Creation and Characterization of a Mitochondrial DNA-Depleted Pancreatic β-Cell Line

Impaired Insulin Secretion Induced by Glucose, Leucine, and Sulfonylureas

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It has been proposed that mitochondrial oxidative phosphorylation in pancreatic β-cells plays an important role in insulin secretion. To examine the impact of mitochondrial dysfunction on insulin secretion, we created a MIN6 cell line that depleted mitochondrial DNA (mtDNA) by treatment with ethidium bromide (EtBr), and studied the response of the cell line to various secretagogues. MIN6 cells cultured with 0.5 μg/ml EtBr for over 2 months (termed MIN6 Δmt cells) revealed a marked (>90%) decrease in mtDNA content and a lack of mRNAs encoded by mtDNA. MIN6 Δmt cells showed the defects of cytochrome *c* oxidase activity, glucose- and leucine-induced increase in cellular ATP content, and respiratory chain–driven ATP synthesis, suggesting that MIN6 Δmt cells lost oxidative phosphorylation activity due to the selective disruption of the subunits of respiratory chain enzymes encoded by mtDNA. MIN6 Δmt cells also showed a decrease in glucose utilization, suggesting the impairment of the glycolytic pathway as well. After stimulation with glucose and leucine, MIN6 Δmt cells showed no response in insulin secretion or intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). On the other hand, arginine stimulated insulin secretion and an increase in $[Ca^{2+}]_i$ in MIN6 Δmt cells as in MIN6 cells. Glibenclamide also stimulated insulin secretion and an increase in $[Ca^{2+}]_i$ in both types of cells, but the responses of MIN6 Δmt cells were significantly lower than those of MIN6 cells. These results suggest the importance of ATP production in insulin secretion and an increase in $[Ca^{2+}]_i$, both induced by glucose and leucine. Moreover, mitochondrial function turns out to be not essential but important for the activation of sulfonylurea-induced insulin secretion. *Diabetes* 47:621–631, 1998

IDDM is a heterogeneous disorder characterized by decreased responsiveness of peripheral tissues to insulin and impairment of insulin secretion from pancreatic β-cells (1). Secretion of insulin is regulated by nutrients, hormones, and neurotransmitters, but the precise mechanisms by which secretagogues regulate insulin secretion are still unclear.

Glucose, a major nutrient secretagogue of insulin, mediates insulin secretion mainly by an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) through the voltage-dependent Ca^{2+} channel (VDCC) (2–5). It is now widely accepted that the process requires glucose metabolism and mitochondrial oxidative phosphorylation leading to the generation of the metabolic product, ATP, and depolarization of the plasma membrane due to closure of the ATP-sensitive K^+ channel (K_{ATP} channel) (2,5–9). Although two main processes—glycolysis and oxidative phosphorylation—are responsible for ATP synthesis, the former process contributes only ~2% to the synthesis, whereas the latter process is the key reaction in islets (6). Insulin secretion induced by other secretagogues (e.g., leucine, glyceraldehyde) is also thought to be mediated by the production of ATP (5,10–12). In this way, mitochondrial oxidative phosphorylation in pancreatic β-cell has been proposed to play an important role in insulin secretion.

Recently, several reports have suggested that mutations in mitochondrial DNA (mtDNA), especially the one involving the substitution of guanine for adenine at nucleotide position (np) 3243 of leucine transfer RNA ($tRNA^{Leu(UUR)}$), might cause diabetes (13–16). This mutation appears to interfere not only with the synthesis of $tRNA^{Leu(UUR)}$, but also with the binding of the transcription termination factor (17), thereby causing defects in the synthesis of proteins encoded by mtDNA (18,19). Because the proteins encoded by mtDNA are the subunits of mitochondrial respiratory chain complexes, the defects in the synthesis of the proteins may cause the failure in ATP synthesis and the impairment of insulin secretion in pancreatic β-cells. However, it is still unclear how the defect in mitochondrial oxidative phosphorylation in pancreatic β-cells could affect their function, especially their response to the various secretagogues.

The pancreatic β-cell line MIN6, established from insuli-

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BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; COX, cytochrome *c* oxidase; COX2, cytochrome *c* oxidase subunit II gene; COX4, cytochrome *c* oxidase subunit IV gene; EtBr, ethidium bromide; K_{ATP} channel, ATP-sensitive K^+ channel; KRH buffer, HEPES-balanced Krebs-Ringer bicarbonate buffer; MIN6 Δmt cells, mitochondrial DNA-depleted MIN6 cells; mtDNA, mitochondrial DNA; ND1, NADH dehydrogenase subunit 1; np, nucleotide position; PBS, phosphate-buffered saline; RIA, radioimmunoassay; SDH, succinate dehydrogenase; SUR, sulfonylurea receptor; $tRNA^{Leu(UUR)}$, leucine transfer RNA; VDCC voltage-dependent Ca^{2+} channel.

nomas obtained by targeted expression of the simian virus 40 T antigen gene in transgenic mice, exhibits glucose-induced insulin secretion comparable with that of normal islet cells (20); further, it has been demonstrated that the characteristics of the glucose transport system and glucose metabolism in MIN6 cells closely resemble those of isolated islet cells (21). MIN6 cells also secrete insulin in response to many other secretagogues as do β-cells, such as sulfonylureas (22), arginine (23), and leucine (23). In this way, the MIN6 cell line retains the physiological characteristics of normal pancreatic β-cells; thus this cell line seems to be a useful model for studying the mechanisms of secretagogue-induced insulin secretion.

In this study, to investigate the impact of mitochondrial dysfunction on insulin secretion in MIN6 cells, we created a MIN6 cell line that lacked functional mitochondrial oxidative phosphorylation after treatment with ethidium bromide (EtBr) and examined its responses to glucose and other secretagogues.

RESEARCH DESIGN AND METHODS

Reagents and chemicals. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan); fetal bovine serum (FBS), from JRH Biosciences (Lenexa, KS); [γ-^{32}P]ATP, [α-^{32}P]dCTP, D-[5-^{3}H]glucose, and [^{3}H]water, from Du Pont-NEN (Boston, MA); bovine serum albumin (BSA), glibenclamide, diazoxide, nitro blue tetrazolium (NBT), HEPES, 3,3′-diaminobenzidine tetrahydrochloride (DAB), digitonin, phenazine methosulfate, and ATP bioluminescent assay kit, from Sigma (St. Louis, MO); L-arginine, L-glutamate, sodium succinate, L-malate, EtBr, and uridine from Wako (Osaka, Japan); fura-2/acetoxymethyl ester from Dojindo (Kumamoto, Japan); Ab Bead Insulin Eiken Radioimmunoassay kit, from Eiken (Tokyo, Japan); Lab-Tec Chamber Slide, from Nunc (Naperville, IL); Bio-Rad protein assay kit, from Bio-Rad (Richmond, CA); TRIzol Reagent and HAM's F-10 medium, from Life Technologies (Gaithersburg, MD); and glass-bottom microwells, from Mat Tec (Ashland, MA).

Cell culture. MIN6 cells that lacked functional mitochondrial oxidative phosphorylation (MIN6 Δmt cells) were produced as follows, as previously reported (24,25). MIN6 cells were grown for over 2 months in DMEM supplemented with 25 mmol/l glucose, 75 mg/l penicillin, 50 mg/l streptomycin, and 15% heat-inactivated FBS, 5 μmol/l 2-mercaptoethanol, 110 μg/ml pyruvate, 50 μg/ml uridine, and various concentrations of EtBr (0, 0.05, 0.25, 0.5, 1.0, 2.5, and 5.0 μg/ml) (pH 7.4) in humidified 5% CO_2/95% air at 37°C. The cells were replated once a week, and the culture medium was changed every 2–3 days. A MIN6 cell line cultured without EtBr was used as a control. For measurements of insulin content, insulin secretion, and $[Ca^{2+}]_i$, 1×10^5 cells were seeded on 35-mm dish or glass-bottom microwells 3 days before the experiments. For measurement of ATP content, cells were seeded on 6-well plates (10^5 cells/well) 2 days before the experiments. For histochemical staining, 1×10^4 cells were seeded on the chamber slides 3 days before the experiments. For other experiments, 1×10^6 cells were seeded on a 100-mm dish and used when the cells became subconfluent.

Southern blot analysis. Total DNA (1 μg) prepared from MIN6 cells cultured without and with EtBr at various concentrations (0.05, 0.25, and 0.5 μg/ml) was digested with a restriction enzyme, *Xho* I. The digested DNA fragments were subjected to Southern blot analysis with a probe of [α-^{32}P]dCTP-labeled mouse mtDNA fragment [np 2671–3287] (26), as previously described (27). The radioactivity of the band was measured with a BAS 100 bioimaging analyzer (Fujix; Fuji Photo Film, Tokyo, Japan).

