



**HAL**  
open science

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

Katrin Bender, Pierre Maechler, Neville H Mcclenaghan, Peter R Flatt, Philip Newsholme

### **► To cite this version:**

Katrin Bender, Pierre Maechler, Neville H Mcclenaghan, Peter R Flatt, Philip Newsholme. Overexpression of the malate-aspartate NADH shuttle member Aralar1 in the clonal beta cell line BRIN BD11 enhances amino acid stimulated insulin secretion and cell metabolism. *Clinical Science*, 2009, 117 (9), pp.321-330. <10.1042/CS20090126>. <hal-00512874>

**HAL Id: hal-00512874**

**<https://hal.science/hal-00512874v1>**

Submitted on 1 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Overexpression of the Malate-Aspartate NADH shuttle member Aralar1 in the clonal beta cell line BRIN BD11 enhances amino acid stimulated insulin secretion and cell metabolism.

Katrin Bender<sup>1</sup> Pierre Maechler<sup>2</sup>, Neville H. McClenaghan<sup>3</sup>, Peter R Flatt<sup>3</sup> and Philip Newsholme<sup>1</sup>

<sup>1</sup>School of Biomolecular and Biomedical Sciences, Conway Institute, UCD Dublin, Belfield, Dublin 4, Ireland.

<sup>2</sup>Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, 1211 Geneva, Switzerland.

<sup>3</sup>School of Biomedical Sciences, University of Ulster, Coleraine, N. Ireland.

Short title: Over-expression of Aralar 1 alters beta-cell metabolism and function.

Abstract word count: 135

Manuscript word count (excluding abstract): 3915

Figures: 4

Tables: 1

Correspondence to be addressed to:

Prof. Philip Newsholme,

School of Biomolecular and Biomedical Sciences, Conway Institute and Health Sciences Centre, UCD Dublin, Belfield, Dublin 4, Ireland.

Tel 353-1-716-6561

Fax 353-1-283-7211

email [philip.newsholme@ucd.ie](mailto:philip.newsholme@ucd.ie)

This work was supported by the Health Research Board of Ireland and UCD Dublin.

There is no conflict of interest in relation to any of the authors of this paper and publication in Clinical Science

#### ABSTRACT

We have investigated the effects of transduction with recombinant adenovirus 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 over-expression increased long-term (24hr) and acute (20min) 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 data indicate that increased malate-aspartate shuttle activity positively shifted beta-cell metabolism, thereby increasing glycolysis capacity, stimulus-secretion coupling, and ultimately enhancing insulin secretion. We conclude that the aspartate/glutamate carrier 1 is a key metabolic control site in insulin secreting cells.

**Key words:** Pancreatic beta-cells, Insulin secretion, Glucose, L-glutamine, L-glutamate, L-alanine, Triacylglycerol, NADH, Malate-Aspartate shuttle.

**Abbreviations:** AdCA- Aralar1, recombinant adenovirus encoding for Aralar1; AdCA- LacZ, recombinant adenovirus encoding for bacterial beta galactosidase; AGC1, aspartate/glutamate carrier 1; GSIS, glucose- stimulated insulin secretion; KRB Krebs- Ringer Bicarbonate buffer; pfu plaque forming unit; TCA cycle, Tricarboxylic acid cycle; TAG, triacylglycerol

## INTRODUCTION

Nutrient metabolism is tightly coupled to insulin secretion in the pancreatic beta-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  $\text{Ca}^{2+}$ , 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  $\text{Ca}^{2+}$  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 beta-cell may generate many, if not all, of the mitochondrial factors described above [7 – 11].

In beta 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 beta cell function [16 – 18]. However, the malate-aspartate shuttle has been proposed as the key physiologic regulator of cytosolic NADH transfer to the mitochondrial matrix in the beta cell [19, 20], thereby contributing to the amplifying pathway of insulin secretion.

Aralar 1 (or aspartate/glutamate carrier 1, AGC1) is a  $\text{Ca}^{2+}$  sensitive isoform of the aspartate/glutamate carrier and an integral part of the malate-aspartate shuttle [21, 22] that is mainly expressed in heart, brain, skeletal muscle [21, 23] and islets [20]. The significance of Aralar1 for beta cell function, glucose metabolism and insulin secretion was previously demonstrated 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 triacylglycerol or glycogen content have not been investigated. We have now over-expressed Aralar1 using the recombinant adenovirus AdCA-Aralar1 in the glucose and amino acid sensitive BRIN-BD11 beta cell line and assessed functional outcomes.

We hypothesized that over-expression 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 triacylglycerol utilization, while lactate formation was reduced. The clonal beta-cell line BRIN BD11 was chosen for this work as metabolic, signaling, 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

All reagents, unless stated otherwise, were purchased from Sigma Aldrich, Ireland. AdCA-Aralar1 and AdCA-LacZ constructs have been described previously [20]. Anti-Aralar1 and Anti-Citrin were donated by Prof. Jorgina Satrustegui, Madrid.

### *Culture of BRIN-BD11 pancreatic beta cells*

Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium supplemented with 10% (v/v) foetal calf serum (FCS), 0.1% antibiotics (100 U/ml penicillin and 0.1mg/ml streptomycin) and 11.1mmol/l D-glucose, pH 7.4. The origin of BRIN-BD11 cells is described elsewhere [24, 27]. These cells provide an appropriate beta-cell model as evidenced by studies of insulin secretion [24, 25, 27], beta-cell metabolism [1, 7, 8],

electrophysiology [26],  $\text{Ca}^{2+}$  handling [28] and cellular defense [29]. INS-1E cells were cultured as detailed previously [30].

The cells were maintained at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  and 95% air using a Forma Scientific incubator (Marietta, OH, USA). The cells were cultured in 50-70ml of RPMI-1640 tissue culture medium in T175 sterile tissue culture flasks. Cells were subsequently seeded into 96, 24 or 6- well plates and allowed to adhere over a 24hr period. For infection, cells were incubated with RPMI medium supplemented with 5% (v/v) foetal calf serum (FCS), 0.1% antibiotics (100 U/ml penicillin and 0.1mg/ml streptomycin) and 11.1mmol/l D-glucose, pH 7.4 containing various concentrations of recombinant adenovirus/cell (AdCA-Aralar1) constructed as described by Rubi [20]. After transduction cells washed in PBS and further cultured for 24hr to allow transgene expression. During this study, 8 pfu/ cell was used as optimum. AdCA-LacZ, which expresses bacterial  $\beta$ -galactosidase, was used for control transductions. After 24hrs incubation, an aliquot of the media was removed, centrifuged and used for quantitation of insulin and metabolites (D-glucose, L-glutamine, L-lactate and L-glutamate). Cells were also analyzed for their respective insulin, glutamate, triacylglycerol and glycogen content (expressed per mg protein).

