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Insights into the role of anaplerosis in insulin secretion: a ^{13}C NMR study

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Abstract *Aims/hypothesis:* Defining mechanisms and enzymatic paths critical to fuel-regulated insulin secretion are key goals of diabetes research. In this study, ^{13}C -nuclear magnetic resonance spectroscopy and isotopomer analysis were used to investigate the link between insulin secretion and metabolic pathways associated with the tricarboxylic acid (TCA) cycle. *Materials and methods:* To this end, four insulinoma cell lines (βTC3 , $\beta\text{TC-tet}$, INS-1 [832/13], R7T1) and porcine islets were examined under a variety of culture conditions (i.e. presence vs absence of amino acids and sera, and low vs high glucose). *Results:* Glucose consumption, insulin release, and glutamate isotopomeric patterns were influenced by media complexity (e.g. PBS, plain culture media, fully supplemented culture media). The ^{13}C -labelled metabolites increased with media complexity and increasing glucose concentration, with the notable exception of aspartate, which was always higher under low-glucose conditions. The ^{13}C -glutamate isotopomeric fractions were fitted to metabolic models to estimate the relative metabolic fluxes to the TCA cycle through key enzymatic processes. These indices of metabolism were compared with insulin secretion to determine correlative links. A model containing a single pool of pyruvate, an entrance to the TCA cycle via the pyruvate dehydrogenase complex, and two anaplerotic entrances, one through pyruvate carboxylase and another through an undefined (by the modelling program) source, provided the best fit to the data under all conditions tested, for all cell lines. *Conclusions/interpretation:* On the basis of our findings, a strong correlation may exist between stimulated insulin secretion and non-pyruvate carboxylase anaplerosis for the four cell lines examined in this study.

Keywords Anaplerosis · Aspartate · Insulin secretion · Metabolism · NMR spectroscopy

Abbreviations α -KG: alpha-ketoglutarate · GSIS: glucose-stimulated insulin secretion · NMR: nuclear magnetic resonance · PC: pyruvate carboxylase · PDH: pyruvate dehydrogenase · S/N: signal-to-noise ratio · TCA: tricarboxylic acid

Introduction

Defining the pathways that are vital to insulin secretion is essential to understanding secretion mechanisms. The ‘Fuel Hypothesis’ [1–4] is an established mechanism linking elevated extracellular glucose concentrations to insulin secretion. Although broadly accepted, this mechanism cannot adequately address all the observed insulin secretion anomalies. Consequently, the importance of specific enzymatic pathways to insulin release is still under investigation [5–11]. Understanding these pathways is an important step towards discovering novel avenues of therapeutic intervention and assisting cellular engineers in tailoring superior beta cell surrogate cell lines.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful method for acquiring cellular metabolic information [5–7, 10, 12–17]. For example, after exposure to ^{13}C -enriched fuel (e.g. glucose), analysis of glutamate carbon isotopomer patterns resulting from ^{13}C – ^{13}C spin-spin interactions [18–20] provides both a quantitative assessment of metabolism and an insight regarding carbon entry to the tricarboxylic acid (TCA) cycle. Figure 1a illustrates how isotopomeric patterns of the fourth glutamate carbon (C4) arise. The first row shows a single resonance generated when the ^{13}C -labelled C4 is surrounded by non- ^{13}C labelled carbons (C3 and C5). The second and third rows show doublets generated when one of adjacent carbons, C3 or C5, respectively, is also labelled with ^{13}C , and the fourth row shows the quartet that is generated when both C3 and C5 in addition to C4 are labelled with ^{13}C . Labelling of non-neighbouring

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carbons is irrelevant. The distribution of these four possibilities (the fractional enrichment) determines the final isotopomeric pattern. Figure 1b,c depicts C4 glutamate patterns for the stated fractional enrichments. Isotopomeric patterns arise for each glutamate carbon and because the paths by which ^{13}C -labelled carbons enter (and re-enter) the TCA cycle dictate the positions where the label is situated, steady-state isotopomeric patterns reflect the relative flux of enzymes associated with these paths.

