Mitochondrial DNA Is Required for Regulation of Glucose-stimulated Insulin Secretion in a Mouse Pancreatic Beta Cell Line, MIN6*

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To determine whether mtDNA and mitochondrial respiratory function in pancreatic beta cells are necessary for the phenotypic expression of glucose-stimulated insulin secretion, we used a cultured mouse pancreatic beta cell line, MIN6, and two derivative lines, mtDNA knockout MIN6 (ρ0 MIN6) and mtDNA repopulated cybrid MIN6. The MIN6 cells retain the property of glucose-stimulated insulin secretion, but their mtDNA knockout induced the loss of mitochondrial transcription, translation, and respiration activity, without inhibition of transcription of the insulin gene or loss of succinate dehydrogenase activity, indicating that the observed mitochondrial dysfunction in ρ0 MIN6 cells was not due to a cytotoxic side effect derived from the mtDNA knockout. Moreover, the mtDNA depletion also inhibited both the glucose-stimulated increase in the intracellular free Ca2+ content and the elevation of insulin secretion. The possibility of the involvement of nuclear genome-encoded factors in this process was excluded by the observation that the missing sensitivity to extracellular glucose stimulation in ρ0 MIN6 cells was restored reversibly by repopulation with foreign mtDNA and isolating cybrid MIN6 clones. Therefore, these findings provide unambiguous evidence for the involvement of the mitochondrial dysfunction induced by mtDNA impairment in developing pathogeneses of some forms of diabetes mellitus.

Diabetes mellitus is characterized as a genetically heterogeneous disorder but exhibits common features, such as glucose intolerance (1) and a relative lack of insulin release (2), which lead to high blood sugar levels. It is generally considered that various genetic factors, as well as unknown ones, could be responsible for the subsets of the disease. They encode factors involved in pancreatic beta cell function, translation, and respiration activity, without inhibition of transcription of the insulin gene or loss of succinate dehydrogenase activity, indicating that the observed mitochondrial dysfunction in ρ0 MIN6 cells was not due to a cytotoxic side effect derived from the mtDNA knockout. Moreover, the mtDNA depletion also inhibited both the glucose-stimulated increase in the intracellular free Ca2+ content and the elevation of insulin secretion. The possibility of the involvement of nuclear genome-encoded factors in this process was excluded by the observation that the missing sensitivity to extracellular glucose stimulation in ρ0 MIN6 cells was restored reversibly by repopulation with foreign mtDNA and isolating cybrid MIN6 clones. Therefore, these findings provide unambiguous evidence for the involvement of the mitochondrial dysfunction induced by mtDNA impairment in developing pathogeneses of some forms of diabetes mellitus.

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secretion of insulin in response to the extracellular glucose concentration (27, 28). The results showed that the property of glucose-stimulated insulin secretion was totally lost on mtDNA knockout, i.e. depletion of mtDNA, in the \( \rho^- \) MIN6 cells, whereas it could be reversibly restored by repopulation with foreign mtDNA through the fusion of the cells with enucleated cytoplasts from a mouse fibroblast cell line, suggesting that mtDNA and the mitochondrial respiratory function are necessary for glucose-stimulated insulin secretion.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—A mouse pancreatic islet beta cell line, MIN6 cells, established from a transgenic mouse harboring an insulin promoter plus SV40 T antigen fusion construct (27), was cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose and 15% fetal calf serum (FCS). In the present study, MIN6 cells collected at passages up to 50 were used in RPMI 1640 medium supplemented with 15% FCS and pyruvate (0.1 mM). The mtDNA knockout MIN6 cell line, \( \rho^- \) MIN6, was isolated by treating MIN6 cells with bis-4-piperidyle-dichloro (150 mM) at 37 °C for 7 days, and were cultured in RPMI 1640 medium supplemented with FCS (15%), pyruvate (0.1 mg/ml), and uridine (50 \( \mu \)g/ml). B82CAP cells, which are deficient in thymidine kinase, i.e. resistant to Evtrelin and resistant to chloramphenicol (CAP) (28), were cultured in RPMI 1640 medium with 10% FCS, pyruvate (0.1 mg/ml), and CAP (100 \( \mu \)g/ml).

