Leucine Culture Reveals That ATP Synthase Functions as a Fuel Sensor in Pancreatic β-Cells*

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Our goal was to investigate whether leucine culture affects β-cell glucose sensing. One-day culture of rat islets with 10 mM leucine had no effect on glucose-induced insulin secretion. One-week leucine culture decreased the threshold for glucose-induced insulin secretion and increased maximal insulin secretion at 30 mM glucose. Glucose-induced cytosolic free Ca2+ was increased at 1 week but not at 1 day of leucine culture. Without glucose, ATP content was not different with or without leucine culture for 1 week. With 20 mM glucose, ATP content was higher by 1.5-fold in islets cultured for 1 week with leucine than those without leucine. Microarray experiments indicated that culture of RINm5F cells with leucine increased expression of ATP synthase β subunit 3.2-fold, which was confirmed by real time reverse transcription-PCR analysis (3.0 ± 0.4-fold) in rat islets at 1 week but not after 1 day with leucine culture. Down-regulation of ATP synthase β subunit by siRNA decreased INS1 cell ATP content and insulin secretion with 20 mM glucose. Overexpression of ATP synthase β subunit in INS1 cell increased insulin secretion in the presence of 5 and 20 mM glucose. In conclusion, one-week leucine culture of rat islets up-regulated ATP synthase and increased ATP content, which resulted in elevated [Ca2+]i levels and more insulin exocytosis by glucose. Depletion of ATP synthase β subunit with siRNA produced opposite effects. These data reveal the fuel-sensing role of mitochondrial ATP synthase in the control of ATP production from glucose and the control of glucose-induced insulin secretion.

Glucose is the main secretagogue of insulin secretion from pancreatic β-cells. The mechanisms of glucose-induced insulin secretion have been studied extensively. Through glycolysis and oxidation, glucose increases pancreatic β-cell ATP/ADP ratio, which closes ATP-sensitive potassium (KATP) channels and depolarizes the cell membrane. This results in an influx of extracellular Ca2+ and increase of free cytosolic [Ca2+] that stimulates exocytosis of insulin granules (1–5). Another mechanism is KATP channel-independent and involves increased effectiveness of [Ca2+] (6, 7). In pancreatic β-cells, most of the intracellular ATP comes from the oxidation of glucose-derived pyruvate and oxidation of NADH in the mitochondria via the electron transport chain. Damage or inhibition of ATP synthesis results in β-cell dysfunction and impairs glucose-stimulated insulin secretion (8, 9). Some recent publications indicate that superoxide produced by hyperglycemia activates uncoupling-protein-2 (UCP2) and destroys the proton gradient between inner and outer mitochondrial membranes. This negatively affects the activity of ATP synthase, decreases ATP production, and impairs glucose-stimulated insulin secretion of pancreatic β-cells resulting in diabetes (8, 9). More recently, it is shown that mitochondrial metabolism sets the maximal limit of fuel-stimulated-insulin secretion in β-cells (10). These findings implicate that mitochondrial ATP synthase may play a vital role in fuel-stimulated insulin secretion of pancreatic β-cells.