Using isotopomeric analysis, a correlation between insulin induction and 'pyruvate cycling' (a previously undocumented relationship for insulin-secreting cells that describes the circular carbon exchange between pyruvate and the TCA cycle), was recently proposed [10]. An important aspect of this correlation was the use of two, largely independent, pyruvate pools. Another study using similar NMR techniques revealed a strong link between

insulin secretion and anaplerosis to the TCA cycle from enhanced flux through pyruvate carboxylase (PC), and a positive correlation between pyruvate cycling and insulin secretion in response to glucose stimulation [7]. The authors also stated that carbon flux through PC and pyruvate dehydrogenase (PDH) is most relevant to insulin secretion.

The aforementioned ^{13}C NMR studies were performed on various clones of INS-1 cells incubated in buffered salt solutions [7, 10] similar to the commonly used buffers applied in islets studies [21–23]. However, insulin secretion, glucose consumption and oxygen consumption are significantly affected by media composition [24, 25]. Consequently, the links between metabolism and secretion may vary as a function of medium composition. Therefore, we acquired ^{13}C NMR and ^{31}P NMR data from four insulin-secreting cell lines and porcine islets exposed to various media conditions and applied metabolic models to glutamate isotopomeric data to determine the role of anaplerosis in insulin secretion.

Materials and methods

Cell lines

βTC3 , $\beta\text{TC-tet}$ and R7T1 murine insulinomas were obtained from S. Efrat (Albert Einstein College of Medicine, Bronx, NY, USA) and cultured in DMEM (22 mmol/l glucose) supplemented with 15% (v/v) equine serum (Hyclone, Logan, UT, USA), 2.5% fetal bovine serum (FBS; Mediatech, Herndon, VA, USA), antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) and 6 mmol/l L-glutamine. R7T1 cells also required 100 $\mu\text{mol}/\text{l}$ doxycycline. INS-1 (832/13) rat insulinoma cells from C. Newgard (Duke University, Durham, NC, USA) were cultured in RPMI-1630 (11 mmol/l glucose) supplemented with 10% FBS, antibiotics, 2 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate and 50 $\mu\text{mol}/\text{l}$ β -mercaptoethanol. Cultures grew at 37°C under humidified (5% CO_2 /95% air) conditions. Porcine islets were provided by C. Weber (Emory University, Atlanta, GA, USA), stored in DMEM (5 mmol/l glucose) with 10% FBS, antibiotics and 6 mmol/l L-glutamine. An extract was generated within 48 h of isolation.

^{13}C -labelled media

Incubation media were: PBS containing no nutrients other than added labelled glucose; 'incomplete' media, DMEM or RPMI (dictated by the cell line) containing labelled glucose, glutamine and antibiotics, but no sera; and 'complete' media, DMEM or RPMI containing labelled glucose, glutamine, antibiotics and sera. Incubation media incorporated either 3 or 15 mmol/l uniformly labelled ^{13}C -glucose (Cambridge Isotopes, Cambridge, MA, USA). To eliminate a potential pH effect on insulin release, the pH of all solutions was 7.2

Fig. 1 Mechanism of ^{13}C isotopic splitting. **a** Depiction of how neighbouring labelled carbon atoms influence the isotopomeric splitting of a selected labelled fourth carbon of glutamate (C_4). Dark circles, labelled carbon; open circles, unlabelled carbon. The possible signals arising from a labelled C_4 as a consequence of labelling of the immediate neighbours are shown. **b**, **c** Illustration of the resultant C_4 glutamate patterns for the fractional enrichments stated on the right

[26, 27]. Cultures were rinsed, incubated in glucose-free PBS for 1.5 h to normalise experiments to the same initial conditions, and rinsed again before incubation for 4 h in ^{13}C -labelled media. Porcine islets (~100,000 islet equivalents) were exposed for 4 h in complete media containing 15 mmol/l uniformly labelled ^{13}C -glucose. The 4-h time-point was chosen to assure that isotopomeric enrichment of the glutamate resonances was at steady-state, as previously reported [10].

Cell extraction

Monolayer culture extractions used four confluent T-175 flasks. Trypan blue exclusion and manual cell counting of a representative flask determined cell number and viability (>95% viable in each extract). After incubation, dual-phase extractions were performed [28]. Aqueous portions were isolated, treated with Chelex-100 (Sigma, St Louis, MO, USA), lyophilised, resuspended in D_2O (Cambridge Isotopes, Cambridge, MA), and placed in 5 mm NMR tubes.