#### *Long-term Chronic (24hr) and acute (20min) insulin secretion*

After 24hrs incubation, an aliquot of the media was collected, cleared by centrifugation at 200 x g for 5min and analysed for insulin using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Uppsala, Sweden). Additionally, some cells were further incubated for 40 min in the presence of 1.1mmol/L glucose followed by 20 min with 16.7mM glucose plus 10mM L- alanine. This stimulus results in a robust and reproducible secretory response in normal conditions [7, 24, 27]. After incubation an aliquot of the buffer was removed, centrifuged and analysed for insulin. In some experiments aminooxyacetate was used to inhibit aminotransferase activity and thereby the malate- aspartate shuttle function.

#### *Determination of metabolites.*

D- glucose, L- glutamine, L- glutamate and L- lactate concentrations 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 12,000 rpm and analysed. The linear range of each assay run was as follows: D- glucose (dynamic range of 0.05- 25.0  $\text{g L}^{-1}$ ), L- lactate (0.05- 2.7 $\text{g L}^{-1}$ ), L- glutamate (15- 1460  $\text{mg L}^{-1}$ ) and L- L-glutamine (30- 1169 $\text{mg L}^{-1}$ ).

L-alanine was determined following a protocol based on enzymatic reactions [41]. In the presence of NAD, L-alanine is oxidized to pyruvate and ammonia by L-alanine dehydrogenase (AlaDH), 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- 10mM L-alanine). L-alanine concentrations were determined relative to a standard curve of 6 known L-alanine concentrations (0, 2, 4, 6, 8, 10 mM L-alanine).

**Triacylglycerol (triglyceride):** Cellular triacylglycerol content was measured as outlined previously (Dixon *et al* 2004). Following 24 hours culture, the Aralar 1 overexpressing or control cells were washed with PBS, trypsinised and resuspended in 2mM NaCl, 20mM EDTA, and 50mM sodium phosphate (pH 7.4) and sonicated for 1-2 min. 10 $\mu\text{l}$  of the homogenate was mixed with 10 $\mu\text{l}$  tert-butyl alcohol and 5 $\mu\text{l}$  triton-X-100/methyl alcohol (1:1, v/v) for the extraction of the lipids. Triacylglycerol was determined using a commercial kit (Humana, Wiesbaden, Germany) and expressed as  $\mu\text{g}/\text{mg}$  protein.

**Glycogen:** Glycogen content was determined as described elsewhere [42]. BRIN-BD11 ( $4 \times 10^6$  cells/ T25 flask) and INS-1E cells ( $0.9 \times 10^6$  cells/ T25 flask) were seeded in 5 ml of media and cultured and transduced as required. Cells then were washed three times with ice- cold PBS and flasks were frozen at  $-20^{\circ}\text{C}$  over night. Cells then were solubilized with 0.4 ml of 0.1 mM NaOH and the cell suspension was transferred into microcentrifuge tubes. Tubes containing the cell suspension were heated at  $80^{\circ}\text{C}$  for 1 h to destroy glucose and glucose-6-phosphate. Samples were cooled to room temperature 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 mM sodium acetate buffer by sonication. 1.4 units of amyloglucosidase suspension were added and samples were stored at room temperature for 1 h. The glucose concentration in each sample was determined using the YSI multiparameter analyser. Concentrations were extrapolated from a glycogen standard curve using rabbit liver- derived glycogen standards (0-1  $\mu\text{g}$  glycogen/ ml). Results were expressed in pmol glycogen/ mg protein.

**ATP:** BRIN-BD11 cells were seeded at  $1.5 \times 10^5$  cells in 1ml media in a 24 well plate and allowed to adhere over night. The following day the cells were transduced with the respective adenovirus and cultured for further 24 h to allow transgene expression. ATP was determined using a time stable ATP determination kit (Biaffin GmbH, Kassel, Germany) based on the luciferase assay.

**Protein determination:** Protein was extracted using RIPA lysis reagent containing 1 X Halt Protease Inhibitor Cocktail (Thermo Scientific). Cellular protein was determined using a BCA protein assay kit (Pierce, kit no. 23225), which utilises a modification of the biuret reaction.

**Western Blot Analysis:** 50ug BRIN BD11 cell protein extracts were prepared using RIPA lysis buffer (Upstate). Samples were subsequently subjected to 7.5% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. After blocking in 5% milk protein membranes then were probed with Anti- Aralar antibodies directed against Aralar1 amino acids 7- 272. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which is abundant in the beta-cell, was used as control antibody. INS- 1E cell protein lysates were used as positive control for Aralar1. For the determination of citrin expression, the nitrocellulose membrane which was probed with antibody directed against amino acids 9- 278. INS- 1E cell protein lysates were used as negative control for citrin and HEK-293 cell protein lysates were used as positive control for citrin and incubated with polyclonal anti Proteins were visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce).

**Statistical analysis:** The results are presented as mean  $\pm$  SD. Groups of data were compared using a Student's unpaired *t* test or ANOVA where appropriate. Differences were considered significant at a *p* value of less than 0.05.

## Results

### *Overexpression of Aralar 1*

BRIN-BD11 cells express Aralar1 protein to equivalent levels to the previously described INS-1E cells (Fig 1). Immunoblotting revealed a band of the expected size (70 kDa) (Fig 1A). Immunolocalization of Aralar1 in insulin secreting cells was determined in a previous study [20]. GAPDH was expressed in both cell lines (Fig 1B). Citrin protein expression was also detected in BRIN-BD11 cells, but not to the same level as in HEK-293 cells, a positive control (Fig 1C).

Aralar1 was successfully over- expressed in the BRIN-BD11 cell line and protein delivery was confirmed using Western blot analysis (Fig 1D). Unlike INS-1E cells and rat islets [20], BRIN-BD11 cells were found to express both isoforms of the aspartate/glutamate carrier, Aralar1 and Citrin. Therefore we assessed whether the adenoviral construct AdCA-Aralar1 acted

specifically to over-express Aralar1 protein levels. AdCA-Aralar1 was designed to express the isoform of the aspartate/glutamate carrier, Aralar1 and not any other form. This was confirmed (Fig 1D).

#### *Cell Viability determinations*

Overexpression of Aralar1 did not result in significant changes in cellular integrity as determined by MTS based cell viability assays (results not shown).

