Overexpression of the malate–aspartate NADH shuttle member Aralar1 in the clonal β-cell line BRIN-BD11 enhances amino-acid-stimulated insulin secretion and cell metabolism

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A B S T R A C T

In the present study, we have investigated the effects of the transduction with recombinant adeno-virus AdCA-Aralar1 (aspartate–glutamate carrier 1) on the metabolism, function and secretory properties of the glucose- and amino-acid-responsive clonal insulin-secreting cell line BRIN-BD11. Aralar1 overexpression increased long-term (24 h) and acute (20 min) glucose- and amino-acid-stimulated insulin secretion, cellular glucose metabolism, L-alanine and L-glutamine consumption, cellular ATP and glutamate concentrations, and stimulated glutamate release. However, cellular triacylglycerol and glycogen contents were decreased as was lactate production. These findings indicate that increased malate–aspartate shuttle activity positively shifted β-cell metabolism, thereby increasing glycolysis capacity, stimulus–secretion coupling and, ultimately, enhancing insulin secretion. We conclude that Aralar1 is a key metabolic control site in insulin-secreting cells.

INTRODUCTION

Nutrient metabolism is tightly coupled to insulin secretion in the pancreatic β-cell [1]. Mitochondrial metabolism is crucial for the coupling of glucose and amino acid recognition to exocytosis of insulin granules. This is illustrated by *in vitro* and *in vivo* observations that mitochondrial dysfunction severely impairs insulin secretion [2,3]. Mitochondria generate ATP which, in addition to cytoplasmic Ca^{2+}, is the main coupling messenger in insulin secretion. Mitochondria generate additional coupling factors, which serve as sensors for the control of exocytosis [2]. Numerous studies have sought to identify the factors that mediate the key amplifying pathway over the Ca^{2+} signal in nutrient-stimulated insulin secretion. Predominantly, these factors are nucleotides (ATP, GTP, cAMP and NADPH), although metabolites have also been implicated, such as long-chain acyl-CoA derivatives, malonyl-CoA [3,4] and glutamate [5,6]. Glucose, alanine and glutamine metabolism in the β-cell may generate many, if not all, of the mitochondrial factors described above [7–11].

In β-cells, the NADH shuttle system is predominantly comprised of the glycerophosphate and malate–aspartate shuttles [12]. However, other redox shuttle mechanisms have been demonstrated [13–15]. Previous studies have demonstrated the significance of the glycerophosphate shuttle for β-cell function [16–18]. However, the malate–aspartate shuttle has been proposed as the key physiological regulator of cytosolic NADH transfer to the mitochondrial matrix in the β-cell [19,20], thereby contributing to the amplifying pathway of insulin secretion.

Aralar1 (aspartate–glutamate carrier 1; AGC1) is a Ca^{2+}-sensitive isoform of the aspartate–glutamate carrier and an integral part of the malate–aspartate shuttle [21,22], which is mainly expressed in the heart, brain, skeletal muscle [21,23] and islets [20]. The significance of Aralar1 for β-cell function, glucose metabolism and insulin secretion has been demonstrated previously in the INS-1E cell line and isolated rat islets [20]. However, amino acid sensitivity to modulation of malate–aspartate capacity, or the effect of increased capacity on TAG (triacylglycerol) or glycogen content have not been investigated. We have now overexpressed Aralar1 using AdCA-Aralar1 (recombinant adenovirus encoding Aralar1) in the glucose- and amino-acid-sensitive BRIN-BD11 β-cell line and assessed functional outcomes.

We hypothesized that overexpression of the malate–aspartate shuttle component Aralar1 in BRIN-BD11 cells would affect cellular nutrient metabolism and insulin secretion. Indeed, we now report that acute insulin secretion was substantially enhanced. This was associated with significant increases in cellular glucose, alanine, glycogen and TAG utilization, whereas lactate formation was reduced. The clonal β-cell line BRIN-BD11 was chosen for the present study as metabolic, signalling, insulin secretory and cell viability responses to glucose and amino acids, as well as other stimuli, are intact and have been well characterized [7,8,24–26].

MATERIALS AND METHODS

Reagents

All reagents, unless stated otherwise, were purchased from Sigma–Aldrich. AdCA-Aralar1 and AdCA-LacZ (recombinant adenovirus encoding bacterial β-galactosidase) constructs have been described previously [20]. Anti-Aralar1 and anti-citrin antibodies were donated by Professor Jorgina Satrústegui (University Autonoma, Madrid, Spain).