Assays

Media samples drawn at the start and conclusion of incubations were assayed for glucose. Samples drawn 20 min into exposures were assayed for insulin. Glucose levels were determined with a VITROS DT60 II Bioanalyzer (Ortho-Clinical Diagnostics, Rochester, NY, USA), and glucose consumption rates were calculated. Insulin levels were assayed with an EIA kit (Linco, St Charles, MO, USA), measured on a Synergy HT plate reader (Bio-Tek, Winooski, VT, USA), and rates of insulin release over the 20-min period were calculated.

NMR spectroscopy

Both ^{31}P and ^{13}C NMR spectra were acquired using a 5-mm broadband probe equipped with a ^1H decoupling channel. A capillary containing dioxane (^{13}C) and trimethylphosphate (^{31}P) was placed in the NMR tube as a chemical shift reference. Data were collected on a 500 MHz vertical bore magnet equipped with a Bruker Avance console (Bruker, Billerica, MA, USA) using the following acquisition parameters: ^{13}P : sweep width 8.09 kHz at 202.56 MHz; repetition time 6 s; number of transients 1,024; and ^{13}C : sweep width 30 kHz at 125.84 MHz; repetition time 6 s; number of transients 10,240. Waltz ^1H decoupling was applied throughout the acquisition sequences.

Spectroscopic analysis

Areas under the ^{13}P resonances of the α -ATP and α -ADP were determined by line-fit analysis (Acorn software,

Fremont, CA, USA) and the ATP/ADP ratios were calculated. ^{13}C NMR spectra were analysed by calculating the multiplets of glutamate carbons (C2, C3, C4) with line-fit analysis. Factors were applied to glutamate signal areas correcting for relaxation and nuclear Overhauser effects. Isotopomeric modelling analysis was performed with tcaCALC to estimate indices of metabolism [18–20], and tcaCALC output values were run with tcaSIM to ensure that tcaCALC did not reach false local minima.

Metabolic models

Metabolic models applied in tcaCALC include a dual-pyruvate pool model (Fig. 2a), and a single-pyruvate pool model with two anaplerotic entrances to the TCA cycle (Fig. 2b). In the dual-pyruvate pool model, glycolysis of uniformly labelled ^{13}C -glucose creates a pyruvate pool that enters the TCA cycle solely through pyruvate dehydrogenase to form uniformly labelled acetyl-CoA. A second pyruvate pool is derived from TCA cycle intermediates that exit through the pathways described below and re-enter the TCA cycle via pyruvate carboxylase, although a small fraction may enter via pyruvate dehydrogenase (dashed line). Pyruvate cycling occurs only between the second pyruvate pool and the TCA cycle. In the single-pyruvate pool model, fully labelled pyruvate is formed by glycolysis from uniformly labelled glucose in the media and enters the TCA cycle via PDH and PC. Carbon from the TCA cycle can replenish the pyruvate pool via oxaloacetate or malate, although replenishment from oxaloacetate via phosphoenol-pyruvate carboxykinase is negligible in islets [29–31]. The single-pyruvate pool model (Fig. 2b) includes a non-PC anaplerotic entrance to the TCA cycle from an undefined carbon source designated as ‘2nd anaplerotic entrance’, shown entering the TCA cycle between α -ketoglutarate (α -KG) and malate for visual simplicity. Pyruvate cycling is defined as the average of the fluxes through PC (anaplerotic entrance into the TCA cycle) and pyruvate kinase (exit from the TCA cycle to the pyruvate pool) [7, 10]. Per cent anaplerosis is defined as the carbon entry from all anaplerotic pathways (model-dependent) relative to the total carbon entering the TCA cycle. Both models assume complete exchange between oxaloacetate and fumarate, and a random orientation transfer between succinyl-CoA and malate in the TCA cycle.