#### *Effect of Aralar 1 overexpression on acute (20min) glucose stimulated insulin secretion*

Raising glucose levels from basal 1.1 mM to 8 mM resulted in a 1.7-fold increase of acute (20min) insulin secretion in Aralar1 over-expressing and 1.8-fold increase in control LacZ transduced cells (Fig 2A). A rise from basal 1.1 mM glucose to 11.1 mM glucose resulted in a 2.0-fold stimulation in Aralar1 over-expressing and 2.1-fold increase in control LacZ transduced cells. Upon stimulation with 16.7 mM glucose, Aralar1 over-expressing cells displayed a 4.5-fold increase of insulin secretion compared to basal. In contrast LacZ transduced control cells only responded with a 3.6-fold increase above basal glucose (1.1mM). This was equivalent to Aralar1 over-expressing cells secreting 24% more insulin than control LacZ transduced cells ( $p < 0.02$ , Fig 2A). As with INS- 1E cells, stimulation of BRIN- BD11 cells over- expressing Aralar1 with 2 mM pyruvate did not result in a difference in insulin secretion compared to control cells (data not shown).

No significant difference was observed in terms of acute insulin secretion between Aralar1 over-expressing and LacZ control cells when cells were stimulated with 30mM KCl over a 20min time period (a positive control). Aralar1 over- expressing cells responded with a 5.37-fold increase and control LacZ cells responded with a 5.12- fold increase in insulin secretion compared to cells incubated in Krebs-Ringer bicarbonate buffer (data not shown).

It is possible to report insulin secretion as a % of total cell insulin content. Upon incubation with 1.1mM glucose, no significant difference between AdCA-Aralar1 transduced cells and AdCA-LacZ transduced cells was observed in term of insulin secretion expressed as percent of total insulin content. When glucose levels were raised to 16.7mM, Aralar1 over-expressing cells responded with a 7.53-fold increase in insulin secretion over basal, whereas LacZ control cells responded with a 5.10- fold increase in insulin secretion expressed as percent of total insulin content ( $p = 0.01$ ). Aralar1 over-expressing cells therefore secreted significantly more insulin than LacZ control cells, upon stimulation with 16.7 mM glucose and the magnitude of increase from Aralar 1 over-expressing cells over Lac Z controls cell was similar between the two methods of reporting secretion.

#### *Effect of Aralar 1 overexpression on acute (20min) 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 enhanced from Aralar1 over-expressing BRIN-BD11 cells when compared to control LacZ transduced cells in an acute insulin secretion test involving 20 min stimulation with 16.7 mM glucose and 10 mM L-alanine (+ 56.7 %,  $p = 0.04$ , Fig 2B). This stimulus produced a robust and reproducible maximum nutrient stimulated secretory response in BRIN-BD11 cells (Fig 2D). When malate-aspartate shuttle function was inhibited by using the aminotransferase inhibitor aminooxyacetate (at concentrations not affecting cell integrity), acute insulin secretion with 16.7 mM glucose and 10 mM L-alanine was significantly reduced in both control LacZ transduced and Aralar1 over-expressing cells (- 30.1 %,  $p = 0.007$  and - 34.9 %,  $p = 0.002$  respectively; Fig 2B).

*Effect of Aralar 1 overexpression on insulin content*

Aralar1 over-expressing BRIN-BD11 cells stored significantly more insulin in the unstimulated (basal) state compared to control LacZ transduced cells ( $14.9 \pm 1.11$  ng/  $10^6$  cells compared to  $11.9 \pm 0.30$  ng/  $10^6$  cells; + 25.3 %;  $p= 0.01$ ; data not shown). After acute (20min) stimulation, with 16.7 mmol/l glucose, insulin content was reduced in both cell types such that there was not significant difference in content. As expected (if stored insulin was elevated), over a 24 h period in 11.1mM glucose, Aralar1 over- expressing BRIN- BD11 cells secreted significantly less insulin than Control LacZ transduced cells in culture ( $187 \pm 52.7$  ng/mg protein/ 24h compared to  $264 \pm 51.0$  ng/mg protein/ 24h ; -34.5%,  $p< 0.05$ ).

*Effect of Aralar 1 overexpression on nutrient consumption and metabolite production*

## 24 hour consumption rates

Glucose consumption (from an initial concentration of 11.1mM) was not modified while glutamine exhibited a trend to increased consumption (although not reaching significance) by Aralar1 overexpression during 24hr post-transduction incubation (Fig 3A, B).

## 1 hour consumption rates

Glucose consumption (from an initial concentration of 16.7mM glucose) was increased in the presence of 10mM alanine over 1hr in Aralar 1 overexpressing cell, an effect blunted by the addition of AOA (Fig 3C)

Over a 1 h period (following 40 min at 1.1 mM glucose), Aralar1 over-expressing cells consumed the same amount of glucose compared to control cells (Fig 3C). However, Aralar1 over-expressing cells consumed significantly more glucose than control LacZ cells when stimulated with 16.7 mM glucose and 10 mM L-alanine (+ 41.8 %,  $p= 0.01$ , Fig 3C). Glucose consumption at 16.7 mM glucose was significantly reduced in Aralar1 over-expressing cells when 1 mM AOA was added (- 42.7 %,  $p= 0.04$ , Fig 3C). Such an effect was not observed in control LacZ transduced cells (Fig 3C).

L-glutamine consumption by Aralar1 over-expressing cells over 1hr was significantly enhanced after exposure to 10 mM L-glutamine and 1.1 mM glucose (+ 59.1 %,  $p= 0.03$ ; Fig 3D). In similar acute exposure conditions (1 hr ) L-alanine consumption during 1hr in Aralar1 over-expressing BRIN BD11 cells was significantly greater than in control LacZ- transduced cells at 16.7 mM glucose (+ 45.3 %,  $p= 0.04$ ; Fig 3E).

BRIN-BD11 cells over-expressing Aralar1 contained significantly more ATP than control LacZ cells subsequent to 24 hr post-transduction incubation (+ 68.9 %,  $p= 0.04$ ; Table 1). Over a 24 h period, BRIN-BD11 cells over-expressing Aralar1 produced significantly less lactate than control LacZ transduced cells (- 55.3%;  $p= 0.02$ ; Table 1). No difference in glutamate output was observed between Aralar1 over- expressing and control LacZ over-expressing BRIN-BD11 cells (Table 1).

In order to determine metabolic fates of glucose in Aralar1 over- expressing cells, we determined intracellular glutamate and lactate concentrations after a brief period of glucose deprivation followed by acute glucose stimulation. In control LacZ- transduced cells, glutamate concentrations were enhanced 1.9-fold by 16.7 mM glucose stimulation compared to 1.1 mM glucose. Aralar1 over-expressing cells exhibited a 2.7-fold increase in cellular glutamate at 16.7 mM glucose compared with 1.1 mM glucose. Aralar1 over-expression potentiated glucose- induced glutamate release by 31.9 % ( $p= 0.02$ ; Table 1). Lactate release was reduced

by 55.3% from Aralar1 over-expressing compared with control LacZ over-expressing BRIN-BD11 cells (Table 1)

#### *Effect of Aralar 1 overexpression on triacylglycerol and glycogen utilization*

Aralar1 over-expressing BRIN-BD11 cells contained significantly less triacylglycerol than control LacZ-transduced cells (- 26.3%,  $p= 0.03$  Table 1). Triacylglycerol synthesis is thought to be cytoprotective in the context of lipotoxicity due to removal of excessive non-esterified fatty acids [39], but hydrolysis may contribute to stimulation of insulin secretion under appropriate conditions (see [34] for review). Aralar1-over-expressing cells contained significantly less glycogen than control LacZ cells (- 35.1%;  $p= 0.04$ ; Table 1).

