Regulation of Leucine-stimulated Insulin Secretion and Glutamine Metabolism in Isolated Rat Islets*

Glutamate dehydrogenase (GDH) is regulated by both positive (leucine and ADP) and negative (GTP and ATP) allosteric factors. We hypothesized that the phosphate potential of β-cells regulates the sensitivity of leucine stimulation. These predictions were tested by measuring leucine-stimulated insulin secretion in perfused rat islets following glucose depletion and by tracing the nitrogen flux of [2-15N]glutamine using stable isotope techniques. The sensitivity of leucine stimulation was enhanced by long time (120-min) energy depletion and inhibited by glucose pretreatment. After limited 50-min glucose depletion, leucine, not α-ketoisocaproate, failed to stimulate insulin release. β-Cells sensitivity to leucine is therefore proposed to be a function of GDH activation. Leucine increased the flux through GDH 3-fold compared with controls while causing insulin release. High glucose inhibited flux through both glutaminase and GDH, and leucine was unable to override this inhibition. These results clearly show that leucine-induced stimulation of insulin by augmenting glutaminolysis through activating glutaminase and GDH. Glucose regulates β-cell sensitivity to leucine by elevating the ratio of ATP and GTP to ADP and P i and thereby decreasing the flux through GDH and glutaminase. These mechanisms provide an explanation for hypoglycemia caused by mutations of GDH in children.

In addition to glucose, amino acids and other metabolic fuels are important stimulators of insulin secretion from pancreatic β-cells. Leucine, which has been studied extensively, may stimulate insulin release through two different mechanisms. The first involves transamination of leucine to α-ketoisocaproate (KIC) and subsequent mitochondrial oxidation. The second promotes insulin release via allosteric activation of glutamate dehydrogenase (GDH) causing oxidation of glutamate to the Krebs cycle intermediate, α-ketoglutarate, plus ammonia. The importance of the latter mechanism has been highlighted recently by the discovery of a dominant form of congenital hyperinsulinism associated with mutations of GDH leading to a gain of enzyme activity, because sensitivity to inhibition by GTP and ATP is impaired (1–3). Affected children have increased β-cell responsiveness to leucine and are susceptible to acute hypoglycemia following a high protein meal (4). The involvement of GDH may explain the observation that, in contrast to other amino acids, leucine-stimulated insulin secretion (LSIS) is suppressed by high glucose. For example, Gao et al. (5) reported that glucose inhibits leucine stimulation of glutaminolysis and insulin secretion in isolated mouse islets, presumably by increasing intracellular ATP and GTP while decreasing ADP and thus inhibiting GDH activity.

GDH has also been proposed by Maechler and Wollheim (6) to play an essential role in glucose-mediated insulin secretion by acting in the reverse direction to catalyze production of glutamate, which is hypothesized to work as a cofactor in the process leading to exocytosis of insulin granules. These investigators have suggested that lower levels of GDH in mouse islets may explain the reduced second phase of insulin secretion of mouse islets compared with the predominant second phase insulin release observed in rat and human islets (7). Their suggestion that net flux through GDH is toward synthesis rather than oxidation of glutamate has been contradicted by other reports (8–10) and is also not consistent with our proposal that the mutations of GDH that cause hyperinsulinism in children act by increasing the oxidation of glutamate and, presumably, the resulting decrease of the intracellular glutamate level. Because of the renewed interest in GDH as a potential key regulatory step in amino acid- and glucose-stimulated insulin secretion and because of the controversy about the direction of net flux through the enzyme, the present experiments were undertaken to test the hypothesis that LSIS is due to increased oxidation of glutamate by GDH and that this process is inhibited by glucose.

EXPERIMENTAL PROCEDURES

Islets Isolation and Culture—Adult male Wistar rat islets were isolated by collagenase digestion and cultured in RPMI 1640 medium (glucose-free; Sigma). The culture medium was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin, and islets were incubated at 37°C in a 5% CO2/95% air humidified incubator. Islets were cultured with different concentrations of glucose, 5, 10, and 25 mM for 3 to 4 days.

