Mitochondria-derived glutamate at the interplay between branched-chain amino acid and glucose-induced insulin secretion

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Abstract In pancreatic β-cells, glutamate has been proposed to mediate insulin secretion as a glucose-derived factor, although it is also considered for its sole catabolic function. Hence, changes in cellular glutamate levels are a matter of debate. Here, we investigated the effects of glucose and the glutamate precursor glutamine on kinetics of glutamate levels together with insulin secretion in INS-1E β-cells. Preincubation at low (1 mM) glucose resulted in reduced cellular glutamate levels, which were doubled by exposure to glutamine. In glutamine-deprived cells, 5 mM glucose restored glutamate concentrations. Incubation at 15 mM glucose increased cellular glutamate, along with stimulation of insulin secretion, following both glutamine-free and glutamine-rich preincubations. Nuclear magnetic resonance (NMR) spectroscopy of INS-1E cells exposed to 15 mM D-[1-^{13}C]glucose revealed glutamate as the major glucose metabolic product. Branched-chain amino acids, such as leucine, reduced cellular glutamate levels at low and intermediate glucose. This study demonstrates that glucose stimulates glutamate generation, whereas branched-chain amino acids promote competitive glutamate expenditure.

Key words: Insulin secretion; Glutamate dehydrogenase; Glutamine; Mitochondrion; Nuclear magnetic resonance

1. Introduction

Glutamate dehydrogenase (GDH; EC 1.4.1.3) is an ubiquitously expressed mitochondrial enzyme, playing a major role in glutamate turnover [1]. GDH catalyses reductive amination from α-ketoglutarate to glutamate as well as the reverse oxidative deamination of glutamate to α-ketoglutarate [2]. Therefore, depending on the flux direction, GDH could either provide an assimilatory pathway for ammonium or play a catabolic role for amino acids [3].

In pancreatic β-cells, some authors favour the concept that the anaplerotic glutamate to α-ketoglutarate flux dominates. Consequently, amino acid-derived glutamate would stimulate insulin secretion through the entry of α-ketoglutarate into the tricarboxylic acid (TCA) cycle, leading to the generation of mitochondrially-derived adenosine triphosphate (ATP). A recent hypothesis suggests that glutamate, generated by cataplerosis from glucose-derived α-ketoglutarate, acts downstream of mitochondrial function, participating in the coupling of glucose metabolism to insulin secretion [4,5]. Such a model implies that cellular glutamate concentrations are increased upon glucose stimulation, which is the source of major debates [6–8]. Several studies have observed an increase in cellular glutamate contents during glucose stimulation in islets from rat [7,9,10], mouse [7] and human [4], as well as in β-cell lines [4,11,12]. Moreover, INS-1E cells and rat islets overexpressing the glutamate decarboxylating enzyme GAD65 exhibit decreased glutamate levels and altered glucose-induced insulin secretion [11].

However, other studies reported the absence of glutamate level changes upon glucose stimulation in islets isolated from ob/ob mice [13,14], or rats [6]. The controversy is also nourished by demonstration that GDH activators, such as 2-aminobicyclo-[2,2,1]heptane-2-carboxylic acid (BCH) and branched-chain amino acids, promote glutamine/glutamate oxidation with concomitant stimulation of insulin secretion [15,16].

Given the importance of the glutamate pathway in glucose-induced insulin secretion [17], the present study reinvestigates the role of GDH and glutamate levels in INS-1E β-cells. First, we describe the effects of glutamine during preincubation periods on the subsequent glucose-stimulated insulin secretion and cellular glutamate levels. Second, we used ^{13}C nuclear magnetic resonance (NMR) to determine carbon fluxes derived from labelled glucose. Third, we report the time-dependent dynamic changes in glutamate contents and insulin release in cells incubated without glutamine but under different conditions of glucose and branched-chain amino acids, both interfering with GDH.

2. Materials and methods

2.1. Cell culture

INS-1E cells cloned from INS-1 were cultured in RPMI-1640 medium supplemented with 5% foetal calf serum, 10 mM HEPES, 1 mM pyruvate, 50 μM β-mercaptoethanol and antibiotics as described [11]. INS-1E cells from passages 52–76 were used in this study.