Some amino acids, particularly leucine and its non-metabolizable analogue 2-amino-2-norbornanecarboxylic acid, have been known to stimulate insulin secretion from pancreatic β-cells by activation of glutamate dehydrogenase (11–15). More recently, the branched-chain amino acids, including leucine, isoleucine, and valine, have been reported to activate the mammalian target of rapamycin (mTOR)1 signaling pathway (16–19) in β-cells. Leucine stimulates protein synthesis and pancreatic β-cell proliferation via the mTOR signaling pathway at physiological concentrations (16). These studies indicate a new role of branched-chain amino acids in pancreatic β-cell biology in addition to serving as fuels or residues for protein synthesis. As the rate-limiting enzyme of glucose metabolism, it is believed that glucokinase sets a strict control on glucose metabolism in pancreatic β-cells. However, overexpression of glucokinase or hexokinase I fails to increase the maximal insulin output induced by glucose, although it decreases the threshold for glucose-induced insulin secretion in pancreatic β-cells (10, 20–22). Thus, glucokinase may not be the only rate-limiting step in glucose-induced insulin secretion of pancreatic β-cells. Other factor(s) may also be rate-limiting and contribute to the tight control of glucose-induced insulin secretion. As the key enzyme catalyzing the conversion of ADP and P, to ATP in the electron transport chain, ATP synthase (complex V) may play an important role in ATP synthesis and hence glucose-induced insulin secretion. Thus, the aim of this study was to investigate the role of ATP synthase in glucose-induced insulin secretion by attempting to change its expression level with leucine culture and siRNA.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Rat Islets—Islets were isolated from 4–6 male Sprague-Dawley rats (220–300 g) by collagenase P digestion and
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Ficol gradient centrifugation as described in detail previously (14). Before culturing, the islets were washed at least three times with leucine-free RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM gluconate, 100 μg/mL streptomycin, and 100 U/mL penicillin in one sterile conical tube. Batch units of 50–100 islets were cultured in 10 ml of RPMI 1640 with or without 10 mM leucine at 37 °C in 5% CO₂, 95% air in a 6-cm dish for 1 day or 1 week. Medium was changed every three days.

Insulin Secretion of Perifused Rat Islets—Fresh or cultured rat islets were washed once with 10 ml of pre-warmed KRB-G0 (0 mM glucose, 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 25 mM Heps (pH 7.4), and 0.1% bovine serum albumin) and perifused with KRB-G0 at 37 °C at a flow rate of 1 ml/min as described in detail previously (14). In each experiment, 30 islets of similar sizes were hand picked under microscopy and put in the perifusion chamber. The perifusion protocol was as follows: pre-perifusion in G0 for 60 min, followed by 15 min in G0, 20 min with G2, 20 min with G5, 20 min with G10, 20 min with G30, and 20 min with 30 mM KCl. Two to three islets of each condition were analyzed for each islet preparation. Data were collected over four islet preparations, and each preparation used four different rats. When calculating the increase of free cytosolic [Ca²⁺] in each experiment, the average concentration of cytosolic [Ca²⁺] between 0–5 min under KRB-G0 was set as baseline. For a given condition, the absolute value of cytosolic [Ca²⁺] was calculated as the mean ± SEM, whereas the net increase of [Ca²⁺] was obtained by subtracting the baseline value from the absolute value.

Insulin Secretion and ATP Content Measurement in Incubated Islets—Islets were washed once with 10 ml of prewarmed KRB-G0 and then incubated in 10 ml of KRB-G0 at 37 °C in 5% CO₂, 95% air for 30 min. Islets were then divided into batches of five islets and transferred into tubes containing 1 ml of KRB with 0, 5, or 20 mM glucose. Islets were then cultured in 10 ml of RPMI 1640 with or without 10 mM leucine at 37 °C in 5% CO₂, 95% air in a 6-cm dish for 1 day or 1 week. Medium was changed every three days. Islets were then washed once with 10 ml of prewarmed KRB-G0 and 200 μl of Opti-MEM I. The DNA-Lipofectamine 2000 complex was added into the well, and the plate was rocked gently back and forth. Cells were then incubated at 37 °C in 5% CO₂, 95% air for 3 h before 1 ml of complete RPMI 1640 was added into each well. The cells were cultured for 2 days.

The cells were washed twice with 1 ml of KRB-G0 before they were preincubated in 1 ml of KRB-G0 for 1 h. They were then incubated in 1 ml of KRB with 0 mM or 20 mM glucose for 1 h. KRB was transferred to the culture tube and centrifuged at 15,000 rpm for 15 min. The supernatant was collected for insulin measurement by radioimmunoassay. Lysis buffer (200 μl/well) (Roche Applied Science) was added, and the cells were scraped on ice. The cell lysate was transferred into a 1.5-ml tube and centrifuged at 4 °C at 15,000 rpm for 15 min. The ATP content in the supernatant was assayed as above, and the data was normalized to its protein content. For real time RT-PCR, 48 h after transfection the cells were washed twice with 1 ml of ice-cold phosphate-buffered saline followed by extraction of total RNA. RT-PCR was performed as described above.

