) regulates GSIS.

Keywords ATP · Insulin secretion · Islets · Malate · Metabolic carriers · Metabolism · NADPH · Pancreas · Pancreatic beta cell · Pyruvate cycling

Abbreviations

Ad-siDIC-2	Adenovirus siDIC-2
CIC	Citrate/isocitrate carrier
DIC	Dicarboxylate carrier
GSIS	Glucose-stimulated insulin secretion
ICDc	Cytosolic NADP ⁺ -dependent isocitrate dehydrogenase
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
siControl	Scrambled control siRNA
siDIC	siRNA targeted against <i>Dic</i>
siRNA	Small interfering RNA
TCA	Tricarboxylic acid

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Introduction

Glucose-stimulated insulin secretion (GSIS) is biphasic and involves K_{ATP} channel-dependent and K_{ATP} channel-independent pathways. The K_{ATP} channel-dependent pathway or consensus model of GSIS holds that an increase in glucose metabolism causes a rise of the cellular ATP/ADP ratio, which promotes closure of K_{ATP} channels, membrane depolarisation and the subsequent activation of L-type voltage-dependent Ca^{2+} channels [1]. Opening of these channels allows the entry of extracellular Ca^{2+} and the concomitant rise in cytosolic Ca^{2+} concentrations triggers insulin release [2]. This K_{ATP} channel-dependent pathway is particularly important during the first phase of insulin release, whereas K_{ATP} channel-independent mechanisms appear to play a significant role in the second and more sustained phase of insulin release.

Experimental evidence for the K_{ATP} channel-independent pathway of GSIS comes from studies showing that glucose can still augment insulin secretion under conditions where the K_{ATP} channels are held open by application of diazoxide and where cytosolic Ca^{2+} was clamped at high levels with high K^+ [3]. Similar observations have been made in beta cells, which lack functional K_{ATP} channels [4, 5]. Our group, as well as others, has shown an apparent disconnect between nutrient-stimulated increase in the ATP/ADP ratio and insulin release [3–21]. Although ATP is a critical factor in the control of GSIS, an important question arises from these studies: what are the other important metabolic signalling molecule(s) for insulin secretion?

The production of candidate coupling factors such as malonyl-CoA, long-chain-acyl-CoA and NADPH has been shown to depend on the export of mitochondrial metabolites via one of the following mitochondrial carrier proteins: (1) the dicarboxylate carrier (DIC), which translocates malate in exchange for a phosphate; (2) the 2-oxoglutarate carrier, which transports 2-oxoglutarate (also known as α -ketoglutarate) in exchange for malate; and (3) the citrate/isocitrate carrier (CIC), which catalyses an electroneutral exchange of one of three tricarboxylic acids (citrate, isocitrate, *cis*-aconitate) plus a proton, for another tricarboxylate- H^+ , a dicarboxylate (malate or succinate) or phosphoenolpyruvate [17, 22]. These mitochondrial carriers play a critical role in intermediary metabolism as they not only provide the necessary source of carbon for fatty acid and cholesterol synthesis, but also facilitate production of sufficient amounts of NADPH, which is required to support these metabolic pathways.

Another important insulin secretion pathway that depends on metabolite transport is pyruvate cycling [12, 15, 17, 19, 23–25]. Pyruvate cycling has been suggested to

generate two potentially important signalling molecules for insulin release, α -ketoglutarate and NADPH. NADPH can be produced via one of three pyruvate cycling pathways, the pyruvate/malate pathway, the pyruvate/citrate pathway, or the pyruvate/isocitrate pathway. A key NADPH-producing enzyme for both the pyruvate malate and pyruvate citrate pathways is the cytosolic NADP⁺-dependent isoform of malic enzyme [12, 26, 27]. The key enzyme for the pyruvate isocitrate pathway is the cytosolic NADP⁺-dependent isocitrate dehydrogenase (ICDc) [23]. Although strong evidence for a role of the pyruvate–isocitrate pathways exists [12, 17, 19, 23, 25–27], others have shown a role for the other two other pyruvate cycling pathways [28–30]. At present it is unknown which pyruvate cycling pathway(s) is (are) critical for insulin release.

Understanding the mechanism of abnormal insulin secretion is essential to developing novel therapies for type 2 diabetes. However, before we can even begin to develop therapies to improve insulin release in the diabetic state, we first must understand how the beta cell senses glucose under normal physiological conditions. Our group has provided evidence for a novel pathway involved in GSIS. We have shown that suppression of ICDc activity strongly impairs GSIS, pyruvate cycling and NADPH production [23]. We have also shown that CIC plays a particularly important role in regulation of insulin secretion possibly by providing isocitrate for use by ICDc [17]. DIC would provide malate as an important counter-ion for citrate transport by CIC. In the current study we show that inhibition of DIC with a DIC inhibitor, butylmalonate, or by small interfering RNA (siRNA)-mediated suppression of its gene expression, results in potent inhibition of NADPH production and GSIS. We also show that the inhibitory effects of butylmalonate on GSIS were reversed in isolated rat islets by adding dimethyl malate.

Methods

Cell lines We used the cell line 832/13 [31], which was derived from INS-1 rat insulinoma cells [32] and was a gift from C. B. Newgard (Duke University, Durham, NC, USA). Insulin secretion and content assays were performed as previously described [17, 25, 31].

Measurement of malate and citrate by gas chromatography/mass spectrometry Cytosolic and mitochondrial malate and citrate levels were measured relative to an added [¹³C₁₃]malate or [²H₄]citrate as internal standards (IsoTec, Toronto, ON, Canada) by gas chromatography/mass spectrometry, as previously described [17]. In brief, at the end of the secretion assay, cells were treated with saponin (80 μ g/ml) to

selectively permeabilise the plasma membrane of cells, followed by centrifugation [17, 33, 34]. The supernatant fraction represented the cytosolic fraction and the cell pellet representing the mitochondrial fraction [17]. For more details, see Electronic supplementary material (ESM), Measurement of malate.

siRNA duplexes and construction of the adenovirus siDIC recombinant adenovirus Three siRNA duplexes were tested against the rat *Dic* (accession number BC081734). The siRNA sequences and transfection protocol can be found in the ESM (siRNA duplexes). Experiments were performed 3 days after duplex transfection.