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Abbreviations: BCH, 2-aminobicyclo-[2,2,1]heptane-2-carboxylic acid; GDH, glutamate dehydrogenase; NMR, nuclear magnetic resonance; KRB, Krebs–Ringer bicarbonate buffer; PBS, phosphate-buffered saline

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2.2. Metabolism of [1-^{13}C]glucose followed by NMR spectroscopy of cell extracts

When confluent, INS-1E cells grown in T175 flasks were washed with phosphate-buffered saline (PBS) and preincubated at 37°C for 2 h in RPMI-1640 with no glucose or glutamine. The cells were then washed with PBS and incubated for 30 min in Krebs–Ringer bicarbonate buffer (KRB) with 2.5 mM D-[1-^{13}C]glucose (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 10 mM $NaHCO_3$, 5 g/l bovine serum albumin, pH 7.4). This was followed by an incubation period of 1 h in KRB supplemented with 15 mM [1-^{13}C]glucose. The cells were then washed with PBS, perchloric acid (6%) was added, and the cells were scraped off the culture flasks. The extracts of five culture flasks were pooled and centrifuged at 1500 rpm for 5 min. As previously described [12], the resulting supernatant was neutralised with KOH (5 M and 0.1 M solutions) and the pellets were soaked overnight in 0.1 M NaOH before protein measurements. The neutralised supernatant was centrifuged (1500 rpm, 5 min) and the supernatant was treated with Chelex-100 resin and then lyophilised. Each experiment was carried out on at least two independent cultures.

The lyophilised cell extracts were dissolved in 3 ml potassium phosphate buffer (100 mM, pH 7.0) and then centrifuged. The supernatant was carefully removed and 10% D_2O was added and the pH checked and adjusted when necessary with 0.1 M NaOH and 0.1 M HCl. An insert containing 5% v/v dioxane in water was used as an external signal intensity reference for quantification of the NMR spectra. A solution of L-alanine, L-glutamate, lactate and D-glucose, each at a concentration of 100 mM, was prepared and used to quantitate concentrations of metabolites in the ^{13}C spectra. Proton-decoupled ^{13}C spectra were acquired on a Bruker DRX 500 spectrometer using a 10 mm broad band probe. Typically spectra were acquired with 32 K data points using 9.4 μs pulses (90° pulse angle), 260 ppm spectral width, 2 s relaxation delay and 12 000 scans. Spectra were recorded at 25°C. Chemical shifts in aqueous media were referenced to tetramethylsilane at 0 ppm as previously described [18]. Exponential multiplications with 2 Hz line broadening were performed using WINNMR software. The assignments of the intermediate metabolites were made by comparison with chemical shift tables in the literature [19]. The amount of ^{13}C in each resonance was evaluated by integration of the extract peaks and the corresponding peaks in the standard sample relative to the dioxane signal. Corrections for the natural abundance signal were made. The absolute enrichments of the glutamate peaks were related to the glutamate concentration in the extracts, determined by enzymatic methods, to give the specific enrichments [20].

2.3. Concomitant static insulin release and cellular glutamate content determination

INS-1E cells were cultured for 4–6 days with complete RPMI-1640 medium in six-well plates coated with polyornithine. KRB HEPES buffer (KRBH) of the following composition was used (in mM): 135 NaCl, 3.6 KCl, 10 HEPES, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, pH 7.4, glucose as indicated, bovine serum albumin 0.1% as carrier. Cells were preincubated for 2 h in 1 mM glucose RPMI-1640 at 37°C either with 2 mM of glutamine or without glutamine, washed twice with glucose-free KRBH and then incubated for 60 min in KRBH plus the indicated conditions. Glucose concentrations ranged from low (1 mM) to high (15 mM), according to the sigmoidal dose response observed in these cells [4]. Incubation was stopped by putting the plates on ice, the supernatant was collected for insulin secretion, and 1 ml of lysis buffer (20 mM Tris–HCl pH 8.0, 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CTDA), 0.2% Tween-20) was added to cells.