Cloning of ATP Synthase β Subunit from INS1 Cells—Two specific primers, 5'-GAC GCC CCC AA ATG TTG AGT CTT GTG GGC CG T GTC-3' containing an Apal site and 5'-GAC GCC GCC CCA TCG CA GAC ATC AGC TTC TGG C-3' (sette D) were used as control.

Overexpression of ATP Synthase β Subunit in INS1 Cells—Forty-eight h after transfection, cells were washed once with cold phosphate-buffered saline. Total RNA was extracted from the cells, and real time RT-PCR was performed as above. For insulin secretion, INS1 cells were seeded in a 24-well plate and transfected with pShuttle-ATP-synthase-β subunit. 48 h after transfection, the cells were washed twice with 1 ml of prewarmed KRB and preincubated in 1 ml of KRB-G0 for 1 h. Cells were then incubated in 1 ml of KRB containing 0, 5, or 20 mM glucose for 1 h, respectively. The cells transfected with empty pShuttle vector were used as control.

RESULTS

Culturing Islets with Leucine Increases Glucose-induced Insulin Secretion—To investigate whether leucine can affect glucose-induced insulin secretion, rat islets were cultured in complete RPMI 1640 (11 mM glucose, 10% fetal bovine serum) with or without 10 mM leucine for 1 day or 1 week. The time course of perifusion was shown in Fig. 1, and the averaged insulin secretion during each condition is presented in Fig. 1B. After a 1-day culture, both untreated (Leu0) and leucine-treated (Leu10) islets had similar basal insulin secretion in the absence of glucose (19.5 ± 4.7 nanounits/islet-min versus 13.7 ± 2.2 nanounits/islet-min, p > 0.05). With 5 mM glucose, insulin secretion of untreated and leucine-treated islets was 21.6 ± 4.1 nanounits/islet-min versus 22.2 ± 8.3 nanounits/islet-min (p > 0.05). With 5 mM glucose, insulin secretion of untreated and leucine-treated islets was 44.4 ± 26.4 nanounits/islet-min versus 42.6 ± 17.1 nanounits/islet-min (p > 0.05). With 10 mM glucose, insulin secretion of untreated and leucine-treated islets was 62.9 ± 20.1 nanounits/islet-min versus 87.9 ± 38.3 nanounits/islet-min (p < 0.05). With 30 mM glucose, insulin secretion of untreated and leucine-treated islets was 75.8 ± 8.8 nanounits/islet-min.
A week (B) were perfused simultaneously. The data are mean ± S.E. of six independent experiments from at least 12 different rats. *, p < 0.05 comparing treated and untreated islets. #: p < 0.05 comparing treated and fresh islets.

The average insulin secretion of the treated islets (470.0 ± 137.5 nanounits/islet-min, p < 0.05) was 3.1-fold higher than that of untreated islets (154.3 ± 25.5 nanounits/islet-min, p < 0.05). With 30 mM KCl, insulin secretion of treated islets (214.1 ± 26.5 nanounits/islet-min, p < 0.05) was 3.1-fold higher than that of untreated islets (90.3 ± 9.5 nanounits/islet-min). With 30 mM glucose, insulin secretion of the treated islets (470.0 ± 137.5 nanounits/islet-min, p < 0.05) was 5.9-fold higher than that of untreated islets (76.0 ± 12.9 nanounits/islet-min, p < 0.05). The KCl-induced insulin secretion in rat islets cultured without leucine was similar to that of fresh islets (Figs. 1 and 2). Thus, treatment of rat islets with 10 mM leucine decreased the threshold for glucose-induced insulin secretion and increased the maximal insulin output significantly in a time-dependent manner.