Northern blot analysis. For analysis of mRNAs transcribed from mtDNA and nuclear DNA, total cellular RNA was purified using a TRIzol reagent from MIN6 cells cultured without and with EtBr at various concentrations (0.05, 0.25, and 0.5 μg/ml). Insulin mRNA content was measured using RNA prepared from MIN6 and MIN6 Δmt cells (MIN6 cells cultured with 0.5 μg/ml EtBr) incubated with either 2.5 or 25 mmol/l of glucose for 6 h. Either 5 or 20 μg of total RNA were subjected to Northern blot analysis with a [α-^{32}P]dCTP-labeled probe corresponding to mouse NADH dehydrogenase subunit 1 cDNA (np 2761–3061) (26), mouse cytochrome *c* oxidase subunit II (COX2) cDNA (np 7016–7500) (26), mouse cytochrome *c* oxidase subunit IV (COX4) cDNA (np 314–660) (28), mouse glucokinase cDNA (np 1–450) (29), rat sulfonylurea receptor (SUR) cDNA (np 3601–3940) (30), hamster insulin I cDNA (np 58–390) (31), or mouse β-actin cDNA (np 25–564) (32), as previously described (27). Autoradiography was performed for 3 days at –70°C, and the radioactivity of the band was measured with a BAS 100 bioimaging analyzer (Fuji).

Histochemical staining of mitochondrial enzymes. Histochemical staining was performed as follows, as previously reported (33). MIN6 and MIN6 Δmt cells on the slides were washed twice with ice-cold phosphate-buffered saline (PBS) (pH 7.4) and air dried at 4°C. For staining of the cytochrome *c* oxidase (COX) activity, the cells were incubated with 0.1 mol/l acetate buffer (pH 5.6) containing 2 mg/ml DAB, 0.1% $MnCl_2$, and 1% H_2O_2 for 60 min at 37°C. Then the cells were rinsed in water, immersed in 1% $CuSO_4$ for 5 min, and counterstained with hematoxylin for another 5 min. For staining of the succinate dehydrogenase (SDH) activity, the cells were incubated with 0.1 mol/l Tris-HCl (pH 7.4), 10 mg/ml sodium succinate, 1 mg/ml NBT, and 25 μg/ml phenazine methosulfate for 30 min at 37°C. Each of the slides was finally rinsed in water and dehydrated with acetone. The samples were investigated in a microscope (magnification ×200) (BX50-PM20; Olympus, Tokyo, Japan).

Measurement of cellular ATP contents and respiratory chain–driven ATP synthesis. ATP content of the cells was measured by the method of Ashcroft et al. (34) with some modifications. MIN6 and MIN6 Δmt cells cultured with 25 mmol/l glucose were washed with HEPES-balanced Krebs-Ringer-bicarbonate (KRH) buffer (119 mmol/l NaCl, 4.74 mmol/l KCl, 2.54 mmol/l $CaCl_2$, 1.19 mmol/l $MgSO_4$, 1.19 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, and 10 mmol/l HEPES; pH 7.4) containing 2.5 mmol/l glucose and 0.5% BSA. After preincubation in the KRH buffer for 30 min, the cells were washed twice with KRH buffer and incubated in 1 ml of KRH buffer with the indicated concentrations of glucose (2.5, 5, 10, and 25 mmol/l) or leucine (0, 5, and 20 mmol/l) in the presence of 2.5 mmol/l glucose for 60 min at 37°C. After the addition of 250 μl of ice-cold 2.5 mmol/l perchloric acid, the cells were scraped and disrupted by sonication. Cell extracts were subsequently neutralized to pH 7.0–7.5 using a solution containing 2 mol/l KOH and 0.6 mol/l 3-(*N*-morpholino)propanesulfonic acid, and the amount of ATP was measured fluorimetrically using the ATP bioluminescent assay kit. Protein content of the samples was determined with a Bio-Rad protein assay kit (Bio-Rad), and ATP content was expressed as nanomoles per milligram of cell protein.

Respiratory chain–driven ATP synthesis in digitonin-permeabilized cells was measured by the method of Wanders et al. (35) with some modifications. MIN6 and MIN6 Δmt cells were washed with PBS and tripsinized. The detached cells were pelleted by centrifugation at room temperature (800*g*, 2 min), gently suspended in HAM's F10 medium, and kept at room temperature for 30 min followed by centrifugation (800*g*, 2 min). The cells were subsequently washed twice and finally taken up in PBS. After determination of the concentration of the cell protein, the cells were incubated at a final concentration of 0.05 mg cell protein/ml in a medium containing the following components: 150 mmol/l KCl, 25 mmol/l Tris-HCl, 2 mmol/l EDTA, 10 mmol/l potassium phosphate, 1 mmol/l ADP, 0.1% BSA, 40 μg/ml digitonin, and the indicated oxidizable substrates and respiratory chain inhibitor. Reactions were proceeded for 10 min at 37°C and terminated by adding perchloric acid (final concentration 0.5 mol/l) and chilled for 15 min in ice water. Then the tubes were centrifuged (12,000*g*, 2 min at 4°C), and the amount of ATP in the supernatants was measured as described above.

Measurement of glucose utilization. Glucose utilization was measured by following the conversion of [5-^{3}H]glucose into $^{3}H_2O$ as described by Ishihara et al. (21). Cells (2×10^5 in 24-well plates) were preincubated in KRH buffer containing 0.5% BSA without glucose for 30 min at 37°C. Cells were then incubated at 37°C in 0.3 ml of KRH buffer with 0.5% BSA and varying concentrations of glucose (0.15, 0.3, 0.6, 1.0, 5.0, 10, 15, 25, and 50 mmol/l) containing 5 and 20 μCi/ml [5-^{3}H]glucose for lower (≤1 mmol/l) and higher (≥5 mmol/l) glucose concentrations, respectively. After a 2-h incubation period, 0.1 ml of the incubation media was collected in a 1.5-ml tube and added with 20 μl of 1 mol/l HCl. The tubes were then placed in scintillation vials containing 0.6 ml of distilled water and kept at 37°C for 36 h to allow the $^{3}H_2O$ in the tube to equilibrate with the water in the vial. Subsequently, the tube was removed and 10 ml of scintillation cocktail was added to each vial and the radioactivity was counted. The rate of glucose utilization was calculated and expressed as described previously (21).

Measurements of insulin secretion and intracellular insulin content. Insulin secretion was examined by the method described previously (22) with some modifications. The cells were washed, pre-incubated for 30 min in KRH buffer with 2.5 mmol/l glucose and 2% BSA, and incubated in KRH buffer with or without the indicated secretagogues for 30 min at 37°C. Then the medium was collected and centrifuged to remove cells. Insulin content in the supernatant was determined by radioimmunoassay (RIA) using an insulin assay kit as indicated in the manufacturer's (Eiken, Tokyo, Japan) instructions . Protein content of the cells was determined with a Bio-Rad protein assay kit (Bio-Rad) after solubilization with 500 μl of 1 mol/l NaOH.

Intracellular insulin content was measured according to the procedure of Moore et al. (36). The cells cultured in 35-mm dishes were washed, incubated in KRH with 2.5 mmol/l glucose and 2% BSA for 30 min at 37°C, and washed again three times with PBS. Ice-cold 5 mol/l acetic acid (3 ml) was added to cell monolayers, and the cells were scraped off with a rubber policeman. The acid-cell mixture was frozen and thawed three times and allowed to stand at 0°C for 12 h. After removal of the cell debris by centrifugation at 3,000*g* for 30 min, the extract was lyophilized and stored at –80°C. The residual acid present in the samples was neutralized with NaOH and insulin was assayed by RIA. The unit of insulin was based on the instructions of RIA assay kit. The insulin concentrations secreted

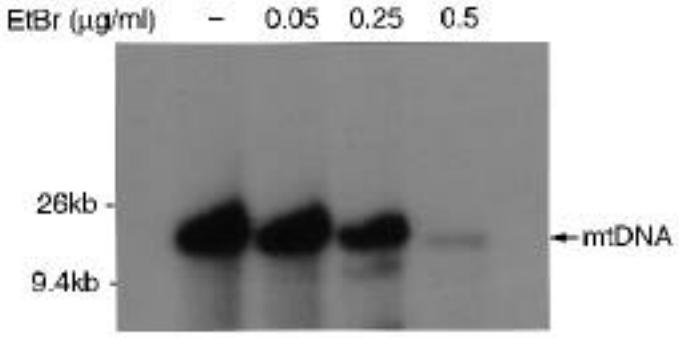

FIG. 1. Southern blot analysis of mitochondrial DNA in MIN6 cells treated with EtBr. Total DNA from MIN6 cells cultured without or with various concentrations of EtBr (0.05, 0.25, and 0.5 μg/ml) was digested with *Xho* I, electrophoresed on a 0.9% agarose gel (1 μg/lane), and transferred onto a nitrocellulose membrane. The membrane was hybridized with a probe of [α-^{32}P]dCTP-labeled mouse mtDNA fragment.