For glutamate determination, cells were collected and rapidly sonicated. Following protein determination with a Bradford's assay, glutamate levels were measured in cell lysates by monitoring reduced nicotinamide adenine dinucleotide (NADH) fluorescence augmentation during oxidation of glutamate in the cell extracts in the presence of an excess of GDH (Boehringer Mannheim, dilution 1/100 000). NADH fluorescence, excited at 340 nm, was measured at 460 nm in an LS-50B fluorimeter (Perkin Elmer) before and after 30 min incubation at room temperature in 1.5 ml buffer (50 mM Tris–HCl pH 9.5, 2.6 mM EDTA, 1.4 mM NAD, 1 mM adenosine diphosphate (ADP)). Basal NADH was subtracted and glutamate levels were evaluated according to internal standards. Glutamate levels measured in this study appear to be higher than in previous reports from the same laboratory [4,11]. This could be explained by a recent re-evaluation of

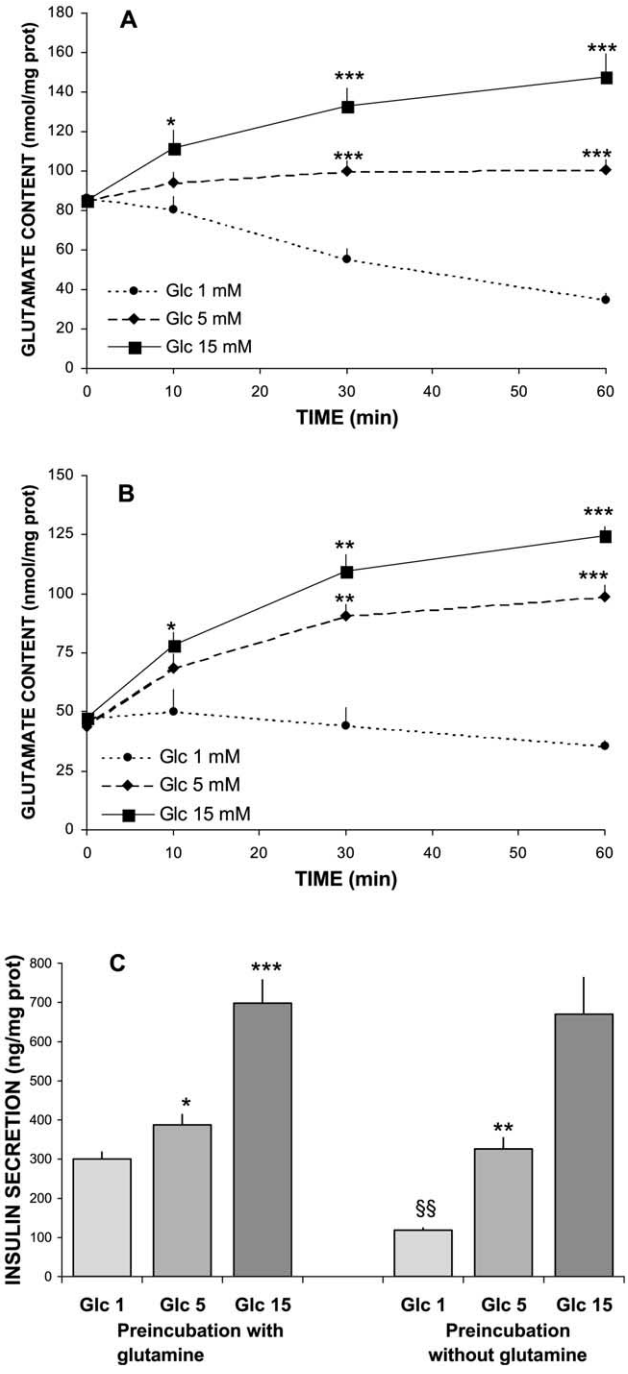

Fig. 1. Modulation of intracellular glutamate levels by glucose in INS-1E cells. A: INS-1E cells were preincubated 2 h in the presence of glutamine in order to maintain the pool of glutamine/glutamate, and then incubated for 0, 10, 30 and 60 min in the absence of glutamine at different glucose concentrations. B: INS-1E cells were preincubated 2 h without glutamine and then incubated for 0, 10, 30 and 60 min in the continuous absence of glutamine at different glucose concentrations. C: Insulin secretion in INS-1E cells preincubated 2 h in either the presence or the absence of glutamine and then incubated 60 min without glutamine at different glucose concentrations. Data are the means+S.E.M. of three to seven individual experiments done in duplicate. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus glucose 1 mM; $^{\S\S}P<0.01$ versus corresponding groups of the glutamine-plus preincubation series.

our internal standards and controls, although the relative values are not modified. Insulin secretion was determined by radioimmunoassay using rat insulin as standard.