**Culturing Islets with Leucine Increases Glucose-induced Rise of Free Cytosolic \( \text{Ca}^{2+} \)——**Free cytosolic \( \text{Ca}^{2+} \) plays a vital role in exocytosis of insulin granules in pancreatic \( \beta \)-cells. The effects of leucine treatment on glucose-induced changes of islet-free cytosolic \( \text{Ca}^{2+} \) were also assayed. The basal \( \text{Ca}^{2+} \) level without glucose of 1-day-cultured islets (68 ± 7 nM) was the same as that of fresh islets (76 ± 5 nM, p > 0.05). The KCl-induced \( \text{Ca}^{2+} \) change was smaller in islets cultured for 1 day without (126 ± 22 nM) or with (125 ± 10 nM) leucine than the change in fresh islets (204 ± 36 nM, p < 0.05, Fig. 3A). However, there was no significant difference between the two culture conditions with all glucose concentrations tested or with 30 mM KCl (Figs. 3A and 4A). Both 1-day-treated and untreated
islets had a smaller Ca$^{2+}$ response and a smaller insulin secretion peak response to KCl than did fresh islets (Figs. 1A and 3A). Therefore, there was no significant difference in the glucose-induced change of free cytosolic [Ca$^{2+}$] between 1-day-treated islets and untreated islets.

Basal [Ca$^{2+}$] without glucose was the same in islets cultured for 1 week with (72 ± 4 nM) or without (68 ± 6 nM) 10 mM leucine ($p > 0.05$). With 2 mM glucose, free cytosolic [Ca$^{2+}$] of treated islets began to increase by 3 ± 1 nM ($p < 0.05$) compared with basal level, whereas that of untreated islets was the same as baseline (68 ± 6 nM) (Fig. 4B). With 5 mM glucose, free cytosolic [Ca$^{2+}$] of treated islets increased by 16 ± 4 nM ($p < 0.05$) when compared with baseline, whereas that of untreated islets was still the same as baseline (Fig. 4B). Glucose at 10 mM increased free cytosolic [Ca$^{2+}$] by 33 ± 6 nM in treated islets but only 3 ± 1 nM in untreated islets. Glucose at 30 mM increased free cytosolic [Ca$^{2+}$] by 47 ± 6 nM ($p < 0.05$) in treated islets but only by 9 ± 1 nM in untreated islets. Treated islets also had a significantly larger increase of [Ca$^{2+}$] (80 ± 7 nM) than did untreated islets (33 ± 2 nM) when stimulated with 30 mM KCl compared with baseline (Figs. 3B and 4B). Therefore, there was a significant elevation of glucose-induced free cytosolic [Ca$^{2+}$] in 1-week-treated islets compared with untreated islets (Figs. 3, B, C, and 4B). The dose-response curve of glucose-induced cytosolic Ca$^{2+}$ rise was shifted to the left by leucine treatment.

**Effect of Leucine Culture on Islet ATP Content and Insulin Secretion**—In islets cultured for 1 day without leucine, ATP content was 2.2 ± 0.2 pmol/islet without glucose and 3.2 ± 0.6 pmol/islet with 5 mM glucose or 3.1 ± 0.3 pmol/islet with 20 mM glucose ($p > 0.05$). In 1-day leucine-treated islets, ATP was 1.8 ± 0.3 pmol/islet without glucose and 2.8 ± 0.5 pmol/islet ($p > 0.05$) at 5 mM glucose or 2.9 ± 0.4 ($p > 0.05$) pmol/islet at 20 mM glucose (Fig. 5A). We did not detect significant difference in basal insulin secretion without glucose in the same islets cultured for 1 day with leucine (337 ± 81 nanounits/islet-h) or without leucine (243 ± 55 nanounits/islet-h). Insulin secretion tended to be higher in the presence of 20 mM glucose, and there was no difference between the two culture conditions (1045 ± 425 nanounits/islet-h versus 1123 ± 290 nanounits/islet-h) (Fig. 5B).