in the medium and cellular content are expressed as pmol · 30 min^{-1} · mg^{-1} cell protein and pmol/mg cell protein, respectively.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured as described previously (22) with slight modifications. The cells were loaded with 5 μmol/l fura-2/acetoxymethyl ester for 15 min at 37°C. Then the cells were washed three times in KRH buffer and allowed to equilibrate for 10 min in KRH buffer containing 2.5 mmol/l glucose and 2.0% BSA. The recording of the fluorescent signal was continued for the indicated times in the presence of the indicated stimuli. The fura-2 fluorescence of the cells was selected for study with ultraviolet light at a wavelength of 340 or 380 nm. An emitted light of wavelength >510 nm was focused on a silicon intensifier tube camera (C2400–08; Hamamatu Photonix, Hamamatu, Japan). The video signal from the camera was directed to a digitized image processor (Argus-50/Ca; Hamamatu Photonix). The data were obtained from seven 3 × 3-μm square windows in the cytoplasm of different cells and averaged.

Statistical analysis. Data are expressed as means ± SD. Differences between two groups were evaluated by unpaired Student's t test. $P < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

Effect of EtBr treatment on the mitochondrial DNA and mRNAs in MIN6 cells. We initially investigated the effect of long-term EtBr treatment on mtDNA in MIN6 cells. Total DNA was prepared from MIN6 cells cultured without or with EtBr at various concentrations (0.05, 0.25, and 0.5 μg/ml) for over 2 months and was subjected to Southern blot analysis using a probe specific for mtDNA. In MIN6 cells cultured without EtBr, 16.5 kilo base pairs (kb) of mtDNA were detected; the content of mtDNA did not show a significant change after treatment with 0.05 μg/ml EtBr. However, in cells treated with 0.25 and 0.5 μg/ml EtBr, mtDNA contents decreased by 24% ($n = 4$; $P < 0.05$) and 90% ($n = 4$; $P < 0.001$), respectively (Fig. 1). To completely deplete the cells of mtDNA, MIN6 cells were cultured with higher concentrations of EtBr. However, 2.5 and 5.0 μg/ml of EtBr treatment resulted in cell death in a few weeks. In 1.0 μg/ml of EtBr, the cells survived but showed morphological changes, which were not observed in the cells treated with 0.5 μg/ml of EtBr.

To examine the effect of EtBr treatment on transcription from mtDNA and nuclear DNA, mtDNA transcripts (NADH dehydrogenase subunit 1 [ND1] and COX2 mRNAs) and nuclear DNA transcripts (COX4, glucokinase, SUR, and β-actin mRNAs) were quantified by Northern blot analysis. In MIN6 cells without EtBr treatment, 0.95 kb of ND1 mRNA was detected. The mRNA decreased significantly by 71 and 79% ($n = 4$; $P < 0.005$) after treatment with 0.05 and 0.25 μg/ml EtBr, respectively, and was undetectable with 0.5 μg/ml EtBr treatment (Fig. 2*A*, *top*). COX2 mRNA transcribed from mtDNA was also undetectable in MIN6 cells with 0.5 μg/ml EtBr

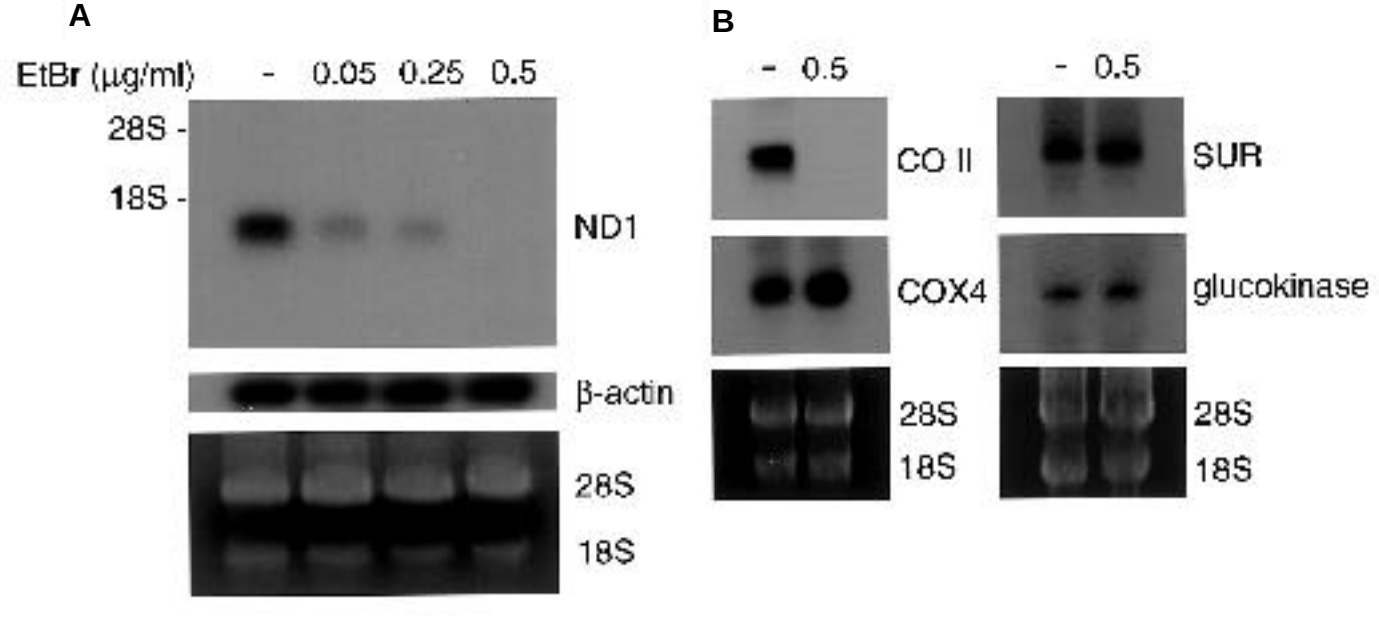

FIG. 2. Northern blot analysis of mRNAs transcribed from mtDNA and nuclear DNA. *A*: Total cellular RNA (5 μg) extracted from MIN6 cells treated with EtBr at various concentrations (0, 0.05, 0.25, and 0.5 μg/ml) was electrophoresed and visualized by EtBr staining (*bottom*). The RNA was transferred onto a nitrocellulose membrane, and hybridized with probes of ND1 cDNA (*top*) and mouse β-actin cDNA (*middle*). *B*: Total RNA (5 or 20 μg) prepared from MIN6 cells treated without or with 0.5 μg/ml EtBr was blotted with probes of mouse COX2 cDNA (CO II), mouse COX4 cDNA (COX4), rat SUR cDNA (SUR), and mouse glucokinase cDNA (glucokinase). For analysis of glucokinase and SUR mRNA, 20 μg of total RNA was used. EtBr stainings are shown at the bottom.