Insulin Secretion by Perfused Islets—100 cultured rat islets were loaded onto nylon filters in a small chamber and perfused in a Krebs-Ringer bicarbonate buffer (115 mmol/liter NaCl, 24 mmol/liter NaHCO3, 1.4 mmol/liter KCl, 1.1 mmol/liter MgCl2, 2.5 mmol/liter CaCl2, pH 7.4) with 0.25% bovine serum albumin at a flow rate of 2 ml/min. Perifusate solutions were gassed with 95% O2/5% CO2 and maintained.
FIG. 1. Effect of run-down duration on islet responsiveness.
Isolated rat islets were cultured with 10 mM glucose for 3 days and then perifused with 2 mM glutamine in the absence of glucose for run-down periods of 50 min (diamonds) or 120 min (circles) prior to stimulation with 10 mM leucine (panel A), 10 mM glucose (panel B), and 10 mM KIC (panel C). Values represent the means ± S.E. for 100 islets from three separate perifusions.

FIG. 2. Effect of run-down duration on islet response to leucine ramp. Isolated rat islets were cultured with 10 mM glucose for 3 days and then perifused with 2 mM glutamine in the absence of glucose for run-down periods of 50 min (triangles) or 120 min (circles) prior to stimulation with a leucine ramp (0 to 25 mM at 0.5 mM/min). Results are presented as means ± S.E. for 100 islets from three separate perifusions.

**RESULTS**

Effects of Glucose on LSIS—Fig. 1 shows the insulin secretory responses of cultured rat islets to leucine, KIC, or glucose after perifusion in glucose-free buffer to allow run-down of energy stores for either a short (50-min) or a long (120-min) period. Islets cultured with medium containing 10 mM glucose followed by a short period of glucose depletion (50-min) failed to respond to 10 mM leucine (Fig. 1A). In contrast, islets exposed to the longer period of glucose depletion (120-min) had a brisk,
Regulation of Islet Glutaminolysis

Fig. 3. Effect of glucose concentration on islet responsiveness to leucine. Isolated rat islets were cultured with 10 mM glucose for 3 days and then perfused with 2 mM glutamine in the presence of different concentrations of glucose for 120 min prior to stimulation with 10 mM leucine. Circles, 0 mM glucose; diamonds, 10 mM glucose; triangles, 25 mM glucose. At the end of the experiment, after removal of leucine for 10 min (glucose, 0 mM), islets were stimulated with 30 mM KCl.

Fig. 4. Effect of leucine and glutamine concentration on islet responsiveness. Isolated rat islets were cultured with 10 mM glucose for 3 days and then perfused in absence of glucose for 20 min prior to exposure to different concentration of glutamine for 30 min prior to stimulation with 10 mM leucine (panel A) or 20 mM leucine (panel B). Solid circles, 0 mM glutamine; solid triangles, 2 mM glutamine; open triangles, 5 mM glutamine; open circles, 10 mM glutamine.

Biphasic secretion of insulin in response to 10 mM leucine. After the longer periods of glucose depletion, base-line insulin release was 0.8 ± 0.1 ng/100 islets/min, and following leucine, the insulin secretion reached a peak of 6.8 ± 1.6 ng/100 islets/min followed by a second phase plateau of 10 ± 0.1 ng/100 islets/min.

In contrast to leucine, both 10 mM KIC and 10 mM glucose were able to stimulate biphasic insulin release in islets exposed to glucose-free medium for 50 min (Fig. 1, B and C). Glucose stimulated nearly identical insulin responses after the short or long periods of energy depletion (Fig. 1B). KIC at 10 mM caused a greater initial insulin peak (21 ± 4 versus 10 ± 4 ng/100 islets/min, p < 0.001) and a higher second phase plateau (11 ± 0.3 versus 6 ± 0.2 ng/100 islets/min, p < 0.001) in islets with 120-min run-down compared with islets with 50-min run-down (Fig. 1C).