In islets cultured for 1 week without leucine, ATP content was increased by 1.6-fold at 5 mM glucose (2.6 ± 0.3 pmol/islet, $p < 0.01$) and by 1.6-fold at 20 mM glucose (2.5 ± 0.1 pmol/islet, $p < 0.01$) when compared with ATP content without glucose.
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4.1 ± 0.9% (without leucine) (p > 0.05) when incubated without glucose, 26.5 ± 8.0% (with leucine) versus 6.8 ± 3.2% (without leucine, p < 0.05) at 5 mM glucose, and 49.3 ± 3.1% (with leucine) versus 15.8 ± 3.7% (without leucine, p < 0.05) at 20 mM glucose.

Effects of Short Term Leucine Treatment on ATP Content and Insulin Secretion—To test the possibility that leucine affects ATP content and insulin secretion because of its role as a fuel or as an allosteric regulator of glutamate dehydrogenase, islets were cultured for 1 week without leucine and then treated with 10 mM leucine for 30 min (Leu30min) before ATP content and insulin secretion were assayed (Fig. 7). The addition of 10 mM leucine for 30 min did not affect glucose-induced ATP content and insulin secretion. ATP content of Leu30min islets increased from 1.5 ± 0.2 pmol/islet at 0 mM glucose to 2.5 ± 0.3 pmol/islet at 5 mM glucose, and 2.5 ± 0.2 pmol/islet at 20 mM glucose. ATP content of untreated islets increased from 1.5 ± 0.2 pmol/islet to 2.6 ± 0.3 pmol/islet (p > 0.05) and 2.5 ± 0.2 pmol/islet (p > 0.05). The values for untreated and Leu30min islets were not different. Leu30min and untreated islets had similar insulin secretion, which was 433 ± 51 nanounits/islet-h (Leu30min) versus 303 ± 49 nanounits/islet-h (untreated, p > 0.05) without glucose, 800 ± 483 nanounits/islet-h (Leu30min) versus 650 ± 327 nanounits/islet-h (untreated, p < 0.05) with 5 mM glucose, and 1413 ± 467 nanounits/islet-h (Leu30min) versus 1847 ± 377 nanounits/islet-h (untreated, p < 0.05) with 20 mM glucose. Culture of islets for 1 week with 10 mM β-leucine did not affect basal ATP level (1.4 ± 0.3 pmol/islet) or glucose-induced ATP content at 5 mM (2.4 ± 0.2 pmol/islet) or at 20 mM (2.5 ± 0.3 pmol/islet). In the same islets insulin secretion was the same as untreated islets when incubated without glucose (323 ± 41 versus 303 ± 49 (untreated islet) nanounits/islet-h, p > 0.05), with 5 mM glucose (732 ± 204 versus 650 ± 327 (untreated islet) nanounits/islet-h, p > 0.05) or with 20 mM glucose (1893 ± 325 versus 1847 ± 377 (untreated islet) nanounits/islet-h, p > 0.05).

