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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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Summary

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 leucine's metabolism contributed to its insulinotropism. Indeed, we found that leucine increased acetoacetate and was metabolized to CO₂ 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 SCCoAs. 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 increase 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.

Keywords

Leucine and BCH; acetoacetate; short chain acyl-CoAs; insulin secretion; glutamate dehydrogenase; ATP citrate lyase

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

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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 release were potentiated (1–4,9). 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 CO₂ (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 the activation of glutamate dehydrogenase enzyme activity, on insulin release and on the levels of acetoacetate, α -ketoglutarate and short chain acyl-CoAs 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.

Materials and Methods

Materials

[U-¹⁴C]leucine and [U-¹⁴C]glucose were from Amersham and [U-¹⁴C]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).

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 μ M β -mercaptoethanol and 10 mM Hepes buffer) and penicillin (100 units/ml) and streptomycin (100 μ g/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 two hours before the insulin release study, the cells were maintained in Krebs Ringer bicarbonate buffer containing 15 mM sodium Hepes and 15 mM NaHCO₃ 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 hour, samples of incubation medium were collected and analyzed for insulin as previously described (21).

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 ¹⁴C-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 ¹⁴CO₂ 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).

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.

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).

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.

Statistical Analysis

Statistical significance was confirmed with student's t-test.

Results

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 (Figure 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 glutamate. In INS-1 832/13 cells leucine-induced insulin release was always larger than BCH-induced insulin release and ranged from 20% higher (Figure 1) to 100% higher (Figure 1 and Figure 2) in within-experiment direct comparisons. This is despite the fact that BCH was equally or slightly more potent as an activator of glutamate dehydrogenase from the beta cell in our hands (Figure 3) or from the brain in a study by Erecinska (25). An observation incidental to the overall purpose of the study was that, relative to glucose, leucine is a stronger stimulant of insulin release in INS-1 cells than in pancreatic islets (compare Figure 1 and Figure 2 to Table 1).

Oxidation of Leucine and Glutamine

Table 2 shows that leucine was metabolized to $^{14}\text{CO}_2$ by pancreatic islets and that glutamine (10 mM) decreased leucine oxidation by 27% when the concentration of leucine was 2 mM and by 49% when the concentration of leucine was 10 mM consistent with glutamine-derived carbon competing with leucine-derived carbon for metabolism in the citric acid cycle. In the presence of added glutamine (10 mM), which provides a very high concentration of glutamate (7,8), leucine and BCH stimulated insulin release from islets equally well and about as strongly as glucose (Table 1). Both leucine and BCH increased glutamine oxidation, but BCH increased glutamine oxidation more (42% vs 20%, Table 2) consistent with both leucine and BCH activating glutamine metabolism, but in addition, leucine-derived carbon competing with glutamine for oxidation in the citric acid cycle.

Metabolite Formation and Insulin Release

Glutamine by itself did not increase acetoacetate or α -ketoglutarate in pancreatic islets (Table 3). Leucine by itself caused a large increase in acetoacetate in pancreatic islets, whereas BCH by itself caused only a small increase in acetoacetate. BCH alone caused increases in α -ketoglutarate that approximately matched those caused by leucine alone. In the presence of a high concentration of glutamine (10 mM), both leucine and BCH stimulated large increases in acetoacetate and α -ketoglutarate. Of all the compounds tested, KIC caused the largest increase in acetoacetate (Table 3) consistent with the fact that KIC at 10 mM is about as potent an insulin secretagogue in pancreatic islets as glucose (Table 1 and (21)). Another indication that acetoacetate might be important for insulin release is that adding β -hydroxybutyrate, a source of acetoacetate, to pancreatic islets increased BCH-induced insulin release to that of leucine-induced insulin release. Adding β -hydroxybutyrate to leucine did not further augment insulin release (Table 1). In INS-1 832/13 cells leucine stimulated more insulin release than BCH in the presence or absence of added glutamine and adding β -hydroxybutyrate or α -ketoisocaproate as a source of acetoacetate, increased leucine- and BCH-induced insulin release even more (Figure 2). Table 4 shows that leucine alone, but not BCH alone, caused increases in short chain acyl-CoAs in INS-1 cells. In the presence of a high concentration of glutamine, both leucine and BCH caused increases in short chain acyl-CoAs, but the increases in the presence of leucine plus glutamine were slightly larger than in the presence of BCH plus glutamine.