#### **Discussion**

We describe here a key regulatory role for the malate-aspartate shuttle in insulin secreting cells based on results generated following Aralar 1 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). Due to the unique metabolic design of the beta cell [34, 35] the activity of NADH shuttles may limit glycolysis due to a limit on NADH reoxidation to  $\text{NAD}^+$  [36, 37] and thus insulin secretion.

A previous study reported that Aralar 1 overexpression in the glucose responsive beta cell line INS-1E potentiated metabolism secretion coupling stimulated by 15 mM glucose, increased NAD(P)H generation, mitochondrial membrane hyperpolarisation, ATP levels and glucose oxidation accompanied by reduced lactate production. In vivo beta 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 beta cell line to investigate the combined effects of glucose and alanine on insulin secretion, amino acid metabolism, metabolic secretion coupling and has added new evidence to support the hypothesis that the Aralar 1 component of the malate aspartate shuttle is a key metabolic control site in the beta cell. Metabolic control is generally accepted to reside at GLUT-2, glucokinase, pyruvate dehydrogenase, pyruvate carboxylase and glutamate dehydrogenase in the beta cell [37].

A generalised scheme depicting involvement of glucose, alanine and glutamine in insulin secretion together with involvement of malate-aspartate shuttle is depicted in Figure 4. This Figure outlines the routes of metabolism for glucose, alanine and glutamine highlighting pathways and metabolites in common. Routes for formation of metabolic end-products such as glycogen and triacylglycerol 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 of important stimulus secretion coupling factors such as ATP and  $\text{Ca}^{2+}$  are evident. Aralar 1, 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 over-expression of a key component of the shuttle (aspartate-glutamate carrier 1), may remove constraint on the glycolytic pathway thus allowing 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 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]. Here, we have demonstrated that the addition of glucose and alanine resulted in the greatest



increase in insulin secretion compared to any other nutrient combination in aralar1 over-expressing 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 Aralar 1 overexpressing cells indicating enhanced metabolism and generation of stimulus secretion coupling factors such as ATP and glutamate as reported in this work. Glucose metabolism may also be stimulated by 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 gamma 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 catalysed 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 this work that Aralar 1 overexpression resulted in a significant increase in intracellular glutamate production compared to control cells.

Accumulation of triacylglycerol in pancreatic beta cells is a consequence of 'glucolipotoxic' conditions *in vitro* and *in vivo* [34, 38] and thus impaired insulin secretion. Indeed the accumulation of triacylglycerol probably reflects the cellular response to excessive non-esterified fatty acid levels, effectively removing fatty acids from adverse metabolic action [39]. 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 triacylglycerol synthesis, [43]) so enhancing pyruvate formation and entry into the TCA cycle. In the latter situation, triacylglycerol hydrolysis will be favoured (as synthesis is attenuated) therefore resulting in lower TAG content (Table 1), higher rates of fatty acid oxidation, ATP synthesis, and insulin secretion.

Glycogen accumulation in beta cells and islet cells in glucotoxic conditions has previously been described [40]. 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 shuttle activity, then glycogen breakdown may be favoured, resulting in enhanced oxidative metabolism, ATP generation and insulin release.

From the above considerations, over-expression of Aralar1 and the subsequent stimulation of the malate-aspartate shuttle appears to shift beta-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 depicted in Figure 4. Enhanced metabolic intermediate generation, a consequence of higher metabolism, may be beneficial for beta cell protection and survival [44]. Further studies are required to explore these possibilities and ultimately their application to primary human beta cell function and integrity.

### Acknowledgements

We thank the Health Research Board of Ireland for funding, UCD School of Biomolecular and Biomedical Science and the Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva for provision of space and resources. We also thank Prof.

Jorgina Satrústegui of University Autonoma, Madrid, Spain for provision of anti-Aralar 1 and anti-citrin antibodies. In addition we also thank Mr Mauricio Krause for his help and advice with respect to the preparation of Figure 9.

Accepted Manuscript

## References

1. McClenaghan NH (2007) Functional insights for understanding and therapy of diabetes Physiological regulation of the pancreatic beta-cell. *Exp Physiol* **92**: 481-496
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. Prentki M. (1996). New insights into pancreatic beta-cell metabolic signaling in insulin secretion. *Eur J Endocrinol* **134**, 272-86.
4. Prentki M. and Tornheim K., Corkey B.E. (1997). Signal transduction mechanisms in nutrient-induced insulin secretion. *Diabetologia* **40 Suppl 2**, S32-41.
5. Maechler P, Wollheim CB (1999) Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* **402**: 595-596
6. Carobbio, S., Frigerio, F., Rubi, B., Vetterli L., Bloksgaard, M., Gjinovci, A., Pournourmohammadi, S., Herrera, P.L., Reith, W., Mandrup, S. and Maechler P. (2009) Deletion of Glutamate Dehydrogenase in beta cells abolishes part of the insulin secretory response not required for glucose homeostasis. *J. Biol. Chem.* **284**: 921-929
7. Brennan, L. Shine, A. Hewage, C., Malthouse, J.P.G., Brindle, K., McClenaghan, N., Flatt, P.R. and Newsholme, P. (2002) A NMR based demonstration of substantial oxidative L-alanine metabolism and L-alanine enhanced glucose metabolism in a clonal pancreatic beta-cell line – Metabolism of L-alanine is important to the regulation of insulin secretion. *Diabetes* **51**: 1714-1721
8. Brennan, L., Corless, M., Hewage, C., Malthouse, J.P.G., McClenaghan, N.H., Flatt P.R. and Newsholme, P. (2003) <sup>13</sup>C NMR analysis reveals a link between L-glutamine metabolism, D-glucose metabolism and gamma-glutamyl cycle activity in a clonal pancreatic beta-cell line. *Diabetologia* **46**: 1512 – 1521
9. Newsholme P, Procopio J, Ramos Lima MM, Pithon-Curi TC, Curi R (2003) Glutamine and glutamate – their central role in cell metabolism and function. *Cell Biochem Funct* **21**:1-9
10. Dixon, G., Nolan, J., McClenaghan, N.H., Flatt, P.R. and Newsholme P. (2003). A comparative study of amino acid consumption by rat islet cells and the clonal beta-cell line BRIN-BD11 – the functional significance of L-alanine. *J. Endocrinol.* **179**, 447-454
11. Corless, M., Kiely A., McClenaghan, N.H., Flatt, P.R. and Newsholme P. (2006) Glutamine regulates expression of key transcription factor, signal transduction, metabolic gene and protein expression in a clonal pancreatic beta cell line. *J. Endocrinology* **190**: 719-727
12. MacDonald M.J. (1982) Evidence for the malate aspartate shuttle in pancreatic islets. *Arch Biochem Biophys.* **213**: 643-9.
13. Malaisse WJ (1995) Non-insulin-dependent diabetes mellitus and islet B-cell mitochondrial glycerophosphate dehydrogenase deficiency. *Diabet Med* **12**, 479- 81
14. MacDonald MJ (1995) Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets. Further implication of cytosolic NADPH in insulin secretion. *J Biol Chem* **270**: 20051-20058
15. Farfari S., Schulz V., Corkey B., Prentki M. (2000) Glucose- regulated Anaplerosis and Cataplerosis in pancreatic islets. Possible implication of a pyruvate/ citrate shuttle in insulin secretion. *Diabetes* **49**, 718- 726
16. Rutter G.A., Pralong W.F., Wollheim C.B. (1992). Regulation of mitochondrial glycerol-phosphate dehydrogenase by Ca<sup>2+</sup> with electropermeabilized insulin- secreting cells (INS-1). *Biochim Biophys Acta* **1175**, 107- 13
17. MacDonald M.J., Marshall L.K. (2000). Mouse lacking NAD<sup>+</sup>- linked glycerol phosphate dehydrogenase has normal pancreatic beta-cell function but abnormal metabolite pattern in skeletal muscle. *Arch Biochem Biophys* **384**, 143- 153