Statistical methods

For groups within data sets (e.g. PBS, incomplete and complete media), means and standard deviations were calculated, and a Student’s *t*-test (two-tailed) for independent samples was performed. Relationships between data (e.g. insulin secretion vs pyruvate carboxylase activity) were tested with the Pearson product-moment correlation. A metabolic model’s ability to derive information from isotopomer data was judged by how well the model-

Fig. 2 Models of metabolism used in this study. **a** The dual-pyruvate pool model. **b** The single-pyruvate pool model. *Dashed line (a)*: the partial communication of the second pyruvate pool with acetyl-CoA via pyruvate dehydrogenase (PDH). PDH, pyruvate carboxylase (PC) and pyruvate kinase (PK) are tcaCALC parameters designed to represent relative fluxes of pyruvate metabolism (PDH and PC) and synthesis from TCA cycle intermediates (PK). Potential pathways from malate and oxaloacetate are included although conversion from oxaloacetate has been shown to be negligible in islets

generated isotopomeric data reflected the actual spectra (defined as the residual difference) and by how well the modelled glutamate enrichment C3/C4 ratio replicated the ratio of the fitted resonances (defined as the % C3/C4 error), although similar ratios can be derived from any two resonances. Student's *t*-tests (two-sample, unequal variance) were performed on model-derived residuals and % C3/C4 errors for each cell line to test whether different metabolic models provide similar values when applied to the same data. *F*-tests were performed on standard deviations of the model-derived residuals and % C3/C4 errors to test whether different metabolic models provide similar precisions when applied to the same data.

Results

The effects of media complexity (15 mmol/l glucose) on the glucose consumption rate, insulin secretion rate and ATP/ADP ratio for the INS-1 (832/13) and βTC3 cell lines

are summarised in Fig. 3. For the INS-1 (and R7T1) cells, the glucose consumption and insulin secretion rates increased in response to increasing media complexity, though ATP/ADP was not influenced by complexity changes under high glucose conditions. However, in the βTC3 (and βTC-tet) cells, the respective insulin secretion data did not follow this trend, despite a large increase in glucose consumption from PBS to either incomplete or complete media. Rather, there was little change in insulin secretion from PBS to incomplete conditions, then a large significant increase under complete conditions. This describes alterations in insulin release unrelated to glucose stimulation. Again, the ATP/ADP ratio was not influenced by complexity changes. Similar changes as a result of complexity were observed under 3 mmol/l glucose conditions, although the ATP/ADP ratio, glucose consumption rates and insulin secretion rates measured in 15 mmol/l glucose conditions were elevated compared to analogous 3 mmol/l glucose conditions, for all categories of media complexity. Correlation analysis of data from all cells under all conditions demonstrated that the glucose consumption rate was a poor indicator of the insulin secretion rate ($r^2=0.144$), and was even worse ($r^2=0.055$) for ATP/ADP ratio.

Media complexity also had an impact on the integration of ^{13}C -labelled glucose carbons into amino acids and other metabolites. Specifically, almost all detectable resonances were more enriched as media complexity increased (i.e. PBS < incomplete media < complete media). When glucose concentration was increased within a given medium composition, enrichment of most resonances was maintained or increased. As shown in the βTC3 spectra in Fig. 4a,b, there were large increases in serine, lactate and alanine resonances, while glutamate, citrate and glycine resonances maintained enrichment levels similar to those found under low glucose conditions. Contrary to other amino acids, the enrichment of aspartate was reduced under high glucose conditions (both under PBS or complete media conditions). The decrease in enriched aspartate associated with increased glucose levels was observed in all four lines, and similar changes were seen under PBS conditions, although the absolute signal-to-noise ratio (S/N) was inferior because of lower fractional enrichment. Figure 4c shows spectra obtained from porcine islets under complete media with high glucose concentrations. Differences from the insulinoma spectra are: (1) the serine C2 resonance was not detected in the islet spectrum possibly because of its lower S/N; (2) the alanine C3 had shifted, possibly because of a pH difference between the samples; and (3) the resonances *x* and *y* (tentatively assigned as C4- and C3-α-KG) were greatly enhanced.