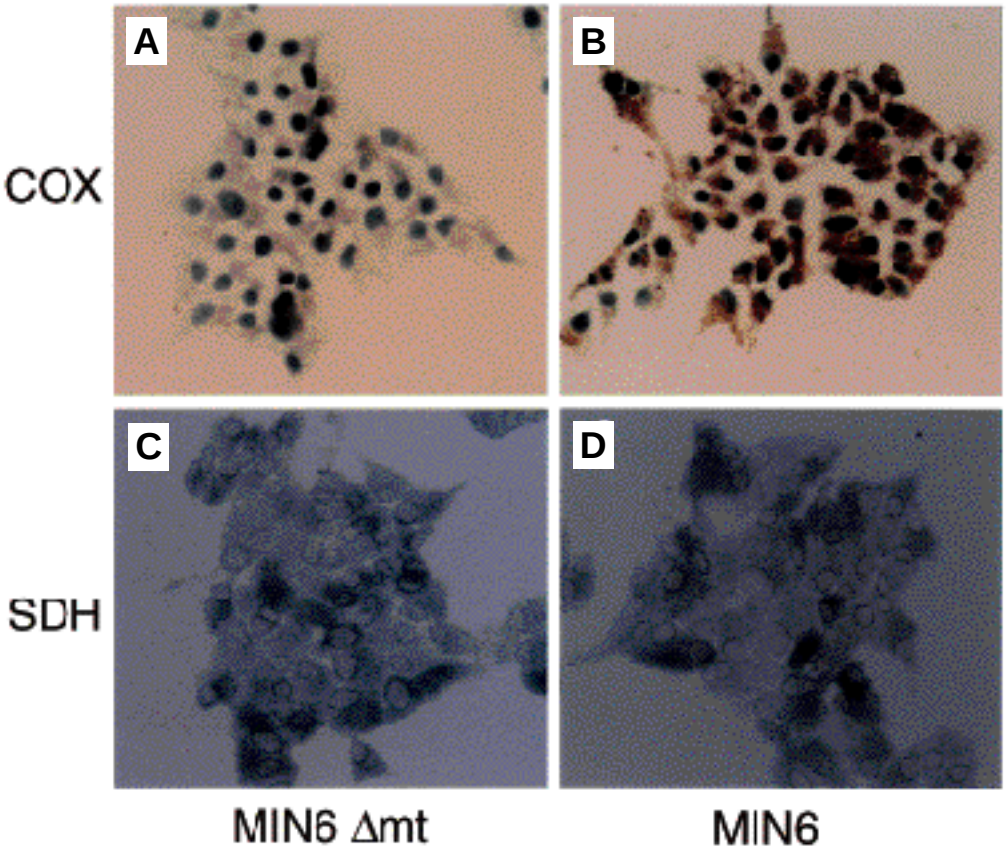

FIG. 3. Histochemical staining of mitochondrial enzymes in MIN6 Δmt cells. MIN6 Δmt cells (*left*) and MIN6 cells (*right*) grown on chamber slides were stained histochemically for COX activity (*A*, *B*) or SDH activity (*C*, *D*). The samples were photographed through a light microscope (magnification ×200). Each pair of photos was taken under identical conditions of exposure and contrast.

treatment (Fig. 2*B*). On the other hand, the level of β-actin mRNA, a nuclear DNA transcript, was not affected by EtBr treatment at any concentration (Fig. 2*A*, *middle*). COX4 mRNA, which encoded one of the respiratory chain subunits but was transcribed from nuclear DNA, was detected in both cells and was slightly increased (~30%) in MIN6 cells treated with 0.5 μg/ml EtBr (Fig. 2*B*). Glucokinase and SUR mRNAs, which both encoded proteins that were related to insulin secretion, were comparable in cells treated with or without EtBr.

On the basis of these results, MIN6 cells cultured with 0.5 μg/ml EtBr (MIN6 Δmt cells) and without EtBr (controls) were used for further studies.

Analysis of mitochondrial respiratory chain complexes activity by histochemical staining. Activities of the mitochondrial respiratory enzymes in MIN6 Δmt cells were examined by histochemical staining. When stained for activity of COX (whose subunits were encoded by both mtDNA and nuclear DNA), MIN6 Δmt cells revealed a marked decrease of activity when compared with MIN6 cells (Fig. 3*A* and *B*). On the other hand, when stained for activity of SDH (whose subunits were encoded only by nuclear DNA), MIN6 Δmt cells showed no significant difference from MIN6 cells (Fig. 3*C* and *D*). Therefore, selective impairment of the mitochondrial enzyme derived from mtDNA was confirmed in MIN6 Δmt cells.

Measurement of cellular ATP contents and respiratory chain–driven ATP synthesis. We then measured cellular ATP content stimulated with glucose and leucine. When the cells were incubated with various concentrations of glucose for 60 min, ATP content in MIN6 cells increased significantly in a dosage-dependent manner, with the amount of ATP in 25 mmol/l glucose being 2.3-fold higher than that in 2.5 mmol/l glucose. However, MIN6 Δmt cells showed no response to increased concentrations of glucose (Fig. 4*A*). By leucine stimulation, ATP content in MIN6 cells also increased significantly in a dosage-dependent manner. In MIN6 Δmt cells, leucine stimulation did not alter ATP content (Fig. 4*B*).

To further examine the functional capacity of the mitochondrial oxidative phosphorylation system, ATP synthesis driven by respiratory chain was measured in digitonin-permeabilized MIN6 and MIN6 Δmt cells. To determine the concentration of digitonin required to permeabilize the plasma membrane, malate-driven ATP synthesis was measured in the presence of various concentrations of digitonin (0–200 μg/ml) in MIN6 cells. As the result, the rate ATP synthesis increased with increasing digitonin concentrations and became constant with 5–80 μg/ml digitonin (data not shown). Therefore we used 40 μg/ml digitonin for further experiments. After two independent stimulations—one with malate (plus glutamate) and the other with succinate (plus rotenone)—MIN6 Δmt cells showed drastic reductions in ATP production compared with MIN6 cells (Fig. 5). Moreover, the level of ATP in MIN6 Δmt cells incubated with each oxidizable substrate was not different from that in MIN6 Δmt

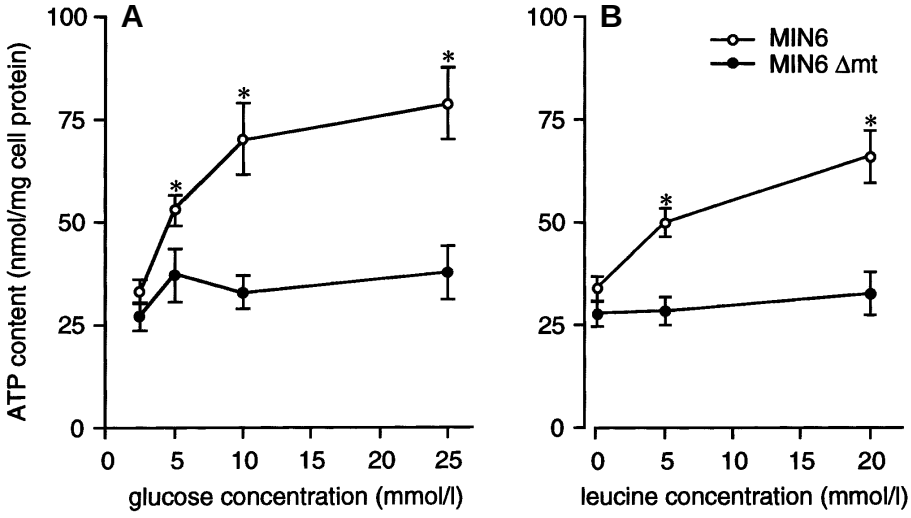

FIG. 4. Measurement of ATP contents in MIN6 Δmt cells. MIN6 cells (○) and MIN6 Δmt cells (●) were incubated with various concentrations of glucose (2.5, 5, 10, and 25 mmol/l) (*A*) or leucine (0, 5, and 20 mmol/l) (*B*) in the presence of 2.5 mmol/l glucose for 60 min at 37°C. The amount of ATP in the cell extracts was measured fluorimetrically. Data are expressed as nmol/mg cell protein. Data are means ± SD (bars); $n = 3$. *$P < 0.005$ vs. MIN6 cells with 2.5 mmol/l glucose alone.

cells incubated without the substrate (data not shown). Thus, the functional capacity of the oxidative phosphorylation system was completely disrupted in MIN6 Δmt cells.

Glucose utilization in MIN6 Δmt cells. Glucose utilization was studied at 0.15–50 mmol/l glucose concentrations in MIN6 and MIN6 Δmt cells. The glucose utilization in MIN6 Δmt cells was comparable with that in MIN6 cells at 0.3 mmol/l or less glucose concentration (Fig. 6*A*), but was significantly lower than that in MIN6 cells at higher glucose concentrations (0.6–50 mmol/l) (Fig. 6*B*).

Analyses of insulin mRNA, intracellular insulin content, and basal insulin secretion in MIN6 Δmt cells. Insulin mRNA was examined using RNA isolated from MIN6 Δmt and MIN6 cells incubated in different glucose concentrations. The levels of insulin mRNA from MIN6 Δmt cells cultured in 2.5 and 25 mmol/l glucose showed slight but significant decreases—20% ($n = 4$; $P < 0.05$) and 24% ($n = 4$; $P < 0.05$), respectively—when compared with those from MIN6 cells (Fig. 7). In each cell, insulin mRNA levels were comparable in the two glucose concentrations.

Next intracellular insulin content and basal insulin secretion in MIN6 and MIN6 Δmt cells were examined. The insulin content in MIN6 Δmt cells was approximately 1.7-fold higher than that in MIN6 cells (3,534.0 ± 346.8 vs. 2,147.4 ± 194.4 pmol/mg cell protein, respectively; $n = 6$; $P < 0.001$). Insulin secretion from cells incubated with 2.5 mmol/l glucose in the absence of any other secretagogues was different in proportion to the insulin content, and was 1.7-fold higher in MIN6 Δmt cells than in MIN6 cells (29.6 ± 1.4 vs. 17.7 ± 1.4 pmol · 30 min^{-1} · mg^{-1} cell protein, respectively; $n = 4$; $P < 0.001$).