Culture of BRIN-BD11 pancreatic β-cells

Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium supplemented with 10 % (v/v) FCS (fetal calf serum), 0.1 % antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin) and 11.1 mmol/l D-glucose (pH 7.4).

The origin of BRIN-BD11 cells is described elsewhere [24,27]. These cells provide an appropriate β-cell model as shown by studies of insulin secretion [24,25,27], β-cell metabolism [1,7,8], electrophysiology [26], Ca^{2+} handling [28] and cellular defence [29]. INS-1E cells were cultured as described previously [30].

The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2/95 % air using a Forma Scientific incubator. The cells were cultured in 50–70 ml of RPMI-1640 tissue culture medium in T175 sterile tissue culture flasks. Cells were subsequently seeded into 96-, 24- or six-well plates and were allowed to adhere over a 24 h period. For infection, cells were incubated with RPMI medium supplemented with 5 % (v/v) FCS, 0.1 % antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin) and 11.1 mmol/l D-glucose (pH 7.4) containing various concentrations of AdCA-Aralar1 constructs as described by Rubi et al. [20]. After transduction, cells were washed in PBS and cultured for a further 24 h to allow transgene expression. During the present study, 8 pfu (plaque-forming units)/cell was used as optimum. AdCA-LacZ, which expresses bacterial β-galactosidase, was used for control transductions. After incubation for 24 h, an aliquot of the medium was removed, centrifuged and used for quantification of insulin and metabolites (D-glucose, L-glutamine, L-lactate and L-glutamate). Cells were also analysed for their respective insulin, glutamate, TAG and glygogen content (expressed per mg of protein).

Long-term chronic (24 h) and acute (20 min) insulin secretion

After incubation for 24 h, an aliquot of the medium was collected, cleared by centrifugation at 200 *g* for 5 min and analysed for insulin using the Mercodia Ultrasenstive Rat Insulin ELISA kit. Additionally, some cells were incubated further for 40 min in the presence of 1.1 mmol/l D-glucose followed by 20 min with 16.7 mmol/l D-glucose + 10 mmol/l L-alanine. This stimulus results in a robust and reproducible secretory response in normal conditions [7,24,27]. After incubation, a portion of the buffer was removed, centrifuged and analysed for insulin. In some experiments, AOA (amino-oxyacetate) was used to inhibit aminotransferase activity and thereby malate–aspartate shuttle function.

Determination of metabolites

D-Glucose, L-glutamine, L-glutamate and L-lactate

Concentrations of D-glucose, L-glutamine, L-glutamate and L-lactate were determined using the YSI 7100 Multiparameter Bioanalytical system. An aliquot of supernatant (either culture medium or Krebs–Ringer bicarbonate buffer) was removed from the tissue culture plate or flask, centrifuged for 5 min at 400 *g* and analysed. The linear range of each assay run was: 0.05–25.0 g/l (dynamic range) for D-glucose, 0.05–2.7 g/l for L-lactate,

15–1460 mg/l for L-glutamate and 30–1169 mg/l for L-glutamine.

L-Alanine

L-Alanine was determined following a protocol based on enzymatic reactions [31]. In the presence of NAD, L-alanine is oxidized to pyruvate and ammonia by AlaDH (L-alanine dehydrogenase), forming NADH, whose absorbance at 340 nm is directly proportional to the amount of L-alanine present. Actual concentrations were extrapolated from a standard curve of known concentrations (0–10 mmol/l L-alanine).

TAG

Cellular TAG content was measured as described previously [32]. Following culture for 24 h, Aralar1-overexpressing cells or control cells were washed with PBS, trypsinized, resuspended in 2 mmol/l NaCl, 20 mmol/l EDTA and 50 mmol/l sodium phosphate (pH 7.4) and sonicated for 1–2 min. A portion (10 μl) of the homogenate was mixed with 10 μl of t-butyl alcohol and 5 μl of Triton X-100/methyl alcohol (1:1, v/v) for the extraction of the lipids. TAG was determined using a commercial kit (Humana) and is expressed as μg/mg of protein.

