Effects of Depletion of Mitochondrial DNA in Metabolism Secretion Coupling in INS-1 Cells

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Mitochondrial dysfunction due to alterations in the mitochondrial genome (mtDNA) has recently attracted much attention, with the finding that mutations in the mitochondrially encoded proteins perturb cell function. Several disorders have been linked to such genetic changes, including a specific diabetic phenotype. Using ethidium bromide (EtBr) that intercalates into mtDNA, we have effectively eliminated functions under the control of mtDNA from the highly differentiated INS-1 insulin-secreting cell line. We have investigated the consequences on insulin secretion, mitochondrial enzyme activity, organelle structure, and membrane polarization in such cells (INS-1 ρ^0). Under these conditions, the mitochondrial membrane potential fails to hyperpolarize in response to either glucose or methylsuccinate. In agreement with this finding, the morphology of the mitochondria is altered in the presence of EtBr, sharing similarities with mitochondria in which the membrane potential has been collapsed with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). In addition, there is no effect of either nutrient secretagogue at the level of the plasma membrane potential, although the effect of the depolarizing agent KCl on membrane depolarization is completely preserved. Similarly, glucose and methylsuccinate fail to increase insulin secretion, whereas KCl is still effective. To test further the effects of mtDNA depletion on exocytosis, we permeabilized INS-1 cells with *Staphylococcus aureus* α-toxin, which forms small holes in the plasma membrane. In contrast to control cells, mitochondrial substrates were incapable of stimulating insulin secretion in mtDNA-deficient cells, emphasizing that the defect in secretion lies at the level of mitochondrial function rather than in the exocytotic process. The results indicate the paramount importance of the mitochondria in the downstream effects elicited by exposure to elevated concentrations of nutrient secretagogue. *Diabetes* 47:374–380, 1998

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$[Ca^{2+}]_c$, cytosolic calcium concentration; $[Ca^{2+}]_m$, mitochondrial calcium concentration; EtBr, ethidium bromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MeS, methylsuccinate; mtDNA, mitochondrial DNA; TCA, tricarboxylic acid; $\Delta\psi_m$, mitochondrial membrane potential; $\Delta\psi_p$, plasma membrane potential.

Glucose-induced insulin secretion from pancreatic β-cells is critically dependent on the generation of metabolic coupling factors, including ATP (1–5). Although poorly understood, lesions in these interdependent signaling pathways are thought to be instrumental in eliciting the pathogenesis of the diabetic phenotype that is characterized by glucose intolerance and an impairment of insulin secretion (4,6). Normal mitochondrial function determines the signal generation by nutrient secretagogues (2). Among these signals, an increase in the cytosolic ATP:ADP ratio in response to glucose results in closure of the ATP-sensitive K^+ channels, leading to a depolarization of the plasma membrane and an influx of Ca^{2+} (7,8). The subsequent increase in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) is the main trigger of insulin exocytosis (9). Increases in $[Ca^{2+}]_c$ also lead to enhanced changes in the mitochondrial calcium concentration ($[Ca^{2+}]_m$) (10–13) that stimulate mitochondrial metabolism through the activation of Ca^{2+}-sensitive NADH-generating dehydrogenases (14–17). This process may contribute to the stimulation of insulin secretion by glucose and other nutrients, which both raise $[Ca^{2+}]_m$ and provide carbons to the tricarboxylic acid (TCA) cycle (3,5,13,18).

Considerable evidence has now accumulated on the molecular basis of disorders linked to mitochondrial dysfunction. Of particular interest is the role of the mitochondrial genome (mtDNA), a small, circular, highly compact DNA structure present in multiple copies within each mitochondrion (19). It encodes for just 13 of the proteins involved in the respiratory chain complexes (20); the rest remain encoded by the nuclear genome. However, since it also codes for the transcription and translation apparatus responsible for mtDNA replication (20), it affords a certain degree of autonomy in this process, allowing mtDNA to replicate essentially independently and at a much higher rate than the corresponding events in the nucleus required for mitosis (21,22). In addition, the frequency of mutation is much more common because the regulatory and repair mechanisms are far less stringent during mitochondrial division. As a result, drifts in the mtDNA population can occur, leading to varying proportions of normal and mutated mtDNA coexisting within cells (23). Such heteroplasmy in the mtDNA population is believed to be instrumental in the progressive worsening of conditions linked to mutations in the mtDNA as mutated mtDNA continues to divide alongside the nonmutated form (24,25).