18. Ravier M.A., Eto K., Jonkers F.C., Nenquin M., Kadowaki T., Henquin J.C. (2000). The oscillatory behavior of pancreatic islets from mice with mitochondrial glycerol- 3- phosphate dehydrogenase knockout. *J Biol Chem* **275**, 1587- 1593.
19. Eto K., Tsubamoto Y., Terauchi Y., Sugiyama T., Kishimoto T., Takahashi N., Yamauchi N., Kubota N., Murayama S., Kasai H., Yazaki Y., Kadowaki T. (1999). Role of NADH shuttle system in glucose- induced activation of mitochondrial metabolism and insulin secretion. *Science* **283**, 981- 985.
20. Rubi, B., del Arco, A., Bartley, C., Satrustegui, J., and Maechler, P. (2004). The malate-aspartate NADH shuttle member Aralar1 determines glucose metabolic fate, mitochondrial activity, and insulin secretion in beta cells. *J Biol Chem* **279**, 55659-55666.
21. Del Arco A., Satrustegui J. (1998). Molecular cloning of Aralar, a new member of of the mitochondrial carrier superfamily that binds calcium and is present in human muscle and brain. *J Biol Chem* **273**, 23327- 23334
22. Palmieri L, Pardo B, Lasorsa FM, del Arco A, Kobayashi K, Iijima M, Runswick MJ, Walker JE, Saheki T, Satrustegui J, Palmieri F. (2004). Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria. *EMBO J* **20**: 5060-9
23. Del Arco A., Satrustegui J. (2004). Identification of a novel subfamily of mitochondrial carriers with calcium- binding domains. *J Biol Chem* **279**, 24701- 24713
24. McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YHA, O'Harte FPM, Yoon T-W, Swanston-Flatt SK, Flatt PR (1996) Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-DB11, produced by electrofusion. *Diabetes* **45**: 1132-1140
25. McClenaghan NH, Flatt PR (1999) Engineering cultured insulin-secreting pancreatic beta-cell lines. *J Mol Med* **77**: 235-243
26. Chapman JC, McClenaghan NH, Cosgrove KE, Hashmi MN, Shepherd RM, Giesberts AN, White SJ, Ammälä C, Flatt PR, Dunne MJ. (1999) ATP-sensitive potassium channels and efaroxan-induced insulin release in the electrofusion-derived BRIN-BD11 beta-cell line. *Diabetes* **48**: 2349-57.
27. McClenaghan NH, Barnett CR, O'Harte FPM, Flatt PR (1996) Mechanisms of amino acid-induced insulin secretion from the glucose-responsive BRIN-BD11 pancreatic beta-cell line. *J Endocrinol* **15**: 349-357
28. Van Eylen F, Horta OD, Barez, A, Kamagate A, Flatt PR, Macianskiene R, Mubagwa K and Herchuelz A (2002) Overexpression of the Na/Ca exchanger shapes stimulus-induced cytosolic Ca<sup>2+</sup> oscillations in insulin producing BRIN BD11 cells. *Diabetes* **51**: 366-375
29. Conroy SJ, Green I, Dixon G, Byrne PM, Nolan J, Abdel-Wahab YH, McClenaghan N, Flatt PR, Newsholme P. (2002). Evidence for a sustained increase in clonal beta-cell basal intracellular Ca<sup>2+</sup> levels after incubation in the presence of newly diagnosed Type-1 diabetic patient sera. Possible role in serum-induced inhibition of insulin secretion. *J Endocrinol* **173**: 53-62.
30. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB and Maechler P (2004) Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* **145**: 667-678
31. Patterson G.H., Knobel S.M., Arkhammer P., Thastrup O., Piston D.W. (2000). Separation of the glucose- stimulated cytoplasmic and mitochondrial NAD(P)H responses in pancreatic islet beta- cells. *Proc Natl Acad Sci USA* **97**, 5203- 5207.
32. Rink T.J., Montecucco C., Hesketh T.R., Tsien R.Y. (1980). Lymphocyte membrane potential assessed with fluorescent probes. *Biochim Biophys Acta.* **595**, 15-30.
33. Baracca A., Sgarbi G., Solaimi G., Lenaz G. (2003). Rhodamine 123 as a probe of mitochondrial membrane: Evaluation of proton flux through F<sub>0</sub> during ATP synthesis. *Biochim Biophys Acta* **1606**, 137- 146.