The siRNA sequences for *Dic* (DIC-1–3) and scrambled control siRNA (siControl) were used to prepare recombinant adenoviruses by previously described methods [17, 25]. The siRNA adenoviruses were purified using a kit (Adeno-X Purification Kit; BD Biosciences, Clontech, Palo Alto, CA, USA) and virus titre was estimated by measuring absorbance at 260 nm.

*Real-time PCR analysis of *Dic* mRNA expression* RNA was isolated from 832/13 cells using a kit (Aurum RNA Mini kit; BioRad, Hercules, CA, USA) and from primary rat islets using MicroRNA kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed using a kit (iScript cDNA synthesis kit; BioRad). *Dic* (also known as *Slc25a10*) mRNA levels were detected by real-time PCR as previously described [17, 25], using prevalidated *Dic* and 18S RNA-specific fluorescent probes obtained from Applied Biosystems (Foster City, CA, USA).

Cell viability assay Cell viability was assessed either by the cytotoxicity assay (SIGMA, Oakville, ON, Canada) or the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) mitochondrial dye method.

DIC immunoblot DIC immunoblots were performed as described in the ESM (DIC immunoblot).

Isolation of mitochondria and measurement of mitochondrial malate transport Mitochondria were prepared and assayed for malate transport from control (untreated), siControl and siDIC-2-treated 832/13 cells as previously described [35]; for more detailed protocol, see corresponding section of ESM. Malate exported from cell mitochondria was assayed enzymatically with alkali enhanced fluorescence of NAD(P)(H) [35, 36] (details, see ESM).

Glucose utilisation, glucose oxidation and glucose incorporation into fatty acids Samples were processed for

measurement of glucose utilisation, glucose oxidation and glucose incorporation into fatty acids as previously described [17, 25] and in ESM (Glucose utilisation, Glucose oxidation). Briefly, 832/13 cells were preincubated for 2 h in KRB secretion buffer with low glucose (2 mmol/l). After the preincubation period, 832/13 cells were cultured in the presence of tracer.

ADP and ATP determination Cells (832/13 cells) were pretreated for 2 h in KRB with 2.8 mmol/l glucose and then incubated for 2 h in KRB with 2.8 or 16.7 mmol/l glucose. At the end of the experiment, cells were collected and snap-frozen in dry ice/ethanol. The cell pellets were stored at -80°C until assayed. ATP and ADP content was determined as described [17, 37].

NADPH and NADP^+ assays Cells (832/13 cells) were pretreated for 2 h in KRB with 2.8 mmol/l glucose and then incubated for 2 h at 2.8 or 16.7 mmol/l glucose. At the end of the experiment, cells were collected and snap-frozen in dry ice/ethanol. The cell pellets were stored at -80°C until assayed. NADP^+ and NADPH levels were measured as described [17, 23].

Islet isolation and insulin secretion Islets were removed from adult male Sprague–Dawley rats weighing approximately 250 to 300 g, and cultured as previously described [17, 25] and detailed in the corresponding ESM section. Insulin assay and insulin content were measured as previously described [17, 25, 37] and in ESM (Islet isolation). Islet butylmalonate studies were performed 24 h after islet isolation. For these studies two groups of islets were used: control (untreated islets) or islets treated with butylmalonate. Butylmalonate was only added during the insulin secretion assay. For the adenovirus studies we used three groups of islets: control (untreated), Ad-siControl and adenovirus siDIC-2 (Ad-siDIC-2). For each experimental group, 100 small to medium-sized islets were either untreated or treated with 1000 particles/ml of adenovirus overnight (final volume 2 ml), washed with growth media and cultured for 2 days. Compared with the untreated group of islets, there were no differences in islet cell viability between islets treated with Ad-siControl and those treated with Ad-siDIC-2.

For high glucose or palmitate treatments, islets were preincubated for 24 h in islet culture medium (described above) and then transferred to control medium (culture medium with 5% FBS [vol./vol.] and 0.25% BSA [0.25%] without NEFA [Sigma]), control medium with high glucose (20 mmol/l) or palmitate medium (control medium with 0.4 mmol/l palmitate) for 48 h. After 48 h, islet cDNA was prepared and *Dic* expression assessed by real-time PCR as described above.

Statistics Statistical significance was assessed by Student's *t* test or by one-way or two-way ANOVA for repeated measures followed by multiple Bonferroni comparisons. All data are expressed as means \pm SEM. All experimental protocols used in this study were approved by the Research Ethics Board of the University of Waterloo.

Results

The DIC inhibitor butylmalonate dose-dependently inhibits GSIS in 832/13 cells Butylmalonate dose-dependently inhibited insulin release in response to glucose in the INS-1-derived 832/13 cells (Fig. 1a). Butylmalonate (2 mmol/l) significantly inhibited insulin secretion stimulated by glucose+KCl (16.7 mmol/l glucose+30 mmol/l KCl) by $8.8\pm 3\%$ as compared with untreated cells (Fig. 1a). To stimulate insulin secretion independently of glucose metabolism, we used 30 mmol/l KCl in these studies. Butylmalonate (2 mmol/l) did not affect basal insulin secretion or GSIS (Fig. 1a). However, addition of 5 mmol/l butylmalonate resulted in a significant inhibition of insulin release stimulated both by glucose and by glucose+KCl ($47\pm 3\%$ and $49\pm 4\%$ respectively; Fig. 1a), whereas 10 mmol/l butylmalonate further suppressed both of the above by $62\pm 2\%$ and $81\pm 1\%$, respectively. KCl may be expected not to restore GSIS, if the role of DIC is independent of the glucose-stimulated rise of Ca^{2+} and is dependent on some other mitochondria-derived signalling molecule that modulates insulin release. At 10 mmol/l butylmalonate basal insulin release was significantly elevated ($p<0.001$).