Leucine Culture Increases ATP Synthase β Subunit Expression—Most of the ATP in pancreatic β-cells comes from oxidation of NADH in the mitochondria by the electron transport chain. ATP is synthesized directly by complex V (ATP synthase). Our preliminary microarray experiments show that treatment with leucine up-regulated ATP synthase β subunit by 3.2-fold in the RINm5F cell line. In the present study, we focused on analyzing ATP synthase (β chain) expression by real time RT-PCR. Our results showed that in 1-day-treated islets, the ATP synthase β subunit mRNA level was 1.4- ± 0.2-fold higher than that of untreated islets, which was not statistically significant (p > 0.05, Fig. 8A). In 1-week-treated islets, the ATP synthase β subunit mRNA level was 3.0- ± 0.2-fold (p < 0.01) higher than that of untreated islets. Our results also showed that culture with 10 mM β-leucine for 1 week failed to affect the ATP synthase β subunit mRNA level in rat islets (Fig. 8B). To test if leucine affects gene expression via the mTOR signaling pathway, rat islets were cultured with 10 nM rapamycin or 10 nM rapamycin plus 10 mM leucine for 1 week. The results showed that 10 nM rapamycin alone had no significant effect on ATP synthase β subunit mRNA level. Rapamycin failed to block the leucine effects on gene expression when tested at 10 nM (Fig. 8B) or 100 nM (data not shown).
**Down-regulation of ATP Synthase β Subunit Using siRNA Cassettes**—Real-time RT-PCR analysis showed that cassette A reduced significantly (p < 0.05) the ratio of Ct value of ATP synthase β subunit mRNA level by about 20% when compared with control. The effect of cassette B was not significant (p > 0.05). The transfection efficiency of GFP-containing plasmid in INS1 cells was about 30–40%. Assuming that the transfection of siRNA cassette DNA had similar efficiency and that the ATP synthase β subunit was decreased by 50% in the transfected cells, the difference of Ct values between these cells and the control cells would not be more than 1. It is difficult to detect such small differences by real-time RT-PCR. At 48 h post transfection, cassette A reduced glucose-induced ATP content significantly (p < 0.05) by 42 and 48% at 0.15 and 0.30 μg/well, respectively (Fig. 9B). Cassette B reduced glucose-induced ATP content significantly (p < 0.05) by 48 and 43% at 0.15 and 0.30 μg/well, respectively (Fig. 9B). The siRNA cassette effects on ATP content also seemed to reach a maximum at 0.15 μg/well. In the same cells, siRNA cassette A reduced 20 mM glucose-induced insulin secretion significantly (p < 0.05) by 27 and 24% at 0.15 and 0.30 μg/well, respectively (Fig. 9A). Cassette B reduced glucose-induced insulin secretion significantly by 27 and 31% at doses of 0.15 and 0.30 μg/well, respectively. The effect seemed to reach a maximum at 0.15 μg/well. The effects on ATP content correlated well with those on insulin secretion. Cassettes C and D did not cause significant changes in ATP content and insulin secretion.

**Effects of ATP Synthase β Subunit Overexpression on Insulin Secretion**—Real-time RT-PCR assay indicated that transfection of pShuttle-ATPase-β subunit increased ATP synthase β subunit mRNA level by 2.3 ± 0.2-fold (p < 0.05). For insulin secretion, all data were normalized to the control value under G0 (control value is 1). Overexpressing cells (0.95 ± 0.03) had similar basal insulin secretion as the control cells (1.00 ± 0.02, p > 0.05) in the absence of glucose. In the presence of 5 or 20 mM glucose, overexpressing cells (1.47 ± 0.07, p < 0.05 or 1.85 ± 0.1, p < 0.05, respectively) secreted more insulin than controls (1.25 ± 0.087 or 1.54 ± 0.08, respectively).