Insulin Release in ATP Citrate Lyase-Deficient Cells

BCH alone, or with added glutamine (2 mM), did not stimulate as much insulin release from the ATP citrate lyase-deficient INS-1 832/13 cells as it stimulated from control INS-1 832/13 cells (Figure 4). However, when either β -hydroxybutyrate, which can supply acetoacetate directly (via reaction 7 shown in Figure 5) or KIC, which also forms acetoacetate (via the HMG-CoA lyase reaction, reaction 4 in Figure 5), were added to the BCH-stimulated ATP citrate lyase deficient cells, insulin release was raised to values equal to those from the control INS-1 832/13 cells (Figure 4). Insulin release by leucine alone or leucine plus glutamine (2 mM) in the ATP citrate lyase cells was equal to those in the control cells and adding β -hydroxybutyrate or α -ketoisocaproate to the ATP citrate lyase deficient cells in the presence of leucine or leucine-plus-glutamine did not further increase insulin release (Figure 4). As expected from previous studies with INS-1 832/13 cells (21,26), by themselves, glutamine, β -hydroxybutyrate and KIC did not stimulate insulin release from the normal or the ATP citrate lyase deficient INS-1 832/13 cells. Glutamine plus either β -hydroxybutyrate or KIC stimulated essentially the same insulin release as either compound alone (Figure 4). In additional experiments with ATP citrate lyase deficient cells (not shown in Figure 4), insulin release in the presence of a high concentration of glutamine (10 mM) along with BCH was decreased compared to that in control cells ($1702 \pm 74 \mu\text{U insulin/ 1 h}$ vs. $2485 \pm 252 \mu\text{U insulin/ 1 h}$, $p < 0.01$) just as in the presence of either no added glutamine or a lower concentration of glutamine (2 mM); and supplying mitochondrial acetoacetate by adding β -hydroxybutyrate or KIC increased the insulin release to near-normal or normal ($2171 \pm 117 \mu\text{U insulin/ 1 h}$ and $2446 \pm 72 \mu\text{U insulin/ 1 h}$, respectively) (means $\pm$ SE of 12–24 replicate incubations).

Discussion

Part of the mechanism by which leucine stimulates insulin secretion is by its allosterically activating glutamate dehydrogenase (Figure 5, reaction 11) which increases metabolism of endogenous glutamate (1–10). This conclusion is supported by experiments in which BCH, a nonmetabolizable analog of leucine, activates glutamate dehydrogenase and stimulates insulin release from pancreatic beta cells (references 1–4 and Table 1 and Figure 1 and Figure 2). However, leucine can be metabolized by islet cells (Table 2 and Table 3 and references 11–15) and the contribution of leucine metabolism to its insulinotropism has not been thoroughly studied. The results of the current study as discussed below suggest two points about insulin secretion. First they indicate that leucine's conversion to acetoacetate and acetyl-CoA and to other short chain acyl-CoAs (Table 4), as well as its oxidation to CO_2 (Table 2), contribute to its ability to stimulate insulin secretion and explain why it is a stronger insulin secretagogue than BCH even though BCH and leucine are equally strong as activators of glutamate dehydrogenase (Figure 3 and reference 25). Second, experiments with agents that supplied acetoacetate to rescue decreased BCH-induced insulin release (Figure 4) in cells made deficient in ATP citrate lyase (Figure 5, reaction 2) strengthen the recent conclusion (16) that the pathways of formation of cytosolic short chain acyl-CoAs in the beta cell involve the transfer of acyl carbon from mitochondria to the cytosol as acetoacetate in addition to citrate.