2.4. GDH activity

The activity of purified bovine GDH was measured in the directions of glutamate deamination as well as α-ketoglutarate amination. In the glutamate deamination direction, the reaction mixture contained 50 mM Tris–HCl pH 9.5, 2.6 mM EDTA, 1.4 mM NAD, 5 mM L-glutamate, and test compounds as indicated. GDH activity in the direction of α-ketoglutarate amination was performed in 50 mM Tris–HCl pH 8.0, 50 mM NH_4Cl, 2.6 mM EDTA, 0.15 mM NADH, 1 mM α-ketoglutarate, and substances as indicated. Following GDH addition (0.2 μg/ml), NADH fluorescence was monitored (excitation at 340 nm and emission at 460 nm) in 1.5 ml cuvette using an LS-50B fluorimeter. Enzymatic activity was then evaluated from the slope of fluorescence according to internal standards.

3. Results

First, the effects of glutamine preincubation on subsequent glucose-stimulated glutamate generation in INS-1E cells were investigated. Cells were preincubated for 2 h in 1 mM glucose RPMI-1640 medium with (Fig. 1A) or without (Fig. 1B) 2 mM glutamine. INS-1E cells were subsequently incubated for 0, 10, 30 or 60 min in a glutamine-free KRBH buffer containing various glucose concentrations; i.e. low (sub-physiological, 1 mM), intermediate (physiological, 5 mM), and high (supra-physiological, 15 mM).

Cells preincubated at low glucose in the presence of 2 mM glutamine contained glutamate levels constantly in the range of 85–90 nmol/mg protein. Further incubation with 1 mM glucose KRBH resulted in a progressive decrease in cellular glutamate levels (−60% after 60 min, $P < 0.01$, Fig. 1A) towards values similar to those obtained in glutamine-depleted cells. Under the same glutamine-rich preincubation conditions, 5 mM glucose KRBH slightly increased glutamate content (+18%, $P < 0.05$). Finally, 15 mM glucose incubation resulted in a progressive and sustained elevation of glutamate levels (+78% after 1 h, $P < 0.001$).

Cells preincubated in glutamine-free low glucose medium contained about half of glutamate compared to cells preincubated in glutamine-rich medium. Subsequent incubation in glutamine-free low glucose (1 mM) conditions maintained glutamate contents at low levels, whereas intermediate (5 mM) and high (15 mM) glucose concentrations markedly increased the initial basal levels of intracellular glutamate (+125% and +163% respectively after 1 h incubation). Interestingly, 5 mM glucose allowed to re-establish similar levels of glutamate obtained in glutamine-rich preincubated INS-1E cells, whereas 15 mM glucose markedly increased glutamate levels. Therefore, depletion of cellular glutamate contents following glutamine-free preincubation could be antagonised by elevation of glucose from 1 to approximately 5 mM.

As shown in Fig. 1C, both in the presence and absence of glutamine during preincubation periods, insulin secretion levels were uniformly glucose dose dependent. Insulin release at low glucose was elevated in the presence of glutamine, although the secretory responses at intermediate or high glucose were not significantly affected by the preincubation conditions.

Second, we performed NMR spectroscopy on [1-^{13}C]-glucose-labelled INS-1E cells in order to determine the metabolic fate of glucose. The ^{1}H-decoupled ^{13}C-NMR spectra of cellular extracts obtained after 1 h incubation of INS-1E cells with 15 mM [1-^{13}C]glucose showed that glucose metabolism produced, apart from CO_2, almost exclusively glutamate (Fig. 2). Glutamate appeared labelled at positions C2 (56.0 ppm), C3 (34.7 ppm) and C4 (28.1 ppm) indicating that pyruvate entered the TCA cycle via both pyruvate dehydrogenase- and pyruvate carboxylase-mediated pathways. The percentage ^{13}C enrichments for glutamate were found to be the following: C2 (25.7 ± 3.1%), C3 (21.5 ± 1.1%) and C4 (46.7 ± 2.9%).