In the following experiments, we defined the insulin secretion at 2.5 mmol/l glucose in the absence of any secretagogues as the basal secretion in each cell line, and expressed the insulin secretion with stimulation as the increase relative to each basal insulin secretion, as previously reported (37).

Measurements of insulin secretion and $[Ca^{2+}]_i$ of MIN6 Δmt cells in response to glucose or amino acids. To analyze the impact of the disruption of mitochondrial function on insulin secretion and increase in $[Ca^{2+}]_i$, we initially examined the effects of the nutrient stimulations in MIN6 Δmt cells. For this purpose, insulin secretion induced by glucose or two amino acids, leucine or arginine, was measured (Fig. 8). After stimulation with 25 mmol/l, insulin secretion increased 2.5-fold relative to basal secretion in MIN6 cells. However, MIN6 Δmt cells showed no response to the glucose stimulation.

When stimulated with 20 mmol/l leucine, insulin secretion in MIN6 cells increased 2.2-fold, but did not change from basal secretion in MIN6 Δmt cells. On the other hand, 20 mmol/l arginine enhanced insulin secretion in both MIN6 cells and MIN6 Δmt cells at almost equal levels (4.9 ± 0.6–fold and 5.9 ± 1.0–fold, respectively).

Because the increase in $[Ca^{2+}]_i$ is an important step for insulin secretion in pancreatic β-cells, we measured the change in $[Ca^{2+}]_i$ in response to glucose or amino acids in MIN6 Δmt cells by using fura-2. $[Ca^{2+}]_i$ in MIN6 cells increased in response to the change of glucose concentration (from 2.5 to 25 mmol/l) as previously described (22) (Fig. 9*A*). On the other hand, in MIN6 Δmt cells, $[Ca^{2+}]_i$ did not increase

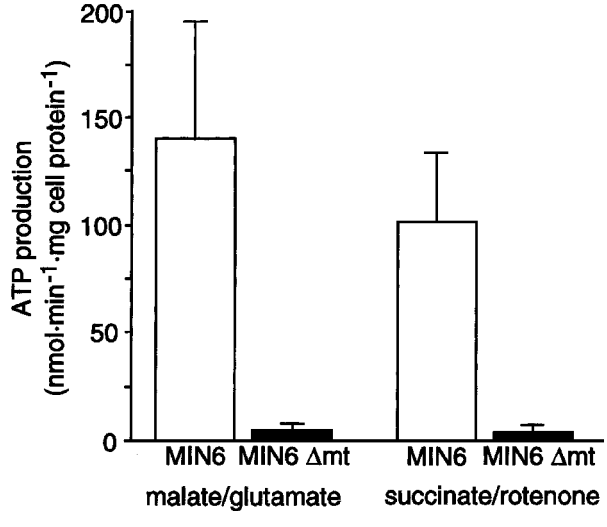

FIG. 5. Measurement of respiratory chain–driven ATP synthesis in MIN6 Δmt cells. Respiratory chain–driven ATP synthesis was examined in MIN6 cells (□) and MIN6 Δmt cells (■) by using two independent stimulations with 20 mmol/l malate (plus 20 mmol/l glutamate) or 20 mmol/l succinate (plus 40 µg/ml rotenone) in the presence of 40 µg/ml digitonine. Reactions were performed for 10 min at 37°C and the amount of ATP was measured fluorimetrically. Data are expressed as nmol · min^{-1} · mg^{-1} cell protein. Data are means ± SD (bars); $n = 4$.

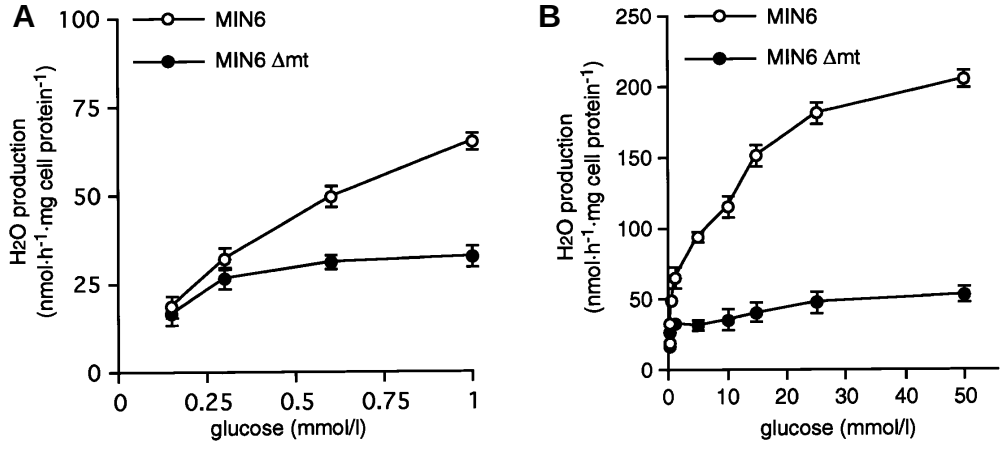

FIG. 6. Measurement of glucose utilization in MIN6 Δmt cells. Glucose utilization was measured in MIN6 cells (○) and MIN6 Δmt cells (●) by following the conversion of [5-^{3}H]glucose into ^3H_2O. *A*: H_2O production rates at low-glucose concentrations (0.15, 0.3, 0.6, and 1.0 mmol/l); *B*: H_2O production rates at all glucose concentrations (0.15, 0.3, 0.6, 1.0, 5.0, 10, 15, 25, and 50 mmol/l). Data are means ± SD of three independent experiments.

after the change of glucose concentration (Fig. 9*B*). Because MIN6 Δmt cells showed a rapid increase in $[Ca^{2+}]_i$ by 25 mmol/l KCl stimulation, the loss of the response was specific for glucose stimulation (Fig. 9*B*). When stimulated with 20 mmol/l leucine, $[Ca^{2+}]_i$ increased in MIN6 cells but not in MIN6 Δmt cells (Fig. 9*C* and *D*). On the other hand, when stimulated with 20 mmol/l arginine, $[Ca^{2+}]_i$ increased by almost the same extent in both cell lines (Fig. 9*E* and *F*). The responses of $[Ca^{2+}]_i$ to the different stimulations paralleled the data of insulin secretion in every secretagogue studied.

Measurements of insulin secretion and $[Ca^{2+}]_i$ of MIN6 Δmt cells in response to sulfonylureas. Finally, we examined the response of MIN6 Δmt cells to sulfonylureas. Sulfonylureas interact with the sulfonylurea receptor of pancreatic β-cells and inhibit the conductance of K_{ATP} channels, which finally leads to insulin secretion by depolarization of the plasma membrane (9,38,39). When MIN6 cells were incubated with the sulfonylurea glibenclamide (10 µmol/l), insulin secretion increased 6.6-fold in the presence of 2.5 mmol/l glucose and 10.1-fold in the presence of 25 mmol/l glucose relative to basal secretion (Fig. 10). The secretion in the high-glucose concentration was significantly higher than that in the low-glucose concentration (10.1 ± 0.8–fold vs. 6.6 ± 0.8–fold, respectively; $P < 0.001$). Insulin secretion of MIN6 Δmt cells increased after glibenclamide stimulation in both glucose concentrations (2.9 ± 1.1–fold and 4.7 ± 1.1–fold in 2.5 and 25 mmol/l glucose, respectively). However, there was no significant difference between the responses in the two glucose concentrations. The increments of insulin secretion in MIN6 Δmt cells were significantly lower than those in MIN6 cells in both glucose concentrations ($P < 0.05$).