Figure 5 shows the glutamate isotopomer patterns generated by βTC3 cells as a function of media complexity but at constant glucose concentration (15 mmol/l). The following differences were observed consistently in all the cell lines examined as media complexity increased from PBS to complete. Under PBS conditions, most glutamate carbons were labelled, leading to more quartets and triplets

Fig. 3 Relationship between **a, b** the glucose consumption rate (*GCR*), **c, d** the insulin secretion rate (*ISR*), and **e, f** the ATP/ADP of monolayer cultures of INS-1 (832/13) (**a, c, e**) and β TC-3 (**b, d, f**) cultures exposed to either PBS, incomplete medium, or complete medium. All solutions contained 15 mmol/l uniformly labelled ^{13}C -glucose. Error bars represent the standard deviation of the mean. * $p < 0.05$ for differences from the value obtained under PBS conditions

(where applicable), and fewer singlets. In complete media, where the unlabelled amino acids and fatty acids present in the media contributed significantly to the cell's metabolic activity, less contiguous labelling occurred, resulting in a rise in singlets and doublets. These isotopomeric patterns hold important information regarding key enzymatic reactions, and were analysed with *tcaCALC* to generate indices of metabolism related to the TCA cycle (e.g. pyruvate cycling, anaplerosis through pyruvate carboxylase, non-PC anaplerosis, and the percentage of carbon entry to the TCA cycle via anaplerosis). Also in Fig. 5 are simulated glutamate spectra (noiseless, narrow line-width, and containing no other species that could give rise to confounding peaks) generated with *tcaSIM* using the output from *tcaCALC*. Visual inspection confirmed that the relative patterns were appropriate when using the single-pyruvate pool metabolic model containing non-PC anaplerosis, ensuring that a false minimum has not been reached in the *tcaCALC* analysis. However, analysis with this model lacking non-PC anaplerosis resulted in substantial differences in the C4 glutamate spectra (e.g. enhanced quartet contribution). This is most evident under complete media conditions, as shown here, where non-PC

anaplerosis is appreciable. Spectral generation using output from the dual-pyruvate pool model also resulted in reasonable patterns. However, the dual-pool simulations lack the C4 singlet and doublet-43 under all conditions (though the real spectra suggest that these exist at low levels), and under complete conditions have an elevated C4 quartet (compared to the real data).

Good fits (i.e. low residuals and low % C3/C4 errors) were obtained using either model, and there were no statistically significant differences. However, analysis of the INS-1 (832/13) cell line with the single-pool model significantly reduced scatter of the % C3/C4 error ($p < 0.01$), indicating that although both models had statistically similar errors (i.e. similar accuracies), use of the single-pool model led to improved precision.

Although both models described in Fig. 2 fit the data with equal statistical confidence, the single-pyruvate pool model was selected for subsequent analysis because: (1) it is consistent with known biochemistry; (2) it allows for both PC and non-PC anaplerotic pathways; and (3) it can be used to explain observed spectral changes in both glutamate and aspartate resonances (the rationale is delineated in the Discussion). Consequently, correlations

Fig. 4 Proton-decoupled ^{13}C NMR spectra. **a, b** Extracts of βTC -3 monolayer cultures exposed to complete media containing 3 and 15 mmol/l uniformly labelled ^{13}C -glucose, respectively. **c** An extract obtained from pig islets (100,000 islet equivalents) exposed to complete media containing 15 mmol/l uniformly labelled ^{13}C -glucose. Assignments are placed above the resonances. Also included are two resonances that were tentatively identified as α -KG C4 (x) and α -KG C3 (y)

between model parameters and metabolic and secretory indices are presented based solely on this model. Table 1 summarises the correlations between the glucose consumption rate, ATP/ADP ratio or the insulin secretion rate and selected indices of metabolism derived from the single-pyruvate pool model (numbers indicate the r^2 value). The top section of Table 1 shows that the R7T1 and INS-1 (832/13) lines displayed the strongest correlations between glucose consumption and tcaCALC-derived anaplerotic parameters, while βTC 3 and βTC -tet lines displayed the weakest correlations. The middle section of Table 1 demonstrates that all four cell lines examined show weak correlations between ATP/ADP and model-derived indices. The bottom section of Table 1 shows that R7T1 and INS-1 (832/13) cells displayed strong correlations between insulin secretion and non-PC anaplerosis and weaker correlations between insulin secretion and either pyruvate cycling or percent anaplerosis. βTC 3 cells show a weak correlation between insulin secretion and non-PC anaplerosis and very weak correlations with other model-derived anaplerotic parameters, while βTC -tet cells display very weak

correlations with all anaplerotic parameters. It is important to note that the dual-pyruvate pool model resulted in similar correlations. Specifically, R7T1 and INS-1 (832/13) cells displayed strong correlations between the insulin secretion rate and pyruvate cycling ($r^2=0.599$ and 0.552 , respectively) and between insulin secretion and percent anaplerosis ($r^2=0.539$ and 0.629 , respectively), corroborating previous observations [10]. However, correlations were very weak for βTC 3 and βTC -tet cells ($r^2=0.108$ and 0.071 , respectively, for pyruvate cycling; and $r^2=0.110$ and 0.071 , respectively, for percent anaplerosis).

