

Leucine Regulation of Glucokinase and ATP Synthase Sensitizes Glucose-Induced Insulin Secretion in Pancreatic β -Cells

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We have recently shown that leucine culture upregulates ATP synthase β -subunit (ATPS β) and increases ATP level, cytosolic Ca^{2+} , and glucose-induced insulin secretion in rat islets. The aim is to test whether glucokinase expression is also affected in rat islets and its role in glucose sensitization during leucine culture. Leucine culture increased glucose-induced NAD(P)H level at 1 and 2 days but not at 1 week. The half-maximal effective concentration of the glucose response curve for NAD(P)H was left-shifted from 5–7 to 2–3 mmol/l. The effect was dose dependent and rapamycin insensitive. Leucine culture did not affect glycer-aldehyde effects on NAD(P)H. Leucine pretreatment for 30 min had no effects on NAD(P)H levels. Leucine culture for 2 days also increased glucose-induced cytosolic Ca^{2+} elevation, ATP level, and insulin secretion. Leucine increase of glucokinase mRNA levels occurred as early as day 1 and lasted through 1 week. That of ATPS β did not occur until day 2 and lasted through 1 week. Leucine effects on both mRNAs were dose dependent. The upregulation of both genes was confirmed by Western blotting. Leucine culture also increased glucose-induced insulin secretion, ATP level, glucokinase, and ATPS β levels of type 2 diabetic human islets. In conclusion, leucine culture upregulates glucokinase, which increases NAD(P)H level, and ATPS β , which increases oxidation of NADH and production of ATP. The combined upregulation of both genes increases glucose-induced cytosolic Ca^{2+} and insulin secretion. *Diabetes* 55: 193–201, 2006

Glucose increases the pancreatic β -cell ATP-to-ADP ratio via glycolysis and oxidation. ATP-sensitive K^+ channels are closed, and the plasma membrane is depolarized. This results in an influx of extracellular Ca^{2+} and an increase of free

cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]$) that stimulates exocytosis of insulin granules (1–5). Insulin secretion can also be increased via an amplifying pathway that involves increased effectiveness of cytosolic $[\text{Ca}^{2+}]$ (6–8). As the rate-limiting enzyme of glycolysis, 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 (9–12). 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. Alternations in ATP synthesis results in changes of β -cell function and glucose-stimulated insulin secretion (13,14). Mitochondrial metabolism may set the maximal limit of fuel-stimulated insulin secretion in β -cells by controlling the rate of ATP production (10). Inhibition of mitochondrial gene transcription, and presumably oxidation of glucose-derived pyruvate in the mitochondria, is found to suppress glucose-induced insulin secretion in βHC9 cells (15,16). 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 inorganic phosphate (Pi) to ATP by proton gradient in the electron transport chain, ATP synthase (complex V) may play an important role in ATP synthesis and hence glucose-induced insulin secretion. It had been established that all of the catalytic sites of F1 ATP synthase are located either exclusively on the β -subunits or at interfaces between β - and α -subunits (17–19), indicating the importance of β -subunit.

We have recently demonstrated that leucine culture upregulates ATP synthase β -subunit (ATPS β) and sensitizes glucose-induced insulin secretion in rat pancreatic islets (20). A selective overexpression of this protein using plasmid transfection in INS1 cells produces similar effects, whereas depletion of this protein using small interfering RNA technique produces opposite effects. We propose that the changes in ATPS β alter the rates of ATP production, Ca^{2+} influx, and insulin secretion. In our previous study, no change in glucokinase mRNA expression was found in RINm5F cells cultured with 10 mmol/l leucine for 4 or 24 h (20). However, the possibility of expression changes of glucokinase in primary islet β -cells has not been tested, and glucokinase involvement in sensitizing glucose effects by leucine culture is unknown (20). Thus, the aim of this

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ATPS β , ATP synthase β -subunit; KRB, Krebs-Ringer bicarbonate.

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study was to investigate the roles of glucokinase and ATP synthase in enhanced glucose-induced insulin secretion in rat and human islets cultured with leucine.

RESEARCH DESIGN AND METHODS

Islets were isolated from 4–6 male Sprague-Dawley rats (220–300 g) by collagenase P digestion and Ficoll gradient centrifugation, as described in detail previously (20,21). Before culture, the islets were washed at least three times with leucine-free RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 μ g/ml streptomycin, and 100 IU/ml penicillin in one sterile conical tube. Batches of 50–100 islets were cultured in 10 ml RPMI 1640 with or without 10 mmol/l leucine at 37°C in 5% CO₂/95% air in a 6-cm dish for 1 day, 2 days, or 1 week. Medium was changed every 3 days.

Culture of INS1 cell line. INS1 cells (passage 10–15) were cultured in RPMI 1640. The cells were split weekly and the medium changed twice weekly. For insulin secretion, the cells cultured with or without leucine were washed twice with prewarmed Krebs-Ringer bicarbonate (KRB) without glucose and then preincubated in KRB without glucose for 1 h, followed by incubating in indicated conditions for 1 h. The supernatant was centrifuged and collected for insulin measurement.

Culture of human islets. Human islets (provided by the Juvenile Diabetes Research Foundation [JDRF]-Penn Islet Transplantation Center) were isolated from donor pancreas, as described previously (22). Batches of 200–300 human islet equivalents (IE) were cultured in 10 ml CMRL 1066 (supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 μ g/ml streptomycin, and 100 IU/ml penicillin) with or without 10 mmol/l leucine at 37°C in 5% CO₂/95% air in a 6-cm dish. Medium was changed every 3 days. Donors were one woman, 63 years old, 127 kg, who died of cerebrovascular accident/intracerebral hemorrhage and who had a 5-year type 2 diabetes history, and one man, 33 years old, 159 kg, who died of anoxia and who had HbA_{1c} 6.4%, which is higher than normal, with no official history of diabetes.

NAD(P)H fluorescence measurement. Islets were washed once with 10 ml prewarmed KRB (115 mmol/l NaCl, 24 mmol/l NaHCO₃, 5 mmol/l KCl, 1 mmol/l MgCl₂, 2.5 mmol/l CaCl₂, 25 mmol/l HEPES, pH 7.4, and 0.1% BSA) without glucose and preincubated in 3 ml KRB without glucose at 37°C in 5% CO₂/95% air for 30 min. The islets were fixed on the tip of a glass micropipette by slight suction and perfused with KRB at 37°C at a flow rate of 1 ml/min. The perfusion protocol was as follows: 5 min for each of these conditions sequentially: 0, 2, 5, 10, and 30 mmol/l glucose and 30 mmol/l KCl. The NAD(P)H fluorescence level was monitored with an excitation wavelength of 320 nm and an emission wavelength at 480 nm, using a Nikon microscope with SimplePCI software (21,23,24). In each condition, 2–3 islets were perfused simultaneously. The same protocol was repeated at least four times. The averaged fluorescence value from 0 to 5 min under 0 mmol/l glucose was set as the baseline value (100%), and all NAD(P)H fluorescence values were normalized to the 0 mmol/l glucose level. The NAD(P)H level under indicated conditions are also presented as the averaged value during the entire 5-min period of perfusion.