Glycogen

Glycogen content was determined as described previously [33]. BRIN-BD11 (4×10^6 cells/T25 flask) and INS-1E cells (0.9×10^6 cells/T25 flask) were seeded into 5 ml of medium and cultured and transduced as required. Cells then were washed three times with ice-cold PBS, and flasks were frozen at $-20\,^{\circ}$C overnight. Cells then were solubilized with 0.4 ml of 0.1 mmol/l NaOH and the cell suspension was transferred into microcentrifuge tubes. Tubes containing the cell suspension were heated at $80\,^{\circ}$C for 1 h to destroy glucose and glucose 6-phosphate. Samples were cooled to room temperature ($25\,^{\circ}$C) and ethanol was added to yield a final ethanol concentration in the sample of 70 % (v/v) to precipitate the glycogen. Samples were centrifuged at $12\,000\,g$ for 10 min. The pellets were dried and subsequently resuspended in 50 mmol/l sodium acetate buffer by sonication. Amylo-glucosidase suspension (1.4 units) was added and samples were stored at room temperature for 1 h. The glucose concentration in each sample was determined using the YSI multi-parameter analyser. Concentrations were extrapolated from a glycogen standard curve using rabbit liver-derived glycogen standards (0–1 μg of glycogen/ml). Results are expressed in pmol of glycogen/mg of protein.

ATP

BRIN-BD11 cells were seeded at 1.5×10^5 cells in 1 ml of medium in a 24-well plate and allowed to adhere overnight. The following day, the cells were transduced with the respective adenovirus and cultured for a further 24 h to allow transgene expression. ATP was determined using a time-stable ATP determination kit (Biaffin) based on the luciferase assay.

Protein determination

Protein was extracted using RIPA lysis reagent containing 1× Halt Protease Inhibitor Cocktail (Thermo Scientific). Cellular protein was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce), which utilizes a modification of the Biuret reaction.

Western blot analysis

A portion (50 μg) of BRIN-BD11 cell protein extract was prepared using RIPA lysis buffer (Upstate). Samples were subsequently subjected to SDS/PAGE on 7.5 % (w/v) polyacrylamide gels. Proteins were transferred on to a nitrocellulose membrane and, after blocking in 5 % (v/v) milk protein, membranes then were probed with anti-Aralar antibodies directed against amino acids 7–272 of Aralar1. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which is abundant in the β-cell, was used as a control. INS-1E cell lysates were used as a positive control for Aralar1. For the determination of citrin expression, the nitrocellulose membrane was probed with an antibody directed against amino acids 9–278 of citrin. INS-1E cell lysates were used as a negative control for citrin, and HEK-293 (human embryonic kidney) cell lysates were used as a positive control for citrin. Membranes incubated with the polyclonal antibodies were visualized using Supersignal West Pico chemiluminescent substrate (Pierce).

Statistical analysis

Results are means $\pm$ S.D. Groups of data were compared using an unpaired Student's t test or ANOVA, where appropriate. Differences were considered significant at a P value < 0.05.

RESULTS

Overexpression of Aralar1

BRIN-BD11 cells expressed Aralar1 protein at equivalent levels to that in INS-1E cells (Figure 1), with immunoblotting revealing a band of the expected molecular mass (70 kDa) (Figure 1A). Immunolocalization of Aralar1 in insulin-secreting cells has been determined in a previous study [20]. GAPDH was expressed in both cell lines (Figure 1B). Citrin protein expression was also detected in BRIN-BD11 cells, but not at the same level as in HEK-293 cells, the positive control (Figure 1C).

Aralar1 was successfully overexpressed in the BRIN-BD11 cell line, and protein delivery was confirmed using Western blot analysis (Figure 1D). Unlike INS-1E cells and rat islets [20], BRIN-BD11 cells were found to express both isoforms of the aspartate–glutamate
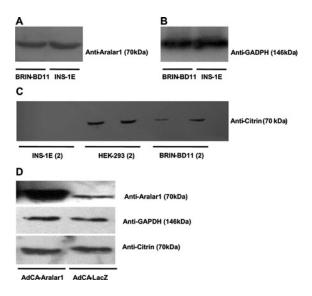
Figure 1   Expression of Aralar1 and citrin in BRIN-BD11 cells prior to (basal expression) and after overexpression with AdCA-Aralar1