Figure 6 represents graphically the correlation between the non-PC anaplerosis and the insulin secretion rate for the R7T1, INS-1 (832/13) and porcine islets. The R7T1 cell line is represented with eight points and the INS-1 (832/13) cell line with ten points. Each point represents an extract derived under a specific culture condition as described in the Materials and methods. Although the islet data form only a single point, this highlights the correlation and supports the importance of non-PC anaplerosis to insulin secretion in these cells.

To assess the relationship between a change in the insulin secretion and a change in model-derived parameters, the following comparisons were considered for each insulinoma cell line: (1) PBS, 3 vs 15 mmol/l glucose; (2) complete media, 3 vs 15 mmol/l glucose; (3) 3 mmol/l glucose, PBS vs complete media; and (4) 15 mmol/l glucose, PBS vs complete media. The relationships are delineated in the Pearson product-moment correlations (r^2) of Table 2. This analysis reveals strong correlations between a change in insulin secretion and a corresponding change in any anaplerosis-related parameter. These correlations are positive with the exception of the INS-1 (832/13) cells that display negative correlations with pyruvate cycling and PC anaplerosis. The anaplerotic parameter that displays strong positive correlation in all four cell lines is non-PC anaplerosis. This can best be illustrated for each of the cell lines graphically (Fig. 7). The four points on the graph correspond to the four comparisons described above.

Discussion

Isotopomer modelling, in concert with metabolic and secretory measurements, can assess the relationship between insulin secretion and enzymatic processes. In this study, two applied models fit the isotopomeric data with similar margin of error, yet point to different key enzymatic processes. Specifically, the dual-pool model suggests that pyruvate cycling is a key component of glucose-stimulated insulin secretion, valid for the R7T1 and INS-1 (832/13) cell lines. Alternatively, the single-pool model suggests that non-PC anaplerosis is the key component to stimulated insulin secretion, and it is valid for all four cell lines tested in this study (see Table 2). This result corroborates a recent study linking mitochondrial anaplerotic substrates to insulin secretion [32], but contradicts a ^{13}C isotopomer study using the INS-1 (832/13)

Fig. 5 Isotopomer patterns of glutamate resonances (Gluta-C2, -C3, -C4) (obtained from extracts of β TC3 monolayer cultures exposed to PBS, incomplete media, or complete media all containing 15 mmol/l uniformly labelled ^{13}C -glucose). The top three rows of spectra are the real NMR data. The next three rows are idealised simulated data (using tcaSIM) using values obtained from tcaCALC analysis with the single-pyruvate pool metabolic model that includes non-PC anaplerosis. The row below shows the simulated data obtained when non-PC anaplerosis is not included in the metabolic model. The lowest three rows show simulated spectra obtained with the dual-pyruvate pool model. The *symbols* beneath the spectra delineate the origin of the peaks: *S*, the singlet; *D*, the doublet; *Q*, the quartet; *T*, the triplet (actually a quartet with the two middle components coincidental with the singlet and each other)

clone and a similar single-pool model examining the role of glutamate as a non-PC anaplerotic source; the authors concluded that flux through PDH and PC, but not glutamate dehydrogenase, correlated to insulin secretion [7].

Potential sources for this non-PC anaplerosis are limited: likely candidates are the amino acids glutamate or aspartate. These amino acids are readily labelled upon incubation with labelled glucose, derive from TCA cycle intermediates α -KG and oxaloacetate, respectively, and can re-enter the TCA cycle at these same points. Glutamine, derived from glutamate, is also a consideration. However, our spectra show little change in labelled glutamine or glutamate levels (Fig. 4), suggesting that these do not play an obvious anaplerotic function. The role of glutamate in insulin release has been debated [33], but is

now thought not to play a direct part [7, 34, 35]. Based on the following evidence, we hypothesise that the key non-PC anaplerotic substrate is aspartate. A prime indicator of the involvement of aspartate in the secretion of insulin comes from our observation that there is a decrease in the aspartate enrichment (Fig. 4) under conditions that induce insulin release (e.g. presence of sera or the traditional glucose-stimulated insulin secretion [GSIS] effect). Outside support for our spectroscopic observation is found in a paper depicting ^{13}C NMR spectral changes from extracts of BRIN-BD11 cells exposed to buffer solutions containing 16.7 mmol/l glucose [6]. When 10 mmol/l alanine was added to the buffer solution, though glucose levels were constant, the insulin secretion was significantly increased (a nearly seven-fold increase), and the aspartate resonances were markedly decreased. Unfortunately, other spectro-