Oxidative damage to the mtDNA may be one of the causal factors involved in the onset of a variety of disease states. In recent years, several mitochondrial disorders associated with forms of type 2 diabetes, such as myoclonic epilepsy and ragged red fiber disease, mitochondrial encephalomyopathy,

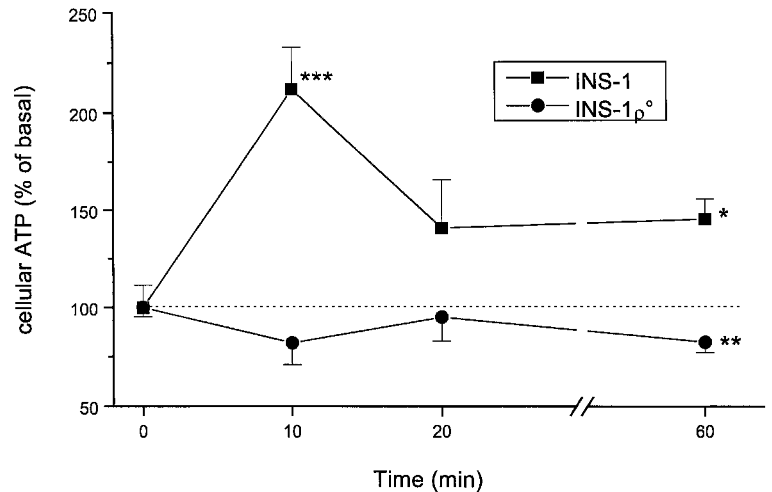

FIG. 1. Comparison of ATP production in INS-1 and INS-1 ρ^0 cells. ATP production in response to 10 mmol/l glucose in control (■) and ρ^0 (□) cells. Data are means ± SE, *n* = 5, **P* < 0.05, ***P* < 0.02, ****P* < 0.002. Control basal values of 100% represent 1.9 ± 0.5 pmol ATP/ µg protein, and the corresponding INS-1 ρ^0 basal represents 4.6 ± 0.5 pmol ATP/µg protein.

lactic acidosis and strokelike syndromes, and maternally inherited diabetes and deafness, have been linked to mutations occurring in the mitochondrial genome (26–28). Indeed, patients with mitochondrial genome mutations often display impaired glucose-stimulated insulin secretion (29).

It is possible to deplete the mtDNA complement of a cell by culturing in the presence of low concentrations of ethidium bromide (EtBr) (0.1–2 µg/ml), which intercalates into circular DNA, resulting in an inhibition of mtDNA replication while (since the concentrations are kept low) having no detectable effect on nuclear DNA division. This procedure takes several days to weeks as the mtDNA population is progressively diluted with continuing cell proliferation. The procedure must be carried out in the continued presence of pyruvate and uridine because, as has been previously noted, mtDNA-less cells become both pyruvate and pyrimidine auxotrophs (30). Using another substance termed bis-4 piperidyle dichloride, Soejima et al. (31) recently reported that elimination of mtDNA abolished glucose-stimulated insulin secretion in the β-cell line MIN6. By treating INS-1 cells, another highly differentiated β-cell line, with EtBr, we have further investigated the impact of mtDNA depletion on mitochondrial morphology, ATP generation, and insulin secretion in intact and permeabilized cells. The latter cellular preparation allows direct assessment of the mitochondrially driven exocytosis of insulin. Using this multiparametric approach to determine the apparent loss of mtDNA, the results reveal a profound effect on the downstream events triggered by the presence of glucose in cells without mtDNA.

RESEARCH DESIGN AND METHODS

Cell culture. INS-1 cells were cultured in RPMI 1640 supplemented with 10% decomplemented fetal calf serum and additions as previously described, including 1 mmol/l pyruvate (32). To deplete the cells of mtDNA, the complete culture medium was supplemented with 0.4 µg/ml EtBr. Because mtDNA-less cells, termed rho (ρ^0) cells, display pyruvate dependency and become pyrimidine auxotrophs, the pyruvate concentration was supplemented with an extra 100 µg/ml. To facilitate continued growth under conditions in which the cellular mtDNA content would gradually become exhausted, 50 µg/ml uridine was also added to the cells. It should be noted, however, that, even in this enriched media, ρ^0 cell growth was compromised compared to control cell growth. This is in contrast to the findings of King and Attardi (33), who found that supplementation of the culture media with pyruvate and uridine could essentially restore ρ^0 cell growth to a rate comparable to that observed in control 143B.TK$^-$ cells.