**DISCUSSION**

**Role of β-Cell Mitochondria in Fuel Sensing**—Pancreatic β-cells secrete insulin in response to glucose through metabolism and production of ATP. Though glycolysis produces small amounts of ATP, the vast majority of ATP is produced through the mitochondrial oxidation of pyruvate, which is produced as the glycolytic product of glucose. The increase in ATP/ADP ratio closes ATP-sensitive potassium channels and causes membrane depolarization. This opens voltage-dependent calcium channels to allow Ca²⁺ influx. The increase of cytosolic Ca²⁺ triggers exocytosis of insulin granules. The amount of secreted insulin is controlled tightly by the glucose concentration. The mechanisms of this control (or glucose sensing) have been the subject of extensive studies (1–7). It is now believed that the high Kₘ hexokinase IV (glucokinase) is the rate-limiting enzyme for glucose phosphorylation and glucose metabolism in the β-cell. However, the sensors for other fuels, such as amino acids, are still unclear. It has recently been shown that a large increase of glycolytic intermediates that bypass the restriction of glucokinase does not affect insulin secretion, whereas mitochondrial membrane hyperpolarization strongly correlates with limitations of insulin secretion (10). It has been suspected that factors downstream of glucokinase play important roles in the restriction of metabolism and insulin secretion in the presence of large amounts of glycolytic intermediates. It is suggested that limitations imposed by the mitochondrial membrane potential are likely related to the thermodynamics of generation of the proton gradient across the mitochondrial inner membrane, which are created by the mitochondrial electron transport chain complex. The limitations of proton gradient formation thus limit ATP production.
It has been shown that inhibitors of the electron transport chain and ATP production all impair insulin secretion (10, 26–30). An increase in mitochondrial metabolism by a Na'-Ca²⁺ exchanger inhibitor also potentiates glucose-induced insulin secretion in rat islets (31). It is thus proposed that the mitochondrial proton gradient sets the limit of insulin secretion.

For the first time, our study demonstrates that culture of pancreatic β-cells with leucine increases their glucose sensitivity as demonstrated by the leftward shift in the threshold of insulin secretion and of cytosolic Ca²⁺ rise and by the increase in the maximal response of insulin secretion and of cytosolic Ca²⁺ rise. Our findings contradict those reported by Anello et al. (32) that 24-h culture with 20 mM leucine impairs glucose-induced insulin secretion and increases ADP level in rat islets. Their findings also show a decrease in ATP/ADP ratio. The lack of changes in the ATP level and glucose utilization and oxidation in their study is difficult to explain. However, we did not detect any changes in glucose-induced insulin secretion, cytosolic Ca²⁺, or ATP level after 24 h culture with leucine in rat islets. In contrast, changes in glucose effects on insulin secretion, cytosolic Ca²⁺, and ATP content were detected after 1 week of culture with leucine. The insulin secretion induced by 30 mM glucose was increased dramatically. Thus, the alteration of the signal transduction is located more upstream of ATP synthase/ATP synthase subunit. ATP synthase subunit was up-regulated by 3.2-fold in leucine-treated RINm5F cells. Changes of other metabolic enzymes have not been detected in these experiments. This enzyme is also called mitochondrial electron transport chain complex V and plays an important role in the production of ATP (33, 34). Energy from the proton gradient is used to generate ATP as the collapse of the gradient through the ATP synthase drives the ATP synthetic machinery (8, 34). Though the screening experiments were first performed in RINm5F cells, the increase in the ATP synthase β subunit mRNA level was subsequently confirmed in rat islets. Rat islet ATP synthase β subunit expression was increased by leucine treatment for 1 week but not for 1 day, which is in agreement with insulin secretion, cytosolic Ca²⁺, and ATP level measure.
The data was normalized to its protein content (for 1 h. The supernatant was collected for insulin secretion (A), and the
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(39). The human gene is identical to the rat gene (P19044)
pathways rather than their well known role in serving as
nuclear stress (up-regulation of two heat shock proteins) and
perturbations in ATP synthesis and mitochondrial metabolisms (down-regulation of ATP synthase β subunit and creatine kinase B). It is demonstrated that the catalytic β subunit of ATP synthase is phosphorylated in vivo and that the levels of a down-regulated ATP synthase β subunit phosphoisoform in diabetic muscle correlated inversely with fasting plasma glucose levels. These data suggest that ATP synthase β subunit plays a role in the regulation of ATP synthase and that alterations in the regulation of ATP synthesis and cellular stress proteins may contribute to the pathogenesis of type-2 diabetes (39). The human gene is identical to the rat gene (P19044) under investigation in this study except for three residues. If it is true that the decrease of ATP synthase protein level and the decrease of phosphorylation level of this enzyme are related to the development of type-2 diabetes (39), the same enzyme in the pancreatic β-cells of type-2 diabetic human may have similar defects, which may result in impaired glucose-induced insulin secretion. A recent study by Deng et al. shows that the threshold of glucose-induced insulin secretion is higher in the islets of type-2 diabetic human islets compared with non-diabetic islets. The amount of insulin secretion during a ramp of glucose stimulation is much smaller in diabetic than non-diabetic islets (40).