**Southern Blot Analyses of mtDNA**—Total DNA (1 \( \mu \)g) extracted from 2 \( \times \) 10\(^6\) cells was digested with a single cut restriction enzyme, XhoI, and the restriction fragments were separated by 0.8% agarose gel electrophoresis. After blotting onto a Nytran membrane, the DNA fragments were hybridized with \([\alpha^32P]dATP\)-labeled mouse mtDNA. The radioactivity of the fragments was measured with a BAS2000 bioimaging analyzer (Fuji, Fuji Photo Film, Tokyo, Japan).

**Northern Blot Analyses of Transcripts of mtDNA and the Insulin Gene**—Total cellular RNA was extracted with an Isogen RNA isolation kit (Nippon Gene, Toyama, Japan). Total denatured RNA (10 \( \mu \)g) was electrophoresed on a 1% agarose gel containing formaldehyde and then transferred onto a Nytran membrane. The membrane was hybridized with a \([\alpha^32P]dATP\)-labeled mouse mtDNA fragment (COI and COII) or rat insulin I cDNA probe (30). The radioactivity of the bands was measured with a BAS2000 bioimaging analyzer (Fuji Photo Film).

**Analysis of Mitochondrial Translation Activity**—Mitochondrial translation products were labeled with \([\beta^35S]\)methionine as described previously (20) with a slight modification. Briefly, semiconfluent cells in a dish were incubated in methionine-free medium containing 10% fetal bovine serum for 1 h at 37 °C. Then, the cells were labeled with \([\beta^35S]\)methionine in methionine-free medium containing 0.2% FCS for 1 h in the presence of emetine (0.2 mg/ml). The mitochondrial fraction was obtained by homogenization in 0.25 M sucrose, 1 mM EGTA, and 10 mM Hepes-NaOH, pH 7.4, followed by differential centrifugation. Proteins in the mitochondrial fraction (15 mg/lane) were separated by SDS 15% polyacrylamide gel electrophoresis. The dried gel was exposed to an imaging plate for 18 h and the labeled polypeptide was analyzed with a BAS2000 bioimaging analyzer.

**Analyses of Mitochondrial Respiration Enzyme Activity**—The activities of mitochondrial respiratory enzymes cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) were assayed as described previously (31 and 32, respectively).

**Measurement of Oxygen Consumption**—MIN6 and \( \rho^- \) MIN6 cells were incubated in the medium with 0.1 or 25 mM glucose for 1 h, and the \( O_2 \) consumption was measured as follows: cells (5 \( \times \) 10\(^4\)) were trypsinized and dispersed in phosphate-buffered saline with 0.25 mM glucose, and \( O_2 \) consumption of cells was recorded in a polarographic cell (1.0 ml) at 37 °C with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) (33).

**Measurement of the Intracellular Free Ca\(^{2+}\) Concentration**—The intracellular free Ca\(^{2+}\) concentration was measured according to the procedure of Yada et al. (34). Briefly, MIN6 cells were cultured on a glass coverslip in a 6-cm dish and then incubated with 10 \( \mu \)M fura-2/AM for 30 min at 37 °C in Heps-balanced Krebs-Ringer bicarbonate (KRB) buffer containing 0.1 mM glucose. The cells were then placed on the stage of an inverted microscope and superfused at 1 ml/min at 37 °C with KRB buffer or stimulated (25 mM) glucose concentration. When delivering and removing superfusate, fura-2 dual excitation (340 and 380 nm) and fluorescence detection (510 nm) were accomplished with an Aruguus-50/CA image analysis system (Hamamatsu Photonics Inc., Hamamatsu, Japan), and the 340-380 and 340-380 nm ratio signals were recorded continuously. The intracellular free Ca\(^{2+}\) concentration is expressed in all records as the 340/380 nm ratio.