Pathways of Leucine and Glutamate Metabolism to Short Chain Acyl-CoAs in the Presence of Endogenous Glutamate or a Low Concentration of Exogenously Added Source of Glutamate

In pancreatic islets, adding β -hydroxybutyrate increased BCH-induced insulin release (Table 1) and in INS-1 832/13 cells, β -hydroxybutyrate and α -ketoisocaproate increased both leucine-induced and BCH-induced insulin release (Figure 2). In ATP citrate lyase deficient cells β -hydroxybutyrate and α -ketoisocaproate increased BCH-induced insulin release to a level equal to leucine-induced insulin release (Figure 4). The enhancement of insulin release by a source of acetoacetate in normal rat pancreatic islets or INS-1 832/13 cells, or in the ATP citrate lyase

deficient cells provides information not only on the pathways of leucine- vs. BCH-induced insulin secretion, but also on essential pathways of metabolism-induced insulin secretion in general and is consistent with the following schemes: Glucose-induced insulin release is normal in ATP citrate lyase deficient cells (reference 16 and Figure 4) because glucose-derived carbon can be transferred to the cytosol to form short chain acyl-CoAs via acetoacetate (via reactions 2, 3, 8, 9, 10 and 13 shown in Figure 5) instead of via citrate (via reactions 1, 12, 9, 10 and 13 shown in Figure 5). It is likely that when BCH stimulates insulin release, the pathways for transfer of acyl groups from mitochondria to the cytosol in INS-1 832/13 cells are more dependent on citrate than acetoacetate as a carrier of acyl groups, while during leucine-stimulated insulin release, either acetoacetate or citrate can carry acyl groups from the mitochondria to the cytosol where they may have roles in supporting insulin secretion. Leucine can be metabolized sequentially to KIC and HMG-CoA. HMG-CoA can be converted to acetoacetate plus acetyl-CoA (Figure 5, reaction 4) catalyzed by HMGCoA lyase, which is present in mitochondria of pancreatic islets and INS-1 cells (16). This is consistent with the fact that leucine alone, but not BCH alone, caused increases in acetoacetate (Table 3) and short chain acyl-CoAs (Table 4). The inability of BCH to increase short chain acyl-CoAs is likely because the formation of short chain acyl-CoAs in the presence of BCH begins with a very circuitous pathway of carbon derived from glutamine or glutamate via the activation of glutamate dehydrogenase by BCH to form α -ketoglutarate (Figure 5, reaction 11). This would be followed by the metabolism of α -ketoglutarate to malate via reactions of part of the citric acid cycle, malate exiting the mitochondria and its conversion to pyruvate catalyzed by malic enzyme in the cytosol. Pyruvate can then enter mitochondria where pyruvate dehydrogenase (PDH in Figure 5) can convert it to acetyl-CoA. The acetyl-CoA can be converted to citrate by citrate synthase (Figure 5, reaction 1) and to acetoacetate via acetyl-CoA acyl transferase and succinyl-CoA:3-ketoacid-CoA transferase (reactions 2 and 3 shown in Figure 5). Mitochondrial citrate can also be formed from glutamate-derived α -ketoglutarate via the reverse of the reactions of citric acid cycle with NADP isocitrate dehydrogenase substituting for the NAD-dependent isocitrate dehydrogenase of the cycle (27). The idea that when BCH stimulates insulin release, flux through citrate (Figure 5, reactions 12, 9, 10 and 13) is more necessary than flux through acetoacetate (Figure 5, reactions 8, 9, 10 and 13) for glutamate carbon to be converted to the pool of cytosolic acetyl-CoA and other short chain acyl-CoAs, is supported by the experiments with the ATP citrate lyase-deficient INS-1 832/13 cells. These experiments showed that BCH alone, or with added glutamine, did not stimulate as much insulin release from these cells as it stimulated from control INS-1 832/13 cells (Figure 4) and that when either β -hydroxybutyrate, which can supply acetoacetate directly (via reaction 7 shown in Figure 5) or α -ketoisocaproate (via the HMG-CoA lyase reaction, Figure 5, reaction 4), were added to the BCH-stimulated ATP citrate lyase deficient cells, insulin release was raised to values similar to those from the ATP citrate lyase cells incubated with leucine (which can be converted to acetoacetate (Figure 5, reaction 4)) or with leucine and/or additional sources of acetoacetate added to control INS-1 832/13 cells incubated without a source of acetoacetate (Figure 4). When the citrate pathway to acetyl-CoA and acetoacetyl-CoA is blocked as in the ATP citrate lyase deficient cells (reactions 12 and 9 in Figure 5), all carbon from mitochondria destined to become short chain acyl-CoAs in the cytosol can only be transferred via acetoacetate and thus the addition to the medium of compounds that supply acetoacetate bring BCH-induced insulin release equal to leucine-induced insulin release and do not further increase leucine-induced insulin release. In other words, leucine and BCH become equal in the presence of added sources of acetoacetate because the acetyl-CoA and acetoacetate from all sources are transferred to the cytosol via only one pathway, which is the same pathway for both leucine and BCH (via reactions 7 to 8 and 9, 4 to 8 and 9 and 4, 2 and 3 to 8 and 9 in Figure 5).