Free cytosolic [Ca²⁺] measurement. Islets were loaded in 3 ml KRB without glucose with 2 μ mol/l fura-2/AM and 0.2 mg/ml pluronic F-127 at 37°C in 5% CO₂/95% air for 30 min. Islets were fixed and perfused as in NAD(P)H experiments (20,21). When calculating the increase of free cytosolic [Ca²⁺] in each experiment, the average concentration of cytosolic [Ca²⁺] between 0 and 5 min under KRB without glucose was set as baseline. For a given condition, the absolute value of cytosolic [Ca²⁺] was also calculated as the mean concentration during the entire 5-min period of stimulation, 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 prewarmed KRB without glucose and then incubated in 10 ml KRB without glucose at 37°C in 5% CO₂/95% air for 30 min. Islets were then divided into batches of five and transferred into tubes containing 1 ml KRB with 0, 5, or 20 mmol/l glucose. Islets were incubated at 37°C in 5% CO₂/95% air for 1 h in a shaking water bath. After incubation, 950 μ l of supernatant was removed for insulin radioimmunoassay. Lysis buffer (450 μ l) for ATP extraction (Roche, Indianapolis, IN) was added. Islets were sonicated on ice for 40 pulses at 60% of maximal output (200 W). Sonicated samples were then frozen-thawed twice on dry ice to ensure complete lysis of cells. Islet lysate insulin content was assayed by radioimmunoassay at the Diabetes Center of the University of Pennsylvania. Islet lysate ATP content was measured using an HS II ATP bioluminescence assay kit (Roche), as detailed previously (20), using 50 μ l in triplicate. Data were collected from at least 15 batches of islets in four independent experiments.

Quantitative real-time RT-PCR assay. Total RNA was extracted from islets using an RNeasy mini kit (Qiagen), and agarose gel electrophoresis was used

to analyze the quality of total RNA. Real-time RT-PCR was performed in duplicate with One-step RT-PCR Master Mix reagents kit (TaqMan). The cycling conditions for RT-PCR were as follows: 48°C for 30 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Quantitative values were obtained as threshold PCR cycle number (Ct) when the increase in fluorescent signal of PCR product showed exponential amplification. Target gene mRNA level was normalized to that of β -actin in the same sample. In brief, the relative expression level of the target gene compared with that of β -actin was calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$. The ratio of relative expression of the target gene in leucine-treated islets to that of untreated islets was then calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{treated islet}} - \Delta Ct_{\text{nontreated islet}}$ (20,25).

Western blot assay. Islets or INS1 cells were lysed in Chaps cell extraction buffer (Cell Signaling) by freezing and thawing on dry ice three times. Lysed solution was centrifuged at 13,000 rpm at 4°C for 10 min, and the pellet was disposed. Electrophoresis was performed at 170 V using 4–20% gradient gel (Pierce). Proteins in the gel were transferred to a Hybond-C Extra membrane (Amersham Biosciences) at 120 V for 2 h at 4°C. The membrane was washed once with 10 ml of 1 \times TBST (1.2 g/l Tris base, 5.84 g/l NaCl, 0.1% Tween-20, pH 7.5) before blocking in 20 ml blocking buffer (1 \times TBST containing 5% skim milk) at room temperature for 1 h. The membrane was incubated at 4°C overnight with 1:500 glucokinase polyclonal antibody (SC-7908; Santa Cruz Biotechnology) or 1:2,000 polyclonal antibody against rat ATP5 β (provided by David A. York) (26). The membrane was washed five times with 1 \times TBST and incubated in 1:5,000 peroxidase-conjugated donkey anti-rabbit antibodies at room temperature for 1 h before washing as above, and then it was developed with enhanced chemiluminescence. After assay of the target proteins, the membrane was stripped with 0.2 N NaOH, and Western blot was repeated using β -actin (1:500) antibody (SC-7210; Santa Cruz Biotechnology).

Quantification of protein. Enhanced chemiluminescence Western blot film was scanned, and the relative amount of target protein was determined using ImageQuant (version 5.2; Amersham Biosciences). All data were normalized to the control value. The result of each experiment represented the average of three independent scans. The data were collected from at least three independent experiments.

Statistical analysis. Data are the means \pm SE. Statistical significance of differences between groups was analyzed by unpaired Student's *t* test or by one-way ANOVA when more than two groups were compared.

RESULTS

Leucine culture sensitized glucose stimulation of NAD(P)H level of rat islets. NAD(P)H level of fresh islets at 2, 5, 10, and 30 mmol/l glucose was 116 ± 2 , 126 ± 2 , 133 ± 2 , and $141 \pm 8\%$ of the baseline at 0 mmol/l glucose, respectively. After 1 day of culture, the NAD(P)H level of leucine-treated islets at 2, 5, 10, and 30 mmol/l glucose was 147 ± 6 , 157 ± 7 , 167 ± 7 , and $181 \pm 8\%$ of its baseline at 0 mmol/l glucose, respectively, whereas that of untreated islets was 119 ± 1 , 128 ± 2 , 139 ± 3 , and $151 \pm 3\%$ of its baseline, respectively (Fig. 1A). After 2 days of culture, NAD(P)H level of untreated and leucine-treated islets was 119 ± 6 versus $150 \pm 5\%$ at 2 mmol/l glucose, 129 ± 5 versus $160 \pm 4\%$ at 5 mmol/l glucose, 137 ± 5 versus $170 \pm 5\%$ at 10 mmol/l glucose, and 148 ± 6 versus $184 \pm 4\%$ at 30 mmol/l glucose of their corresponding baselines, respectively (Fig. 1B). The untreated islets had similar NAD(P)H levels as the fresh islets at all glucose concentrations tested. After 1 week of culture, the NAD(P)H level of untreated and leucine-treated islets was 118 ± 8 versus $122 \pm 2\%$ at 2 mmol/l glucose, 125 ± 6 versus $125 \pm 4\%$ at 5 mmol/l glucose, 132 ± 3 versus $127 \pm 3\%$ at 10 mmol/l glucose, and 150 ± 3 versus $132 \pm 4\%$ ($P < 0.05$) of their corresponding baselines (Fig. 1C). Leucine culture thus increased glucose-induced NAD(P)H level at day 1 and day 2, but not at 1 week.