(A and B) Expression of Aralar1 in BRIN-BD11 cells was determined. BRIN-BD11 cells were seeded and allowed to adhere overnight. Protein was extracted using RIPA lysis reagent containing $1\times$ Halt Protease Inhibitor Cocktail (Thermo Scientific). Proteins were probed with an anti-Aralar antibody directed against amino acids 7–272 of Aralar1 (A). Bands correspond to a protein of 70 kDa. INS-1E cell lysates were used as a positive control for Aralar1. Membranes then were stripped and probed with an anti-GAPDH antibody (B). (C) Expression of citrin in BRIN-BD11 cells. For the determination of citrin expression, an antibody directed against amino acids 9–278 of citrin was used. INS-1E cell lysates were used as a negative control, and HEK-293 cell lysates as a positive control for citrin. (D) Protein expression after adenoviral transduction. For determination of adenoviral overexpression, BRIN-BD11 cells were seeded, allowed to adhere overnight prior to transduction with adenovirus and cultured for a further 24 h to allow transgene expression. Proteins were probed with the anti-Aralar antibody. Bands correspond to a protein of 70 kDa. Each blot was also incubated with the anti-citrin and anti-GAPDH antibodies.
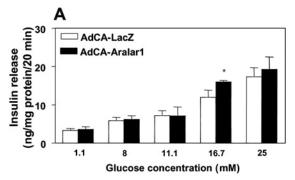
carrier Aralar1 and citrin. Therefore we assessed whether AdCA-Aralar1 acted specifically to overexpress Aralar1 protein levels. AdCA-Aralar1 was designed to express the Aralar1 isoform and not any other form. This was confirmed as shown in Figure 1(D).

Cell viability determination

Overexpression of Aralar1 did not result in any significant changes in cellular integrity, as determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based cell viability assays (results not shown).

Effect of Aralar1 overexpression on acute (20 min) glucose-stimulated insulin secretion

Raising glucose levels from 1.1 mmol/l at basal to 8 mmol/l resulted in a 1.7-fold increase in acute (20 min)
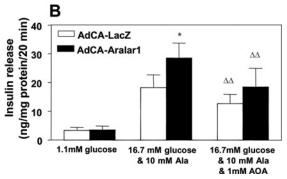

Figure 2   Effect of Aларar1 overexpression on nutrient-stimulated insulin secretion

BRIN-BD11 cells were cultured, allowed to adhere overnight prior to transduction with the recombinant adenovirus and then cultured for a further 24 h to allow transgene expression prior to being studied for acute (20 min) insulin secretion. Values are means $\pm$ S.D. of three independent experiments performed in quadruplicate *$P < 0.05$ compared with AdCA-LacZ at the corresponding conditions; $^{\triangle\triangle}P < 0.01$ compared with the response to glucose + alanine. Ala, alanine.

insulin secretion in Aralar1-overexpressing cells and a 1.8-fold increase in control LacZ-transduced cells (Figure 2A). A rise from the basal glucose level at 1.1 mmol/l to 11.1 mmol/l D-glucose resulted in a 2.0-fold stimulation in Aralar1-overexpressing cells and a 2.1-fold increase in control LacZ-transduced cells. Upon stimulation with 16.7 mmol/l D-glucose, Aralar1-overexpressing cells displayed a 4.5-fold increase in insulin secretion compared with basal. In contrast, control LacZ-transduced cells only responded with a 3.6-fold increase above basal glucose (1.1 mmol/l). This was equivalent to Aralar1-overexpressing cells secreting 24% more insulin than control LacZ-transduced cells ($P < 0.02$; Figure 2A). As with INS-1E cells, stimulation of BRIN-BD11 cells overexpressing Aralar1 with 2 mmol/l pyruvate did not result in a difference in insulin secretion compared with control cells (results not shown).

No significant difference was observed in terms of acute insulin secretion between Aralar1-overexpressing cells and control LacZ-transduced cells when cells were stimulated with 30 mmol/l KCl over a 20 min time period (a positive control). Aralar1-overexpressing cells

responded with a 5.37-fold increase and control LacZ-transduced cells responded with a 5.12-fold increase in insulin secretion compared with cells incubated in Krebs–Ringer bicarbonate buffer (results not shown).

It is possible to report insulin secretion as a percentage of total cell insulin content. Upon incubation with 1.1 mmol/l D-glucose, no significant difference between AdCA-Aralar1- and AdCA-LacZ-transduced cells was observed in terms of insulin secretion expressed as percentage of total insulin content. When glucose levels were raised to 16.7 mmol/l, Aralar1-overexpressing cells responded with a 7.53-fold increase in insulin secretion over basal, whereas control LacZ-transduced cells responded with a 5.10-fold increase in insulin secretion expressed as percentage of total insulin content ($P = 0.01$). Aralar1-overexpressing cells therefore secreted significantly more insulin than control LacZ-transduced cells upon stimulation with 16.7 mmol/l D-glucose, and the magnitude of the increase from Aralar1-overexpressing cells over LacZ-overexpressing control cells was similar between the two methods of reporting secretion.