Butylmalonate effects on cytosolic and mitochondrial malate and citrate levels Cytosolic and mitochondrial malate and citrate concentrations were measured in 832/13 cells cultured at either low or high glucose concentrations in the absence or presence of the specific DIC inhibitor butylmalonate. As expected, incubation of 832/13 cells in the presence of 5 mmol/l butylmalonate significantly reduced glucose-stimulated mitochondrial export of malate by $39\pm 5\%$ (Fig. 1b). In contrast, butylmalonate-mediated inhibition of DIC did not cause any significant changes in mitochondrial malate levels of 832/13 cells incubated at low or high glucose concentrations. In addition, 5 mmol/l butylmalonate significantly reduced glucose-stimulated mitochondrial citrate export into the cytosolic compartment by $35\pm 4\%$ without affecting mitochondrial citrate levels (Fig. 1c). The lack of an increase in mitochondrial malate levels was not unexpected as levels of tricarboxylic acid (TCA) intermediates are highly regulated. These results suggest that cytosolic malate provided by DIC is essential for export of

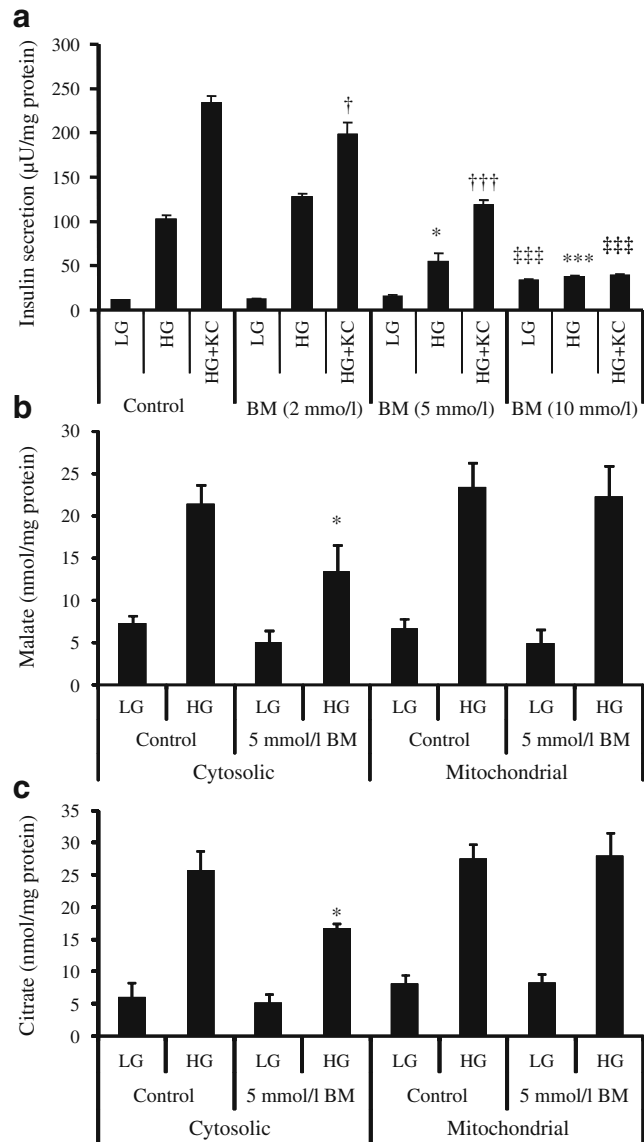


Fig. 1 The DIC inhibitor butylmalonate (BM) dose-dependently inhibits GSIS, and inhibits mitochondrial malate and citrate transport in 832/13 cells. **a** GSIS in response to 2, 5 and 10 mmol/l butylmalonate and glucose as labelled ($n=4$), and **(b)** malate and **(c)** citrate transport in response to glucose stimulation ($n=5$ for both) as labelled: LG, low glucose (2.8 mmol/l); HG, high glucose (16.7 mmol/l); HG+KCl, high glucose plus KCl (16.7 mmol/l glucose, 30 mmol/l KCl). Cytosolic and mitochondrial malate and citrate were separated as described in Methods section. * $p<0.05$ and *** $p<0.001$ for HG control vs HG butylmalonate; † $p<0.05$ and ††† $p<0.001$ for HG+KCl control vs HG+KCl plus butylmalonate; ††† $p<0.001$ for LG control vs LG butylmalonate

mitochondrial citrate by CIC (CIC transports citrate in exchange for malate [17]).

The effects of butylmalonate on insulin secretion did not affect glucose-stimulated changes in the ATP/ADP ratio (data not shown) and insulin content from 832/13 cells (control 8934 ± 145 ; butylmalonate 2 mmol/l 8780 ± 231 ; butylmalonate 10 mmol/l 8611 ± 579 μ U insulin/mg pro-

tein). None of the effects of butylmalonate could be ascribed to cytotoxicity, as treatment of 832/13 cells for 3 h with 10 mmol/l butylmalonate had no effect on cell viability as assessed by ToxiLight cytotoxicity assay or the MTS mitochondrial dye method (data not shown).

Transfection-based siRNA-mediated suppression of *Dic* expression inhibits GSIS Because we cannot rule out the possibility that butylmalonate targets other proteins besides DIC, we next used siRNA technology to ensure that specific targeting of DIC had occurred and tried to repeat some of our earlier findings done with the pharmacological inhibitor. To this end, 832/13 cells were transfected with three siRNA duplexes directed against different regions of *Dic* mRNA or a control, non-specific siRNA duplex (siControl). The three DIC-specific siRNA duplexes reduced the number of *Dic* gene transcripts by approximately 48% to 92% (Fig. 2a), causing a reduction in GSIS (5–69%) that was proportionate to the degree of *Dic* knockdown by each of the siRNA duplexes (Fig. 2b).