Insulin secretion in the presence of the K_{ATP} channel opener, diazoxide (250 µmol/l), or diazoxide plus KCl (25 mmol/l) was also examined. After treatment with diazoxide, the increase in insulin secretion observed in 25 mmol/l glucose stimulation alone was completely inhibited in MIN6 cells (Fig. 10). In MIN6 Δmt cells, no response was observed, as in stimulation with glucose alone. After treatment with diazoxide plus KCl, insulin secretion from MIN6 cells increased by

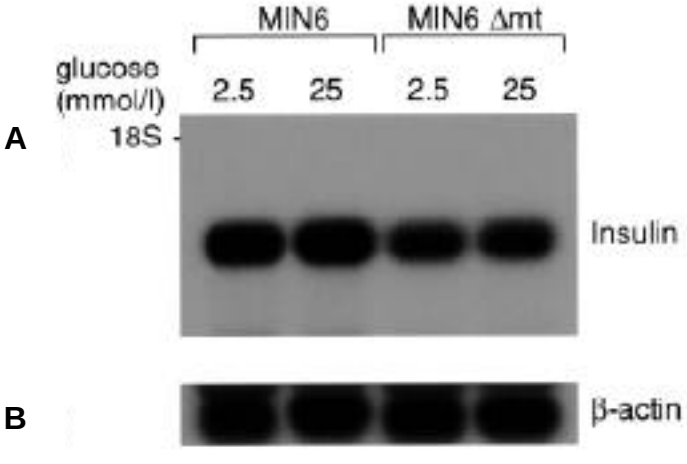

FIG. 7. Northern blot analysis of insulin gene mRNA. Total RNA was prepared from MIN6 and MIN6 Δmt cells incubated with 2.5 or 25 mmol/l glucose for 6 h. The RNA (5 µg/lane) was electrophoresed, transferred, and hybridized with [α-^{32}P]dCTP-labeled hamster insulin I cDNA probe (*A*) or mouse β-actin cDNA probe (*B*).

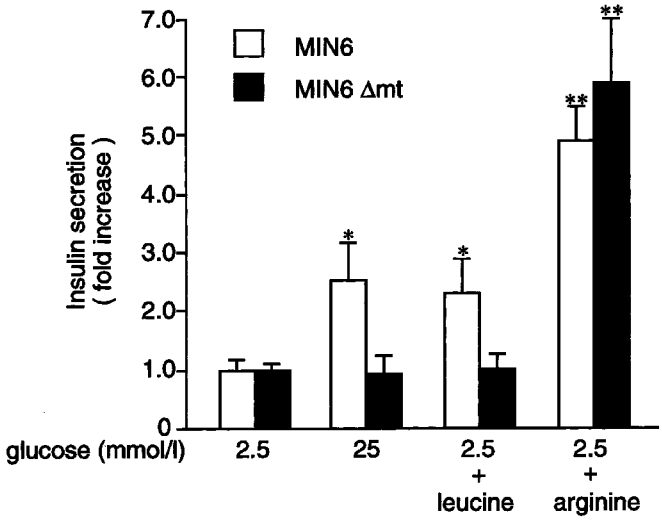

FIG. 8. Insulin secretion in response to glucose or amino acids in MIN6 Δmt cells. MIN6 cells (□) and MIN6 Δmt cells (■) were incubated with 2.5 mmol/l glucose (basal secretion), 25 mmol/l glucose, or two amino acids (20 mmol/l leucine or 20 mmol/l arginine), both in the presence of 2.5 mmol/l glucose at 37°C for 30 min. Immunoreactive insulin secreted into medium was measured by RIA. The response to secretagogue in each cell line is expressed as *x*-fold increase relative to each basal secretion. Data are means ± SD (bars); $n = 4$. *$P < 0.05$; **$P < 0.005$ vs. each basal secretion.

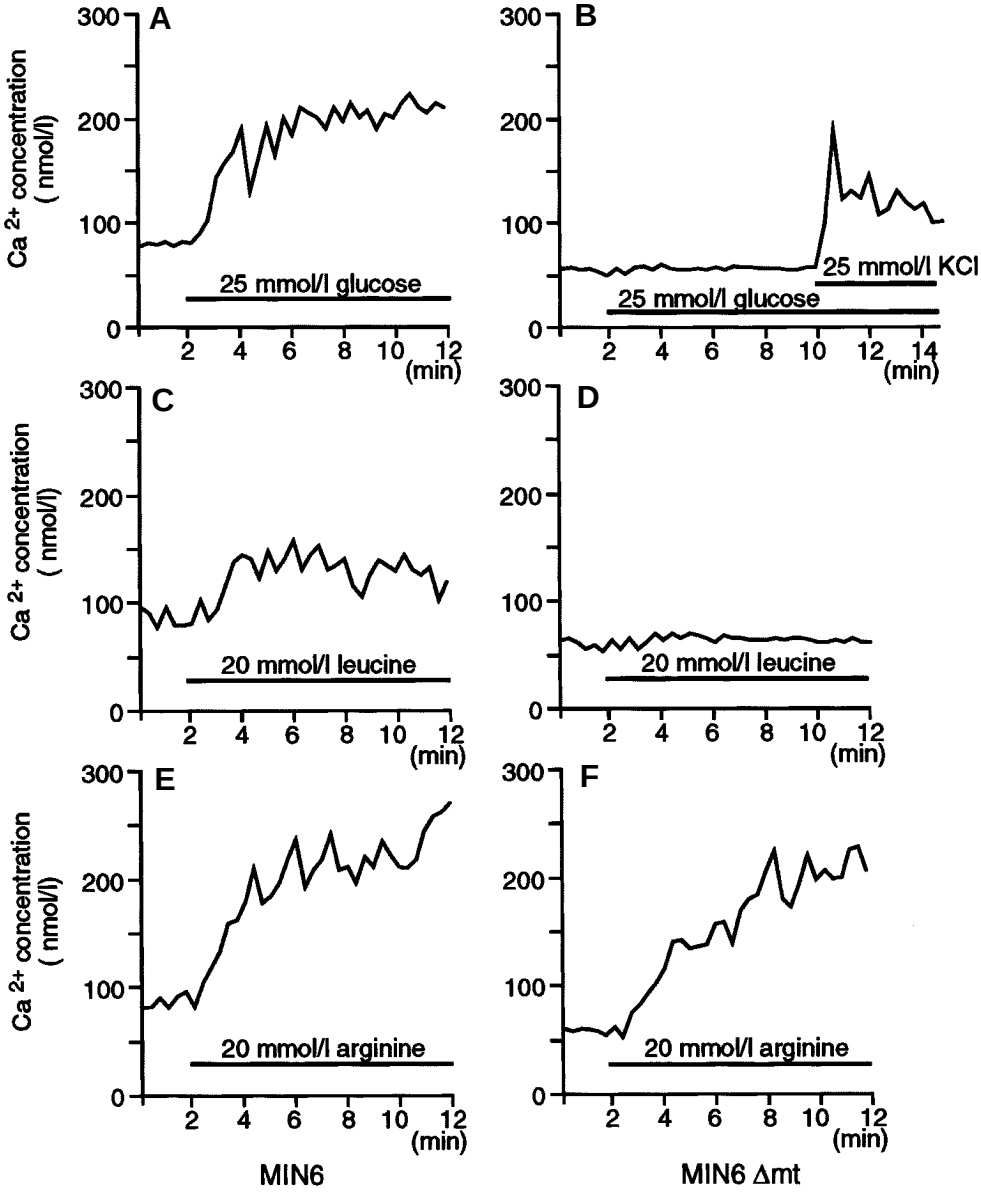

FIG. 9. Measurement of $[Ca^{2+}]_i$ in MIN6 Δmt cells stimulated with glucose or amino acids. Changes in $[Ca^{2+}]_i$ in MIN6 cells (*left*) and MIN6 Δmt cells (*right*) in response to 25 mmol/l glucose (*A*, *B*), 25 mmol/l KCl (*B*), 20 mmol/l leucine (*C*, *D*), or 20 mmol/l arginine (*E*, *F*) were measured using fura-2. Data were obtained from seven 3 × 3-μm square windows in the same microscopic field and averaged. One point was taken in every 20 s. The addition of each secretagogue is indicated by the black bar in the panel.

~11-fold in 2.5 mmol/l glucose and 17-fold in 25 mmol/l glucose. The increment in 25 mmol/l glucose was significantly higher than that in 2.5 mmol/l glucose (16.8 ± 3.5–fold vs. 11.1 ± 1.3–fold, respectively; $P < 0.05$). Insulin secretion from MIN6 Δmt cells treated with diazoxide plus KCl also increased in both glucose concentrations, but the increments were almost equal in both glucose concentrations (15.2 ± 3.1–fold in 2.5 mmol/l glucose and 14.7 ± 1.6–fold in 25 mmol/l glucose). There were no significant differences in the increments between MIN6 and MIN6 Δmt cells in both glucose concentrations. According to this result, MIN6 Δmt cells retained the capacity of insulin secretion induced by membrane depolarization.

To further investigate the effect of glibenclamide, we measured the changes in $[Ca^{2+}]_i$ in response to glibenclamide in MIN6 and MIN6 Δmt cells. In both MIN6 cells and MIN6 Δmt cells, although $[Ca^{2+}]_i$ increased after the addition of 10 μmol/l glibenclamide (Fig. 11*A* and *B*), the response was weaker in the MIN6 Δmt cells than in the MIN6 cells. Therefore it was suggested that the decrease in insulin secretion induced by glibenclamide in MIN6 Δmt cells reflected the decreased elevation of $[Ca^{2+}]_i$.