Effect of Aralar1 overexpression on acute (20 min) glucose- plus alanine-induced insulin secretion

BRIN-BD11 cells are known to be sensitive to amino-acid-induced insulin secretion [1]. Insulin secretion was significantly ($P = 0.04$) enhanced from Aralar1-overexpressing cells when compared with control LacZ-transduced cells in an acute insulin secretion test involving 20 min of stimulation with 16.7 mmol/l D-glucose and 10 mmol/l L-alanine (+57 %; Figure 2B). This stimulus produced a robust and reproducible maximum nutrient-stimulated secretory response in BRIN-BD11 cells (Figure 2B). When malate–aspartate shuttle function was inhibited by using the aminotransferase inhibitor AOA (at concentrations not affecting cell integrity), acute insulin secretion with 16.7 mmol/l D-glucose and 10 mmol/l L-alanine was significantly reduced in both control LacZ-transduced cells (−30 %; $P = 0.007$) and Aralar1-overexpressing cells (−34.9 %; $P = 0.002$) (Figure 2B).

Effect of Aralar1 overexpression on insulin content

Aralar1-overexpressing cells stored significantly ($P = 0.01$) more insulin in the unstimulated (basal) state compared with control LacZ-transduced cells (14.9 ± 1.11 compared with 11.9 ± 0.30 ng/10^6 cells respectively; +25 %). After acute (20 min) stimulation, with 16.7 mmol/l D-glucose, insulin content was reduced in both cell types such that there was no significant difference in content. As expected (if stored insulin was elevated), over a 24 h period in 11.1 mmol/l D-glucose, Aralar1-overexpressing cells secreted significantly ($P < 0.05$) less insulin than control LacZ-transduced cells

in culture (187 ± 52.7 compared with 264 ± 51.0 ng · mg^{-1} of protein · 24 h^{-1}; −30 %).

Effect of Aralar1 overexpression on nutrient consumption and metabolite production

24 h consumption rates

Glucose consumption (from an initial concentration of 11.1 mmol/l) was not modified, whereas L-glutamine exhibited a trend to increased consumption (although not reaching significance) by Aralar1 overexpression during 24 h post-transduction incubation (Figures 3A and 3B).

1 h consumption rates

Glucose consumption (from an initial concentration of 16.7 mmol/l D-glucose) was increased in the presence of 10 mmol/l L-alanine over 1 h in Aralar1-overexpressing cells, an effect blunted by the addition of AOA (Figure 3C).

Over a 1 h period (following 40 min at 1.1 mmol/l D-glucose), Aralar1-overexpressing cells consumed the same amount of glucose compared with control cells (Figure 3C). However, Aralar1-overexpressing cells consumed significantly ($P = 0.01$) more glucose than control LacZ-transduced cells when stimulated with 16.7 mmol/l D-glucose and 10 mmol/l L-alanine (+42 %; Figure 3C). Glucose consumption at 16.7 mmol/l D-glucose was significantly ($P = 0.04$) reduced in Aralar1-overexpressing cells when 1 mmol/l AOA was added (−43 %; Figure 3C). Such an effect was not observed in control LacZ-transduced cells (Figure 3C).

L-Glutamine consumption by Aralar1-overexpressing cells over 1 h was significantly ($P = 0.03$) enhanced after exposure to 10 mmol/l L-glutamine and 1.1 mmol/l D-glucose (+71 %; Figure 3D). In similar acute exposure conditions (1 h), L-alanine consumption during 1 h in Aralar1-overexpressing cells was significantly ($P = 0.04$) greater than in control LacZ-transduced cells at 16.7 mmol/l D-glucose (+45 %; Figure 3E).

BRIN-BD11 cells overexpressing Aralar1 contained significantly ($P = 0.04$) more ATP than control LacZ-transduced cells subsequent to 24 h post-transduction incubation (+69 %; Table 1). Over a 24 h period, BRIN-BD11 cells overexpressing Aralar1 produced significantly ($P = 0.02$) less L-lactate than control LacZ-transduced cells (−38 %; Table 1). No difference in L-glutamate output was observed between Aralar1-overexpressing and control LacZ-transduced BRIN-BD11 cells (Table 1).

