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Studies with leucine, β-hydroxybutyrate and ATP citrate lyase-deficient beta cells support the acetoacetate pathway of insulin secretion

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ABSTRACT

We hypothesized that contrasting leucine with its non-metabolizable analog 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) might provide new information about metabolic pathways involved in insulin secretion. Both compounds stimulate insulin secretion by allosterically activating glutamate dehydrogenase, which enhances glutamate metabolism. However, we found that leucine was a stronger secretagogue in rat pancreatic islets and INS-1 cells. This suggested that leucine's metabolism contributed to its insulinotropism. Indeed, we found that leucine increased acetoacetate and was metabolized to CO_2 in pancreatic islets and increased short chain acyl-CoAs (SC-CoAs) in INS-1 cells. We then used the leucine–BCH difference to study the hypothesis that acyl groups derived from secretagogue carbon can be transferred as acetoacetate, in addition to citrate, from mitochondria to the cytosol where they can be converted to SC-CoAs. Since BCH cannot form sufficient acetoacetate from glutamate, transport of any glutamate-derived acyl groups to the cytosol in BCH-stimulated cells must proceed mainly via citrate. In ATP citrate lyase-deficient INS-1 cells, which are unable to convert citrate into cytosolic acetyl-CoA, insulin release by BCH was decreased and adding β-hydroxybutyrate or α-ketoisocaproate, which increases mitochondrial acetoacetate, normalized BCH-induced insulin release. This strengthens the concept that acetoacetate-transferred acyl carbon can be converted to cytosolic SC-CoAs to stimulate insulin secretion.

1. Introduction

Next to glucose, leucine is the most potent physiological insulin secretagogue and it is the only amino acid that supports insulin secretion in the absence of glucose or another amino acid. One of the mechanisms by which leucine stimulates insulin secretion is by allosterically activating glutamate dehydrogenase [1–7]. This enhances metabolism of glutamate which can act as a fuel to supply energy and metabolites for anaplerosis which is also believed to be important for insulin secretion [8]. The evidence for leucine stimulating insulin release via activation of glutamate dehydrogenase is quite strong and comes in part from studies with 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), a non-metabolizable analog of leucine. Like leucine, BCH has been shown to both activate glutamate dehydrogenase and stimulate insulin release [1–3]. In addition, in *in vitro* experiments, when the insulin release incubation medium was supplemented with glutamine, which is a source of glutamate (By itself glutamine cannot stimulate insulin release [4,5].), both leucine-induced and BCH-induced insulin releases were potentiated [1–5,9]. Proof that activation of glutamate dehydrogenase can stimulate insulin secretion in vivo in humans is known from the hypoglycemic disorder that is characterized by dietary leucine sensitivity combined with increased blood insulin and ammonia. The disorder is associated with amino acid alterations in glutamate dehydrogenase in the region of the enzyme where GTP interacts and normally inhibits the enzyme. These mutations cause the enzyme to be constitutively active and the beta cell inappropriately metabolizing glutamate and secreting insulin and larger body tissues converting glutamate to ammonia [10].

In our studies of leucine and BCH in pancreatic islets and clonal insulin cell lines, we noticed that leucine was a slightly stronger stimulant of insulin release. Previous studies showed that leucine can be metabolized to CO_2 [11–14] and can increase acetoacetate in pancreatic islets [15] and multiple short chain acyl-CoAs in INS-1 cells [16], but were not meant to directly answer the question of whether leucine's metabolism contributes to its insulinotropism. No one has previously reported that leucine is a stronger insulin stimulant than BCH. We thought that the idea of leucine's metabolism to acetoacetate might converge with a concept about the role of acetoacetate as a carrier of acyl groups from mitochondria to cytosol in the beta cell [16]. We hypothesized that discerning the differences between leucine and BCH might reveal more information about the metabolic pathways involved in insulin secretion. In the present project, we studied the response of pancreatic beta cells to incubation with leucine and BCH in more detail. We compared the effects of BCH and leucine on

Abbreviations: BCH, 2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid, KIC, α-ketoisocaproate