Third, time-dependent effects of leucine on INS-1E intracellular glutamate contents were measured. INS-1E cells were preincubated for 2 h in 1 mM glucose plus 2 mM glutamine medium, then incubated in a glutamine-free KRBH containing different glucose concentrations in the presence or absence of 5 mM leucine, serving as nutrient as well as potent GDH activator [17]. In the presence of 1 mM glucose (Fig. 3A), leucine rapidly decreased the glutamate content towards a near total depletion after 1 h (−91%, $P < 0.001$). In the presence of 5 mM glucose (Fig. 3B), leucine induced a progressive decrease in glutamate levels, although at 15 mM glucose (Fig. 3C) glutamate levels were maintained at the elevated values obtained during the preincubation period. Insulin secretion was stimulated both by glucose and leucine (Fig. 3D). Therefore, in INS-1E cells incubated in glutamine-free buffer, 5 mM leucine promoted glutamate consumption and disrupted equilibrium obtained with 5 mM glucose alone. Noteworthy, when expressed in percentage, the decreasing effect of leucine is more important as the glucose concentration is low.

Next, we tested the effects of different branched-chain amino acids on cellular glutamate levels and insulin release in the presence of different glucose concentrations. INS-1E cells

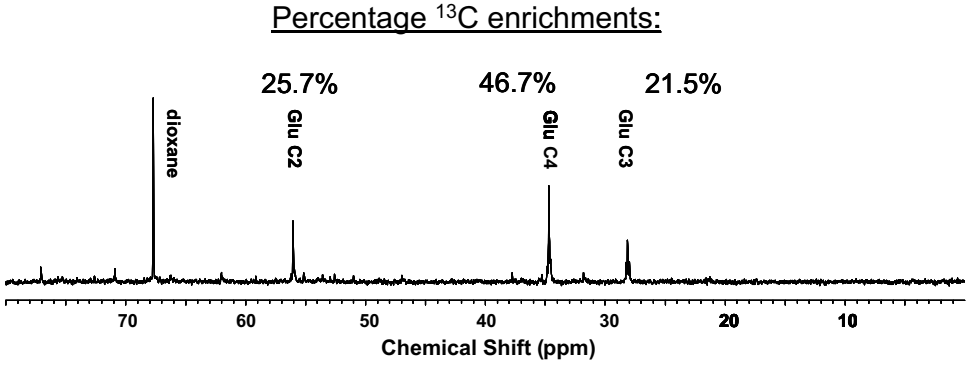

Fig. 2. Metabolism of glucose followed by NMR spectroscopy of cell extracts. Proton-decoupled ^{13}C-NMR spectrum of extracts from INS-1E cells obtained after incubation for 1 h with 15 mM glucose.