34. Newsholme P, Keane D, Welters H and Morgan N. (2007). Life and death decisions for the pancreatic beta cell - The role of fatty acids *Clin Sci* **112**: 27-42
35. Newsholme P., Brennan L., Maechler P. (2005). New insights into amino acid metabolism, beta- cell function and diabetes. *Clin Sci* **108**, 185- 194.
36. Bender K., Newsholme P., Brennan L., Maechler P. (2006). The importance of redox shuttles to pancreatic beta-cell energy metabolism and function. *Biochem Soc Trans* **34**, 811-814.
37. Maechler, P., Carobbio, S., and Rubi, B. (2006). In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. *Int J Biochem Cell Biol* **38**, 696-709.
38. Poitout V. (2008) Glucolipototoxicity of the pancreatic beta-cell: myth or reality? *Biochem Soc Trans* **36**: 901-904
39. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG (2001) Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* **50**,1771-1777
40. Newman ME (1977). Glycogen metabolism and cyclic AMP levels in isolated islets of lean and genetically obese mice. *Horm Met Res* **9**, 358- 361.
41. Bergmeyer HU, 1983 *Methods of Enzymatic Reactions Vol VIII*, p.341
42. Dringen R. and Hamprecht B. (1992). Glucose, Insulin and Insulin- like growth factor I regulate the glycogen content of astroglia- rich primary cultures. *J Neurochem* **58**, 511- 517.
43. Briaud I., Harmon J. S., Kelpel C. L., Segu V. B. G., and Poitout V. (2001) Lipotoxicity of the pancreatic beta-cell is associated with glucose-dependent esterification of fatty acids into neutral lipids. *Diabetes* **50**: 315-321
44. Broca C, Brennan L, Petit P, Newsholme P, Maechler P. (2003) Mitochondria-derived glutamate at the interplay between branched-chain amino acid and glucose-induced insulin secretion. *FEBS Lett.* **545**: 167-72.

## Figure legends

### Figure 1

Fig 1A - D.

Fig 1A, B. Expression of Aralar1 in BRIN-BD11 cells was determined. BRIN-BD11 cells were seeded and allowed to adhere over- night. Protein was extracted using RIPA lysis reagent containing 1 X Halt Protease Inhibitor Cocktail (Thermo Scientific). Proteins were probed with Anti- Aralar antibody directed against Aralar1 amino acids 7- 272. Bands correspond to 70 kDa. INS- 1E cell protein lysates were used as positive control for Aralar1 (Fig 1A). Membranes then were stripped and probed with Anti- GAPDH (Fig 1B).

Fig 1C. Expression of Citrin in BRIN-BD11 cells. For the determination of citrin expression, citrin antibody directed against amino acids 9- 278 was used. INS- 1E cell protein lysates were used as negative control for citrin and HEK-293 cell protein lysates were used as positive control for citrin.

Fig 1D. Protein expression after adenoviral transduction. For determination of adenoviral over-expression, BRIN-BD11 cells were seeded, allowed to adhere over- night prior to transduction with adenovirus and cultured for further 24 h to allow transgene expression. Proteins probed with Anti- Aralar antibody directed against Aralar1 amino acids 7- 272. Bands correspond to 70 kDa. Each blot was also incubated with Anti- Citrin directed against amino acids 9- 278, and with Anti- GAPDH.

### Figure 2

Fig 2A, B. For nutrient stimulated insulin secretion, BRIN-BD11 cells were cultured, allowed to adhere over night prior to transduction with the recombinant adenovirus and then cultured for further 24h to allow transgene expression prior to being studies of acute (20 min) insulin secretion. Values are the mean +/- SD of 3 independent experiments in quadruplicate \* $p < 0.05$  AdCA- Aralar1 versus control AdCA- LacZ at corresponding conditions,  $\Delta\Delta p < 0.01$ , + L- alanine vs. +L- alanine+ AOA .

### Figure 3 A - D

Fig 3A, B. BRIN-BD11 cells were allowed to adhere over night prior to transduction with adenovirus and cultured for further 24 h to allow transgene expression. The sample media was collected after 24hr and analysed for D-glucose and L-glutamine. Values are the mean +/- SD of 3 independent experiments in quadruplicate.

Fig 3C. BRIN-BD11 cells were allowed to adhere over night prior to transduction with adenovirus and cultured for further 24 h to allow transgene expression. Cells were challenged nutrients for 1 h and the supernatant was collected for analysis. Values are the mean +/- SD of 3 independent experiments in quadruplicate. \* $p < 0.05$ , AdCA- Aralar1 versus control AdCA- LacZ at corresponding conditions,  $\Delta\Delta p < 0.005$  +alanine vs.+ alanine + AOA.

Fig 3D, E. BRIN-BD11 cells were allowed to adhere over- night and then transduced with the respective adenovirus. Cells were challenged with 10mM L- glutamine or 10 mM L- alanine respectively in combination with 1.1mM or 16.7 mM glucose for 1 h. The supernatant was collected for analysis. Values are the mean +/- SD of 3 independent experiments in quadruplicate. \* $p < 0.05$ , AdCA- Aralar1 versus control AdCA- LacZ

**Figure 4**

Pancreatic beta cell metabolic stimulus secretion scheme depicting involvement of glucose, alanine and glutamine in insulin secretion together with involvement of the malate-aspartate shuttle. See text for further description. Abbreviations of enzymes and transporters are as follows: 1: glucokinase, 2: cytosolic aspartate aminotransferase, 3: cytosolic malate dehydrogenase, 4: glycerol kinase, 5: glyceraldehyde-3-phosphate dehydrogenase, 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: triacylglycerol lipase, 15: amino acid transporter, 16: GLUT2, 17: voltage dependent calcium channel.

**TABLE 1**

ATP content. BRIN-BD11 cells were allowed to adhere over- night and then transduced with adenovirus. Cells were cultured for further 24h to allow transgene expression. Cells were lysed and analysed for ATP content. Values are the mean +/- SD of 3 independent experiments in quadruplicate. \* $p < 0.05$ , AdCA- Aralar1 versus control AdCA- LacZ

Lactate production. BRIN-BD11 cells were allowed to adhere over night prior to transduction with adenovirus and cultured for further 24 h to allow transgene expression. The sample media was collected for analysis. Values are the mean +/- SD of 3 independent experiments in quadruplicate. \* $p < 0.05$ , AdCA- Aralar1 versus control AdCA- LacZ

Glutamate production. BRIN-BD11 cells were allowed to adhere over night prior to transduction with adenovirus and cultured for further 24 h to allow transgene expression. The sample media was collected for analysis. Values are the mean +/- SD of 3 independent experiments in quadruplicate.

Intracellular glutamate. BRIN-BD11 cells were allowed to adhere over- night and then transduced with adenovirus. Cells were challenged with 16.7 mM glucose for 20 min. Cells then were extracted and used for glutamate determination. The results are expressed as mean  $\pm$  SD for 3 separate determinations

Triacylglycerol content. BRIN-BD11 cells were allowed to adhere over night prior to being transduced with adenovirus. Cells were cultured for further 24 h to allow transgene expression prior to extraction of triglycerides. . Values are the mean +/- SD of 3 independent experiments in quadruplicate. \* $p < 0.05$ , AdCA- Aralar1 versus control AdCA- LacZ,  $\Delta\Delta p < 0.01$  AdCA- Aralar1 vs. control at 24 h.