Pathways for Insulin Secretion in the Presence of a High Concentration of Exogenously Added Glutamine, a Source of Glutamate

When a very high concentration of glutamine (10 mM) is added to the normal cells to supply a large amount of glutamate, the mass action effect of the large amount of glutamate is what apparently enables BCH to convert glutamate into essential metabolites and to stimulate insulin release as strongly as leucine (Table 1). Glutamate-derived α -ketoglutarate can be metabolized in the citric acid cycle as far as oxaloacetate, producing some ATP, and converted to acetyl-CoA and citrate via the circuitous pathway involving malic enzyme or via the pathway originating with the reverse of the NADP-dependent isocitrate dehydrogenase. In the presence of a high concentration of glutamine, BCH stimulated the formation of nearly as much acetoacetate and an amount of α -ketoglutarate equal to that formed in the presence of leucine plus glutamine (Table 3). In addition, certain short chain acyl-CoAs were increased by BCH in the presence of a high concentration of glutamine, almost to the extent caused by leucine in the presence of a high concentration of glutamine (Table 4). The incorporation of glutamate carbon into mitochondrial acetoacetate can occur from acetyl-CoA via reactions 2 and 3 shown in Figure 5 as described above. However, the idea that glutamine-derived glutamate carbon is exported from mitochondria in large part as citrate is supported by the fact that even in the presence of a high level of glutamine, BCH-induced insulin release was decreased in the ATP citrate lyase deficient cells and was normalized by β -hydroxybutyrate or KIC which supply acetoacetate. The normalization of BCH-induced insulin release by these compounds strengthens the theory that acetoacetate is a carrier of acyl groups from mitochondria to the cytosol where it is converted to short chain acyl-CoAs.

Conclusion

The results of the current study indicate that leucine is a more potent insulin secretagogue than BCH because leucine is metabolized to acetoacetate plus acetyl-CoA (Figure 5, reaction 4) in addition to its activating glutamate dehydrogenase (Figure 5, reaction 11). Besides being used to make short chain acyl-CoAs, the acetoacetate and acetyl-CoA can also be metabolized to CO₂ in the citric acid cycle to produce ATP. The results further indicate that the normal beta cell translocates carbon from physiologic insulin secretagogues from the mitochondria to the cytosol as acetoacetate and/or citrate to form short chain acyl-CoAs.

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Abbreviations

BCH, 2-Aminobicyclo[2,2,1]heptane-2-carboxylic acid; KIC, α -ketoisocaproate.