To further examine the role of DIC on GSIS, one of the highly efficient siRNA duplexes (siDIC-2) was used as a tool to suppress DIC in our subsequent studies. The siDIC-2 duplex reduced *Dic* mRNA by $76\pm 4\%$ and protein levels by $70\pm 8\%$ (Fig. 2c). These effects on gene and protein levels caused a $64\pm 3\%$ reduction in GSIS and a $33\pm 6\%$ reduction in insulin release stimulated by glucose+KCl (Fig. 2d). As with the butylmalonate studies, GSIS was not restored with KCl in siDIC-2-treated cells suggesting that DIC may play a role in generating a unique mitochondria-derived signalling molecule.

Effects of butylmalonate and siDIC-2 on malate transport from isolated mitochondria Mitochondria were isolated from 832/13 cells and used to assess the effects of butylmalonate and siDIC-2 on pyruvate-stimulated malate export from isolated mitochondria. Mitochondria were treated with no substrate (background malate export without a stimulus) or with pyruvate. Pyruvate enters the mitochondrial TCA cycle and is converted to malate. No significant differences were seen for

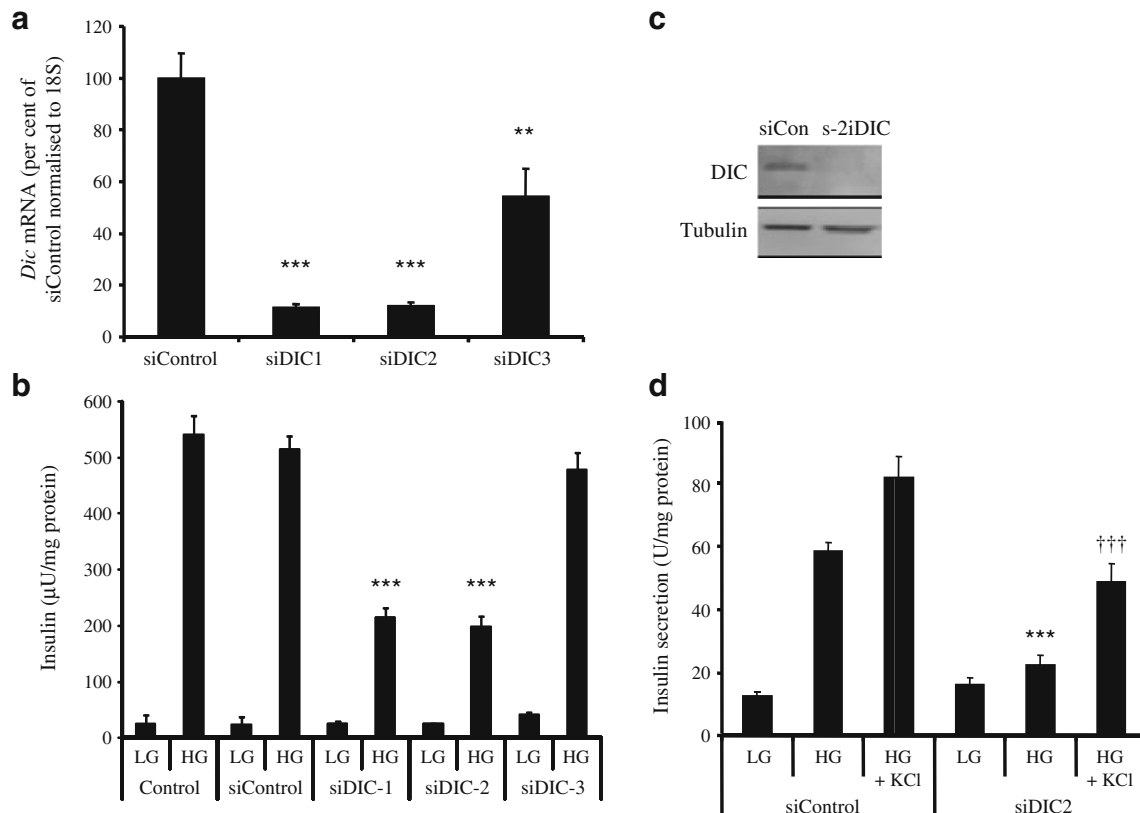


Fig. 2 Three different siRNA duplexes against *Dic* (DIC-1–3) inhibited GSIS in 832/13 cells. **a** The effects of the three siRNA duplexes on *Dic* gene expression ($n=4$) and **(b)** on GSIS ($n=4$) with glucose stimulation as labelled: LG, low glucose (2.8 mmol/l); HG, high glucose (16.7 mmol/l). Control cells **(b)** were untreated; siControl cells were treated with an siRNA that has no known

target. **c** Western blot data (siCon, cells treated with siControl; siDIC-2, cells treated with siDIC-2) ($n=6$). **d** Effects on KCl-stimulated insulin secretion ($n=6$), with glucose stimulation as labelled. HG+KCl, high glucose plus KCl (16.7 mmol/l glucose, 30 mmol/l KCl). ** $p<0.01$ and *** $p<0.001$ for HG control vs HG siDIC-2; ††† $p<0.001$ for HG+KCl control vs HG+KCl plus siDIC-2

pyruvate-stimulated malate transport in control mitochondria and mitochondria treated with the control siRNA (siControl; Fig. 3). Butylmalonate dose-dependently inhibited pyruvate-stimulated malate transport as compared with control mitochondria (Fig. 3). Mitochondria treated with siDIC-2 also inhibited pyruvate-stimulated malate transport from isolated mitochondria as compared with control mitochondria (Fig. 3). These results suggest that pharmacological or molecular biology-based methods of inhibiting DIC reduce malate transport in isolated mitochondria.

Metabolic effects of DIC suppression We next examined several potential metabolic mechanisms by which DIC suppression may inhibit GSIS. Treatment of 832/13 cells with siDIC-2 did not affect glucose utilisation (Fig. 4a) or glucose oxidation (Fig. 4b) relative to untreated (control) or siControl treatment, but did significantly reduce incorporation of radiolabelled glucose into fatty acids by $26\pm 5\%$ (Fig. 4c). Similarly to findings for the pharmacological DIC inhibitor, ATP, ADP or ATP:ADP levels were not significantly altered by siDIC-2-mediated suppression of DIC activity (Fig. 5).