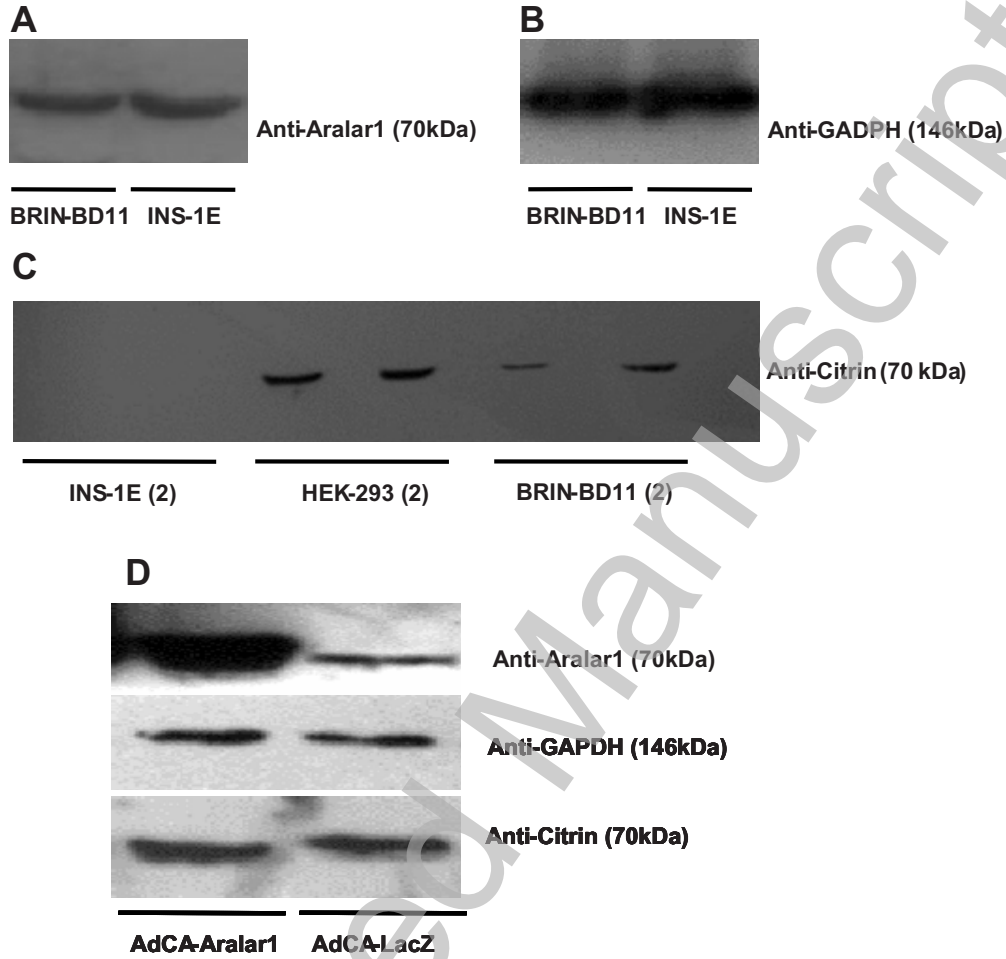
Glycogen content. BRIN-BD11 cells were allowed to adhere over- night and then transduced with adenovirus. Cells were cultured for further 24h to allow transgene expression and used for glycogen determination. Values are the mean +/- SD of 3 independent experiments in quadruplicate. \* $p < 0.05$ , AdCA- Aralar1 versus control AdCA- LacZ

Table 1

Parameter	AdCA- Aralar1	AdCA- LacZ	Percent change
ATP content (nmol/ mg protein)	78.1 ± 15.5	46.2 ± 11.7	+ 68.9%
Lactate production (µmol/ mg protein * 24h)	27.4 ± 3.08	44.3 ± 6.83	-55.3%
Glutamate production (µmol/ mg protein + 24h)	1.99 ± 0.60	1.75 ± 0.30	Not significant
Intracellular glutamate (µmol/ mg protein)	0.49 ± 0.08 (1.1mM gluc) 1.31 ± 0.07 (16.7 mM gluc)	0.44 ± 0.06 (1.1mM gluc) 0.87 ± 0.19 (16.7mM gluc)	+31.9% (16.7mM glucose)
Triacylglycerol content (µmol/ mg protein)	0.19 ± 0.02	0.30 ± 0.08	-26.3%
Glycogen content (µmol/ mg protein)	1.33 ± 0.69	2.06 ± 0.34	-35.1%