Table 1 Pearson product-moment correlations (r^2) between glucose consumption, ATP/ADP and insulin secretion, and select parameters from tcaCALC analysis

Cell line	Correlations between glucose consumption and: ...			
	Pyruvate cycling	PC anaplerosis	Non-PC anaplerosis	Percent anaplerosis
β TC3	0.228	0.342	0.012	0.338
β TC-tet	0.044	0.483	0.032	0.136
R7T1	0.454	0.266	0.507	0.545
INS-1	0.546	0.593	0.282	0.642
Correlations between ATP/ADP and:				
Cell line	Pyruvate cycling	PC anaplerosis	Non-PC anaplerosis	Percent anaplerosis
β TC3	0.034	0.109	0.000	0.085
β TC-tet	0.003	0.021	0.000	0.005
R7T1	0.218	0.038	0.452	0.300
INS-1	0.201	0.141	0.062	0.202
Correlations between insulin secretion and:				
Cell line	Pyruvate cycling	PC anaplerosis	Non-PC anaplerosis	Percent anaplerosis
β TC3	0.050	0.024	0.321	0.109
β TC-tet	0.074	0.024	0.069	0.104
R7T1	0.328	0.122	0.768	0.496
INS-1	0.340	0.186	0.542	0.518

The single-pyruvate pool model shown in Fig. 2b was used. Model-derived terms include pyruvate cycling, anaplerosis through pyruvate carboxylase, non-pyruvate carboxylase anaplerosis, and the percent anaplerosis

scopic studies failed to show spectra or mention changes in aspartate resonances [7, 10].

The decrease of enriched aspartate is probably not the result of a reduction in the activity of aspartate transaminase and the conversion of labelled oxaloacetate to labelled aspartate under stimulatory conditions. This would require a specific reduction of aspartate transaminase activity under conditions where other transaminase enzyme activities increase (e.g. the observed increase of alanine with glucose stimulation). Alternatively, the aspartate transaminase activity may be increased, but in the opposite direction (i.e. conversion of aspartate to oxaloacetate, with a commensurate change of α -KG to glutamate). Support for an enhanced aspartate-to-oxaloacetate conversion comes from an NMR-spectroscopic study of labelled aspartate metabolism in cultured astrocytes [36]. They observed that labelled aspartate gave rise to labelled

pyruvate, alanine and lactate, and therefore must have initially labelled oxaloacetate through aspartate transaminase activity. They further found that glycolysis influenced aspartate metabolism; i.e. inhibition of glycolysis reduced aspartate metabolism [36]. We speculate that the converse is also true: enhanced glycolysis, caused by increased glucose levels or supplements found in complete media, increases aspartate metabolism. Further evidence of the association between aspartate and insulin release involves aspartate aminotransferase. When an inhibitor of aspartate aminotransferase (aminooxyacetate) is introduced, insulin secretion stimulated by succinate esters is not affected. However, this compound is known to completely inhibit GSIS [37]. In other words, when aspartate levels are not allowed to drop, insulin release is inhibited.

We propose the following metabolic scheme, which is consistent with our data and known biochemical pathways (Fig. 8). First, aspartate and α -KG are converted to glutamate and oxaloacetate, respectively, by aspartate aminotransferase activity (enzyme 1). The glutamate can then convert back to α -KG through glutamate dehydrogenase (enzyme 2), along with NAD(P)H and NH_4^+ . The net result is that aspartate is lost as an anaplerotic substrate to the TCA cycle, no net change occurs in the glutamate or α -KG levels, and the electron carrier NAD(P)H is produced. All steps in this mechanism can proceed in the mitochondrial matrix and would not require energy-depleting shuttles. The succinate hypothesis of insulin secretion states that secretagogues form NAD(P)H and mevalonate [8]. Our scheme introduces a mechanism by which NAD(P)H may be generated. Of course, the presence of a pathway does not indicate that it is the sole path, or even a dominant path of metabolism.