Fig. 2 shows the effect of short or long periods of glucose depletion on islet sensitivity to stimulation with a leucine ramp. The threshold for stimulation of insulin release by leucine was lower in islets with 120-min run-down than islets with 50-min run-down (6 versus 14 mM leucine). Furthermore, maximum leucine-stimulated insulin secretion was doubled by extended glucose depletion (15 ± 1 versus 8 ± 3 ng/100 islets/min, p < 0.001).

As shown in Fig. 3, the suppression of leucine-stimulated insulin release by glucose was concentration-dependent. When glucose was added to the perfusion buffer during the 120-min equilibration period and then removed for 20 min prior to stimulation with leucine, insulin release was partially inhibited by 10 mM glucose and completely suppressed by 25 mM glucose. In contrast, exposure to glucose had no effect on insulin release in response to depolarization with potassium chloride. When the glucose concentration of the medium used to culture isolated rat islets for 3 days prior to perfusion was reduced from 10 to 5 mM, a biphasic insulin response to stimulation by 10 mM leucine was observed even after very brief glucose depletion (data not shown).

Fig. 4 shows the effect of increasing glutamine concentrations on glucose suppression of leucine-mediated insulin re-
lease. As shown in panel A, glucose suppression of LSIS was overcome in a dose-dependent manner by increasing the concentration of glutamine from 0 to 10 mM. In the absence of leucine, glutamine alone, even at 10 mM, did not stimulate insulin release. As shown in panel B, increasing the concentration of leucine from 10 to 20 mM increased insulin secretion in the presence of 5 and 10 mM glutamine but had very little added effect in the presence of 2 mM glutamine. These results showed that high leucine in the presence of high glutamine could overcome glucose inhibition of LSIS.

To test whether the inhibitory effect of glucose on LSIS was on the pathway of leucine oxidation via KIC, the effect of glucose pretreatment on oxidation of [1-14C]leucine in the absence of glucose was examined (Fig. 5). Maximum rates of leucine oxidation were lowered by 30% with increasing concentrations of glucose pretreatment (p < 0.01). However, oxidation rates of 20 mM leucine by islets cultured in 10 mM glucose were 80% the rate with 10 mM leucine in islets cultured at 5 mM glucose. Studies of insulin secretion showed that islets cultured in 10 mM glucose did not respond to 20 mM leucine, whereas islets cultured with 5 mM glucose responded to 10 mM leucine. Thus, it seems that direct oxidation of leucine contributes little or nothing to the process responsible for LSIS.

Effects of Glucose Depletion on Islet ATP and GDH—To determine the factors responsible for altering leucine sensitivity, we measured the effects of incubation in glucose-free medium for varying time on islet ATP concentrations and on basal and maximal GDH activity. As shown in Table I, withdrawal of glucose for 120 min resulted in a nearly 50% fall in islet ATP concentration. Basal GDH enzyme activity was 20% of the ADP-stimulated maximal value, suggesting carryover of inhibitors, such as GTP, in the crude islet homogenate. Basal GDH activity increased during the 120 min of glucose depletion by 40% without a change in maximal enzyme activity, consistent with a decline in islet content of GTP and ATP, which are potent allosteric inhibitors of the enzyme.

Effects of Leucine and Glucose on Flux through GDH—The above experiments identified GDH as the likely site for glucose suppression of leucine-mediated insulin release. To examine in detail the effects of leucine and glucose on rates of flux through GDH, experiments were carried out using 2,15N-labeled glutamate to trace the flow of the amino nitrogen into glutamate and ammonia. A 90-min pre-incubation in unlabeled 10 mM glutamate was used to mimic the condition of prolonged glucose depletion. Incubations with 10 mM [2-15N]glutamine alone or together with leucine, glucose, or leucine plus glucose were then carried out for 2 h using batches of 1,000 isolated rat islets per tube. As shown in Table II, both leucine and glucose stimulated insulin secretion by these islets. However, the combination of the two gave the same rates of insulin release as glucose alone, consistent with glucose inhibition of LSIS seen previously. The intracellular concentrations of glutamine, glutamate, aspartate, alanine, and γ-aminobutyric acid (GABA) were measurable under these conditions. Islet glutamine, glutamate, aspartate, and alanine concentrations remained relatively constant during the 2-h control incubation with 10 mM glutamine. Incubation with 10 mM leucine did not change islet glutamate concentrations but caused a 30% decrease in aspartate concentrations compared with control. Incubation with 25 mM glucose caused a 40% decrease of glutamate and a 70% decrease of aspartate concentrations. The combination of leucine plus glucose further decreased aspartate concentrations and partly reversed the glucose-induced depression of glutamate concentrations. The intracellular alanine concentrations were very small compared with the glutamate and aspartate pools. Alanine concentrations rose in the presence of glucose, consistent with increased glycolytic flux to pyruvate and subsequent transamination to yield alanine.

