Chronic exposure to high leucine impairs glucose-induced insulin release by lowering the ATP-to-ADP ratio

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Chronic exposure to high leucine impairs glucose-induced insulin release by lowering the ATP-to-ADP ratio. Am J Physiol Endocrinol Metab 281: E1082–E1087, 2001.—Exposure of rat pancreatic islets to 20 mM leucine for 24 h reduced insulin release in response to glucose (16.7 and 22.2 mM). Insulin release was normal when the same islets were stimulated with leucine (40 mM) or glyburide (1 μM). To investigate the mechanisms responsible for the different effect of these secretagogues, we studied several steps of glucose-induced insulin secretion. Glucose utilization and oxidation rates in leucine-precultured islets were similar to those of control islets. Also, the ATP-sensitive K⁺ channel-independent pathway of glucose-stimulated insulin release, studied in the presence of 30 mM K⁺ and 250 μM diazoxide, was normal. In contrast, the ATP-to-ADP ratio after stimulation with 22.2 mM glucose was reduced in leucine-exposed islets with respect to control islets. The decrease of the ATP-to-ADP ratio was due to an increase of ADP levels. In conclusion, prolonged exposure of pancreatic islets to high leucine levels selectively impairs glucose-induced insulin release. This secretory abnormality is associated with (and might be due to) a reduced ATP-to-ADP ratio. The abnormal plasma amino acid levels often present in obesity and diabetes may, therefore, affect pancreatic islet insulin secretion in these patients.

Adenosine triphosphate; adenosine diphosphate; β-cell desensitization; amino acids; adenine nucleotides; adenosine triphosphate potassium channel-independent pathway

Type 2 diabetes is characterized by inadequate pancreatic β-cell insulin release in response to glucose and by impaired insulin action (5, 6, 16, 19, 20, 31). In these patients, the altered insulin secretory pattern depends, at least in part, on the negative influence of chronic high glucose and/or free fatty acids (FFA) plasma concentrations (gluco- or lipotoxicity). These metabolites are believed to affect pancreatic β-cells function by chronic stimulation (by either glucose or FFA or both) and consequent “desensitization” to glucose (2, 11, 23, 26, 28, 30, 33, 37).

In contrast to the effects of glucose and FFA, less studied are the effects of amino acids, the third class of nutrient insulin secretagogues. Increased amino acid levels (and in particular leucine, isoleucine, and valine in the concentration range of 0.1–0.3 mM) have been described in both type 1 and type 2 diabetes and in obesity (12, 13, 38) as a consequence of impaired insulin effect on amino acid uptake and metabolism. Also, in animal models of diabetes, insulin action deficiency increased amino acid plasma concentrations up to 1.5 mM (4, 35).

The aim of this study was to investigate whether chronic (24 h) exposure of cultured rat pancreatic islets to an elevated leucine concentration (20 mM) might affect insulin secretion stimulated by glucose or other secretagogues. Leucine at 20 mM was used, because this is a concentration that gives a maximal insulin stimulation, similar to that obtained with 16.7 mM glucose in vitro (22).

Materials and methods

Materials. Crude collagenase was obtained from Boehringer Mannheim (Mannheim, Germany). Culture medium CMRL-1066, heat-inactivated fetal calf serum, glutamine, and gentamycin were obtained from Gibco (Glasgow, UK). D-[5-3H]glucose and D-[U-14C]glucose were from Amersham (Amersham, Buckinghamshire, UK). Leucine, diazoxide, glyburide, ATP, ADP, phosphoenolpyruvate, and pyruvate kinase were from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Islet preparation and culture conditions. Pancreatic islets were isolated by the collagenase method from fed male Wistar rats (200–250 g) injected intraperitoneally with 0.2 ml of a 0.2% pilocarpine solution 2 h before being killed by decapitation. Purified islets were cultured overnight at 5.5 mM glucose in CMRL-1066 medium containing 10% fetal calf serum, 2 mM l-glutamine, and gentamycin at 37°C in a 95% air-5% CO₂ atmosphere and then at 5.5 mM glucose with or without 20 mM leucine for 24 h.