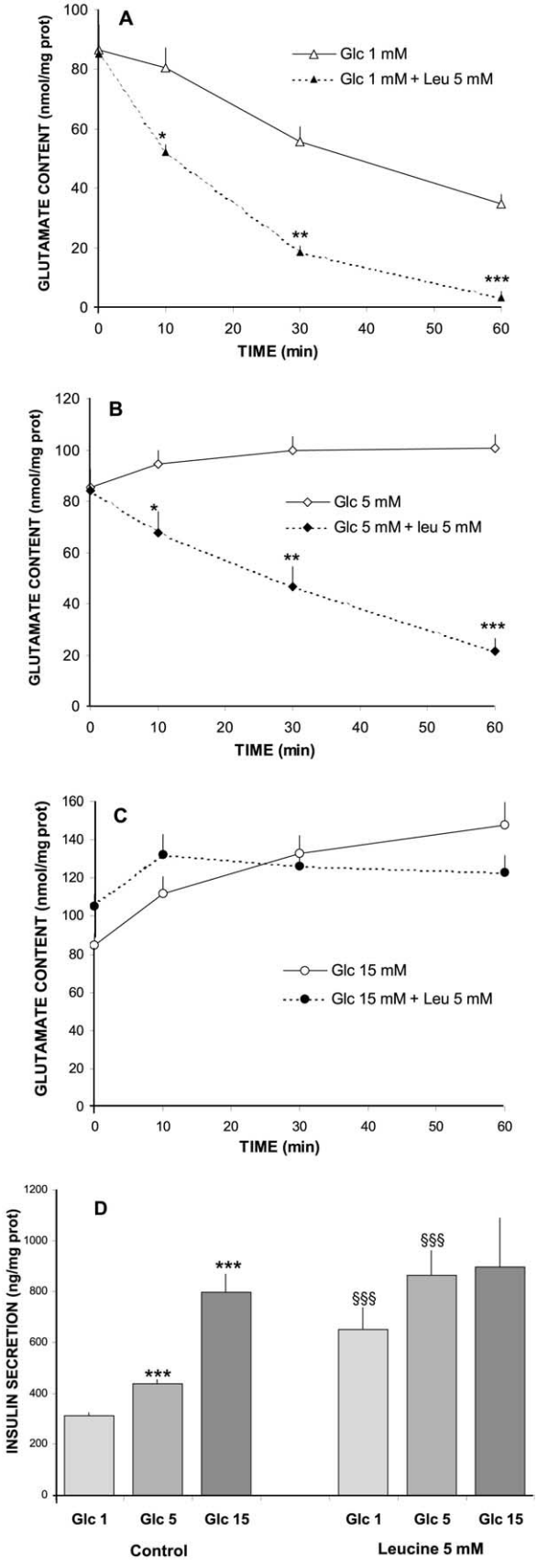

Fig. 3. Modulation of intracellular glutamate levels by leucine in INS-1E cells. A–C: INS-1E cells were preincubated 2 h in the presence of glutamine and then incubated for 0, 10, 30 and 60 min without glutamine at 1 mM (A), 5 mM (B), 15 mM (C) glucose concentrations in the presence (black symbols) or absence (white symbols) of 5 mM leucine. Data in A, B and C are the means +S.E.M. of three to seven individual experiments done in duplicate. D: Insulin secretion in INS-1E cells preincubated 2 h in the presence of glutamine, then incubated for 60 min without glutamine at 1, 5, and 15 mM glucose in the presence (leucine 5 mM) or absence (control) of leucine. Data are the means+S.E.M. of four to nine individual experiments. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus corresponding controls without leucine. C: $^{***}P<0.001$ versus glucose 1 mM; $^{\S\S\S}P<0.001$ versus corresponding controls without leucine.
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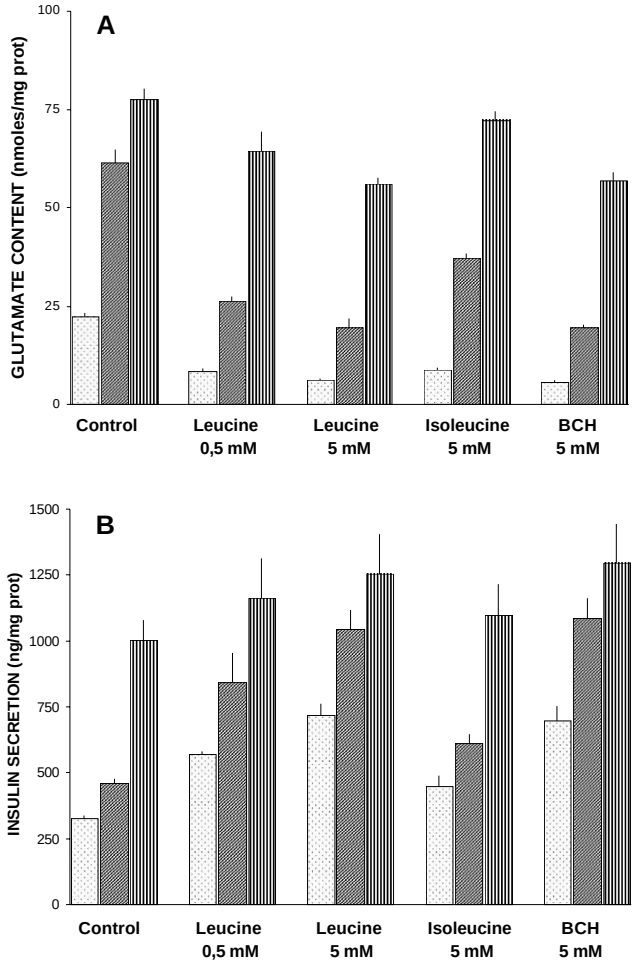