References

1. Gylfe E. Comparison of the effects of leucine, non-metabolizable leucine analogues and other insulin secretagogues on the activity of glutamate dehydrogenase. *Acta Diabetol* 1976;13:20–24.
2. Sener A, Malaisse WJ. L-Leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature* 1980;288:187–189. [PubMed: 7001252]
3. Sener A, Malaisse-Lagae F, Malaisse WJ. Stimulation of pancreatic islet metabolism and insulin release by a nonmetabolizable amino acid. *Proc Natl Acad Sci* 1981;78:5460–5464. [PubMed: 6117857]
4. Malaisse-Lagae F, Sener A, Garcia-Morales P, Valverde I, Malaisse WJ. The stimulus-secretion coupling of amino acid-induced insulin release. Influence of a nonmetabolized analog of leucine on

the metabolism of glutamine in pancreatic islets. *J Biol Chem* 1982;257:3754–3758. [PubMed: 7037786]

5. Fahien LA, MacDonald MJ, Kralotek EH, Mertz RJ, Fabien CM. Regulation of insulin release by factors which also modify glutamate dehydrogenase. *J Biol Chem* 1988;263:13610–13614. [PubMed: 3047128]
6. Li C, Najafi H, Daikhin Y, Nissim IB, Collins HW, Yudkoff M, Matschinsky FM, Stanley CA. Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets. *J Biol Chem* 2003;278:2853–2858. [PubMed: 12444083]
7. MacDonald MJ, Fahien LA. Glutamate is not a messenger in insulin secretion. *J Biol Chem* 2000;275:34025–34027. [PubMed: 10967090]
8. MacDonald MJ. The export of metabolites from mitochondria and anaplerosis in insulin secretion. *Biochim Biophys Acta* 2002;1619:77–88. [PubMed: 12495818]
9. Liu YJ, Cheng H, Drought H, MacDonald MJ, Sharp GW, Straub SG. Activation of KATP channel-independent signaling pathway by the nonhydrolyzable analog of leucine, BCH. *Am J Physiol Endocrinol Metab* 2003;285:E380–E389. [PubMed: 12709398]
10. Li C, Matter A, Kelly A, Petty TJ, Najafi H, MacMullen C, Daikhin Y, Nissim I, Lazarow A, Kwagh J, Collins HW, Hsu BY, Nissim I, Yudkoff M, Matschinsky FM, Stanley CA. Effects of a GTP-insensitive mutation of glutamate dehydrogenase on insulin secretion in transgenic mice. *J Biol Chem* 2006;281:15064–15072. [PubMed: 16574664]
11. Lenzen S, Formanek H, Panten U. Signal function of metabolism of neutral amino acids and 2-keto acids for initiation of insulin secretion. *J Biol Chem* 1982;257:6631–6633. [PubMed: 7045091]
12. Giroix MH, Sener A, Bailbe D, Portha B, Malaisse WJ. Impairment of the mitochondrial oxidative response to D-glucose in pancreatic islets from adult rats injected with streptozotocin during the neonatal period. *Diabetologia* 1990;33:654–660.
13. Giroix MH, Saulnier C, Portha B. Decreased pancreatic islet response to L-leucine in the spontaneously diabetic GK rat: enzymatic, metabolic and secretory data. *Diabetologia* 1999;42:965–977. [PubMed: 10491757]
14. MacDonald MJ, McKenzie DI, Kaysen JH, Walker TMM, Moran SM, Fahien LA, Towle HC. Glucose regulates leucine-induced insulin release and the expression of the branched chain ketoacid dehydrogenase E1 alpha subunit gene in pancreatic islets. *J Biol Chem* 1991;266:1335–1340. [PubMed: 1985951]
15. Malaisse WJ, Sener A, Malaisse-Lagae F, Welsh M, Matthews DE, Bier DM, Hellerstrom C. The stimulus-secretion coupling of amino acids-induced insulin release. Metabolic response of pancreatic islets of L-glutamine and L-leucine. *J Biol Chem* 1982;257:873–8737
16. MacDonald MJ, Smith AD, Hasan NM, Sabat G, Fahien LA. Feasibility of pathways for transfer of acyl groups from mitochondria to the cytosol to form short chain acyl-CoAs in the pancreatic beta cell. *J Biol Chem* 2007;282:30596–30606. [PubMed: 17724028]
17. Hohmeier HE, Mulder H, Chen G, Hekel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1 derived cells lines with robust ATP-sensitive K⁺ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 2000;49:424–430. [PubMed: 10868964]
18. MacDonald MJ, Fahien LA. Glyceraldehyde phosphate and methyl esters of succinic acid. Two “new” potent insulin secretagogues. *Diabetes* 1988;37:997–999. [PubMed: 3290012]
19. MacDonald MJ, Fahien LA, McKenzie DI, Moran SM. Novel effects of insulin secretagogues on capacitation of insulin release and survival of cultured pancreatic islets. *Am J Physiol* 1990;259:E548–E554. [PubMed: 2221056]
20. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 1992;130:167–178. [PubMed: 1370150]
21. MacDonald MJ. Synergistic potent insulin release by combinations of weak secretagogues in pancreatic islets and INS-1 cells. *J Biol Chem* 2007;282:6043–6052. [PubMed: 17210580]
22. MacDonald MJ. Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets: Further implication of cytosolic NADPH in insulin secretion. *J Biol Chem* 1995;270:20051–20058. [PubMed: 7650022]