DISCUSSION

In the present study, we created a pancreatic β-cell line in which mtDNA was depleted by EtBr treatment (MIN6 Δmt cells) and characterized the insulin secretion patterns stimulated with various secretagogues. EtBr is known to intercalate in double-strand nucleic acids. When mammalian cells were cultured in the presence of EtBr, the synthesis of mtDNA and RNA encoded by mtDNA was selectively inhibited and closed-circular mtDNA was broken down; after long-term culture in the presence of sublethal EtBr concentrations, the cells lacked mtDNA and its transcripts (40). At first we tried to construct MIN6 cells completely deficient in mtDNA according to methods described for other cell lines (24,25). In our study, the mtDNA content was markedly decreased by treatment with 0.5 μg/ml EtBr, which was a 10-fold higher amount than that reported in previous studies. Recently, one ρ^0 cell line was created from neuroblastoma

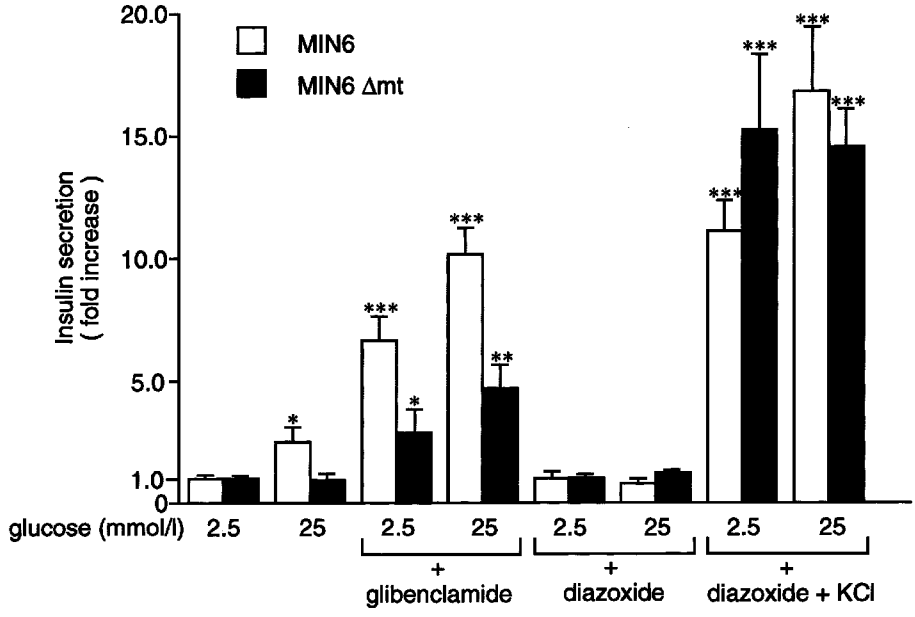

FIG. 10. Insulin secretion in response to glibenclamide or KCl stimulation in MIN6 Δmt cells. MIN6 cells (□) and MIN6 Δmt cells (■) were stimulated with 10 µmol/l glibenclamide, 250 µmol/l diazoxide, or 250 µmol/l diazoxide plus 25 mmol/l KCl in the presence of 2.5 or 25 mmol/l glucose. Immunoreactive insulin secreted into the medium was measured by RIA. Data were expressed as *x*-fold increase relative to each basal secretion, as in Fig. 8. Data are means ± SD (bars); *n* = 4. *P < 0.05; **P < 0.005; ***P < 0.001 vs. each basal secretion.

cells by treatment with 5.0 µg/ml of EtBr (40), thus suggesting that the sensitivity to EtBr treatment could be different in cell types. We therefore tested higher concentrations of EtBr in MIN6 cells. However, in 2.5 and 5.0 µg/ml of EtBr, the cells died within a few weeks, and in 1.0 µg/ml of EtBr, the cells survived but showed morphological changes.

We then examined the effect of EtBr treatment on the level of mRNAs transcribed from mtDNA and nuclear DNA. In MIN6 cells treated with EtBr, ND1 mRNA decreased significantly with 0.05 µg/ml of EtBr and was undetectable with 0.5 µg/ml EtBr. These results suggested that the mRNAs transcribed from mtDNA were affected by EtBr, even at the lower concentration at which the change of the amount of mtDNA was undetectable in Southern blot analysis. COX2 mRNA, which was another transcript of mtDNA, was also undetectable with 0.5 µg/ml EtBr. On the other hand, COX4, SUR, glucokinase, and β-actin mRNAs, transcripts of nuclear DNA, were not decreased in cells treated with EtBr, suggesting that the effect of EtBr on transcription was specific for mtDNA. Therefore, for further analysis, we used the MIN6 cells treated with 0.5 µg/ml EtBr (MIN6 Δmt cells) in which mRNAs encoded by mtDNA were completely depleted and no morphological changes were observed.

In histochemical staining, MIN6 Δmt cells were confirmed to show the defect only in the enzyme activity whose subunits were encoded by mtDNA. This result further suggested that both transcription and translation of proteins encoded by mtDNA were selectively inhibited in MIN6 Δmt cells.

Next, to assess the capacity of MIN6 Δmt cells to produce ATP, we first measured the cellular ATP contents stimulated with glucose and leucine. The glucose- or leucine-stimulated increase in ATP content was completely blunted in MIN6 Δmt cells. We then studied the respiratory chain–driven ATP-synthesis using sets of oxidizable substrates and examined the glucose utilization in the cells. In the ATP synthesis assay, MIN6 Δmt cells showed an impairment of ATP production after all of the stimulations examined. The measurement of respiratory chain–driven ATP synthesis enabled us to estimate the total function of the mitochondrial oxidative phospho-

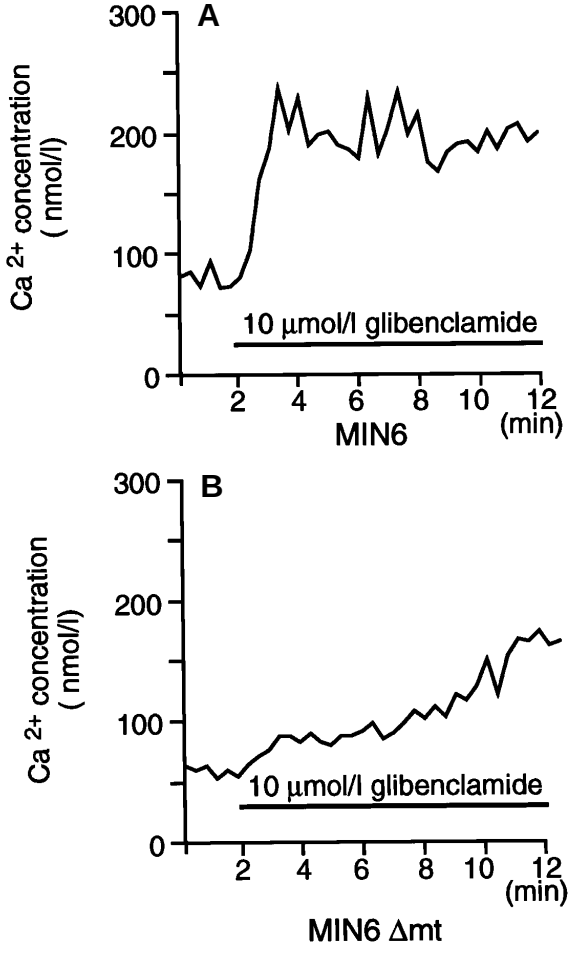

FIG. 11. Measurement of $[Ca^{2+}]_i$ in MIN6 Δmt cells in response to glibenclamide. Changes in $[Ca^{2+}]_i$ in MIN6 (*A*) and MIN6 Δmt cells (*B*) in response to 10 µmol/l glibenclamide were measured by using fura-2, as in Fig. 9. The addition of glibenclamide is indicated by the black bar in the panel.

rylation system (35). Although the majority of subunits that form the respiratory chain enzymes are encoded by nuclear DNA, 13 subunits are encoded by mtDNA. Therefore, the disruption of the mitochondrial oxidative phosphorylation system in MIN6 Δmt cells was probably due to the absence of the subunits encoded by mtDNA. In the glucose utilization assay, marked reduction in glucose usage in MIN6 Δmt cells was confirmed at ≥0.6 mmol/l glucose concentrations, suggesting that not only the mitochondrial oxidative phosphorylation pathway but also the glycolytic pathway was impaired in MIN6 Δmt cells. The mechanism by which EtBr treatment could impair the glycolytic pathway is not clear. It has been suggested that glucokinase, as well as hexokinases I–III, bind to the mitochondrial pore protein at the mitochondrial surface, which could regulate their enzymatic activities (see review in 13). Because the binding affinities of the mitochondrial pore protein for hexokinases were regulated by an electric potential of the mitochondrial membrane that was the energy of the redox reactions during the mitochondrial electron transport (13), the reduced glucose utilization rate observed in MIN6 Δmt cells might be related in part to the reduced glucokinase activity and/or hexokinase activity caused by impaired mitochondrial function. Further analysis of the enzymes and metabolites involved in glycolytic pathway in MIN6 Δmt cells is necessary to clarify this mechanism.