Fig. 4. Modulation of intracellular glutamate levels and insulin release by branched-chain amino acids in INS-1E. A, B; INS-1E cells were preincubated 2 h in the presence of glutamine, then incubated for 60 min without glutamine at 1 mM (dotted bars), 5 mM (diagonally stripped bars) or 15 mM glucose concentrations (vertically stripped bars) in the presence of leucine, isoleucine or BCH as indicated. Intracellular glutamate levels are shown in A and insulin release in B. Data are means+S.E.M. of three to eight individual observations done in duplicate. At 1 mM and 5 mM glucose: $P<0.001$ for all values with branched-chain amino acids versus control. At 15 mM glucose, $P<0.01$ for all values with branched-chain amino acids versus control in A except for leucine 0.5 mM ($P<0.05$) and isoleucine (not significant (NS)), whereas in B all values are NS versus control.

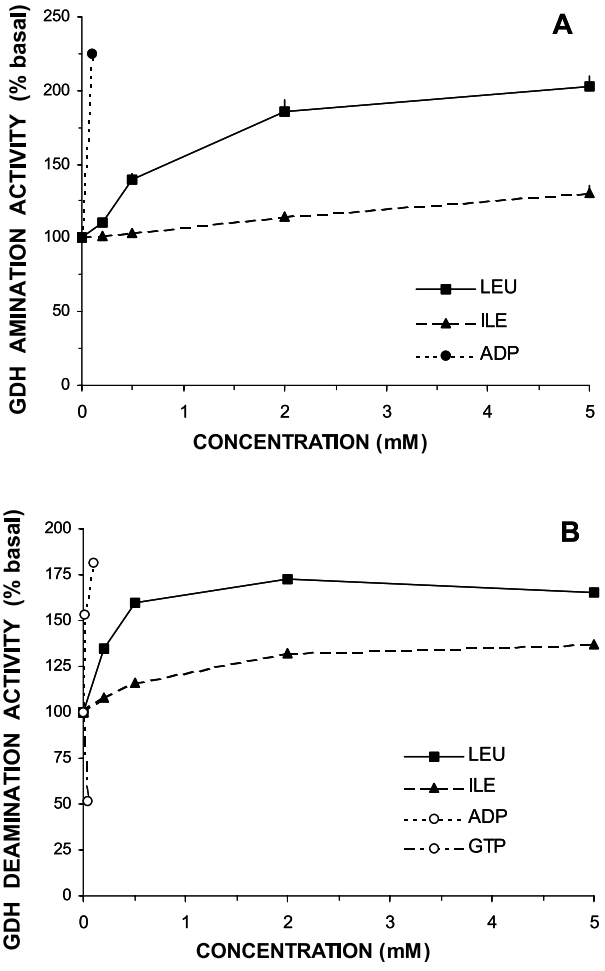

Fig. 5. Effects of branched-chain amino acids and nucleotides on in vitro GDH activity. Bovine liver GDH activity was measured in the direction of reductive amination (A) or in the direction of oxidative deamination (B). α-Ketoglutarate (1 mM) and L-glutamate (5 mM) were used as substrates in A and B respectively. Data are means+S.E.M. of three to five individual observations.

were preincubated at low glucose 2 mM glutamine medium and subsequently incubated for 60 min in glutamine-free KRBH. At 1 and 5 mM glucose, leucine dose dependently decreased cellular glutamate contents and stimulated insulin release (Fig. 4). BCH (5 mM), the non-metabolisable analogue of leucine, mimicked the effects of 5 mM leucine on glutamate levels and insulin release, whereas 5 mM isoleucine exhibited only moderate effects compared to those of leucine.