Leucine effects on glucose-stimulated NAD(P)H were dose dependent in rat islets. Islets were cultured with 0, 1, 2, 5, and 20 mmol/l leucine for 2 days before NAD(P)H measurement. At 2 mmol/l glucose, NAD(P)H of 0, 1, 2, 5, and 20 mmol/l leucine was 111 ± 3 , 120 ± 2 , 122 ± 2 , 132 ± 5 , and $145 \pm 5\%$ of the baseline at 0 mmol/l glucose,

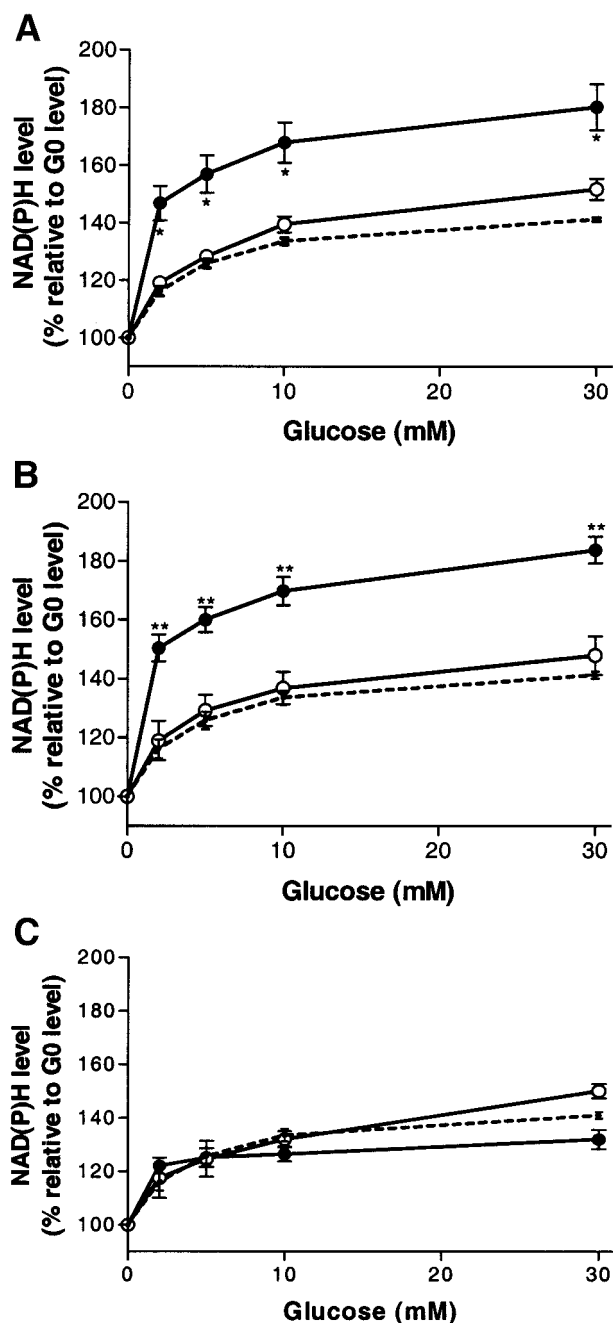


FIG. 1. Effects of leucine culture on NAD(P)H level of rat islets. We measured NAD(P)H levels of fresh islets (dashed line) or islets cultured in RPMI 1640 with (●) or without (○) 10 mmol/l leucine for 1 day (A), 2 days (B), and 1 week (C), respectively. Data are normalized to the basal line as described in RESEARCH DESIGN AND METHODS. * $P < 0.05$, ** $P < 0.01$ when compared with the corresponding control point. Data are means \pm SE of at least eight islets in at least four independent experiments from four different rats. G0, 0 mmol/l glucose.

respectively. At 5 mmol/l glucose, it was 118 ± 5 , 133 ± 4 , 132 ± 2 , 143 ± 5 , and $154 \pm 6\%$ of the baseline, respectively. At 10 mmol/l glucose, it was 131 ± 5 , 151 ± 8 , 159 ± 8 , 160 ± 7 , and $168 \pm 7\%$ of the baseline, respectively. At 30 mmol/l glucose, it was 146 ± 6 , 170 ± 8 , 174 ± 11 , 178 ± 5 , and $186 \pm 5\%$ of the baseline, respectively (Fig. 2A). Leucine culture dose-dependently increased glucose-induced NAD(P)H in pancreatic islets. The half-maximal effective concentration (EC_{50}) of the glucose-response curve of NAD(P)H was ~ 5 – 7 mmol/l in control islets,

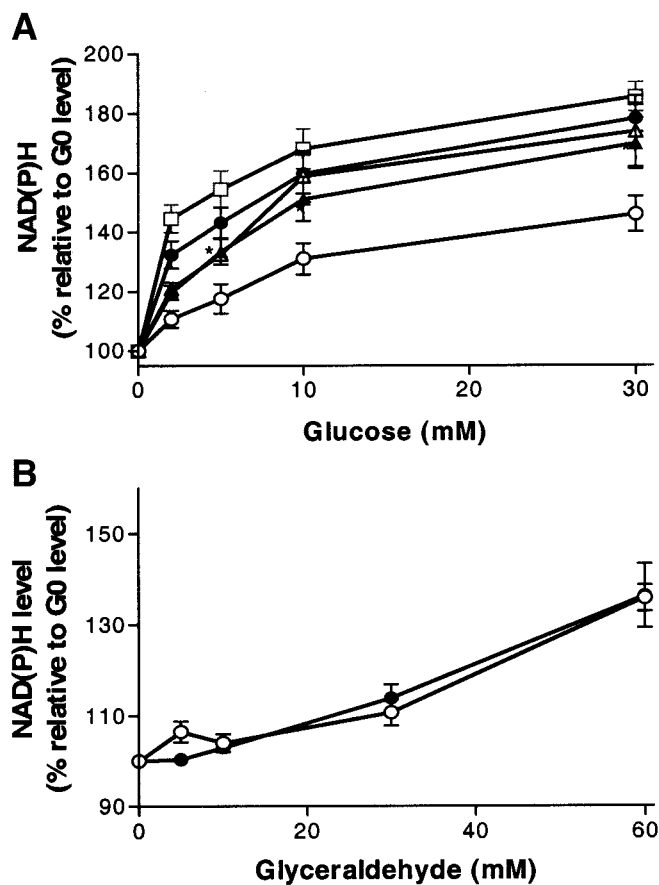


FIG. 2. Dose dependence of leucine effects on glucose-induced NAD(P)H level. A: Islets were cultured with different leucine concentrations for 2 days, and then the NAD(P)H levels were measured as above. ○, 0 mmol/l leucine; ▲, 1 mmol/l leucine; △, 2 mmol/l leucine; ●, 5 mmol/l leucine; □, 20 mmol/l leucine. B: Islets were cultured with 10 mmol/l leucine for 2 days, and then the glyceraldehyde-induced NAD(P)H level was assayed. ○, 0 mmol/l leucine; ●, 10 mmol/l leucine. The data are the means \pm SE of at least six islets in at least three independent experiments using three different rats. * $P < 0.05$ when compared with control. G0, 0 mmol/l glucose.