Insulin secretion. Islets cultured with or without leucine were washed twice in Krebs-Ringer HEPES buffer (containing in mM: 136 NaCl, 5.4 KCl, 2.5 CaCl₂, 0.8 MgSO₄, 0.3
Na₂HPO₄, 0.4 KH₂PO₄, and 10 HEPES and 0.25% BSA, pH 7.35).

In the experiments with 30 mM KCl (high K⁺ medium), NaCl was reduced to 111.4 mM. Batches of five purified islets were then incubated in 1 ml of buffer with appropriate concentration of glucose and test substances (30-min incubation at 37°C). Insulin in the medium was then measured by radioimmunoassay. Results are expressed as insulin released in the medium (pg·islet⁻¹·30 min⁻¹).

Glucose utilization. Islet utilization of glucose was determined by measuring the formation of ³H₂O from D-[5-³H]glucose, as previously described (27). Groups of 15 islets were incubated in 40 μl of Krebs-Ringer bicarbonate buffer (KRBB) (in mM: 118 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃) supplemented with 10 μM HEPES (pH 7.4) containing 2 μCi D-[5-³H]glucose at 2.8 or 16.7 mM glucose. The incubation was carried out in 1.5-ml Eppendorf tubes inside an airtight, sealed 20-ml glass scintillation vial that contained 500 μl of distilled water. After 2 h at 37°C, the reaction was stopped by adding 0.5 M HCl (100 μl) followed by the rubber seal. Scintillation vials were then incubated overnight at 50°C, and water radioactivity was measured. Under these conditions, the recovery from known amount of ³H₂O was fairly constant, ranging from 50 to 60%.

Glucose oxidation. Glucose oxidation was determined by measuring the formation of ¹⁴CO₂ from D-[U-¹⁴C]glucose (29). Groups of 15 islets were incubated in a 1.5-ml Eppendorf tube containing 100 μl of KRBB containing 3 μCi D-[U-¹⁴C]glucose (specific activity: 302 mCi/mM) plus nonradioactive glucose to a final concentration of either 2.8 or 16.7 mM. The tubes, suspended in standard 20-ml glass scintillation vials, were gassed with O₂-CO₂ (95:5) and capped airtight. The vials were then shaken continuously at 37°C for 120 min. The islets’ metabolism was stopped by injection of 100 μl of 0.05 M amytocin A (dissolved in 70% ethanol) into the tube. This was immediately followed by an injection of 250 μl of hyamine hydroxide (New England Nuclear, Boston, MA) into the vials. ¹⁴CO₂ was liberated from the incubation medium by subsequent injection into the tubes of 100 μl of 0.4 mM Na₂HPO₄ solution adjusted to pH 6.0. After 2 h at room temperature (to allow the liberated ¹⁴CO₂ to be trapped by hyamine hydroxide), the cup was removed, 8 ml of a scintillation fluid were added to each flask, and the radioactivity was measured in a scintillation counter.

Measurements of adenine nucleotides in incubated islets. Adenine nucleotides were measured according to Dötimary et al. (9). After the incubation with 2.8 or 22.2 mM glucose, an aliquot of medium was taken for insulin assay while the tubes remained at 37°C. The islets were incubated for another 5 min. The incubation was stopped by the addition of 0.125 ml of tricarboxylic acid (TCA) to a final concentration of 5%. The tubes were then vortexed, left on ice for 5 min, and centrifuged in a microfuge (Beckman). A fraction (0.4 ml) of the supernatant was mixed with 1.5 ml of diethyl ether, and the ether phase containing TCA was discarded. This procedure was repeated three times to ensure complete elimination of TCA. The extracts were then diluted with 0.4 ml of a buffer containing 20 mM HEPES, 3 mM MgCl₂, and KOH as required to adjust pH to 7.75 (assay buffer). The diluted extracts were frozen at −70°C until the day of the assay, which started with an appropriate further dilution. ATP and ADP were assayed in triplicate by a luminometric method (18). To measure total ATP + ADP, ATP was first converted into ATP by mixing 100 μl of the diluted extract with 300 μl of assay buffer supplemented with 1.5 mM phosphoenolpyruvate and 2.3 U/ml pyruvate kinase with incubation at room temperature for 15 min. Samples with known concentrations of ADP, without ATP, were run in parallel to check that the transformation was complete. ATP was measured by the addition of 100 μl of an ATP-monitoring reagent containing luciferase and luciferin (Sigma). The emitted light was measured in a luminometer (Turner TD-20/20). To measure only ATP, the same previously described procedure was followed, except that, in the first incubation step, pyruvate kinase was lacking. ADP levels were then calculated by subtracting ATP from the total ATP + ADP. Blanks and ATP standards were run through the entire procedure, including the extraction steps.