At high glucose (15 mM), leucine slightly decreased glutamate levels, still in a dose-dependent manner, although to lesser extents than those observed at lower glucose concentrations, and leucine stimulated insulin release. Isoleucine had no significant effects on glutamate levels and insulin secretion under elevated glucose, whereas 5 mM BCH was still nearly as effective as 5 mM leucine.

At fixed concentrations of branched-chain amino acids, glucose always increased glutamate levels concomitantly with stimulation of insulin release, whereas at fixed glucose concentrations, amino acids decreased cellular levels of glutamate.

Finally, in vitro measurements of GDH activity in the direction of α-ketoglutarate amination as well as in the direction of glutamate deamination showed that the flux through GDH is similarly controlled in both directions (Fig. 5). As expected [21,22], ADP increased, whereas guanosine triphosphate (GTP) decreased GDH activity. GDH was activated in both directions by leucine, as low as micromolar concentrations, however isoleucine was a weak activator. BCH effects were comparable to those of leucine, while glucose did not modify GDH activity (data not shown).

4. Discussion

Effects of glucose on glutamate levels in β-cells are a matter of debate. In the present study, we monitored cellular glutamate levels in INS-1E β-cells incubated under different culture conditions. In particular, we tested glucose concentrations ranging from sub- to supra-physiological levels (1 to 15 mM respectively). The results demonstrate that glutamate levels are highly modulated, either increased by glucose and glutamine, or decreased by branched-chain amino acids. Similar glutamate levels can be obtained either in the presence of 5 mM glucose without glutamine or by exposure to glutamine (2 mM) at low glucose (1 mM).

Leucine decreased glutamate levels and its non-metabolisable analogue BCH displayed similar effects. This strongly suggests that most of leucine effects are associated with its activation of GDH rather than its direct metabolism as nutrient. It also shows the implication of GDH in the branched-chain amino acid-mediated lowering effects of glutamate levels. Leucine depleted the glutamate pool at low glucose, but established a steady state at 15 mM glucose when glucose and leucine effects on GDH antagonise [23]. These data are in agreement with previous reports showing that glucose regulates glutaminolysis, glutamate fate and insulin secretion through inhibition of GDH [23,24].

The present study reveals that glucose and branched-chain amino acids interact on β-cell glutamate content through their respective opposite effects on GDH. Whereas branched-chain amino acids clearly reduced glutamate levels at low and intermediate glucose concentrations, elevation of glucose was accompanied by an increase in cellular glutamate pool in all conditions. Therefore, GDH could act in anaplerotic and cataplerotic directions, i.e. stimulation of glutaminolysis to form glutamate and consequently α-ketoglutarate, as well as generation of glutamate from α-ketoglutarate. The latter model is supported by NMR spectroscopy showing that glutamate, upon glucose stimulation, is the major metabolic product from labelled glucose in INS-1E cells (Fig. 2), which is in accordance with results obtained in BRIN-BD11 cells [12]. Another NMR study performed in INS-1-832/13 cells also reported enrichment of glutamate with glucose carbons, although the total glutamate concentration was unchanged [25].

Taken together, these results suggest that glutamate might have at least two effects in β-cells. At low glucose, GDH favours the flux of oxidative deamination of glutamate to α-ketoglutarate, glutamate being preferentially used as an anaplerotic fuel for the β-cell due to its position at the crossroad of amino acid catabolism. This would ensure minimal requirement for TCA cycle activity and ATP generation. At elevated glucose, GDH and/or transamination reactions would favour glutamate generation that could then play a messenger role outside mitochondria in the amplification pathway of glucose-induced insulin secretion [4,26].

Recent reports showed either an increase [7] or no change [6] in islet glutamate contents upon glucose stimulation. The present data demonstrate that both effects could be observed depending on culture conditions imposed to the cells. Therefore, the flux direction depends on the nutritional level of the cell, i.e. the concomitant concentrations of glucose, glutamine, and branched-chain amino acids. In vitro, drastic and specific experimental conditions restrict the observation to one unique GDH flux direction. In vivo, β-cell nutritional environment does not vary so drastically and glutamate pool could be near equilibrium, changes in substrate levels inducing only subtle changes of GDH flux and glutamate pool. Investigating this complex equilibrium will contribute to our understanding of β-cell function.

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