which is similar to 7–8 mmol/l reported elsewhere (24). In leucine-treated islets, the EC_{50} was left-shifted to ~ 2 – 3 mmol/l (Figs. 1–2). Consistent with our previous study (20), rapamycin failed to block the effects of leucine culture on glucose-induced NAD(P)H level (data not shown). To test the role of glucokinase on the elevated NAD(P)H level, glyceraldehyde was used as a fuel to bypass glucokinase. The NAD(P)H level of untreated and treated islets for 2 days was 107 ± 2 versus $101 \pm 0\%$ at 5 mmol/l glyceraldehyde, 104 ± 2 versus $103 \pm 1\%$ at 10 mmol/l glyceraldehyde, 111 ± 3 versus $114 \pm 3\%$ at 30 mmol/l glyceraldehyde, and 136 ± 3 versus $136 \pm 7\%$ at 60 mmol/l glyceraldehyde of their corresponding baselines, respectively (Fig. 2B). The glyceraldehyde effects on NAD(P)H were thus not affected by leucine culture. To test the possibility that the increased glucose-induced NAD(P)H is caused by the fuel roles of leucine, we assayed the effects of short-term pretreatment (30 min) with leucine on NAD(P)H level of the fresh islets or islets cultured without leucine for 2 days. Short-term pretreatment had no effect on NAD(P)H level (data not shown). **A 2-day leucine culture increased ATP, cytosolic $[Ca^{2+}]$, and insulin secretion in rat islets.** Islets were cultured with or without 10 mmol/l leucine for 2 days. This

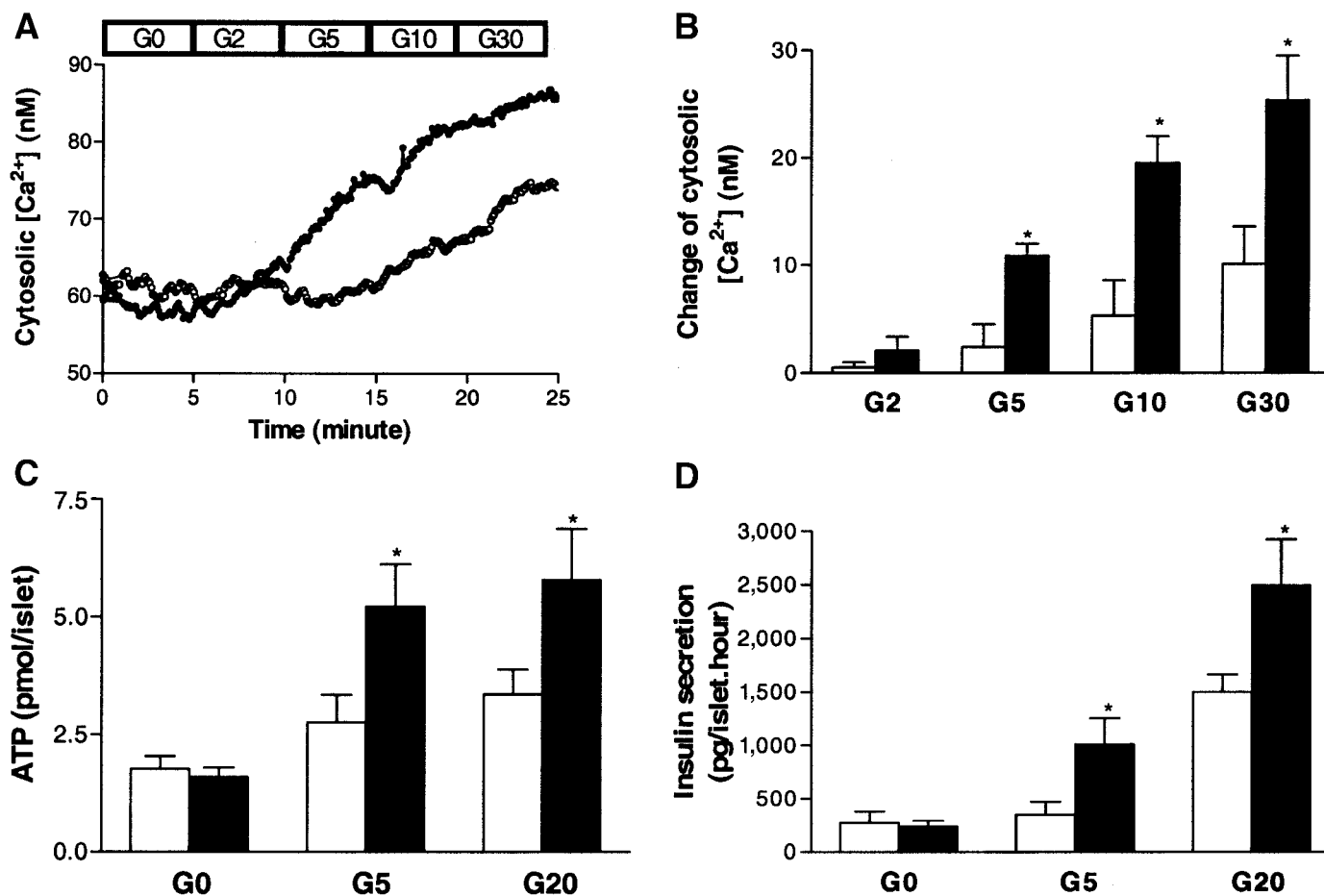


FIG. 3. Effects of leucine culture on free cytosolic $[Ca^{2+}]$, ATP content, and insulin secretion in rat islets. $[Ca^{2+}]$, ATP content, and insulin secretion of islets cultured with (■) or without (□) 10 mmol/l leucine for 2 days were measured as detailed in RESEARCH DESIGN AND METHODS. **A:** Measurement of free $[Ca^{2+}]$. **B:** Average change of $[Ca^{2+}]$. **C:** ATP content measurement. **D:** Insulin secretion. Data are the means \pm SE of >10 batches of three independent experiments using three different rats. * $P < 0.05$ compared with the untreated group. G0, 0 mmol/l glucose; G2, 2 mmol/l glucose; G5, 5 mmol/l glucose; G10, 10 mmol/l glucose; G20, 20 mmol/l glucose; G30, 30 mmol/l glucose.

increased glucose response of rat islet $[Ca^{2+}]$ (Fig. 3A), which was similar to previous reports of islets cultured with leucine for 1 week (20). With 2, 5, 10, and 30 mmol/l glucose, the $[Ca^{2+}]$ increase in treated islets was 2 ± 1 , 11 ± 1 , 20 ± 3 , and 25 ± 4 nmol/l, respectively, whereas in control islets it was 1 ± 1 , 2 ± 2 , 5 ± 3 , and 10 ± 4 nmol/l, respectively (Fig. 3B). It should be noted that the changes in rat islets' $[Ca^{2+}]$ induced by glucose was smaller than those in mouse islets, as reported by other research groups (27–29) and by our group (30). $[Ca^{2+}]$ oscillations have not been observed in rat islets, in contrast to mouse islets. Very few groups have used rat islets to study glucose-induced $[Ca^{2+}]$ responses. In one such study (31), the data for rat islet $[Ca^{2+}]$ measurements were presented as a ratio of fura-2 fluorescence (F340/F380) instead of nanomoles per liter and thus cannot be used for comparison. Another study by Henquin and colleagues (32) showed $[Ca^{2+}]$ responses of cultured rat islets in nanomoles per liter. By using fura-PE3 instead of fura-2, their rat islet $[Ca^{2+}]$ responses to glucose were slightly larger than those of our rat islets, but they were smaller than those of mouse islets by the same group (28). The response patterns were also different between their rat islets and mouse islets published by that group, and $[Ca^{2+}]$ oscillations have not been demonstrated (32).