In order to determine the metabolic fates of glucose in Aralar1-overexpressing cells, we determined intracellular L-glutamate concentration and L-lactate release after a brief period of glucose deprivation followed by acute glucose stimulation. In control LacZ-transduced cells, L-glutamate concentrations were enhanced 1.9-fold by 16.7 mmol/l D-glucose stimulation compared with
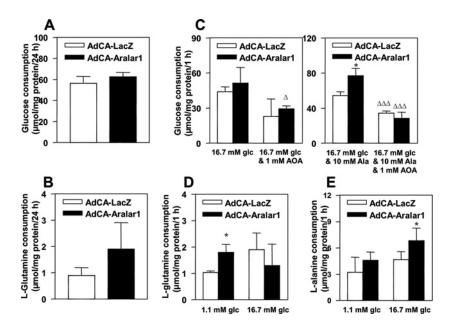
Figure 3   Effect of Aralar1 overexpression on D-glucose, L-glutamine and L-alanine consumption

BRIN-BD11 cells were allowed to adhere overnight prior to transduction with adenovirus and cultured for further 24 h to allow transgene expression. (A and B) The sample medium was collected after 24 h and analysed for D-glucose and L-glutamine. (C) Cells were challenged with nutrients for 1 h and the supernatant was collected for analysis. (D and E) Cells were challenged with 10 mmol/l L-glutamine or 10 mmol/l L-alanine respectively, in combination with 1.1 or 16.7 mmol/l D-glucose for 1 h. The supernatant was collected for analysis. Values are means $\pm$ S.D. of three independent experiments performed in quadruplicate. $*P < 0.05$ compared with AdCA-LacZ; $^{\triangle\triangle\triangle}P < 0.005$ compared with the response to glucose + alanine. Ala, alanine; glc, glucose.

Table 1   Effect of Aralar1 overexpression on nutrient consumption, intracellular metabolite levels and metabolite production

BRIN-BD11 cells were allowed to adhere overnight and then transduced with adenovirus. Cell culture medium was collected for analysis of lactate and glutamate production, and cells were lysed prior to the determination of ATP, TAG, L-glutamate and glycogen content after 24 h. Values are means $\pm$ SD of three independent experiments performed in quadruplicate. Significant differences are as stated in the text.

Parameter	AdCA-Aralar1	AdCA-LacZ	Percentage change
ATP content (μmol/mg of protein)	78.1 ± 15.5	46.2 ± 11.7	$+ 69\%$
L-Lactate production (μmol $\cdot$ mg^{-1} of protein $\cdot$ 24 h^{-1})	27.4 ± 3.08	44.3 ± 6.83	$- 38\%$
L-Glutamate production (μmol/mg^{-1} of protein $\cdot$ 24 h^{-1})	1.99 ± 0.60	1.75 ± 0.30	Not significant
Intracellular L-glutamate (μmol/mg of protein)			
With 1.1 mmol/l D-glucose	0.49 ± 0.08	0.44 ± 0.06	Not significant
With 16.7 mmol/l D-glucose	1.31 ± 0.07	0.87 ± 0.19	
TAG content (μmol/mg of protein)	0.19 ± 0.02	0.30 ± 0.08	$- 37\%$
Glycogen content (nmol/mg of protein)	1.33 ± 0.69	2.06 ± 0.34	$- 35\%$

1.1 mmol/l D-glucose. Aralar1-overexpressing cells exhibited a 2.7-fold increase in cellular L-glutamate at 16.7 mmol/l D-glucose compared with 1.1 mmol/l D-glucose ($P < 0.05$ compared with LacZ control). Aralar1 overexpression potentiated glucose-induced L-glutamate release by 13% (Table 1). L-Lactate release was reduced 38% in Aralar1-overexpressing compared with control LacZ-transduced cells (Table 1).

Effect of Aralar1 overexpression on TAG and glycogen utilization

Aralar1-overexpressing cells contained significantly ($P = 0.03$) less TAG than control LacZ-transduced cells

($- 37\%$; Table 1). TAG synthesis is thought to be cytoprotective in the context of lipotoxicity due to the removal of excessive NEFAs (non-esterified fatty acids) [34], but hydrolysis may contribute to the stimulation of insulin secretion under appropriate conditions (see [35] for review). Aralar1-overexpressing cells contained significantly ($P = 0.04$) less glycogen than control LacZ-transduced cells ($- 35\%$; Table 1).