Cytochrome C oxidase assay. Cytochrome C oxidase activity was measured according to the protocol of Appelmans et al. (34). Briefly, crude mitochondrial preparations (10–50 µg protein) were prepared by Potter-Elvehjem homogenization and the homogenate spun at 1,300 rpm for 15 min to remove unbroken cells, nuclei, and other large debris. Then the supernatant was centrifuged at 9,200 rpm, and the resultant mitochondrial pellet was resuspended and diluted 1:1 for 30 min at 4°C in mitochondrial activation buffer (1 mmol/l EDTA, 1 mmol/l $NaHCO_3$, 0.01% Triton X-100). The assay was then performed in cuvettes containing 40 µmol/l cytochrome C (Sigma, St. Louis, MO) in 75 mmol/l sodium phosphate buffer, pH 7.2. Cytochrome C was reduced with $Na_2S_2O_4$, and the kinetics of oxidation was monitored at 550 nmol/l for 5 min.

ATP measurements. According to the protocol of Stanley and Williams (35), INS-1 control and ρ^0 cells were stimulated as shown and scraped into 1 ml 0.4N $HClO_4$ to terminate the reaction. Following neutralization with 2N K_2CO_3, cell extracts were incubated with an enzymatic mixture (10 mg firefly luciferin-luciferase/ml arsenate buffer [0.1 mol/l $Na_2HAsO_4 \cdot 7H_2O$, 20 mmol/l $MgSO_4 \cdot 7H_2O$]), and the resultant luminescence was measured.

Membrane potential measurements. The plasma membrane potential ($\Delta\psi_p$) was measured using the fluorescent probe bis-oxonol (32). The corresponding mitochondrial membrane potential ($\Delta\psi_m$) was measured in cells that had been preloaded in 10 µg/ml rhodamine 123 (Rh123) as described previously (5).

Immunofluorescence. INS-1 or INS-1 ρ^0 cells were plated at a density of ~50,000 cells on polyornithine-coated glass coverslips. MitoTracker (Molecular Probes, Eugene, OR) was used at a final concentration of 100 nmol/l at 37°C in the presence or absence of FCCP (1 µmol/l) for 30 min to stain the mitochondria. Cells were then fixed in 4% paraformaldehyde and washed extensively in phosphate-buffered saline before mounting on glass slides. Cells were viewed using a Zeiss laserscan confocal 410 microscope.

Cell permeabilization. INS-1 control and ρ^0 cells were grown on extracellular matrix. This matrix is obtained by treating a confluent monolayer of A431 cells with 1% Triton X-100 to detach the cells (36,37). Following a wash in Ca^{2+} free, HEPES-balanced Krebs-Ringer bicarbonate buffer (135 mmol/l NaCl, 3.6 mmol/l KCl, 10 mmol/l HEPES, 2 mmol/l $NaHCO_3$, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgCl_2$, 0.4 mmol/l EGTA, pH 7.4), attached INS-1 cells were permeabilized with *Staphylococcus aureus* α-toxin (1 µg/4–5 × 10^5 cells; kindly provided by Dr. M. Palmer, University of Mainz, Germany) for 8 min at 37°C in intracellular buffer adjusted to approximately 100 nmol/l free calcium (140 mmol/l KCl, 5 mmol/l NaCl, 7 mmol/l $MgSO_4$, 20 mmol/l HEPES, 1 mmol/l ATP, 10.2 mmol/l EGTA, 1.65 mmol/l $CaCl_2$, pH 7.0) (5).

Insulin secretion measurements. For dynamic analyses, cells were used at 1 × 10^6 cells/chamber and perifused with a flow rate of 1 ml/min in Krebs-Ringer buffer as previously described (13). For static incubations, cells set at 4–5 × 10^5 were stimulated for 30 min with the test compound (32). For permeabilized cells, the protocol was as described above except that for static incubations, cells were pre-incubated for 20 min, and then the medium was changed for a further 10 min to 500 nmol/l Ca^{2+} –10 mmol/l ATP stimulatory intracellular buffer before stimulation with test compounds in the same buffer (5). Bovine serum albumin (Sigma) at a final concentration of 0.1% was used as carrier, and the insulin content was measured by radioimmunoassay using rat insulin as standard (32).