As shown in Table III, about 60% of islet glutamate and aspartate were replaced from [15N]glutamine in the control and glucose incubations. The isotopic enrichment of glutamate and aspartate was decreased by 10–20% in the presence of leucine, consistent with some contribution of unlabeled nitrogen from leucine through transamination. Glucose decreased the concentrations but not the isotopic enrichment of both glutamate and aspartate, indicating that glucose suppressed flux from glutamine through glutaminase into these amino acids. Isotopic enrichment of GABA was not determined, but turnover of this pool was likely to have been small, because the concentrations of GABA remained essentially unchanged under all of the incubation conditions (Table II).

As shown in Table III, total ammonia production from the combination of the glutaminase and GDH reactions was stimulated by leucine and suppressed by glucose. In the presence of glucose, the stimulation of ammonia release by leucine was inhibited. The isotopic enrichment of ammonia was significantly increased by incubation with leucine, confirming increased flux through both the glutaminase and glutamate dehydrogenase steps.

Table III shows the calculated rates of [15N]labeled glutamate and aspartate production from glutamine and of the flux rates through the glutaminase and GDH steps. Production of glutamate and, especially, aspartate were decreased by incubation with leucine compared with control and further suppressed by incubation with glucose or glucose plus leucine. Leucine stimulation produced a 350% increase in flux through GDH and a 140% increase in flux through glutaminase compared with the control islets. In contrast, glucose stimulation of islets was associated with a 50% reduction in flux through both GDH and glutaminase. The stimulatory effects of leucine on GDH and glutaminase flux were blocked in the presence of glucose, consistent with the observation that glucose also blocked leucine-stimulated insulin release.
DISCUSSION

The discovery of the GDH linked form of hyperinsulinism has made it important to explore the mechanisms of increased protein and leucine sensitivity of insulin secretion observed in patients with this disorder. The present report describes a robust experimental model in isolated cultured rat islets using a paradigm of energy depletion, or run-down, to test the sensitivity for LSIS. The results show that the energy potential regulates glutaminolysis and modifies the sensitivity of \( \beta \)-cells to leucine stimulation. A prolonged period of run-down to produce a state of energy depletion sensitizes the islet to leucine stimulation. In contrast, with a short period of run-down after withdrawal of high glucose, islets maintain a high energy potential and are insensitive to leucine stimulation. The present results show that the mechanism of LSIS is not primarily through the leucine oxidation pathway, but, as illustrated in Fig. 6, is because of increased glutamine catabolism with enhanced flux through the glutaminase and GDH reactions. Glucose suppression of LSIS involves inhibition of both of these two enzyme steps in glutamine oxidation by an indirect mechanism involving changes in the concentrations of high energy phosphates, GTP and ATP, and of ADP and Pi in \( \beta \)-cells.