DISCUSSION

In the present study, we describe a key regulatory role for the malate–aspartate shuttle in insulin-secreting

cells based on results generated following Aralar1 overexpression in the BRIN-BD11 cell line, which resulted in enhanced glucose and amino acid utilization, mitochondrial activation and insulin secretion (at robust insulinotropic glucose and amino acid concentrations). Owing to the unique metabolic design of the β-cell [35,36], the activity of NADH shuttles may limit glycolysis due to a limit on NADH re-oxidation to NAD$^+$ [37,38] and thus insulin secretion.

A previous study [20] reported that Aralar1 overexpression in the glucose-responsive β-cell line INS-1E potentiated metabolism secretion coupling stimulated by 15 mmol/l D-glucose, increased NADPH generation, mitochondrial membrane hyperpolarization, ATP levels and glucose oxidation, accompanied by reduced lactate production. *In vivo*, β-cells are rarely exposed to raised levels of glucose in isolation, but various combinations of glucose and amino acids will be experienced following a mixed meal. The present study has extended earlier work [20] by employing an alternative β-cell line to investigate the combined effects of glucose and alanine on insulin secretion, amino acid metabolism and metabolic secretion coupling, and has added new evidence to support the hypothesis that the Aralar1 component of the malate–aspartate shuttle is a key metabolic control site in the β-cell. Metabolic control is generally accepted to reside at GLUT2 (glucose transporter 2), glucokinase, pyruvate dehydrogenase, pyruvate carboxylase and glutamate dehydrogenase in the β-cell [38].

A generalized scheme depicting the involvement of glucose, alanine and glutamine in insulin secretion, together with involvement of the malate–aspartate shuttle, is shown in Figure 4. This Figure outlines the routes of metabolism for glucose, alanine and glutamine, highlighting pathways and metabolites in common. Routes for the formation of metabolic end-products, such as glycogen and TAG, are indicated. The generation of cytosolic NADH and its oxidation by the malate–aspartate shuttle is also shown, as are the other components of the shuttle that allow regeneration of NADH in the mitochondrial matrix and promotion of insulin release. The key involvement in important stimulus–secretion coupling factors, such as ATP and Ca^{2+}, are evident. Aralar1, the aspartate–glutamate exchanger, is shown at the inner mitochondrial membrane, together with links to the various stimulus–secretion coupling pathways as elaborated below.

Increasing shuttle activity, by overexpression of a key component of the shuttle (Alarar1), may remove the constraint on the glycolytic pathway thus allowing a higher flux through glycolysis and NADH production. Subsequent enhancement of metabolism of the glycolysis-derived pyruvate, by both pyruvate dehydrogenase and pyruvate carboxylase, may result in higher activity of the TCA (tricarboxylic acid) cycle and, eventually, greater stimulus–secretion coupling factor generation (including ATP). Stimulation of glucose metabolism at both the pyruvate dehydrogenase and pyruvate carboxylase steps by alanine has been described [7]. In the present study, we have demonstrated that the addition of glucose and alanine resulted in the greatest increase in insulin secretion compared with any other nutrient combination in Aralar1-overexpressing cells. Glutamate derived from either glucose or alanine metabolism may increase shuttle substrate concentration and thus may enhance transfer of glycolysis-derived NADH to the mitochondrial matrix. Indeed, glucose, alanine and glutamine utilization were increased in Aralar1-overexpressing cells, indicating enhanced metabolism and generation of stimulus–secretion coupling factors, such as ATP and glutamate, as reported in the present study. Glucose metabolism may also be stimulated by the addition of glutamine, as glutamine carbon may enter the TCA cycle via glutamate, under the action of glutamate dehydrogenase, thus increasing TCA cycle intermediate concentration via an anaplerotic action. Additionally, glutamine may enter the γ-glutamyl cycle via glutamate, which indirectly stimulates mitochondrial electron transport chain activity and thus ATP production [8]. The anaplerotic action of glutamine may enhance glucose-derived NADH oxidation and subsequent stimulation of insulin secretion. As glutamine gives rise to glutamate via the reaction calaysed by glutaminase, then activity of the malate–aspartate shuttle may be increased via shuttle substrate concentration effects.