**Insulin Assay**—MIN6 cells (1 \( \times \) 10\(^5\) cells) were seeded into 1 ml of Dulbecco’s modified Eagle’s medium containing glucose (25 mM) in 24-well plates. Insulin secretion was determined using a static incubation method in 5% CO\(_2\) at 37 °C with KRB buffer as described previously (28). The glucose concentration was 0.1 mM, and the glucose stimulation was carried out in 25 mM glucose. The secreted insulin levels in media were assayed for immunoreactive insulin by radioimmunoassay using rat insulin as a standard. To each well, 200 \( \mu \)l of 1 mM NaOH was added to solubilize the cells for determination of the cellular protein content with a Bio-Rad protein assay kit. For measurement of intracellular insulin content, 1 mM acetic acid ethanol was added to the wells, which were then sealed with pressure-sensitive film. The extract was collected after 24 h incubation at 4 °C, diluted, and assayed by radioimmunoassay.

**Intercellular Transfer of the Exogenous mtDNA of B82CAP Cells into \( \rho^- \) MIN6 Cells and Isolation of Cybrid Clones**—Intercellular mtDNA transfer was carried out by fusion of enucleated B82CAP cells with \( \rho^- \) MIN6 cells as described previously (16) with slight modifications. Briefly, B82CAP cells grown on round glass discs were enucleated by centrifugation (13,000 rpm at 34 °C for 10 min) in the presence of cytochalasin B (10 \( \mu \)g/ml; Sigma). The resulting cytoplasts were mixed with \( \rho^- \) MIN6 cells, and then fusion was carried out in the presence of 50% (v/v) polyethylene glycol 1500 (Boehringer Mannheim). The fusion mixture was cultivated in a selective medium without glucose (DM170; Kyokuto Kagaku, Tokyo, Japan) supplemented with hypoxanthine-aminopterin-thymidine and 15% FCS (16). In this selective medium the residual nonenucleated parental B82CAP cells were completely eliminated with hypoxanthine-aminopterin-thymidine. Unfused parental \( \rho^0 \) MIN6 cells were removed with DM170 medium, since they could not grow in the medium without glucose due to the absence of mtDNA. On days 35–50 after the fusion, colonies that had grown in the selective medium were cloned by the cylinder method. The clones were cultivated in Dulbecco’s modified Eagle’s medium (25 mM glucose and 15% FCS) containing 100 \( \mu \)g/ml CAP.

**Chromosome Analysis**—The chromosome compositions of hybrid clones were analyzed immediately after cloning using air-dried chromosome preparations as described previously (35).

**RESULTS**

**Effect of mtDNA Depletion of MIN6 Cells on the Mitochondrial Respiratory Function**—A mtDNA knockout MIN6 cell line, \( \rho^- \) MIN6, was used to investigate the influence of a deficiency of mtDNA on the mitochondrial respiratory function and on the phenotypic expression of glucose-stimulated insulin secretion observed in parental MIN6 cells. In mtDNA-depleted \( \rho^- \) MIN6 cells, mtDNA and its transcripts, such as those of the COI and COII genes encoding COX subunits, were not detected (Fig. 1, a and b). The overall absence of mitochondrial translation products was confirmed by \([\beta^35S]\)methionine incorporation into mitochondrial synthetized polypeptides in the presence of emetine; no labeled polypeptides were detectable in \( \rho^- \) MIN6 cells (Fig. 2). On the other hand, the amount of transcripts of the insulin gene did not change substantially (Fig. 1b). Therefore, complete inhibition of transcription and translation in mitochondria was attained through the mtDNA depletion in the \( \rho^- \) MIN6 cells without inhibition of transcription of the insulin gene.