Statistical analysis. The statistical significance of differences between means was assessed by Student’s t-test for unpaired data when only two groups of data were compared or by an analysis of variance (ANOVA) followed by Newman-Keul’s test for comparison of dose-response curve.

RESULTS

Insulin release. A 24-h incubation with increasing leucine concentrations resulted in a dose-dependent decrease of glucose-induced insulin release. The effect was significant at 20 mM leucine (915.2 ± 149.9 vs. 1,719.7 ± 156.0 pg·islet⁻¹·30 min⁻¹, means ± SE, n = 6, P < 0.01) that was then used for the further experiments (Fig. 1). In islets cultured for 24 h with 20 mM leucine, basal insulin release (in the absence of glucose) was higher than in control islets (110 ± 13.3 vs. 46 ± 12.1 pg·islet⁻¹·30 min⁻¹, n = 5, P < 0.01). The insulin response to increasing glucose concentrations was statistically different in the two groups of islets (P < 0.0001 by the ANOVA test). In particular, in islets preexposed to leucine, the secretory response to 16.7 and 22.2 mM glucose was reduced compared with control islets (711.9 ± 70°C until the day of the assay, 22.2 mM glucose, and insulin release was measured in the medium. Results are means ± SE of 6 separate experiments (** P < 0.01).

Fig. 1. Effects of 24-h culture with increasing leucine (Leu) concentrations on glucose-stimulated insulin release. Islets were cultured for 24 h in medium containing 5.5 mM glucose without control (open bar) or with (filled bars) the indicated leucine concentrations. Thereafter, batches of 5 islets were incubated for 30 min in the presence of 22.2 mM glucose, and insulin release was measured in the medium. Results are means ± SE of 6 separate experiments (**P < 0.01).
Because in islets preexposed to leucine intracellular ATP levels were significantly lower than in control islets (Table 1). In control islets, both ATP and ADP increased in response to glucose stimulation, and therefore, the ATP-to-ADP ratio clearly increased. In contrast, in leucine-preexposed islets, the ATP-to-ADP ratio was unchanged and significantly lower than in control islets (Table 1). In leucine-exposed islets, these changes in the ATP-to-ADP ratio were associated with a reduced insulin release in response to glucose (Table 1).

We also measured adenine nucleotide content and insulin secretion in the two groups of islets in response to an acute leucine (5 or 40 mM) stimulation (Table 2). Both in control islets and in islets preexposed to 40 mM leucine the ATP-to-ADP ratio was significantly lower than in control islets, respectively, n = 5).

Because in islets preexposed to leucine intracellular insulin content was reduced (47.1 ± 5 vs. 75.6 ± 9 ng/islet in control islets, n = 5, P < 0.05), we investigated whether the impaired insulin release was related to the lower intracellular insulin content. Accordingly, in islets preexposed to leucine, we tested the secretory effects of glyburide or leucine, two secretagogues that stimulate insulin secretion with mechanisms different from the mechanism of glucose. No difference between islets preexposed to leucine or control islets was observed in the secretory response to glyburide 1 μM (843.3 ± 44.2 vs. 926 ± 176.5 pg·islet⁻¹·30 min⁻¹ in control and leucine-exposed islets, respectively, n = 4) or to leucine (40 mM) itself (775.2 ± 153.6 vs. 713.2 ± 123.0 pg·islet⁻¹·30 min⁻¹, n = 4) (Fig. 3).

Glucose utilization and oxidation. To investigate whether the reduced secretory response to glucose was due to changes in glucose metabolism, we measured glucose utilization and oxidation. Preexposure to leucine significantly increased basal glucose utilization, measured at 2.8 mM glucose (59.3 ± 3.9 vs. 43.4 ± 1.9 pmol·islet⁻¹·120 min⁻¹ in control islets, P < 0.05). Glucose utilization at 16.7 mM was not different in the two groups of islets. Glucose oxidation was unaffected by islet preexposure to leucine at both 2.8 and 16.7 mM glucose (Fig. 4).