The results of the present experiments demonstrate that LSIS is conditional, in contrast to the commonly held concept that leucine is comparable with glucose in potency as an insulin secretagogue. These studies clarify previous observations in the isolated perfused rat pancreas showing that LSIS is induced by fuel depletion (13). The conditional nature of LSIS is also consistent with observations in humans showing that normal healthy subjects do not become hypoglycemic in response to intravenous leucine but become sensitive to leucine after treatment with a sulfonylurea drug, such as tolbutamide (14). Because LSIS is mediated by allosteric activation of GDH (15), \( \beta \) islet responsiveness to leucine is directly related to the state of GDH enzymatic activity. Thus, patients with the GDH-linked form of hyperinsulinism are hypersensitive to leucine stimulation (1–3), because of mutations that impair responsiveness to GTP and result in a loss of negative allosteric regulation. In isolated mouse islets, Gao et al. (5) have reported that leucine-induced elevation of cytosolic calcium is blocked after treatment with high glucose. In addition, in mouse islets, the enhancement of [U-\( ^{14} \)C]glutamine oxidation by the leucine analog 2-amino-2-norbornane-carboxylic acid, was inhibited by glucose treatment (5). In the present study, the run-down paradigm clearly showed that the dynamic changes in sensitivity of LSIS reflect the regulation of GDH enzymatic activity by the \( \beta \)-cells energy potential.

Fig. 6 illustrates some of the interactions revealed by the present experiments in the glucose and leucine regulation of glutaminolysis and flux through GDH. During post-prandial hyperglycemia, glucose is the predominant energy source of \( \beta \)-cells, and glucose metabolism increases the levels of ATP and GTP while decreasing the concentrations of ADP, GDP, and Pi (16, 17). The half-maximal inhibitory concentrations of GTP and ATP for allosteric inhibition of GDH are 50–100 nM and 10–20 \( \mu \)M, respectively (3). These values are well below the intramitochondrial concentrations of these nucleotides, implying that GDH activity might be totally inhibited by the enhanced glucose metabolism following a meal. Under such conditions of high energy potential, GDH becomes refractory to

| Table III  |
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| Rates of production of ammonia, [\( ^{15} \)N]glutamate, and [\( ^{15} \)N]aspartate: [\( ^{15} \)N] isotopic enrichments and flux through GDH and glutaminase in cultured rat islets |
| Ammonia production | [\( ^{15} \)N]APE | Production | Flux |
|---|---|---|---|
| nmol/1000 islets/2h | nmol/1000 islets/2h | nmol/1000 islets/2h |
| Control | 130 ± 11 | 7 ± 1.2 | 63 ± 3.3 | 66 ± 2.3 | 14 ± 0.5 | 26 ± 0.4 | 16 ± 3 | 57 ± 4 |
| Leucine 10 mM | 217 ± 7 | 14 ± 0.7 | 50 ± 0.7 | 55 ± 1.3 | 10 ± 1.1 | 14 ± 1.1 | 56 ± 2 | 81 ± 3 |
| Glucose 25 mM | 93 ± 9 | 5 ± 0.8 | 62 ± 0.8 | 62 ± 1.3 | 9 ± 0.3 | 7 ± 0.4 | 7 ± 1 | 22 ± 2 |
| Leucine 10 mM and glucose 25 mM | 80 ± 17 | 5 ± 0.3 | 43 ± 0.1 | 46 ± 0.4 | 7 ± 1.0 | 4 ± 0.4 | 9 ± 2 | 20 ± 3 |

a Compared with control, \( p < 0.01 \).

b Compared with leucine 10 mM, \( p < 0.05 \).
c Compared with control, \( p < 0.05 \).
d Compared with leucine 10 mM, \( p < 0.05 \).
activation by leucine. During energy depletion, the ratio of ATP and GTP to ADP and $P_i$ decreases, and the sensitivity of GDH to allosteric stimulation becomes augmented. The data shown in Table I suggest that the phosphate potential gradually decreases during islet run-down until it reaches a critical threshold, at which point islets become sensitive to leucine.