Then, we analyzed the mitochondrial respiratory enzyme activities of COX, in which subunits were encoded by both mtDNA and nuclear DNA, and of SDH, in which all subunits were relatively encoded by nuclear DNA. The results showed that COX activity was completely inactivated in practically due to the absence of three COX subunits (COI, COII, and COIII) encoded by mtDNA (Fig. 2), suggesting inhibition of the mitochondrial respiratory chain function, whereas SDH activity

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1. The abbreviations used are: FCS, fetal calf serum; CAP, chloramphenicol; COX, cytochrome c oxidase; SDH, succinate dehydrogenase; KRB, Krebs-Ringer bicarbonate.
2. K. Inoue, D. Takai, A. Soejima, and J.-I. Hayashi, manuscript in preparation.
remained at normal or slightly elevated levels in MIN6 and ρ0 MIN6 cells (Fig. 3). Thus, the inhibition of mitochondrial respiratory enzyme activities was limited to enzymes that include subunits encoded by mtDNA. Moreover, the observations that MIN6 cells showed normal SDH activity (Fig. 3) and normal transcription of the insulin gene (Fig. 1) suggest that the deficiency of the mitochondrial respiratory function in the cells is not due to a cytotoxic side effect derived from the mtDNA knockout.

Effect of mtDNA Depletion on the Glucose-stimulated Increases in the O2 Consumption, Intracellular Free Ca2+ Concentration, and Insulin Secretion—First, we confirmed that the glucose oxidation deduced from the O2 consumption was increased by the glucose stimulation in MIN6 cells but not in ρ0 MIN6 cells (Fig. 4). On the other hand, the amounts of transcripts of the insulin gene in both cells did not change by the glucose stimulation (Fig. 1). Moreover, intracellular insulin contents in MIN6 and ρ0 MIN6 cells in the low glucose concentration were similar (4436.3 ± 201.7 and 4734.7 ± 297.9 pmol/mg protein, respectively) and did not change substantially by the glucose stimulation.

Then, we examined whether the inhibition of the mitochondrial respiratory chain function induced by mtDNA depletion in ρ0 MIN6 cells could affect the phenotypic expression of glucose-stimulated increases in the intracellular free Ca2+ concentration and insulin secretion, which had been observed in the parental MIN6 cells. Measurement of the intracellular free Ca2+ concentration by loading fura-2 revealed that the intracellular free Ca2+ concentration in the parental MIN6 cells increased in response to extracellular glucose stimulation (25 mM glucose) (Fig. 5) in a similar way to that observed in pancreatic β cells isolated from islets of mice and rats (34, 36). On the other hand, the intracellular free Ca2+ concentration in the ρ0 MIN6 cells did not change substantially with glucose stimulation (Fig. 5a). These observations suggest that the glucose-stimulated increase in intracellular free Ca2+ is totally inhibited by the mtDNA deficiency in ρ0 MIN6 cells and thus support the idea that the mitochondrial respiratory chain function is necessary for the phenotypic expression of glucose-stimulated influx of Ca2+ in normal pancreatic β cells.
Since it has been generally proposed that the glucose-stimulated influx of Ca\(^{2+}\) into cells is necessary to induce the metabolic processes for insulin secretion, we compared the properties of insulin secretion in response to the extracellular glucose concentration between the parental MIN6 and \(\rho^0\) MIN6 cells. The results shown in Fig. 6 suggest that the basal secreted amount of insulin in the normal MIN6 cells was extremely low, but the amount of insulin secretion progressively increased to about 3–7-fold greater than the basal amount with glucose stimulation (25 mM glucose) \((28, 30)\). On the contrary, the progressive enhancement of insulin secretion by glucose stimulation was not observed in \(\rho^0\) MIN6 cells, even though the basal insulin secretion level was very similar to that in normal cells (Fig. 6).

These observations consistently support the idea that the increase in glucose oxidation activity via the mitochondrial respiratory function plays significant roles in the glucose-stimulated influx of Ca\(^{2+}\) into pancreatic beta cells and the subsequent induction of insulin secretion.

Effect of Introduction of Mouse Fibroblast mtDNA into \(\rho^0\) MIN6 Cells on Glucose-stimulated Insulin Secretion—The intercellular transfer of exogenous mtDNA into \(\rho^0\) MIN6 cells and their mtDNA repopulation were carried out to determine whether the depletion of mtDNA was specifically responsible for the impairment of glucose-stimulated insulin secretion in \(\rho^0\) MIN6 cells or whether nuclear factors were also involved in this process.