We have previously shown that the mitochondrial export of citrate/isocitrate regulates GSIS via the ICDC-mediated production of cytosolic NADPH [23]. It has been proposed that the pyruvate/isocitrate cycle depends on a cytosolic source of malate for efficient mitochondrial export of citrate/isocitrate via CIC [17]. Consistent with this hypothesis, siDIC-2 treatment of 832/13 cells resulted in a significant reduction of glucose-stimulated increments of the NADPH/NADP⁺ ratio (Fig. 6).

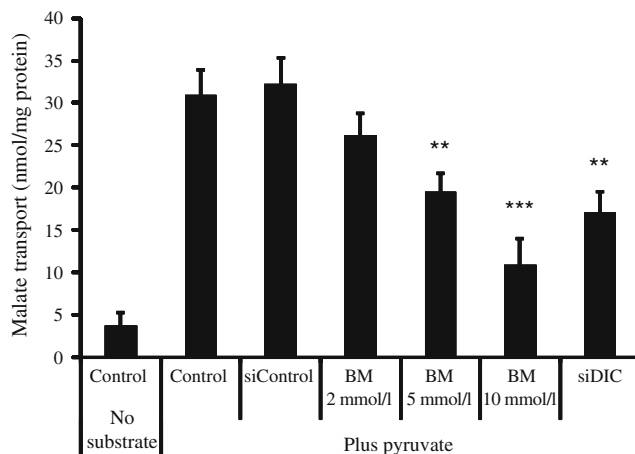


Fig. 3 Malate transport measured in isolated mitochondria from 832/13 cells. Control mitochondria were incubated with no pyruvate (no substrate-treated mitochondria). Mitochondria treated with pyruvate (a TCA substrate that generates mitochondrial malate) were untreated or treated with siControl, siDIC-2 or butylmalonate (BM, $n=4$). ** $p<0.01$ and *** $p<0.001$ for control vs treatment

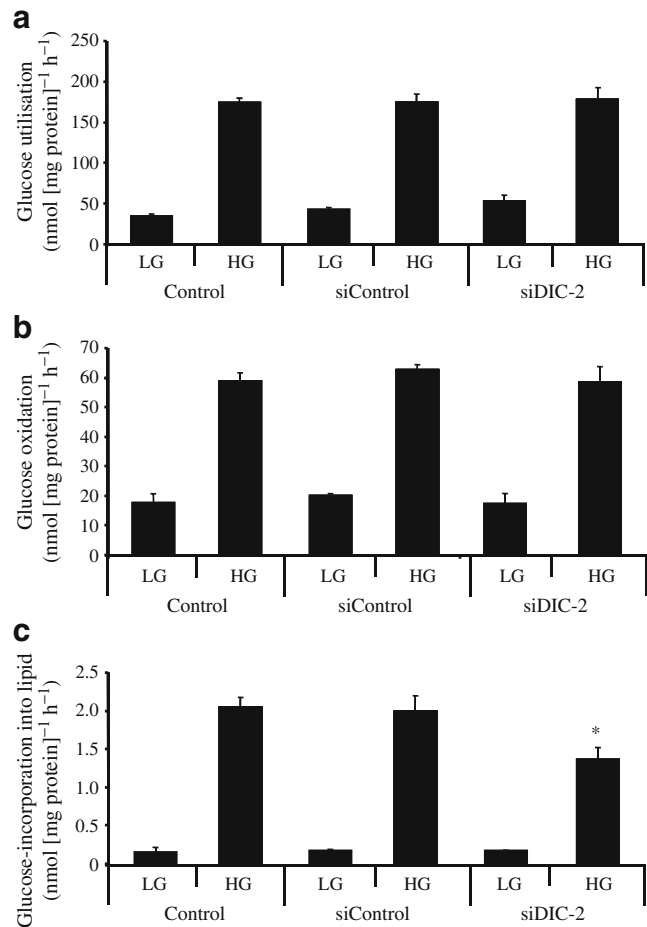


Fig. 4 Metabolic effects of siDIC-2 in 832/13 cells. **a** Glycolytic rate ($n=4$), **b** glucose oxidation ($n=4$) and **c** [$U\text{-}^{14}\text{C}$]glucose incorporation into fatty acids ($n=4$) with glucose stimulation as labelled: LG, low glucose (2.8 mmol/l); HG, high glucose (16.7 mmol/l). * $p<0.05$ for HG siControl vs HG siDIC-2

Adenovirus-mediated delivery of a Dic-specific siRNA inhibits insulin release in 832/13 cells To further investigate the effects of *Dic* knockdown in isolated rat islets, we constructed three recombinant adenoviruses (Ad-siDIC-1–3) containing the same siRNA sequences as described in **ESM** for siDIC-1–3. Treatment of 832/13 cells with Ad-siDIC-2 resulted in a significant decrease in *Dic* mRNA by $87\pm 4\%$ and GSIS as compared with cells treated with Ad-siControl. Similar results were found for Ad-siDIC-1 ($76\pm 5\%$ reduction in GSIS). Consistent with the effects of siDIC-3, the Ad-siDIC-3 did not reduce DIC expression levels ($42\pm 5\%$ reduction) enough to significantly inhibit GSIS. These results confirm the siRNA duplex experiments in 832/13 cells and suggest that DIC plays an important role in insulin secretion and DIC is also a major conduit for malate export from mitochondria.