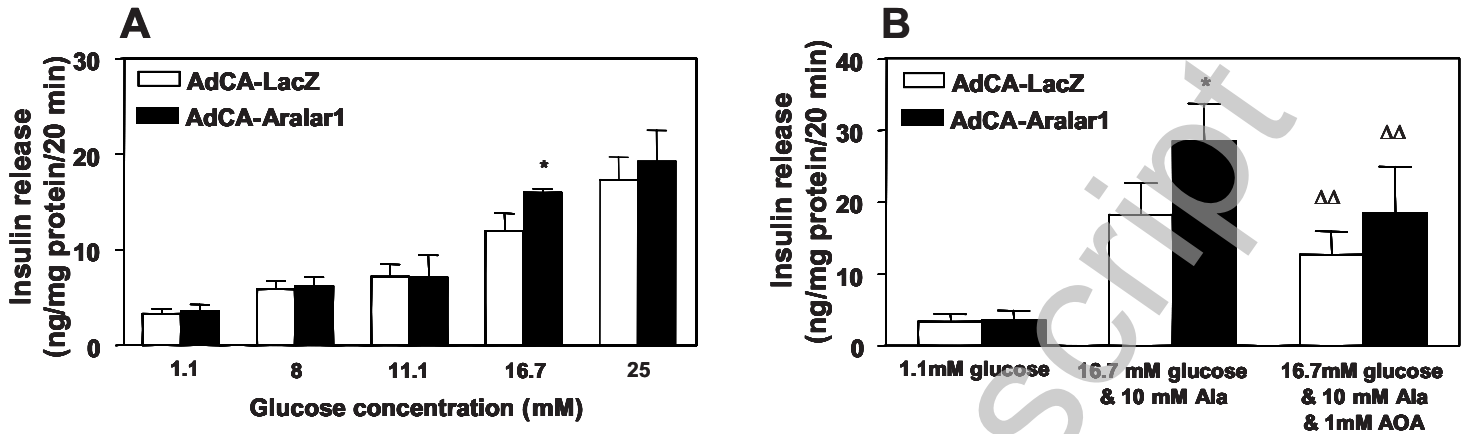


Figure 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090126

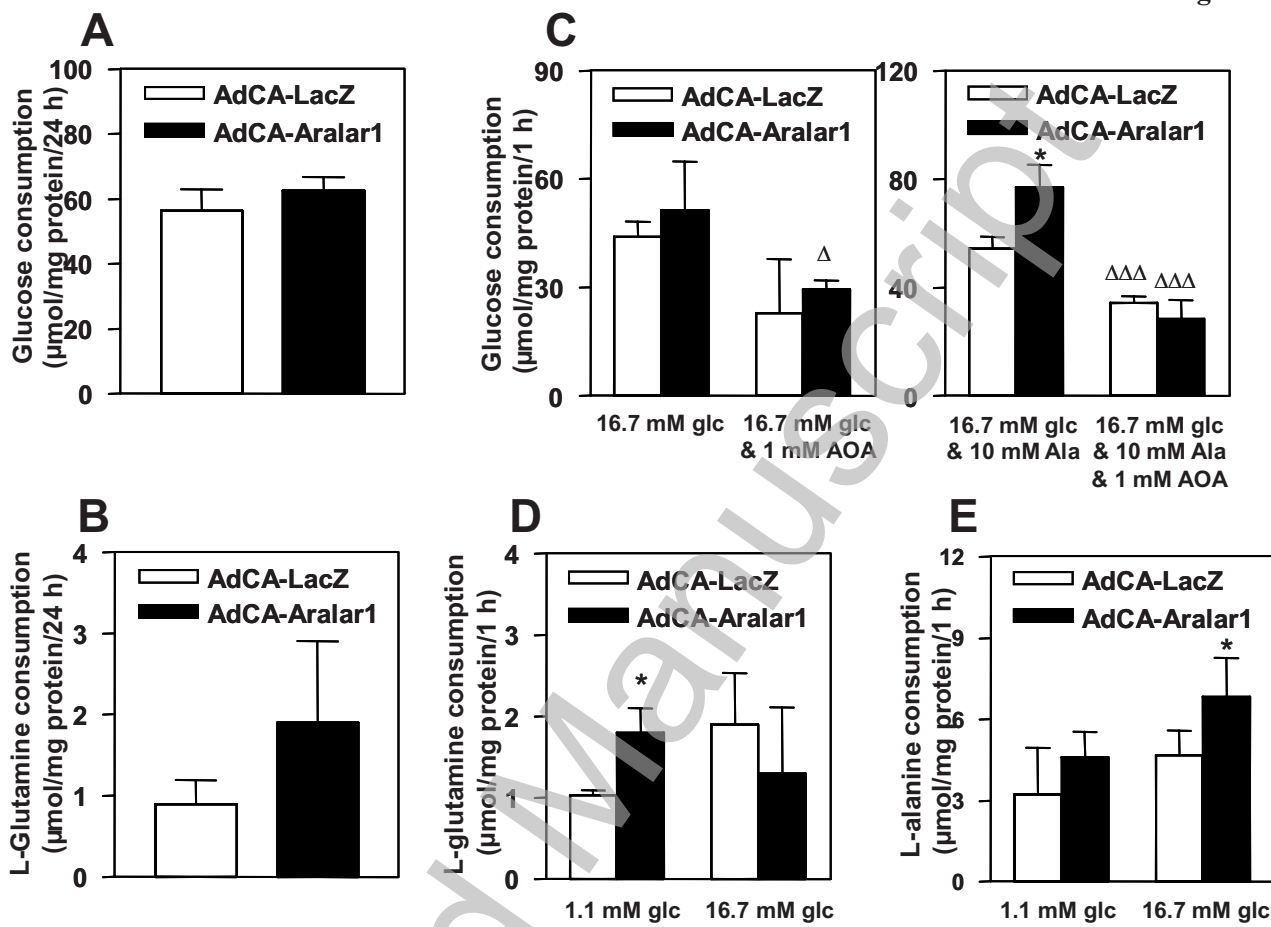
Figure 2



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090126

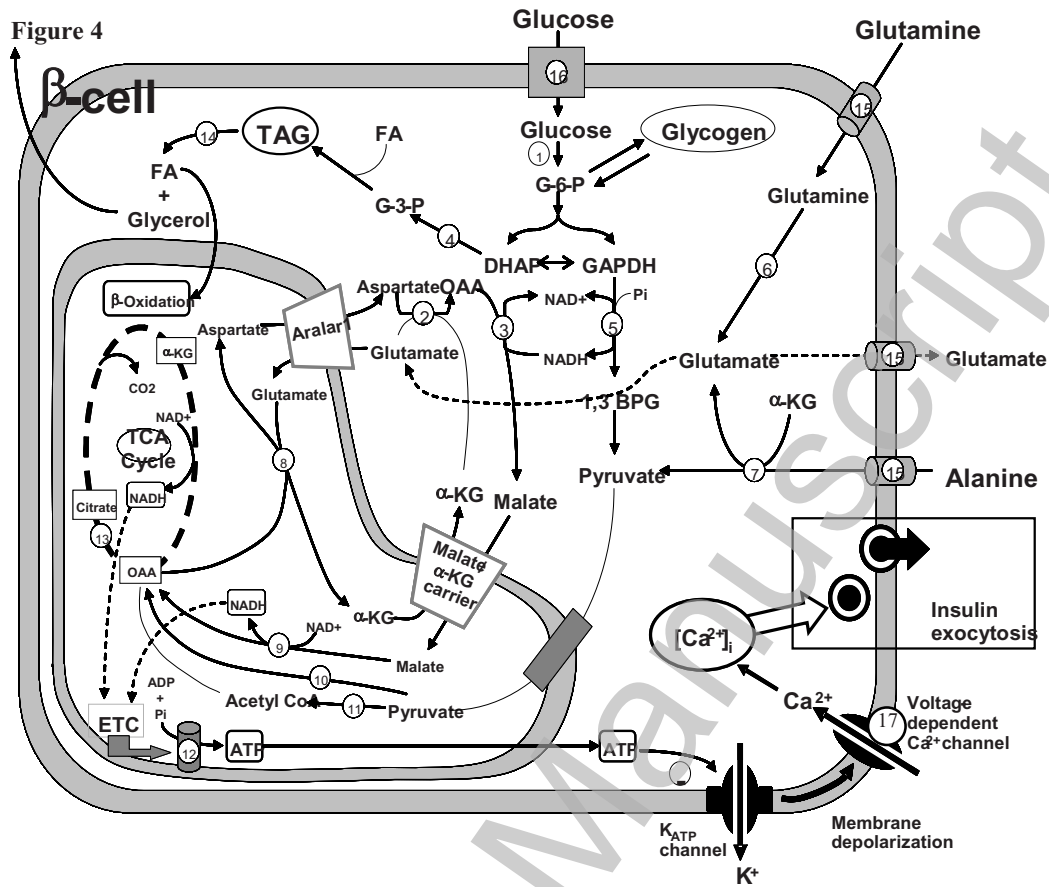
Accepted Manuscript

Figure 3



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090126

Accepted Manuscript



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090126

Accepted Manuscript