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the activation of glutamate dehydrogenase enzyme activity, on insulin release and on the levels of acetoacetate, α-ketoglutarate and short chain acyl-CoA levels in pancreatic islets and/or the INS-1 832/13 cell line. We also compared leucine's and BCH's ability to stimulate insulin release in an INS-1 832/13 cell line we made deficient in ATP citrate lyase so that the cells were unable to convert citrate exported from the mitochondria into acetyl-CoA in the cytosol. The results support the idea that in addition to leucine enhancing flux through glutamate dehydrogenase, leucine's metabolism to acetoacetate and acetyl-CoA contributes to its insulinotropism. Besides being informative about leucine-induced insulin release, the results strengthen the theory that the normal beta cell can export acyl carbon as acetoacetate, in addition to citrate, from the mitochondria to the cytosol to form short chain acyl-CoAs which may have roles in insulin secretion.

2. Materials and methods

2.1. Materials

[U-^{14}C]leucine and [U-^{14}C]glucose were from Amersham and [U-^{14}C]glutamine was from American Radiolabeled Chemicals. Sprague–Dawley rats were from Harlan Sprague–Dawley (Madison, WI). The INS-1 832/13 cell line was from Chris Newgard [17].

2.2. Insulin release

Insulin release from freshly isolated rat pancreatic islets was studied as previously prescribed [18,19]. INS-1 832/13 cells were cultivated as monolayers in INS-1 medium [16,20] (RPMI 1640 tissue culture medium (the glucose concentration in this medium is 11.1 mM) supplemented with 10% fetal bovine serum, 1 mM pyruvate, 50 μM β-mercaptoethanol and 10 mM HEPES buffer) and penicillin (100 units/ml) and streptomycin (100 μg/ml) in 24-well tissue culture plates. Twenty-two hours before an insulin release experiment was to be performed, the glucose concentration in the medium was reduced to 5 mM. For 2 h before the insulin release study, the cells were maintained in Krebs Ringer bicarbonate buffer containing 15 mM sodium HEPES and 15 mM $NaHCO_3$ buffers, pH 7.3, (with the NaCl concentration adjusted to maintain the osmolarity at 300–310 mM), 0.5% bovine serum albumin and 3 mM glucose [17,21]. The plates were washed twice with phosphate-buffered saline and 1 ml of the modified Krebs Ringer solution containing secretagogue with or without inhibitor, or no secretagogue as a control, was added to each well. After 1 h, samples of incubation medium were collected and analyzed for insulin as previously described [21].

2.3. Fuel secretagogue metabolism

Rat pancreatic islets (100/test tube) maintained for 22 h in RPMI 1640 tissue culture medium modified to contain 5 mM glucose and 10% fetal bovine serum were washed and were incubated in the presence of ^{14}C-labeled fuel secretagogues in 0.1 ml Krebs Ringer bicarbonate buffer, pH 7.3 in an Eppendorf microfuge test tube contained inside a rubber-stoppered scintillation vial for 90 min at 37°. Metabolism was stopped by adding 50 μl trichloroacetic acid to the microfuge test tube, and $^{14}CO_2$ was collected in 0.5 ml tissue solubilizer (Solvable, Packard) added to the scintillation vial outside the microfuge test tube. After 3 h, the microfuge test tube was removed and BioSafe scintillation fluid was added to the scintillation vial and radioactivity was estimated by liquid scintillation spectrometry as previously described [22,23].

Table 1
Stronger insulin release caused by leucine than by BCH in rat pancreatic islets and augmentation of BCH-induced insulin release by β-hydroxybutyrate

Incubation	Insulin release (μU insulin/5 islets/1 h)
No addition	18±2 (33)
Glucose	558±43 (18)
Leucine	104±8 (27)
Leucine+β-hydroxybutyrate	104±14 (6)
BCH	51±4 (33)
BCH+β-hydroxybutyrate	103±8 (6)
Leucine+glutamine	565±54 (11)
BCH+glutamine	544±29 (11)
Glutamine	14±3 (6)
β-Hydroxybutyrate	20±4 (6)
KIC	556±31 (14)

Islets were incubated with no addition and with 16.7 mM glucose as negative and positive controls, respectively, or with 10 mM concentrations of leucine alone, BCH alone, glutamine alone or glutamine plus either leucine or BCH or KIC alone for 1 h. Note that β-hydroxybutyrate (5 mM) increased BCH-induced insulin release to equal that of leucine alone but did not further increase leucine-induced insulin release. Results are expressed as mean±SE insulin released per 5 islets per 1 h with number of replicate incubations in parentheses.