ATP-sensitive K⁺ channel-independent pathway. According to recent data (1, 3, 15), the regulation of insulin secretion by glucose involves both ATP-sensitive K⁺ (KₐTP) channel-dependent and KₐTP channel-independent pathways. Because in leucine-exposed islets glucose at high concentrations was less effective in stimulating insulin secretion (Fig. 2), we investigated also the effect of glucose on the KₐTP channel-independent pathway by blocking the KₐTP channels with 250 μM diazoxide and 30 mM K⁺. Under these conditions, glucose effect was similar in the two experimental groups: islets preexposed to leucine showed a secretory response to 22.2 mM glucose similar to that of control islets (1,818.4 ± 297.2 vs. 1,830.8 ± 264.7 pg·islet⁻¹·30 min⁻¹, n = 4) (Fig. 5).

Relationship between insulin release and adenine nucleotide levels. Because β-cell intracellular adenine nucleotides, and in particular the ATP-to-ADP ratio, is believed to play a critical role in glucose-induced insulin release (10), we measured both adenine nucleotide content and insulin secretion in the two groups of islets in the presence of basal (2.8 mM) or stimulating (22.2) glucose concentrations (Table 1). In control islets, in response to glucose concentration increase, ATP levels increased, ADP levels decreased, and as a consequence, the ATP-to-ADP ratio clearly increased. In contrast, in leucine-preexposed islets, both ATP and ADP levels increased in response to glucose stimulation, and therefore, the ATP-to-ADP ratio was unchanged and significantly lower than in control islets (Table 1). In leucine-exposed islets, these changes in the ATP-to-ADP ratio were associated with a reduced insulin release in response to glucose (Table 1).

We also measured adenine nucleotide content and insulin secretion in the two groups of islets in response to an acute leucine (5 or 40 mM) stimulation (Table 2). Both in control islets and in islets preexposed to

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**Fig. 2.** Effect of chronic exposure to high leucine on insulin release in response to glucose. Islets were cultured for 24 h in medium containing 5.5 mM glucose without (○) or with (■) 20 mM leucine. At the end of culture, batches of 5 islets were incubated for 30 min in the presence of increasing glucose concentrations. Results are presented as absolute values. Data represent means ± SE of 5 separate experiments (**P < 0.01; ***P < 0.001).

**Fig. 3.** Effect of chronic exposure to high leucine on leucine (A) or glyburide (B)-stimulated insulin release. Islets were cultured for 24 h in medium containing 5.5 mM glucose without (open bars) or with (filled bars) 20 mM leucine. Thereafter, batches of 5 islets were incubated for 30 min in the absence of glucose with either leucine (A) or glyburide (B) at the indicated concentrations, and insulin release was measured in the medium. Results are means ± SE of 4 separate experiments.
leucine, the acute stimulation with 40 mM leucine greatly stimulated insulin release but was not associated with a significant increase of ATP levels. Only in control islets did we observe an increase of the ATP-to-ADP ratio.

**DISCUSSION**

Our data indicate that the chronic exposure of rat pancreatic islets to high (20 mM) leucine levels selectively impairs glucose-induced insulin release. Insulin secretion induced by other secretagogues like glyburide or leucine itself is unaffected, despite the reduced insulin content of leucine-preexposed islets. We found that the mechanism of the selective secretory impairment of glucose-stimulated secretion in islets exposed to high leucine concentrations is unrelated to

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Values are means ± SE from 4 separate experiments. ATP/ADP, ratio of ATP to ADP. Islets were cultured for 24 h in medium containing 5.5 mM glucose ± 20 mM leucine. Batches of 5 islets were incubated in 1 ml of medium containing the indicated glucose concentrations. At the end of the incubation, insulin release was measured, and the islets were processed for measuring of adenine nucleotides. *P > 0.05 vs. control islets. †P > 0.05 vs. 2.8 mM glucose.