The DIC inhibitor butylmalonate inhibits GSIS in isolated rat islets We next sought to confirm the effects of

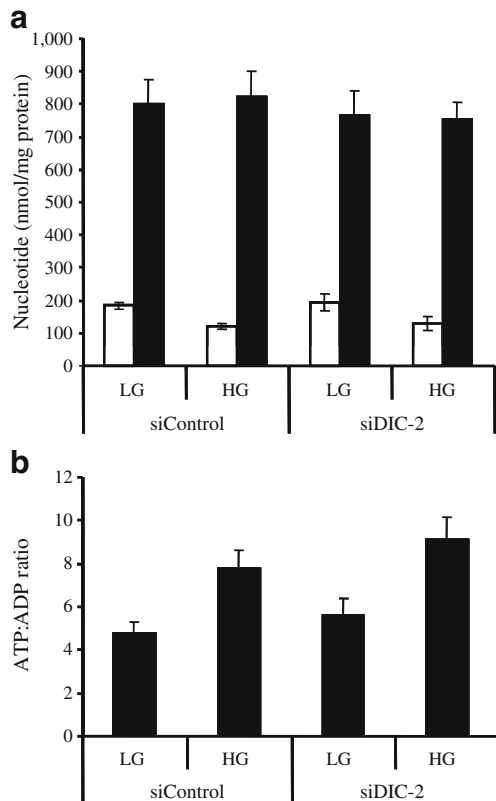


Fig. 5 The effects of siDIC-2 on (a) ATP (black bars) and ADP levels (white bars), and (b) ATP/ADP ratio in 832/13 cells ($n=4$). LG, low glucose (2.8 mmol/l); HG, high glucose (16.7 mmol/l)

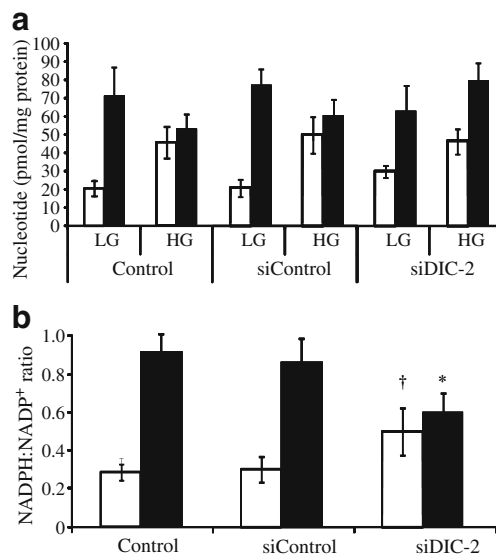


Fig. 6 The effects of siDIC-2 on glucose-stimulated alterations in (a) NADPH (white bars) and $NADP^+$ (black bars), and (b) NADPH/ $NADP^+$ ratio in 832/13 cells ($n=6$). Glucose stimulation (a) as labelled: LG, low glucose (2.8 mmol/l); HG, high glucose (16.7 mmol/l). b White bars, low glucose; black bars, high glucose. * $p<0.05$ for NADPH HG siControl vs NADPH HG siDIC-2; † $p<0.05$ for LG siControl vs LG siDIC-2

butylmalonate in 832/13 cells in primary rat islets. In static incubation experiments, addition of 2 mmol/l butylmalonate to rat islets inhibited insulin secretion in response to 16.7 mmol/l glucose by $29\pm 8\%$ as compared with islets incubated without the drug (Fig. 7a). In rat islets, butylmalonate (5 mmol/l) inhibited insulin release by $39\pm 7\%$ in response to 16.7 mmol/l glucose. These experiments revealed that butylmalonate inhibited insulin secretion in response to 16.7 mmol/l glucose. The inhibitory effects of butylmalonate could be reversed with dimethyl malate (Fig. 7b). Dimethyl malate is a membrane permeable

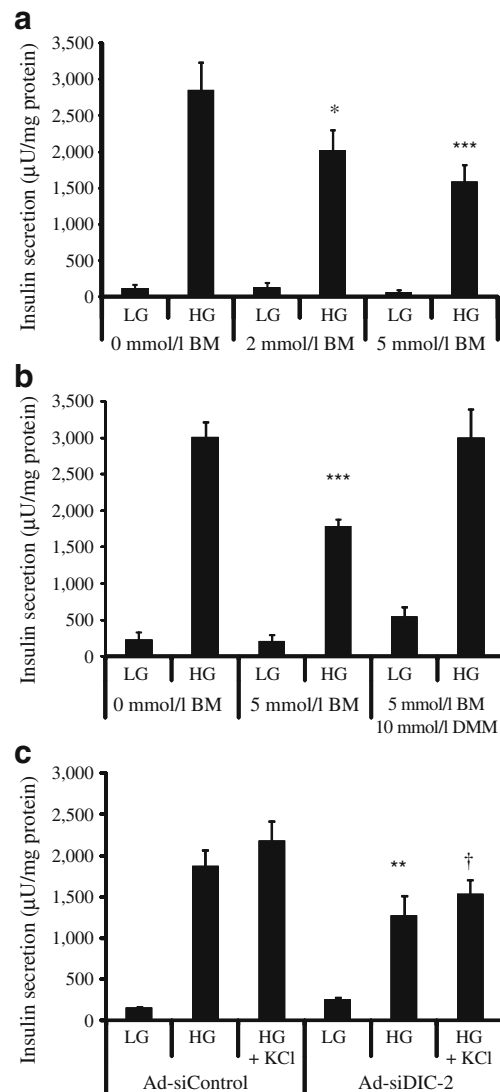


Fig. 7 The effects of (a) butylmalonate (BM) ($n=6$), (b) butylmalonate and rescue with dimethyl malate (DMM), and (c) Ad-siDIC-2 ($n=5$) on insulin secretion in isolated rat islets, with glucose stimulation as labelled: LG, low glucose (2.8 mmol/l); HG, high glucose (16.7 mmol/l); HG+KCl, high glucose plus KCl (16.7 mmol/l glucose, 30 mmol/l KCl). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for HG Ad-siControl vs HG butylmalonate or Ad-siDIC-2; † $p<0.05$ for HG+KCl Ad-siControl vs HG+KCl plus Ad-siDIC-2

analogue of malate and a known activator of pyruvate cycling and NADPH production [15, 38].