Fig. 6 The relationship between model-derived non-pyruvate carboxylase anaplerotic entrance to insulin secretion for the R7T1 and INS-1 (832/13) insulinoma cells, along with the value from the pig islet preparation. INS1 (▲); R7T1 (○); pig islet (⊗)

Table 2 Pearson product-moment correlations (r^2) between the change in insulin secretion as a result of stimulation and the change in *tea*CALC model-derived parameters associated with a given stimulation

	Correlations between change in insulin secretion rate and change in:			
	Pyruvate cycling	PC anaplerosis	Non-PC anaplerosis	Percent anaplerosis
β TC3	0.382	0.452	0.879	0.349
β TC-tet	0.982	0.958	0.413	0.752
R7T1	0.814	0.729	0.722	0.865
INS-1	0.560*	0.619*	0.829	0.198

The stimulating conditions considered were: (1) PBS, 3 vs 15 mmol/l glucose; (2) complete media, 3 vs 15 mmol/l glucose; (3) 3 mmol/l glucose, PBS vs complete media; (4) 15 mmol/l glucose, PBS vs complete media
*Negative correlation

However, the argument outlined above suggests that aspartate metabolism plays an important role in insulin secretion and will be pursued in future studies. There are two important caveats to this study. First, insulin data and model-derived indices are derived from different timeframes. Insulin release is determined during the first 20 min of exposure to reduce the contribution of constitutive insulin release and obtain a measure of stimulated insulin release, but the isotopomer analysis is performed on data acquired over a 4-h period to ensure steady-state dynamics. The ^{13}C analysis is therefore not necessarily reflective of the instantaneous or even early

Fig. 8 Mechanism for the role of aspartate as a source of non-pyruvate carboxylase anaplerosis. Aspartate (*Asp*) and α -KG are converted to glutamate (*Glut*) and oxaloacetate by aspartate aminotransferase activity (1). α -KG is regenerated from *Glut* through glutamate dehydrogenase (2), which generates NAD(P)H and an amine group. Via this mechanism, aspartate levels are reduced, glutamate and α -KG levels are maintained, and oxaloacetate levels increase and enter the TCA cycle as an anaplerotic agent. Potential pathways from malate and oxaloacetate to pyruvate are included, although conversion from oxaloacetate has been shown to be negligible in islets

time-period of release, but rather represents the bioenergetic state over the long-term insulin release phase. The

Fig. 7 The relationship between the change in insulin secretion as a result of stimulation and the change in non-pyruvate carboxylase anaplerosis, as described in Table 2. These are depicted for the following cell lines INS-1 (832/13) (a), R7T1 (b), β TC-3 (c), and β TC-tet (d). Pearson product-moment correlation values (r^2): 0.829 (a); 0.722 (b); 0.879 (c); 0.413 (d)

second caveat is that we observed a two- to three-fold increase in glucose-stimulated insulin secretion with the INS-1 (832/13) cell line, much less than that reported for this particular clone [10]. This may stem from measuring insulin released from cells, where previous studies observed the total insulin (both intra- and extracellular). Additionally, our INS-1 (832/13) cell line may no longer be identical to the original clone because of genetic drift. Regardless, the correlation between insulin secretion (as defined here) and the non-PC anaplerotic input to the TCA cycle is strong.

To summarise, isotopomer analysis indicates that change in non-PC anaplerotic entrance to the TCA cycle correlates with stimulated insulin release for all insulinoma cell lines studied; a correlation more remarkable in that it holds despite the wide variety of media compositions. Contrary to the fate of other labelled compounds, enrichment of the amino acid aspartate decreases under certain stimulatory conditions (e.g. induction with glucose). Based on these results, we hypothesise that the source of the non-PC anaplerotic entrance to the TCA cycle is aspartate, and propose a scheme by which our spectroscopic observations, modelling results, and insulin secretion data are reconciled. Analysis of spectroscopic data obtained from extracts of native porcine islets corroborates the correlation between insulin release and non-PC anaplerosis.

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