Previous studies of glutaminolysis in islets that have used $^{15}$C-glutamine as a tracer to follow the flux of glutamine into CO$_2$ were unable to distinguish between transamination of glutamate and oxidative deamination. In the present study, by using $^{2-15}$N glutamine we were able to directly follow the fate of the amino nitrogen of glutamine and the changes associated with leucine and glucose stimulation. The results highlight the fact that both glutaminase and GDH are regulated by the phosphate potential and are involved in the suppression of LSIS by glucose. Glutaminase, the pathway-controlling step in glutaminolysis, is a phosphate-dependent enzyme in islets (18). Thus, glucose may inhibit glutaminase by decreasing the inorganic phosphate level. Because glutamate is a strong inhibitor of glutaminase, leucine may indirectly stimulate the enzyme by removing glutamate as a result of activation of GDH (see Table III). Thus, regulation of glutaminolysis is the result of the inhibition or activation of these two enzymes.

Although the present experiments were designed to investigate ammonia release from GDH deamination and the glutaminase reactions, some of the alterations in islet amino acid metabolism observed during incubation with glucose and leucine involve transamination reactions. For example, under conditions in which islets were incubated with glutamine as the sole fuel, the $^{15}$N tracer studies indicate that glutamine is oxidized through glutamate, enters the Krebs cycle at $\alpha$-keto-glutarate, and exits at oxaloacetate (OAA) by transamination to aspartate through aspartate aminotransferase (AST) (Fig. 6). This partial Krebs cycle pathway has also been demonstrated in brain (19). In this study, glutamate carbon accumulated in the form of aspartate when glutamate was the sole carbon source, and the transfer of carbons from glutamate to aspartate could be blocked by the aminotransferase inhibitor, amino oxoacetate. In the present experiments, addition of glucose or glucose plus leucine decreased the ratio of aspartate to glutamate in islets, because the generation of acetyl-CoA from glycolysis allows the Krebs cycle to proceed past oxaloacetate, thus reducing the ratio of oxaloacetate to $\alpha$-keto-glutarate and shifting the aspartate aminotransferase reaction toward glutamate formation. The reduction of $^{15}$N labeling of the glutamate and aspartate pools by addition of leucine probably reflects donation of the leucine amino group by transamination to glutamate and KIC. The fact that glucose inhibits LSIS but has little effect on KIC-stimulated insulin release indicates that allosteric activation of GDH is the primary mechanism by which leucine produces insulin release, under the conditions of the present experiments.

The present experiments were designed to measure the oxidative deamination of glutamate through GDH. The results are compatible with the concept that the predominant direction of the GDH reaction in intact islets incubated with glutamine plus or minus leucine or glucose is toward glutamate oxidation. The present observations are consistent with studies by Cooper and co-workers (20, 21) in liver and by Yudkoff et al. (22) in brain indicating that the GDH reaction runs exclusively in the oxidative direction under normal conditions. As these investigators have pointed out, flux toward glutamate synthesis is not likely, because normal concentrations of ammonia are over 100 times lower than the $K_m$ for ammonia of the GDH reaction. The present experiments do not lend support to the hypothesis of Maechler and Wollheim (6) that the GDH reaction runs toward glutamate formation during glucose-stimulated insulin secretion. Indeed, under the conditions used, addition of glucose suppressed both glutamate concentrations and the activity of the GDH reaction. However, the present experiments cannot completely exclude the hypothesis put forth by Maechler and Wollheim (6), because direct measurements were not made of reductive amination flux through GDH.

The present experiments highlight the importance of a GDH-linked metabolic network in $\beta$-cells as illustrated in Fig. 6. The key enzymes in this network include phosphate-dependent glutaminase (PDG), GDH, and aspartate aminotransferase. An increased phosphate potential leads to inhibition of both PDG and GDH, indicating that PDG and GDH may serve as intracellular energy sensors to regulate amino acid metabolism. The metabolism of glucose may control the intracellular amino acid homeostasis by changing the phosphate potential. The changing of aspartate indicates that the transamination reaction plays an important role in glucose and amino acid metabolism in islets.

In conclusion, GDH and glutaminase play important roles in insulin secretion stimulated by a mixture of glutamine and leucine. The sensitivity of islets to leucine stimulation is tightly regulated by the energy potential. GDH and glutaminase may serve as intracellular energy sensors to control the islet responsiveness to leucine stimulation.

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