ATP content of control and treated islets was 1.8 ± 0.2 vs. 1.6 ± 0.1 pmol/islet without glucose, 2.8 ± 0.5 vs. $5.2 \pm$

0.7 pmol/islet with 5 mmol/l glucose, and 3.4 ± 0.5 vs. 5.8 ± 0.9 pmol/islet with 20 mm glucose, respectively (Fig. 3C). In the same islets, insulin secretion (Fig. 3D) for control versus treated was 277 ± 104 vs. 243 ± 54 pg/islet \cdot h without glucose, 350 ± 120 vs. $1,010 \pm 245$ pg/islet \cdot h with 5 mmol/l glucose, and $1,501 \pm 162$ vs. $2,499 \pm 427$ pg/islet \cdot h with 20 mmol/l glucose, respectively. A 2-day culture with leucine thus increased glucose-induced ATP content, $[Ca^{2+}]$ response, and insulin secretion in pancreatic islets.

Leucine culture upregulated glucokinase and ATP5 β mRNA and protein in rat islets. In our previous report (20), leucine upregulated the mRNA level of ATP5 β at 1 week, but not 1 day, of culture and increased glucose-induced insulin secretion in rat islets. Glucokinase gene mRNA levels after 1 day, 2 days, and 1 week of culture were 2.1 ± 0.4 , 3.2 ± 0.6 , and 2.5 ± 0.4 times higher than the control value, respectively. ATP5 β mRNA levels after 1 day, 2 days, and 1 week of culture were 1.4 ± 0.3 , 2.4 ± 0.4 , and 3.0 ± 0.3 times higher than the control value, respectively (Fig. 4A). Thus, glucokinase gene was upregulated faster (in 24 h), with the strongest effect at day 2 (Fig. 4A). On the contrary, ATP5 β was upregulated slower, without a significant increase until 48 h, and with a continuous increase during 1 week of culture (Fig. 4A). The ratio of ATP5 β to glucokinase gene in leucine-treated islets was 0.7 ± 0.1 and 0.9 ± 0.1 after 1 and 2 days culture,

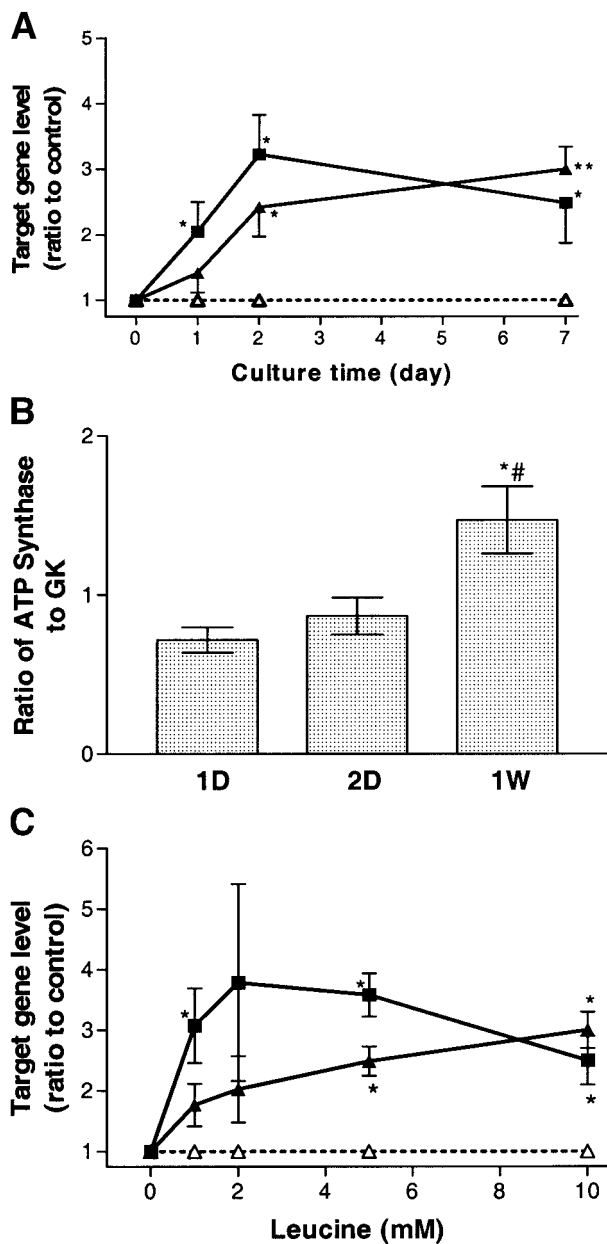


FIG. 4. Leucine culture upregulated glucokinase (GK) and ATP5 β . **A:** Batches of 100 rat islets were cultured with or without 10 mmol/l leucine for 1 day (1D), 2 days (2D), and 1 week (1W), respectively. Total RNA was extracted, and RT-PCR was performed as described in RESEARCH DESIGN AND METHODS. * $P < 0.05$, ** $P < 0.01$ compared with control. **B:** The ratio of ATP synthase to glucokinase calculated from the data of leucine-treated islets in panel A. * $P < 0.05$ compared with 1 day, # $P < 0.05$ compared with 2 days. **C:** Batches of 100 rat islets were cultured with 0, 1, 2, 5, and 10 mmol/l leucine for 1 week, respectively. Total RNA was extracted, and RT-PCR was performed as described in RESEARCH DESIGN AND METHODS. * $P < 0.05$ compared with the control value. Data are the means \pm SE of at least five independent experiments using >10 rats. ▲, ATP synthase; ■, glucokinase; △, control.

respectively. The ratio was significantly increased to 1.5 ± 0.2 after 1 week of culture because of the progressive elevation of ATP5 β and a smaller increase of glucokinase (Fig. 4B). ATP5 β mRNA levels in islets cultured with 1, 2, 5, and 10 mmol/l leucine for 1 week were 1.8 ± 0.4 , 2.0 ± 0.6 , 2.5 ± 0.2 , and 3.0 ± 0.3 times higher than the control value cultured without leucine, respectively. Glucokinase mRNA level was 3.1 ± 0.6 , 3.8 ± 1.6 , 3.6 ± 0.4 , and 2.5 ± 0.6 times higher than the control value without leucine,