The cytoplasmic transfer of exogenous mtDNA into \(\rho^0\) MIN6 cells was attained through the fusion of \(\rho^0\) MIN6 cells with enucleated B82CAP, which is a mouse fibroblast-derived cell line and is resistant to BrdUrd and CAP \((29)\). Then, the fusion mixture was selected with DM170 and hypoxanthine-aminopterin-thymidine (see “Experimental Procedures”) to remove parental B82CAP and \(\rho^0\) MIN6 cells, respectively. Consequently, six clones (CBM-1–CBM-6) that grew in the selective medium were collected (Table I). Since these clones expressed the CAP-resistant phenotype and grew in the presence of CAP, the possibility that these clones were revertant MIN6 cells that had recovered MIN6 mtDNA can be excluded. In this mtDNA transfer system, however, as no selection method to remove hybrid cells (fusion products between \(\rho^0\) MIN6 and nonenucleated B82CAP cells) was available, the six clones that grew in the selective medium with DM170 and hypoxanthine-aminopterin-thymidine were either cybrid clones (fusion products between \(\rho^0\) MIN6 and enucleated B82CAP cells, i.e. revertant MIN6 cells by the introduction of mtDNA from B82CAP cells) or hybrid clones. Then, karyotype analysis was carried out, and the results showed that the modal chromosome number of all six clones we isolated was 42 \((40–44)\), which was comparable to that of parental \(\rho^0\) MIN6 cells, indicating that these are not hybrid clones (Table I). Recovery of the mtDNA population and COX activity by the six clones was confirmed by Southern blot analysis and by means of a biochemical assay (Table I). These observations suggest that the clones we isolated were cybrid clones.

Using two cybrid clones, CBM-1 and CBM-2, we examined whether the property of glucose-stimulated insulin secretion, which was missing in \(\rho^0\) MIN6 cells, could be restored by the introduction and repopulation of mtDNA from fibroblast cell line B82CAP. As shown in Fig. 6, a progressive increase in the amount of secreted insulin in response to the extracellular glucose concentration was observed in both CBM-1 and CBM-2, suggesting the recovery of the missing glucose sensitivity through the introduction of mtDNA.
Therefore, our observations in this study clearly provided unambiguous evidence that mtDNA and the mitochondrial respiration function in pancreatic beta cells are necessary for the phenotypic expression of glucose-stimulated insulin secretion using a pancreatic beta cell line, MIN6 (a normal line), and two of its derivative lines, i.e. mtDNA knockout (ρ0 MIN6, a mutant line) and mtDNA repopulated hybrid MIN6 (a revertant line).

Recently, many pathogenic mtDNA mutations, such as large scale deletions (21, 37, 38), duplications (39–41), and point mutations (23–26), observed preferentially in patients with mitochondrial encephalomyopathies, were also found in patients with diabetes mellitus. Moreover, the clinical association of diabetes mellitus with deafness and various neurological disorders, including chronic progressive external ophthalmoplegia (CPEO)/Kearns-Sayre syndrome; Pearson’s bone marrow pancreas syndrome; mitochondrial encephalopathy, lactic acidosis, and strokelike episodes (MELAS); and myoclonus epilepsy with ragged red fibers (MERRF), was also reported (for review, see Ref. 8). Evidence of the involvement of mtDNA mutations in the pathogenesis of diabetes mellitus was reported in two pedigrees with maternally transmitted diabetes mellitus and deafness, one pedigree had a 10.4-kilobase large scale deletions (21, 37, 38), and the other had a mitochondrial encephalopathy, lactic acidosis, and strokelike episode (MELAS)-specific mitochondrial tRNA\textsubscript{Lys(UUR)} mutation at nucleotide position 3243 (23). The former mutation was subsequently shown to be derived from partially duplicated mtDNA through rearrangement (41). On the other hand, the latter tRNA\textsubscript{Lys(UUR)} 3243 mutation was prevalently found in peripheral leukocytes and muscle of more than 1% patients with diabetes mellitus (8), indicating that these mutations could contribute to the pathogenesis of the disease. More recently, several point mutations other than that at nucleotide position 3243 in the tRNA\textsubscript{Lys(UUR)} gene (42), and point mutations in the tRNA\textsubscript{Lys} gene at 8344 (43) and the tRNA\textsubscript{Glu} gene at 14,709 (44, 45) were also observed in patients with diabetes mellitus. However, there is as yet no convincing evidence that the mitochondrial dysfunction induced by these mtDNA mutations is responsible for the pathogenesis of the disease, although it had already been shown that the accumulation of some of the mtDNA mutations in cultured somatic cell lines was sufficient to induce the mitochondrial dysfunction through inhibition of overall mitochondrial translation (16–20).