An siRNA adenovirus expressing an siRNA against Dic inhibits GSIS in isolated rat islets An adenovirus that expresses an siRNA against *Dic* (Ad-siDIC-2) reduced *Dic* mRNA levels by $59\pm 9\%$ ($p < 0.01$) as compared with Ad-siControl-treated islets. In isolated rat islets, this amount of *Dic* knockdown resulted in $36\pm 5\%$ inhibition of GSIS and $33\pm 4\%$ inhibition of insulin secretion stimulated by glucose+KCl (Fig. 7c), a finding consistent with those for 832/13 cells. There was no statistical significance for GSIS between untreated control islets and Ad-siControl-treated islets (results not shown). Ad-siDIC-2 treatment did not affect islet insulin content relative to Ad-siControl-treated islets (results not shown). Taken together, these results show that the observed effects of DIC suppression in INS-1 832/13 cells can be repeated in primary cultured isolated rat islets.

Effects of palmitate and high glucose on Dic gene expression Treatment of islets with 0.4 mmol/l palmitate for 48 h, but not with 20 mmol/l glucose, reduced *Dic* expression as compared with control cells (Fig. 8). These results suggest that diet-induced obesity may lead to a reduction of DIC in islets.

Discussion

In our previous studies, we have demonstrated that CIC-mediated export of citrate/isocitrate [17] and the cytosolic production of NADPH by cytosolic NADP⁺-dependent isocitrate dehydrogenase [23] plays a critical role in GSIS. Here, we examined the role of DIC in cytosolic NADPH production and GSIS. We hypothesised that DIC would be

required to provide sufficient amounts of cytosolic malate, which can serve as a counter substrate in CIC-mediated translocation of mitochondrial citrate/isocitrate to the cytosol (Fig. 9). These studies demonstrate that DIC plays an important role in regulating insulin release in response to glucose. Inhibition of DIC activity by butylmalonate resulted in a reduction of cytosolic malate and citrate levels. Butylmalonate and the use of siRNAs against *Dic* both resulted in inhibition of KCl-stimulated insulin secretion, suggesting that the effects of DIC on insulin secretion are independent of Ca²⁺-mediated exocytosis. Knockdown of *Dic* caused a significant lowering of NADPH/NADP⁺ ratio, the inhibitory effects of butylmalonate being reversible by dimethyl malate, a known activator of pyruvate cycling that can promote NADPH production [15, 38]. These findings support the concept that the ability of glucose to stimulate an increase in cytosolic malate levels plays an important role in the control of GSIS. Moreover, since *Dic* knockdown had no effect on glycolytic flux, glucose oxidation or ATP/ADP ratio, this suggests that no non-specific or global effect of *Dic* knockdown on beta cell glucose metabolism occurred, and no change in cell viability.

Several theories on alternative signalling molecules involved in GSIS revolve around a key concept in metabolism called anaplerosis. A role for anaplerosis in insulin release has been suggested, as approximately 40% to 50% of pyruvate entering mitochondrial pathways at high glucose does so via the key anaplerotic enzyme, pyruvate carboxylase [39–41]. Several anaplerosis-derived metabolites have been suggested to act as important signalling molecules regulating insulin secretion; these include glutamate, malonyl-CoA, long-chain-acyl-CoA, α -ketoglutarate and NADPH [15, 41–44].

Malonyl-CoA and long-chain-acyl-CoA have been proposed to play an important role in GSIS. Glucose-dependent production of malonyl-CoA and long-chain-acyl-CoA depends on pyruvate carboxylase-mediated anaplerotic elevation of cytosolic citrate levels [45, 46]. Citrate can be processed into malonyl-CoA and subsequently to long-chain-acyl-CoA. In addition, malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase I [47], an action that could divert long-chain-acyl-CoA away from oxidation in the mitochondria towards accumulation in the cytosol [46]. All these steps lead to an elevation of cytosolic malonyl-CoA and long-chain-acyl-CoA. This model has its pros and cons, which have been reviewed extensively elsewhere [12, 48]. Our recent studies [25, 38], as well as others [49–51] have now shown a consistent lack of evidence for a direct role of malonyl-CoA in regulation of GSIS, whereas its potential role in lipid-mediated potentiation of GSIS remains a possibility. Our results showing that inhibition of DIC

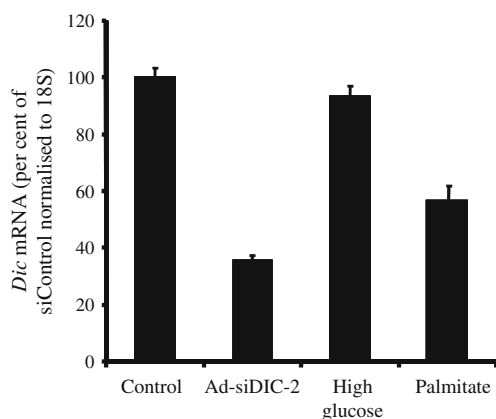


Fig. 8 The effects of Ad-siDIC-2, high glucose (48 h treatment with 20 mmol/l glucose) or palmitate (48 h treatment with 0.4 mmol/l palmitate) on *Dic* gene expression in isolated rat islets