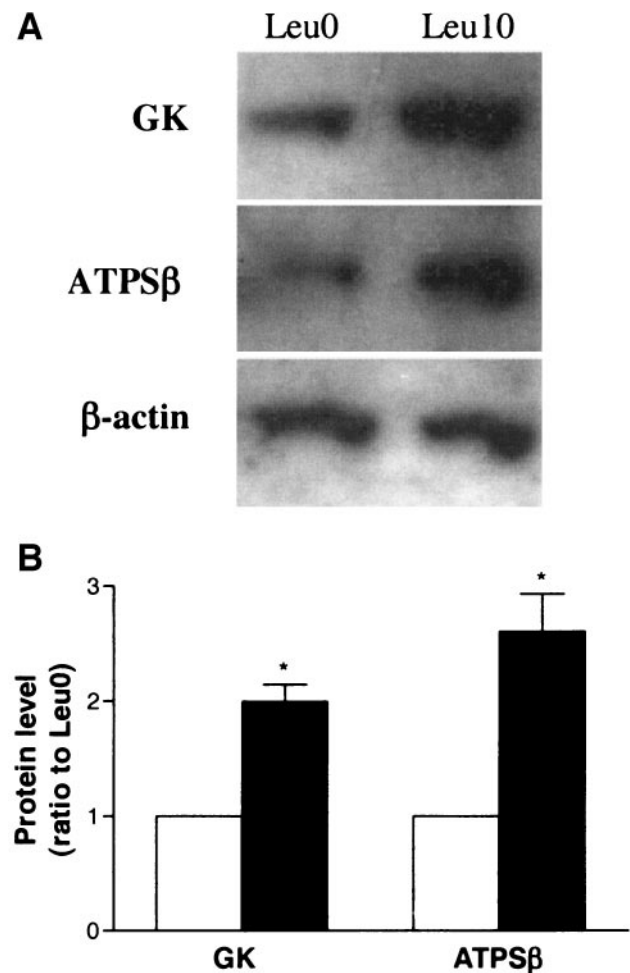


FIG. 5. Western blot assay of glucokinase (GK) and ATP5 β in rat islets. Batches of 200–250 islets were cultured with or without leucine for 2 days, respectively. Islets were lysed, and Western blots were performed as described in RESEARCH DESIGN AND METHODS. **A:** Representative Western blot gel. **B:** Quantification of protein level; data are normalized to control values without leucine treatment. Data are the means \pm SE of three independent experiments using six different rats. * $P < 0.05$ compared with the untreated group. Leu0, 0 mmol/l leucine, □; Leu10, 10 mmol/l leucine, ■.

respectively (Fig. 4C). Thus, leucine culture increased the expression mRNA levels of both genes dose-dependently. Glucokinase and ATP5 β protein in leucine-treated islets was 2.0 ± 0.2 and 2.6 ± 0.3 times higher than the control value without leucine, respectively (Fig. 5A–B).

Leucine culture increased glucose-induced ATP and insulin secretion of type 2 diabetic human islets. Type 2 diabetic human islets (IE was used for measurement) were cultured with or without leucine for 1 week before ATP level, cytosolic $[Ca^{2+}]$, and insulin secretion were measured. For control islets without leucine culture, insulin secretion at 0, 5, and 20 mmol/l glucose was 11.1 ± 0.8 , 12.6 ± 0.7 , and $15.3 \pm 0.8 \mu\text{U} \cdot \text{IE}^{-1} \cdot \text{h}^{-1}$, respectively. Data show impaired glucose-induced insulin secretion in these type 2 diabetic human islets, consistent with a previous report (22). In leucine-treated islets, insulin secretion was $12.1 \pm 0.6 \mu\text{U} \cdot \text{IE}^{-1} \cdot \text{h}^{-1}$ in the absence of glucose, $20 \pm 0.8 \mu\text{U} \cdot \text{IE}^{-1} \cdot \text{h}^{-1}$ in the presence of 5 mmol/l glucose, and $31.4 \pm 2.0 \mu\text{U} \cdot \text{IE}^{-1} \cdot \text{h}^{-1}$ in the presence of 20 mmol/l glucose (Fig. 6A). In the same islets, ATP content of control and leucine-treated islets was 7.9 ± 0.5 vs. 8.0 ± 0.7 pmol/IE at 0 mmol/l glucose, 10.0 ± 0.6 vs. 11.1 ± 0.9