23. MacDonald MJ. Estimates of glycolysis, pyruvate (de)carboxylation, pentose phosphate pathway and methyl succinate metabolism in incapacitated pancreatic islets. *Arch Biochem Biophys* 1993;305:205–214. [PubMed: 8373157]
24. Fahien LA, Teller JK, MacDonald MJ, Fahien CM. Regulation of glutamate dehydrogenase by Mg^{2+} and magnification of leucine activation by Mg^{2+} *Mol Pharmacol* 1990;37:943–949. [PubMed: 2359406]
25. Erecinska M, Nelson D. Activation of glutamate dehydrogenase by leucine and its nonmetabolizable analogue in rat brain synaptosomes. *J Neurochem* 1990;54:1335–1343. [PubMed: 1968960]
26. MacDonald MJ, Longacre MJ, Stoker SW, Brown LJ, Hasan NM, Kendrick MA. Acetoacetate and β -hydroxybutyrate in combination with other metabolites release insulin from INS-1 cells and provide clues about pathways of insulin secretion. *Am J Physiol Cell Physiol*. 2007In press
27. MacDonald MJ, Fahien LA, Brown LJ, Hasan NM, Buss JD, Kendrick MA. Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. *Am J Physiol Endocrinol Metab* 2005;288:E1–E15. [PubMed: 15585595]
28. Gao Z, Young RA, Li G, Najafi H, Buettger C, Sukamvanich SS, Wong RK, Wolf BA, Matschinsky FM. Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells. *Endocrinology* 2003;144:1949–1957. [PubMed: 12697702]

Figure 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.

Figure 2. Leucine with or without glutamine stimulates more insulin release than BCH stimulates in INS-1 832/13 cells and compounds that supply additional acetyl-CoA and acetoacetate augment insulin release in the presence of either leucine and BCH

INS-1 832/13 cells were incubated with either leucine (10 mM) or BCH (10 mM) and without or with D- β -hydroxybutyrate (5 mM), glutamine (2 mM), α -ketoisocaproate (KIC) (2 mM) or glucose (11.1 mM) for 1 h. There was 75 μ g of total cell protein per incubation. Results are expressed as the mean $\pm$ SE with a minimum of 12 incubations for each condition. Differences between leucine versus BCH without or with glutamine were highly significant ($p \leq 0.001$) and the augmentation of insulin release with β -hydroxybutyrate or KIC versus the same condition without these components was also significant ($p \leq 0.001$ or 0.01). Insulin release

in the presence of glutamine, β -hydroxybutyrate and KIC (controls) alone was negligible as shown in Figure 4 and previously in references 21 and 26.