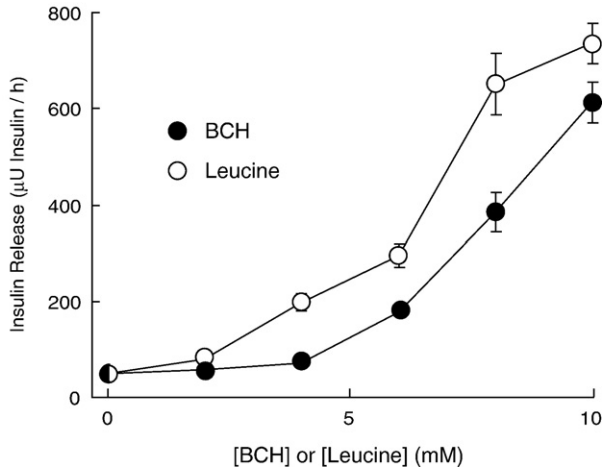

Fig. 1. Leucine is stronger than BCH as a stimulant of insulin release from INS-1 cells. INS-1 832/13 cells (70 μg total cell protein per incubation) were incubated with various concentrations of leucine or BCH for 1 h and insulin release was measured.

2.4. Metabolite measurements

Acetoacetate and α-ketoglutarate were measured as previously described [8,21]. Briefly islets were maintained for 22 h in RPMI 1640 tissue culture medium containing 5 mM glucose and 10% fetal bovine serum and then washed and incubated with insulin secretagogues in Krebs Ringer bicarbonate buffer, pH 7.3. After 30 min with an insulin secretagogue or a control compound, the incubation medium was quickly removed and 50–60 μl of 6% perchloric acid was added. The perchlorate extract was neutralized to ≈pH 7 with about 12 μl of 15% KOH and the metabolites in the extract were assayed on the same day.

2.5. Measurement of short chain acyl-CoAs

INS-1 832/13 cells were maintained as monolayers on 150 mm diameter plates in INS-1 medium [21]. Twenty-four hours before an experiment was to be performed this medium was replaced with fresh medium modified to contain 5 mM glucose. On the day of the experiment, plates were washed twice with 10 ml of phosphate-buffered saline (PBS) and once with 10 ml of Krebs Ringer bicarbonate solution modified to contain 15 mM sodium bicarbonate and 15 mM sodium HEPES buffer, pH 7.3. Secretagogues were added to the plates in 10 ml of the modified Krebs Ringer solution and after 30 min at 37°, this solution was quickly withdrawn and replaced with 2 ml of 1% trifluoroacetic acid in 50% methanol. Plates were quickly chilled to −80°. After 5–10 min at −80° cells were scraped off the plates and acyl-CoA thioesters were measured by LC–MS/MS at the Mass Spectrometry Facility of the University of Wisconsin Biotechnology Center as described previously [16,21].

2.6. Glutamate dehydrogenase

Glutamate dehydrogenase activity was measured in the presence of 5 mM glutamate, 1 mM NAD, 100 μM EDTA, 2 μM rotenone, 1 mM KCN, 0.1% Triton X-100 and 80 mM Tris-hydrochloride buffer, pH 7.5 at 30° [24]. NADH formation was monitored spectrophotometrically at 340 nm. After a background rate was obtained, glutamate was added to obtain a rate attributable to the enzyme.

2.7. Statistical analysis

Statistical significance was confirmed with Student's *t*-test.

3. Results

3.1. Insulin release by leucine and BCH

Leucine was twice as potent as BCH in stimulating of insulin release in pancreatic islets (Table 1) and BCH-induced insulin release was right-shifted compared to leucine-induced insulin release in INS-1 832/13 cells (Fig. 1), even though, prior to the experiments, the INS-1 cells were maintained in standard tissue culture medium which contains a fairly high concentration of glutamine (3 mM), a source of