**Target(s) of Leucine—** Recently, the role of amino acids in regulating protein translation by metabolism-linked signaling pathways rather than their well known role in serving as precursors for protein synthesis has become the subject of extensive studies (16, 18, 19, 41). Leucine has been reported to mediate the phosphorylation of eukaryotic initiation factor 4-E-binding protein-1 (eIF-E-BP1), which is also designated as phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-1). Leucine also causes the phosphorylation of p70S6 kinase (p70s6k) through mTOR signaling pathway in a

**Type-2 Diabetes and Mitochondrial Function—** Type-2 diabetes is characterized by hyperglycemia, insulin resistance, and impaired glucose-stimulated insulin secretion from pancreatic β-cells. The exact mechanisms of the pathogenesis of type-2 diabetes are still unclear and likely involve many different genes. As the rate-limiting enzyme of glucose metabolism, glucokinase is considered the glucose sensor in pancreatic β-cells. Loss of function mutations of glucokinase causes diabetes (35, 36). However, recent studies demonstrated that the metabolic impairment in type-2 diabetics may also involve other enzymes. It has been shown that NADH-O2 oxidoreductase activity and citrate synthase activity of skeletal muscle are lower in type-2 diabetics than lean subjects and non-diabetic obese subjects (37). The same study demonstrates that the skeletal muscle mitochondria are smaller in type-2 diabetic and obese subjects than those in muscle from lean volunteers (37). It has been shown that type-2 diabetic subjects have decreased oxidative enzyme activity and increased lipid content in skeletal muscles (38). A recent study (39) using a proteomics approach has identified eight potential protein markers for type-2 diabetics in human skeletal muscle biopsies in the fasting state. The observed changes in protein expression indicate increased cellular stress (up-regulation of two heat shock proteins) and impaired glucose-stimulated insulin secretion from pancreatic β-cells of type-2 diabetic human may have similar defects, which may result in impaired glucose-induced insulin secretion. A recent study by Deng et al. shows that the threshold of glucose-induced insulin secretion is higher in the islets of type-2 diabetic human islets compared with non-diabetic islets. The amount of insulin secretion during a ramp of glucose stimulation is much smaller in diabetic than non-diabetic islets (40).

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variety of cell lines and primary cells (18, 19). Leucine enhances protein synthesis via the mTOR signaling pathway in pancreatic β-cells. Some important transcriptional regulators might be among those affected proteins. We thus propose that leucine might regulate β-cell glucose sensitivity by regulating some key metabolic genes involved in glucose metabolism and ATP synthesis. A genome-wide screening of 40,000 genes in RINm5F cells using microarray analysis showed that leucine culture up-regulated ATP synthase at the mRNA level. This is a key enzyme controlling ATP synthesis within the mitochondria. ATP synthase might not be the only gene affected by leucine. The direct target of leucine is still unknown. However, its effects are structure-dependent, because D-leucine has no effect. Presumably, leucine may interact with one or multiple transcription factors or regulatory protein(s) to affect the transcription of multiple genes.

To preclude the possibility that the increase of ATP content and insulin secretion in leucine-treated islets resulted from the fuel effects of leucine, the islets were cultured for 1 week without leucine and then treated with 10 mM leucine for 30 min before experiments. Then, ATP content and insulin secretion were assayed. The experimental results showed that a short term leucine treatment did not affect glucose sensing and thus exclude the fuel effects of leucine (Fig. 7).

In conclusion, leucine culture up-regulates ATP synthase β subunit and ATP production in a time-dependent manner. Mitochondrial ATP synthesis is under the control of ATP synthase and plays a vital role in glucose-induced insulin secretion in pancreatic β-cells.

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