Intracellular insulin content in MIN6 Δmt cells was 1.7-fold higher than that in MIN6 cells, although the insulin mRNA level in MIN6 Δmt cells was slightly lower than that in MIN6 cells. The reason for this is unclear. It could be that the MIN6 Δmt cells stored more insulin than MIN6 cells in the high-glucose culture condition before the preincubation period, because in the high-glucose condition MIN6 cells released insulin continuously whereas the MIN6 Δmt cells did not secrete insulin. Basal insulin secretion in 2.5 mmol/l glucose in MIN6 Δmt cells was higher than that in MIN6 cells in proportion to the insulin content, which could reflect the difference of the insulin content in these cells.

In measurements of insulin secretion and $[Ca^{2+}]_i$, MIN6 Δmt cells showed no response to glucose or leucine stimulation. Leucine is metabolized to produce ATP only through the mitochondrial oxidative phosphorylation pathway (10,41); thus the blunted response to leucine stimulation in MIN6 Δmt cells was probably due to the disruption of the mitochondrial oxidative pathway. On the other hand, glucose is metabolized to ATP in both the glycolytic and mitochondrial oxidative pathways. Therefore, not only the lack of ATP generation through the mitochondrial oxidative pathway but also the impaired glycolytic pathway might be responsible for the blunted responses of MIN6 Δmt cells to glucose stimulation, although the former pathway was the key reaction in normal islets (6).

Another nutrient secretagogue, arginine, enhanced insulin secretion and an increase in $[Ca^{2+}]_i$ in MIN6 Δmt cells. These responses induced by arginine were dependent on the influx of extracellular Ca^{2+} via the VDCC in pancreatic β-cells (42–44). However, the mechanism involved in this response is still unclear. Several mechanisms for arginine-induced insulin secretion and increase in $[Ca^{2+}]_i$ have been proposed, such as the metabolism of arginine leading to the formation of ATP (45,46), the generation of nitric oxidase (47), and the direct depolarization of the plasma membrane potential due to the accumulation of the cationic amino acid (44,46). The arginine-induced actions require the presence of nutrients (e.g., glucose), and arginine alone could not induce either an increase in $[Ca^{2+}]_i$ or closure of K_{ATP} channels (41,44,48). Because the arginine-induced increase in $[Ca^{2+}]_i$ was inhibited by addition of the K_{ATP} channel opener, diazoxide (11,44,45), the process could be related to closure of the K_{ATP} channels. In our present study, arginine stimulated an increase in $[Ca^{2+}]_i$ and insulin secretion in MIN6 Δmt cells as in MIN6 cells. Therefore, we suggest that mitochondrial oxidative phosphorylation and metabolites passing through the glycolytic pathway are not required in arginine-induced insulin secretion or increase in $[Ca^{2+}]_i$ in MIN6 cells. Further studies are needed to define the precise pathway of arginine-induced insulin secretion.

Sulfonylureas interact with the sulfonylurea receptor and inhibit the conductance of K_{ATP} channels (9,38,39). In this study, glibenclamide stimulated an increase in $[Ca^{2+}]_i$ and insulin secretion in MIN6 Δmt cells, but the responses were significantly lower than those in MIN6 cells. On the other hand, there was no significant difference in insulin secretion induced by membrane depolarization with KCl between two cells. These data suggest that in MIN6 Δmt cells, the signaling of insulin secretion induced by glibenclamide was partially impaired, although the signaling system after membrane depolarization was not. Sulfonylurea receptors have two nucleotide-binding folds and nine potential phosphorylation sites in the folds (39). Niki and Ashcroft (38,49) reported that the specific binding of glibenclamide to the sulfonylurea receptor was decreased when intracellular ATP was depleted in HIT-T15 cells, and suggested that phosphorylation of the sulfonylurea receptor or of some functionally related protein(s) might play a role in the binding between glibenclamide and its receptor. Because we confirmed that ATP production was depleted in MIN6 Δmt cells, the reduced effect of glibenclamide shown in MIN6 Δmt cells may be caused by the impaired function and/or decreased binding of the sulfonylurea receptor to glibenclamide due to the loss of its phosphorylation.

In MIN6 cells, insulin secretion stimulated with KCl plus diazoxide showed a glucose-dependent increase, whereas with diazoxide alone, the cells did not show the response to the high-glucose concentration. Therefore the glucose-dependent increase shown in MIN6 cells with KCl plus diazoxide is thought to be a K_{ATP} channel–independent event, which would be in accordance with the previous reports on rat and mouse pancreatic β-cells (50–52). In contrast, this glucose-dependent increase with KCl plus diazoxide stimulation was not observed in MIN6 Δmt cells, which suggests that this K_{ATP} channel–independent glucose action observed in MIN6 cells may also require mitochondrial oxidative phosphorylation and/or the glycolytic pathway.

Recently, mtDNA less MIN6 cells (ρ^0 MIN6 cells) were constructed by treatment with bis-4-piperidyledichloride (53). The cells revealed similar characteristics with MIN6 Δmt cells, such as lack of COX activity without influence on SDH activity, and impaired glucose-induced insulin secretion and increase in $[Ca^{2+}]_i$. In our present study, the response to leucine, arginine, and glibenclamide was examined as well as the response to glucose, which enabled us to analyze further the mechanisms of insulin secretion induced by these secretagogues. In this way, an MIN6 Δmt cell line as well as ρ^0

MIN6 cells seem to be good tools for the study of insulin secretion signaling.

Several investigators have examined the role of mitochondrial function in pancreatic β-cells by using various inhibitors and stimulators (54–57), and have suggested the importance of mitochondrial oxidative phosphorylation in closure of the K_{ATP} channel, $[Ca^{2+}]_i$ elevation, or insulin secretion. In the present study using MIN6 Δmt cells, we were able to confirm the importance of mitochondrial function in β-cells without any inhibitors, which might alter cell functions other than mitochondria.

In diabetic patients with the mtDNA mutation, especially the mutation at np 3243 in the mitochondrial $tRNA^{Leu(UUR)}$ gene, the delayed and insufficient insulin responses to a glucose load, together with reduced urinary excretion of C-peptide, have been reported (see review in 58). Recently, the analysis of the β-cells of diabetic patients with the 3243 mutation was reported, in which markedly decreased COX activity and increased SDH activity were confirmed by histochemical staining (59). The function of this 3243 mutation has been examined in vitro by using mtDNA-less (ρ^0) human cell lines, which were derived from human osteosarcoma cells (17,18). The hybrid cells, obtained by transfer of mitochondria with 3243 mutation into ρ^0 cells, had shown defects in the synthesis of proteins encoded by mtDNA and decreases in the activity of respiratory chain complexes and the capacity of oxidative phosphorylation. However, the function of the 3243 mutation in pancreatic β-cells has not yet been examined. It has been reported that most of NIDDM patients with the 3243 mutation preserve the secretory response to arginine (15) and are more likely to show sulfonylurea-resistant and insulin-requiring diabetes than NIDDM patients without the mutation (14–16). These clinical characteristics are consistent with the data observed in MIN6 Δmt cells. Thus the phenomena shown in MIN6 Δmt cells might be similar to that in β-cells with the 3243 mutation. Further analysis of MIN6 Δmt cells may provide us with some clues for the treatment and understanding of these patients.

In conclusion, MIN6 Δmt cells, depleted of mitochondrial DNA by EtBr treatment, showed impaired ATP production through the mitochondrial oxidative phosphorylation pathway and glycolytic pathway. MIN6 Δmt cells lacked the glucose- and leucine-induced insulin secretion, suggesting the importance of ATP production in insulin secretion induced by these secretagogues. In contrast, arginine-induced insulin secretion and increase in $[Ca^{2+}]_i$ did not require these pathways. Moreover, MIN6 Δmt cells showed the relatively weak response to glibenclamide, suggesting that ATP production through these pathways was also important for the stimulation of insulin secretion induced by sulfonylureas.

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