Figure 3. BCH is equal to or slightly stronger than leucine as an activator of INS-1 cell glutamate dehydrogenase

A homogenate of INS-1 832/13 cells was incubated with various concentrations of BCH or leucine and glutamate dehydrogenase enzyme activity was measured.

Figure 4. KIC and β -hydroxybutyrate, which can provide an alternative source of acetoacetyl-CoA, rescue a blunted insulin release response to BCH alone and BCH plus glutamine in ATP citrate lyase deficient INS-1 832/13 cells

The ATP citrate lyase (ATP-CL) deficient cells used in these experiments are 88% deficient in ATP-CL enzyme activity and were the ATP-CL 940-12 cell line described in reference 16. The concentrations of BCH and leucine were 10 mM and D- β -hydroxybutyrate (BHB), α -ketoisocaproate (KIC), and glucose were 5 mM, 2 mM and 11.1 mM, respectively. Insulin release by single or two agents is shown as controls. Insulin release by glucose alone is shown as a positive control. There was 75 μ g of total cell protein per incubation. Results are the mean $\pm$ SE of 8–12 replicate incubations for the ATP-CL deficient cells and 12 replicate incubations for the control cells. Control incubations were untransfected INS-1 832/13 cells or INS-1 832/13 cells transfected with an empty vector. Insulin release from the native INS-1 832/13 cells (N = 6) and those transfected with empty vector (N = 6) were equal and results were averaged together. Differences between ATP-CL deficient cells and control cells, for BCH alone or BCH plus glutamine were highly significant ($p < 0.001$).

Figure 5. Pathways of formation of short chain acyl-CoAs in mitochondria from secretagogue carbon, transfer of acyl group carbon to the cytosol as acetoacetate and citrate and their conversion to short chain acyl-CoAs in the cytosol

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

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 that of leucine alone but did not further increase leucine-induced insulin release. Results are expressed as mean $\pm$ SE insulin released per 5 islets per 1 h with number of replicate incubations in parentheses.

Incubation	Insulin Release (μ U Insulin/5 islets/1 h)
No Addition	18 $\pm$ 2 (33)
Glucose	558 $\pm$ 43 (18)
Leucine	104 $\pm$ 8 (27)
Leucine + β -Hydroxybutyrate	104 $\pm$ 14 (6)
BCH	51 $\pm$ 4 (33)
BCH + β -Hydroxybutyrate	103 $\pm$ 8 (6)
Leucine + Glutamine	565 $\pm$ 54 (11)
BCH + Glutamine	544 $\pm$ 29 (11)
Glutamine	14 $\pm$ 3 (6)
β -Hydroxybutyrate	20 $\pm$ 4 (6)
KIC	556 $\pm$ 31 (14)

Leucine and BCH augment glutamine oxidation and glutamine decreases leucine oxidation in rat pancreatic islets
Rat pancreatic islets (100 islets/test tube) were incubated in the presence of a uniformly ¹⁴C-labeled compound (0.25 mCi/mmol) and without or with an unlabeled compound for 90 min and ¹⁴CO₂ was collected. The concentration of glutamine, BCH and α-ketoisocaproate (KIC) were each 10 mM and the concentration of leucine was 2 mM or 10 mM as indicated. Oxidation of glucose (16.7 mM) is shown for comparison. Results are the mean ± SE with the number of replicate incubations shown in parentheses.