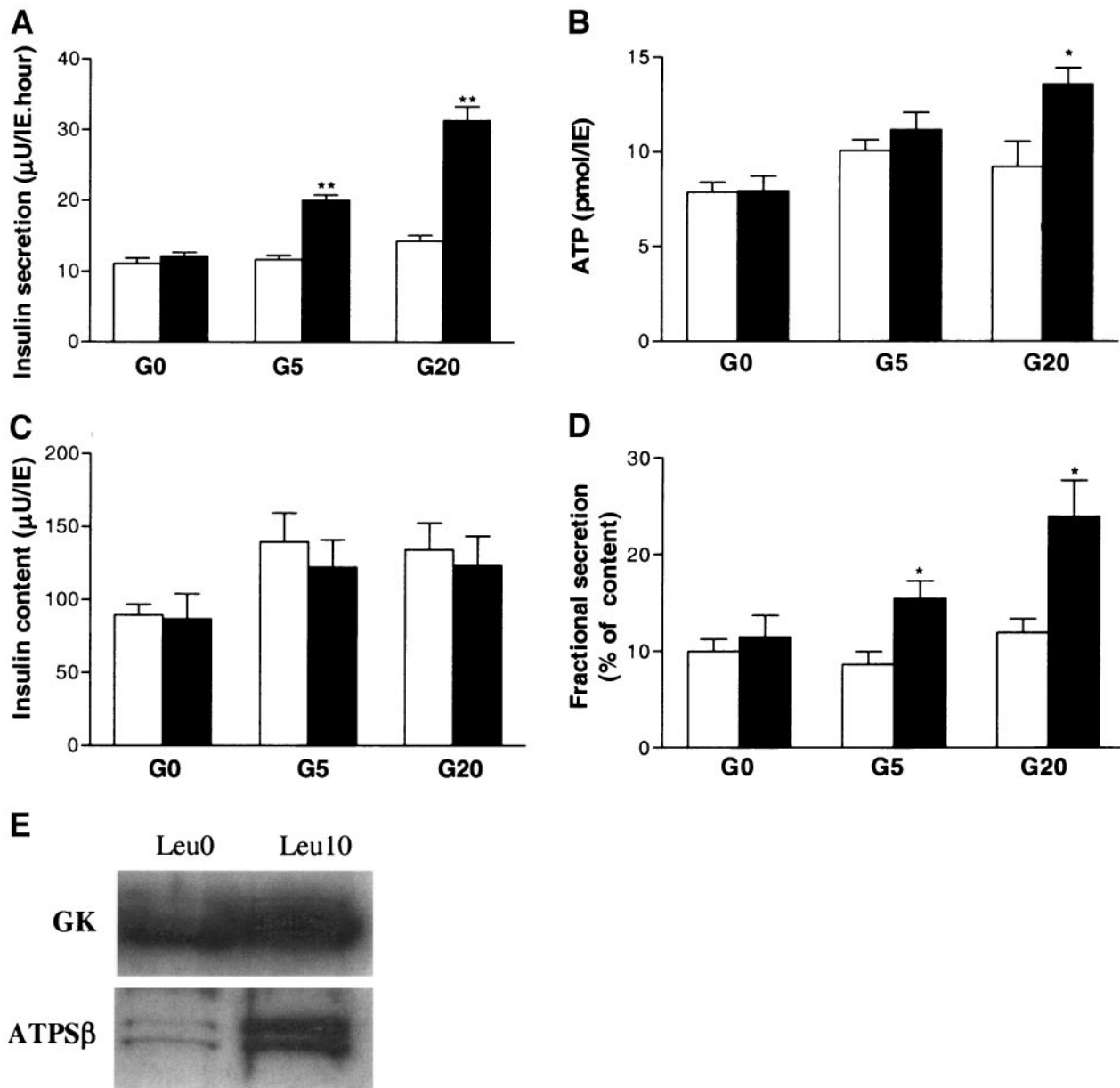


FIG. 6. Effects of leucine culture on insulin secretion and ATP content of type 2 diabetic human islets. Human diabetic islets were cultured in CMRL 1066 with (■) or without (□) 10 mmol/l leucine for 1 week, respectively. Insulin secretion (A), ATP content (B), insulin content (C), and fractional insulin secretion (D) were measured as described in RESEARCH DESIGN AND METHODS. The effect of leucine culture on glucokinase and ATP5 β protein was assayed by Western blot (E). Data are the means \pm SE, using two different pancreata. * $P < 0.05$, ** $P < 0.01$ compared with the untreated group. G0, 0 mmol/l glucose; G5, 5 mmol/l glucose; G20, 20 mmol/l glucose.

pmol/IE at 5 mmol/l glucose, and 9.2 ± 1.3 vs. 13.6 ± 0.8 pmol/islet (Fig. 6B). There was no difference in insulin content between nontreated islets and islets treated with 10 mmol/l leucine, which was 89 ± 9 vs. 87 ± 20 $\mu\text{U}/\text{IE}$ without glucose, 149 ± 22 vs. 122 ± 22 $\mu\text{U}/\text{IE}$ at 5 mmol/l glucose, and 134 ± 23 vs. 124 ± 23 $\mu\text{U}/\text{IE}$ (Fig. 6C). Fractional insulin (insulin secretion divided by the corresponding insulin content) between nontreated islets and islets treated with 10 mmol/l leucine was 10.0 ± 1.3 vs. $11.5 \pm 2.2\%$ in the absence of glucose, 8.6 ± 1.4 vs. $15.4 \pm 1.8\%$ at 5 mmol/l glucose, and 11.9 ± 1.4 vs. $23.9 \pm 3.7\%$ at 20 mmol/l glucose (Fig. 6D). Similar to rat islets, leucine culture unregulated both glucokinase and ATP synthase by two- to threefold in diabetic human islets (Fig. 6E). Basal $[\text{Ca}^{2+}]$ of control human type 2 diabetic islets without glucose was ~ 150 nmol/l. The addition of 5 mmol/l glucose

gradually decreased $[\text{Ca}^{2+}]$ levels. The addition of 20 mmol/l glucose or 30 mmol/l KCl did not affect $[\text{Ca}^{2+}]$ in these islets. In leucine-treated diabetic islets, basal $[\text{Ca}^{2+}]$ was ~ 120 nmol/l, which was significantly lower than that of control islets. Glucose at 5 or 20 mmol/l did not affect $[\text{Ca}^{2+}]$ levels. A significant increase of ~ 25 nmol/l in cytosolic $[\text{Ca}^{2+}]$ was induced by 30 mmol/l KCl in leucine-treated islets (data not shown). In summary, leucine culture increased glucose-induced ATP content and insulin secretion in type 2 diabetic human islets by upregulating glucokinase and ATP synthase.

A 2-day leucine culture upregulated glucokinase and ATP5 β protein in INS1 cells. After culture with 10 mmol/l leucine for 2 days, glucokinase and ATP5 β proteins were upregulated 2.0 ± 0.1 and 2.6 ± 0.6 times higher in the rat-derived INS1 cell line, respectively (Fig. 7A and

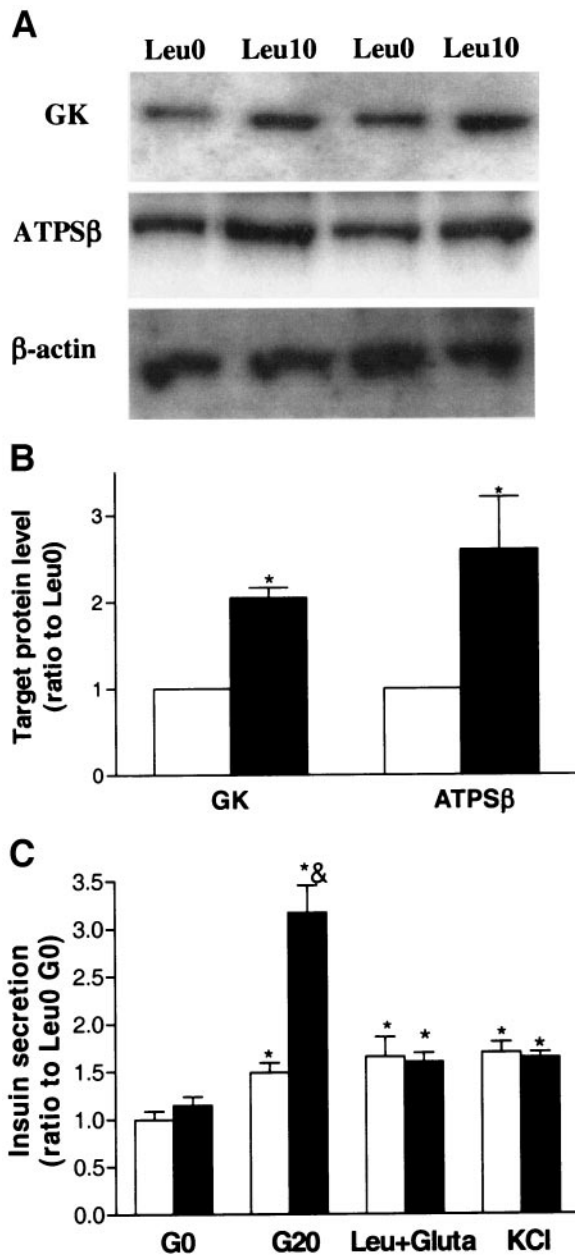


FIG. 7. Leucine culture upregulated glucokinase (GK) and ATP synthase in INS1 cells. INS1 cells were cultured in RPMI 1640 with (■) or without (□) 10 mmol/l leucine for 2 days, and then Western blot (**A** and **B**) and insulin secretion (**C**) were performed as detailed in RESEARCH DESIGN AND METHODS. The data were normalized to control 0 mmol/l glucose level. The data are the means \pm SE of at least 12 observations from four independent experiments. * $P < 0.05$ when compared with 0-mmol/l glucose value of control group; & $P < 0.05$ when compared with 20-mmol/l glucose value of control group. G0, 0 mmol/l glucose; G20, 20 mmol/l glucose; Leu0, 0 mmol/l leucine; Leu10, 10 mmol/l leucine; Gluta, glutamine; Leu, leucine.