In addition to the pathogenic mtDNA mutations, mtDNA depletion could also be a candidate pathogenic factor for the development of diabetes mellitus. Actually, there are several lines of evidence that support this possibility. First, the inhibition of overall mitochondrial translation followed by impairment of the mitochondrial respiratory chain function was similarly induced irrespective of whether the accumulation of mtDNA mutations or mtDNA deletion occurred in cells (16). Second, depletion of mtDNA in various tissues (muscle, liver, and kidney) was reported in fatal mitochondrial encephalomyopathies (46), suggesting that mtDNA depletion could be responsible for the clinical features of diabetes mellitus in the same way as in the case of the accumulation of the pathogenic mtDNA mutations, such as the tRNA\textsubscript{Leu(UUR)} 3243 mutation. Third, one of the diabetogenic agents, streptozotocin, which has been widely used for the induction of pancreatic islet injury and experimental diabetes mellitus, was found to reduce the contents of mtDNA and its transcripts in pancreatic islets (47). Furthermore, in Goto-Kakizaki rats, which have frequently been used as genetic model rats of impaired insulin secretion, a progressive and selective decrease of the mtDNA content (about 30%) was also observed in adult pancreatic islets but not in fetal ones (48).

In this study, we examined whether impairment of the respiratory chain function in mitochondria induced by the mtDNA knockout, i.e. mtDNA depletion, could be a pathogenic factor for the development of some forms of diabetes mellitus. The results showed that the mtDNA depletion induced overall impairment of mitochondrial translation (Fig. 2), and the resultant loss of mitochondrial respiration activity (Fig. 3), without inhibition of transcription of the insulin gene (Fig. 1b) or loss of SDH activity (Fig. 3), indicating that the induced mitochondrial dysfunction was not due to a cytotoxic side effect derived from the mtDNA depletion. Moreover, the mtDNA depletion also inhibited the glucose-stimulated increases in both the intracellular free Ca\textsuperscript{2+} content and insulin secretion in MIN6 cells. These observations suggest that mtDNA and the mitochondrial respiratory function are necessary to express the phenotypes of glucose-stimulated Ca\textsuperscript{2+} influx and insulin secretion in MIN6 cells.

The possibility of the involvement of nuclear genome-encoded factors as well as a cytotoxic side effect in this process was excluded completely by the observation that the missing sensitivity to the extracellular glucose level observed in the ρ0 MIN6 cells was restored reversibly by the repopulation of the cells with foreign mtDNA through fusion with cytoplasts from a mouse fibroblast cell line, B82CAP (Fig. 6). Therefore, these findings constitute unambiguous evidence for the involvement of mtDNA in the pathogenesis of some forms of diabetes mellitus. Moreover, our results substantially support the proposed glucose-signaling hypothesis (9) that mitochondria in pancreatic beta cells play a significant role in such a way that the ATP production and subsequent increase in the ATP/ADP ratio block ATP-sensitive K\textsuperscript{+} channels, resulting in depolarization of the membrane potentials of pancreatic beta cells, followed by the influx of extracellular Ca\textsuperscript{2+} and insulin secretion.

We are now investigating whether the predominance of mutant mtDNA molecules with large scale deletion mutations also induce disruption of glucose-stimulated insulin secretion in MIN6 cells by isolating mouse cell lines containing large scale deletion mutant mtDNA molecules and subsequently transferring them cytoplasmically into ρ0 MIN6 cells.

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