**Table 2. Influence of culture with high leucine on leucine-induced insulin release and adenine nucleotide content**

| Leucine            | 5 mM | 40 mM |
| Insulin release, pg·islet⁻¹·30 min⁻¹ |        |        |
| Control            | 88.9 ± 18.7 | 929 ± 124.2 |
| Leucine            | 111.9 ± 36.5 | 834.3 ± 115.3 |
| Adenine nucleotides, pmol/islet |        |        |
| Control ATP        | 46.3 ± 2.9   | 48.7 ± 1.6 |
| Control ADP        | 7.3 ± 0.7    | 5.1 ± 0.4* |
| Control ATP/ADP    | 6.3 ± 0.5    | 10.3 ± 0.6** |
| Leucine ATP        | 55.8 ± 1.9   | 58 ± 2.4 |
| Leucine ADP        | 6.4 ± 0.3    | 5.3 ± 0.7 |
| Leucine ATP/ADP    | 9 ± 0.5      | 11 ± 1.0 |

Values are means ± SE from 6 separate experiments. Islets were cultured for 24 h in medium containing 5.5 mM glucose ± 20 mM leucine. Batches of 5 islets were incubated in 1 ml of medium containing the indicated leucine concentrations. At the end of the incubation, insulin release was measured, and the islets were processed for measuring of adenine nucleotides. *P > 0.05 vs. 5 mM leucine.
the $K_{ATP}$ channel-independent pathway (14, 34) or to changes in intracellular glucose metabolism, as indicated by similar glucose utilization (marker of the first steps of cytoplasmic glycolysis) and similar glucose oxidation (marker of mitochondrial glucose metabolism) values in respect to control islets. Because leucine is known to acutely affect intracellular adenine nucleotide levels and to mimic the glucose effect on $^{38}$Rb$^+$ and $^{45}$Ca$^{2+}$ handling by pancreatic islets (22), we measured ATP and ADP levels after chronic islet exposure to leucine. In response to glucose stimulation, ADP levels were higher, and more important, the ATP-to-ADP ratio was $\sim$50% lower, in leucine-preexposed islets with respect to control islets. Because ADP may antagonize the effect of ATP on KATP channels with respect to control islets. Because ADP may antagonize the effect of ATP on KATP channels (24), and the ATP-to-ADP ratio plays a critical role and tightly regulates glucose-induced insulin release in both rodent (8) and human islets (7), these alterations may be the cause of the reduced insulin secretion in islets chronically exposed to high leucine levels. In contrast, nucleotide levels were only slightly affected by the acute leucine stimulation, despite the stimulatory effect on insulin secretion.

The cause of increased ADP levels in response to the glucose stimulation in leucine-exposed islets is unknown. An increase of ATP hydrolysis (with subsequent ADP formation) or a decrease of ATP formation from ADP (with subsequent ADP accumulation) are among the various possibilities. Exposure to high leucine levels did not affect the response of pancreatic islets to leucine itself or to the sulfonylurea glyburide. These observations suggest that the secretory pathway distal to the ATP-sensitive $K^+$ channels is normal in leucine-preexposed islets and indicate that the reduced intracellular insulin content of leucine-preexposed islets is not a limiting factor for a quantitatively normal insulin release after appropriate stimulation.

Leucine enters the pancreatic $\beta$-cell and is metabolized in the mitochondria. The mechanism by which it affects insulin release is still under debate. It has been reported that it may increase ATP levels by its own catabolism (21, 22, 25) and allosterically activate glutamate dehydrogenase (17, 36). The effect on nucleotide levels is controversial. Under our experimental conditions, we did not observe an increase of ATP levels in response to acute leucine stimulation. Moreover, in a recent study of Ronner et al. (32), the addition of amino acids did not affect the concentration of ATP and free ADP, yet amino acids greatly stimulated insulin release. The authors proposed that amino acids may regulate insulin release both by an unknown amino acid sensor and by the $K_{ATP}$ channels and that this putative sensor depends on the energized state of the $\beta$-cell.

In conclusion, we found that a prolonged exposure to high leucine levels selectively impairs glucose-induced insulin release from isolated rat pancreatic islets. This secretory alteration is associated with (and might be due to) a reduced ATP-to-ADP ratio. Because elevated amino acid levels, including leucine, have been described in both type 1 and type 2 diabetes, this mechanism might contribute to the $\beta$-cell secretory abnormalities observed in diabetic patients in poor metabolic control.

REFERENCES