Glutamate has been suggested as a glucose-derived stimulus–secretion coupling factor with respect to insulin secretion [1–3,6,8]. We have reported in the present study that Aralar1 overexpression resulted in a significant increase in intracellular glutamate production compared with control cells.

Accumulation of TAG in pancreatic β-cells is a consequence of 'glucolipotoxic' conditions *in vitro* and *in vivo* [35,39] and, thus, impaired insulin secretion. Indeed the accumulation of TAG probably reflects the cellular response to excessive NEFA levels, effectively removing fatty acids from adverse metabolic action [40,41]. In conditions of enhanced catabolic metabolism, such as enhanced malate–aspartate shuttle activity, then glucose metabolism can be diverted away from glycerol 3-phosphate formation (essential for TAG synthesis [41]), so enhancing pyruvate formation and entry into the TCA cycle. In the latter situation, TAG hydrolysis will be favoured (as synthesis is attenuated), therefore resulting in lower TAG content (Table 1), and higher rates of fatty acid oxidation, ATP synthesis and insulin secretion.

Glycogen accumulation in β-cells and islet cells in glucotoxic conditions has been described previously [41]. Glycogen may normally act as a 'buffer' for excess glucose 6-phosphate formation, especially after a rapid rise in extracellular glucose concentration, thus ensuring a more controlled flux through glycolysis. In catabolic conditions, such as higher malate–aspartate
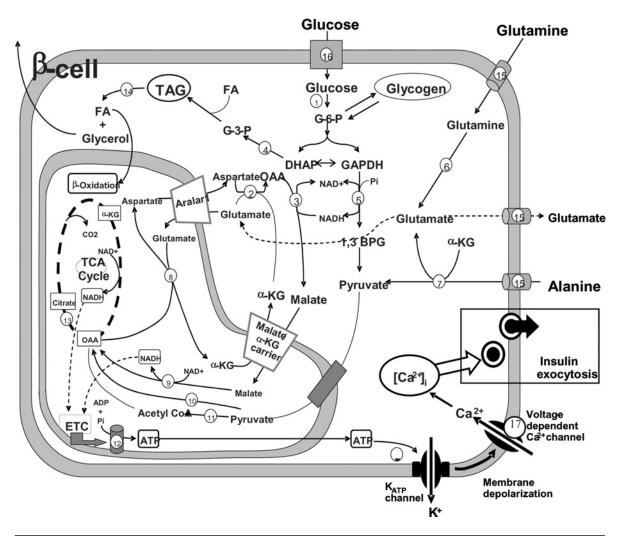
Figure 4 Schematic representation of the pancreatic β-cell metabolic stimulus–secretion showing the involvement of glucose, alanine and glutamine in insulin secretion, together with the involvement of the malate–aspartate shuttle

See text for a further description. Abbreviations of enzymes and transporters are as follows: 1, glucokinase; 2, cytosolic aspartate aminotransferase; 3, cytosolic malate dehydrogenase; 4, glycerol kinase; 5, GAPDH; 6, glutaminase; 7, alanine aminotransferase; 8, mitochondrial aspartate dehydrogenase; 9, mitochondrial malate dehydrogenase; 10, pyruvate carboxylase; 11, pyruvate dehydrogenase; 12, ATP synthase; 13, citrate synthase; 14, TAG lipase; 15, amino acid transporter; 16, GLUT2; 17, voltage-dependent Ca^{2+} channel. Other abbreviations: 1,3 BPG, 1,3 bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; FA, fatty acid; G-3-P, glucose 3-phosphate; G-6-P, glucose 6-phosphate; α-KG, α-ketoglutarate; OAA, oxaloacetate.

shuttle activity, then glycogen breakdown may be favoured, resulting in enhanced oxidative metabolism, ATP generation and insulin release.

From the above considerations, overexpression of Aralar1 and the subsequent stimulation of the malate–aspartate shuttle appears to shift β-cell metabolism towards higher rates of insulinotropic amino acid and glucose consumption, greater stimulus–secretion coupling factor generation and insulin secretion. We conclude that enhanced malate–aspartate shuttle capacity (in terms of protein constituents and metabolic substrates) will result in higher levels of stimulus–secretion coupling factors and thus insulin secretion, as shown in Figure 4. Enhanced metabolic intermediate generation, a consequence of higher metabolism, may be beneficial for β-cell protection and survival [42]. Further studies are required to explore these possibilities and ultimately their application to primary human β-cell function and integrity.

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