B). Insulin secretion at 0 mmol/l glucose, 20 mmol/l glucose, 10 mmol/l leucine plus 2 mmol/l glutamine, and 30 mmol/l KCl was 1.0 vs. 1.1 ± 0.1 (10 mmol/l leucine), 1.5 ± 0.1 vs. 3.2 ± 0.3 (10 mmol/l leucine), 1.7 ± 0.2 vs. 1.6 ± 0.1 , and 1.7 ± 0.1 vs. 1.6 ± 0.1 , respectively (Fig. 7C). The results indicated leucine culture only enhanced insulin secretion stimulated by glucose, but it did not change its secretion stimulated by leucine plus glutamine or high potassium (Fig. 7C). The results also indicated that leucine culture did not affect the basal insulin secretion at 0 mmol/l glucose.

DISCUSSION

To our knowledge, this is the first study showing that leucine culture upregulates glucokinase expression in rat pancreatic islets, INS1 cells, and human islets, following our previous discovery that leucine culture upregulates ATP synthase (complex V of the respiratory chain) in rat islets (20). An important finding in this study is that leucine culture sensitizes glucose-induced insulin secretion in normal rat islets and restores defective glucose-induced insulin secretion in type 2 diabetic human islets. However, it is unfortunate that leucine's effects on nondiabetic human islets cannot be tested due to the lack of islets because the priority for transplantation into type 1 diabetic patients is higher than that of basic scientific research. High basal $[Ca^{2+}]$ and impaired or abolished $[Ca^{2+}]$ response to glucose in type 2 diabetic human islets observed in this study were consistent with observations from other research groups (33,34). It has been reported that long-term exposure of human or rodent islets to high glucose resulted in high basal $[Ca^{2+}]$ and impaired or abolished $[Ca^{2+}]$ response to glucose. Interestingly, the same studies (33,34) show that long-term exposure to high glucose increased glucose sensitivity but decreased maximal insulin secretion in human and rodent islets, whereas we show that exposure to high leucine increased both glucose sensitivity and maximal insulin secretion (20) (Figs. 3 and 6). Thus, leucine and glucose may regulate metabolism and gene expression via different mechanisms. However, it is unclear how leucine stimulates glucokinase and ATP synthase expression. Glucose also inhibits leucine's effects on insulin secretion via allosteric inhibition of glutamate dehydrogenase (21,35), which does not involve changes in gene expression and is an acute effect (within minutes). These multidimensional interactions between glucose and amino acid actions in the pancreatic β -cells are revealing a signaling network that is much more complicated than proposed previously.

Glucokinase is the glucose sensor in pancreatic β -cells. Mutations, decreased expression, or decreased activity have been proposed as causes of diabetes (12,36–39). Recent evidence has shown that glucokinase is not the only rate-limiting step in glucose-induced insulin secretion of pancreatic β -cells. Other factor(s) may also be rate-limiting and may contribute to the tight control of glucose-induced insulin secretion (10–12). It is proposed that mitochondria set the limit of glucose-induced insulin secretion from pancreatic islets. In our previous work, we showed that ATP synthase may function as a rate-limiting factor in pancreatic islets by changing its expression level with leucine culture, small interfering RNA depletion, and plasmid overexpression (20). After 1 day of leucine culture, glucokinase expression is already upregulated; however, insulin secretion is not changed in pancreatic islets. Thus, it may need the combined increase of glucokinase and ATP synthase expression after 48 h of leucine culture to augment insulin secretion. Leucine culture also upregulated glucokinase and ATP synthase in rat-derived INS1 cells (Fig. 7) and enhanced insulin secretion stimulated by glucose, but it did not affect secretion stimulated by leucine plus glutamine or high potassium. The results supported our hypothesis that it is the combined upregulation of glucokinase and ATP synthase that resulted in enhanced glucose-stimulated insulin secretion in leucine-treated islets, expanding our previous demonstration of the important roles of ATP synthase in glucose-stimulated

insulin secretion in pancreatic β -cells. However, it cannot be excluded that other factor(s) are also affected by leucine culture and play some roles in glucose sensitization.

Changes in glycolysis and Krebs' cycle activity, as a result of changes in glucokinase activity, are expected to change the production and thus the cellular level of NAD(P)H, as demonstrated previously using downregulation of glucokinase (40), using tacrolimus inhibition (41), using transgenic mice overexpressing glucokinase (42), or using a sorted subset of pancreatic β -cells with higher glucokinase activity (43). However, the glucose-induced changes of NAD(P)H are more complicated in islets cultured with leucine for different lengths of time because of the changes of multiple genes, which affect the production as well as the consumption of NAD(P)H. After 1 day of leucine culture, glucose-induced NAD(P)H levels were much higher compared with control values without leucine. However, ATP content, cytosolic Ca^{2+} , and insulin secretion were not changed (20). The best explanation is that at day 1 only glucokinase expression is upregulated by leucine, whereas ATP synthase expression remains the same. This increases glycolysis and Krebs' cycle activity and thus NAD(P)H production. Because of the limitation of ATP synthase, little or none of the overproduced NAD(P)H is consumed, and thus it is accumulated intracellularly. After 2 days of leucine culture, glucokinase expression is increased even more. ATP synthase expression is increased by now. NAD(P)H remains elevated at day 2 because its production (controlled by glucokinase) is larger than its consumption (controlled by ATP synthase). After 1 week, glucose-induced NAD(P)H stimulation was similar with or without leucine culture. This is explained by increased, but equal, amounts of consumption (controlled by ATP synthase) and production (controlled by glucokinase expression). This hypothesis is further supported by experiments using glyceraldehyde. Glyceraldehyde increased NAD(P)H levels without glucokinase involvement, and its effects were not affected by leucine culture at day 2, which supports the role of glucokinase upregulation in elevated NAD(P)H levels in leucine-treated islets. It should be noted that the current method cannot demonstrate in which compartments NAD(P)H levels are changed. It is assumed that NAD(P)H levels are not affected by leucine culture when tested in the absence of glucose (23,24,40–42).

In conclusion, it is proposed that ATP synthase is a rate-limiting enzyme in glucose-stimulated insulin secretion, and both ATP synthase and glucokinase contribute to tight control of insulin secretion in pancreatic β -cells: upregulation of glucokinase sensitizes glucose and translates the signal to NADH, whereas upregulation of ATP synthase couples NADH to ATP production and augments insulin secretion.

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