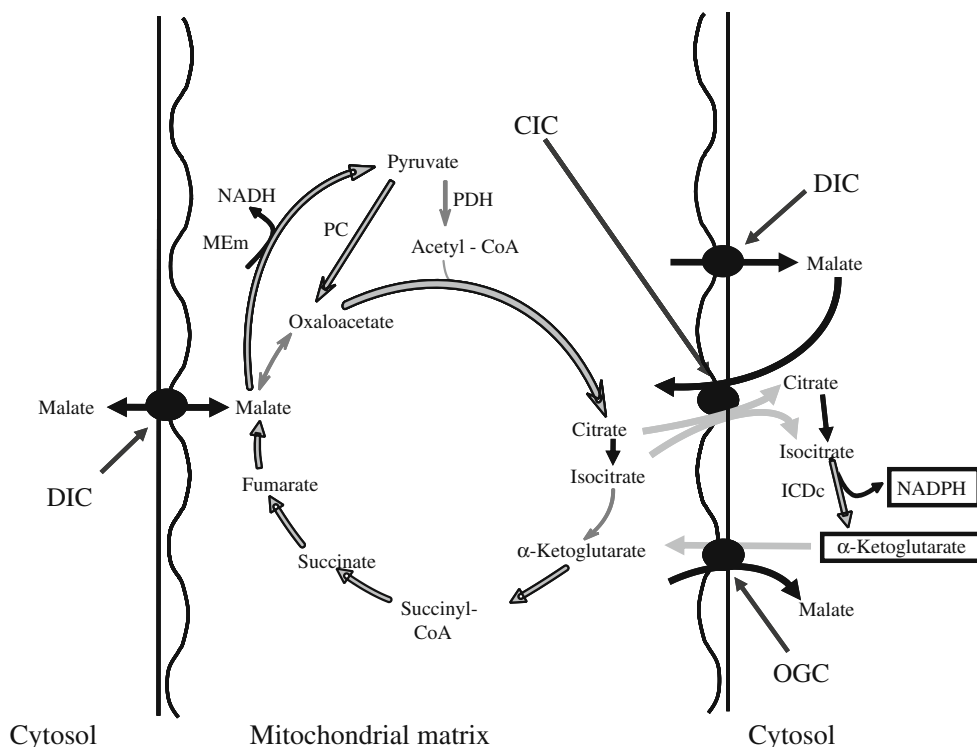


Fig. 9 Schematic diagram showing the potential role of DIC in GSIS. This study supports a role for DIC in insulin secretion. DIC may provide cytosolic malate that is required for CIC to export citrate or isocitrate from the mitochondrial matrix. This process fits into a bigger theme on nutrient-regulated insulin secretion involving pyruvate cycling via the pyruvate–isocitrate pathway (grey). For the pyruvate–isocitrate pathway, oxaloacetate, the product of pyruvate carboxylase, is converted to citrate and isocitrate. Both intermediates are capable of exiting the mitochondria via the CIC carrier in exchange for cytosolic malate. Cytosolic aconitase catalyses conversion of citrate

to isocitrate and, in turn, isocitrate can be converted to α -ketoglutarate via ICDc. α -Ketoglutarate may enter the mitochondria for conversion to malate by tricarboxylic acid cycle enzymes, with subsequent conversion to pyruvate by malic enzyme (MEm), thus completing the pyruvate cycle. The cycling of pyruvate via the pyruvate–isocitrate pathway probably results in generation of signalling molecule(s) that modulate insulin secretion. Two possible signalling molecules that can modulate insulin release are NADPH and α -ketoglutarate. OGC, 2-oxoglutarate carrier

leads to a reduction of glucose incorporation into lipid support this hypothesis.

NADPH has also been suggested to play an important role as a metabolic messenger regulating insulin release [15, 17, 19, 22–24, 52]. A key piece of evidence supporting a role for NADPH in insulin release is that addition of NADPH to patch-clamped beta cells stimulated exocytosis, whereas NADH had no effect. These studies also suggest that the NADPH/NADP⁺ ratio may be the relevant signal, since addition of NADP⁺ reversed the stimulatory effect obtained with NADPH alone [52]. We have also provided additional support for a role of NADPH from studies showing that suppression of ICDc (a key NADPH-producing enzyme) by adenovirus-mediated delivery of an siRNA construct caused a coordinate reduction in the NADPH/NADP⁺ ratio and inhibition of GSIS [23]. We have also shown that suppression of CIC inhibits NADPH production and GSIS [17]. Our current data support the concept that DIC provides a cytosolic source of malate that could be used as a counter ion for citrate/isocitrate export by CIC for use

by ICDc to produce NADPH. Our current findings also support a role for NADPH in GSIS, as we found that siRNA-mediated knockdown of *Dic* also significantly reduced the increment in NADPH/NADP⁺ ratio at high glucose levels. A major question in the field is whether NADPH is involved in insulin secretion, and if so, what is it targeting? Voltage-gated K⁺ channels are one potential target of NADPH [53]; other potential targets have been reviewed previously [12, 42].

Other by-products of anaplerosis and pyruvate cycling could also play a critical role in regulating insulin secretion. For example, a signalling role for cytosolic α -ketoglutarate or an intermediate produced from its further metabolism has been suggested [54]. Our findings from the present study, looking at the role of DIC in insulin secretion, supports this possibility. The 2-oxoglutarate carrier transports α -ketoglutarate out of mitochondria in exchange for cytosolic malate [55]. We have recently shown that dimethyl α -ketoglutarate is able to stimulate insulin secretion and that knockdown of the gene encoding α -ketoglutarate carrier caused a significant impairment of this response [55]. We

have also shown that siRNA-mediated knockdown of *Icdc* which metabolises isocitrate to α -ketoglutarate, also inhibits insulin release [23]. These two studies suggest that either transport of or generation of cytosolic α -ketoglutarate is a key event in control of insulin secretion.

It remains unclear exactly how α -ketoglutarate regulates insulin release, but it could regulate beta cell insulin secretion by serving as a substrate for α -ketoglutarate hydroxylases. α -Ketoglutarate hydroxylases modify proline residues on hypoxia-inducible transcription factors and possibly other substrates, promoting their degradation and inactivation [56]. Support for this concept comes from studies showing that acute inhibition of α -ketoglutarate hydroxylases causes impairment of insulin secretion [57].

In summary, the current study investigates the role of glucose-stimulated export of mitochondrial malate in the production of cytosolic NADPH and in the regulation of insulin secretion. We have shown that the DIC inhibitor, butylmalonate, and siRNA-mediated knockdown of *Dic* inhibited GSIS from INS-1-derived 832/13 cells and isolated rat islets. The effects of DIC inhibition could be reversed by dimethyl malate, which restores cytosolic malate levels, and promotes pyruvate cycling and NADPH production. These results support a role for DIC in insulin secretion, consisting possibly of provision of cytosolic malate, which may serve as a counter substrate for CIC-mediated export of citrate/isocitrate from the TCA cycle. In combination with our earlier work on CIC [17] and ICDC [23], the present study on the role of DIC suggests a novel mechanism in regulation of insulin release that involves pyruvate cycling via the pyruvate/isocitrate pathway (Fig. 8b).

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