Labeled Compound	Unlabeled Compound	Oxidation of Labeled Compound (nmol compound oxidized CO ₂ /100 islets/90 min)	Percent Change in Oxidation Caused by Unlabeled Compound (%)
Leucine, 2 mM	None	1.1 ± 0.08 (3)	-
Leucine, 2 mM	Glutamine, 10 mM	0.79 ± 0.01 (3)	- 27
Leucine, 10 mM	None	3.7 ± 0.6 (11)	-
Leucine, 10 mM	Glutamine, 10 mM	1.9 ± 0.1 (8)	- 49
Glutamine	None	3.8 ± 0.2 (9)	-
Glutamine	Leucine, 10 mM	4.5 ± 0.3 (8)	+ 48
Glutamine	BCH, 10 mM	6.4 ± 0.3 (3)	+ 68
Glucose	None	3.6 ± 0.2 (9)	-

Table 3
Effect of combinations of insulin (non)secretagogues on concentrations of acetoacetate and α -ketoglutarate in rat pancreatic islets
Islets were incubated in Krebs Ringer solution in the presence of (a) secretagogue(s) for 30 min after which metabolism was stopped and metabolites were measured. Results are the mean $\pm$ SE with the number of observations in parentheses.

Secretagogue added (Concentration)	Metabolite Concentration (nmol/mg islet protein)	
	Acetoacetate	α -Ketoglutarate
None	0.06 $\pm$ 0.03 (37)	0.15 $\pm$ 0.04 (22)
Leucine (10 mM)	0.62 $\pm$ 0.07 (8)	0.65 $\pm$ 0.10 (8)
BCH (10 mM)	0.24 $\pm$ 0.05 (13)	0.46 $\pm$ 0.11 (17)
Leucine (10 mM) + Glutamine (10 mM)	0.70 $\pm$ 0.05 (6)	0.92 $\pm$ 0.11 (7)
BCH (10 mM) + Glutamine (10 mM)	0.52 $\pm$ 0.11 (10)	0.88 $\pm$ 0.10 (11)
Glucose (16.7 mM)	0.30 $\pm$ 0.11 (14)	0.37 $\pm$ 0.14 (19)
KIC (10 mM)	1.2 $\pm$ 0.12 (14)	0.68 $\pm$ 0.09 (9)
Glutamine (10 mM)	0.04 $\pm$ 0.08 (3)	0.20 $\pm$ 0.05 (6)

Increases in various short chain acyl-CoAs in INS-1 832/13 cells stimulated with leucine or BCH with or without glutamine
INS-1 cells were incubated in the presence of (non)secretagogues for 30 min and short chain acyl-CoAs were measured. The concentrations of leucine, BCH and glutamine were 10 mM. Results are the mean $\pm$ SE with the number of replicate incubations in parentheses.

Incubation Condition	Short Chain Acyl-CoA Concentration (pmol acyl-CoA / mg cell protein)			
	Acetyl-CoA	Acetoacetyl-CoA	Succinyl-CoA	HMG-CoA
1.5 mM Glucose (Control)	101 $\pm$ 20(9)	10 $\pm$ 3(6)	43 $\pm$ 7(9)	69 $\pm$ 13(11)
Leucine	217 $\pm$ 31(8) ^c	29 $\pm$ 4(9) ^b	106 $\pm$ 12(10) ^c	89 $\pm$ 16(9)
BCH	118 $\pm$ 10(8)	11 $\pm$ 2(9)	69 $\pm$ 7(9) ^a	73 $\pm$ 7(8)
Leucine + Glutamine	213 $\pm$ 22(8) ^b	39 $\pm$ 9(6) ^b	100 $\pm$ 11(10) ^c	78 $\pm$ 9(10)
BCH + Glutamine	137 $\pm$ 12(5)	32 $\pm$ 5(4) ^b	75 $\pm$ 6(5) ^b	55 $\pm$ 4(6)
Glutamine	105 $\pm$ 11(8)	12 $\pm$ 3(4)	34 $\pm$ 3(4)	42 $\pm$ 6(4)
16.7 mM Glucose	196 $\pm$ 16(4) ^c	19 $\pm$ 2(4) ^b	66 $\pm$ 10(4)	76 $\pm$ 14(4)

^a p $\leq$ 0.05
^b p $\leq$ 0.01
^c p $\leq$ 0.0001 vs the 1.5 mM glucose control