Statistical analysis. Values are expressed as means ± SE, and *n* values refer to independent experiments. Where applicable, the significance of difference was calibrated using Student's *t* test for unpaired data.

RESULTS

Prolonged culture of INS-1 cells in EtBr (3–12 months) resulted in a complete loss of cytochrome C oxidase activity

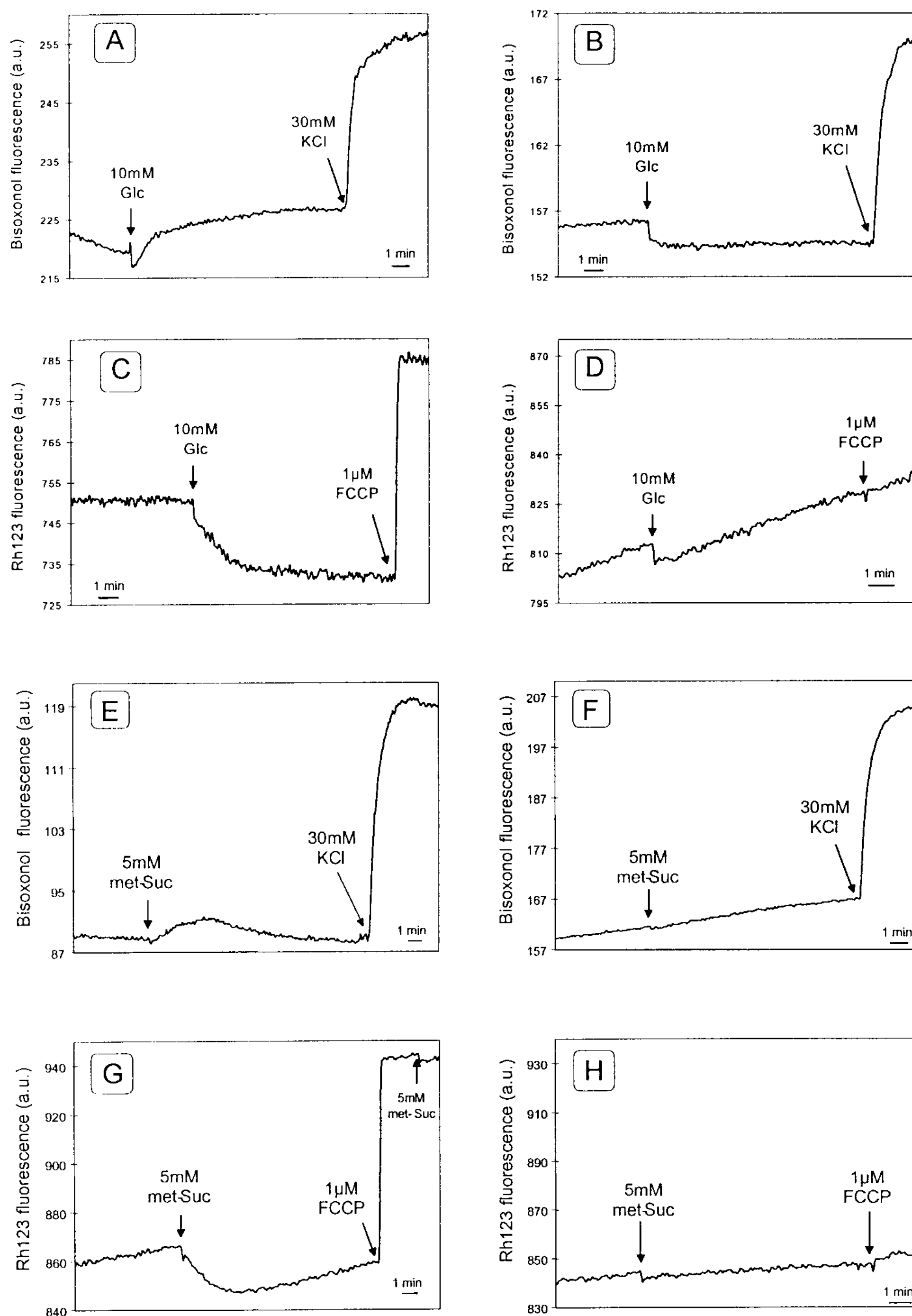

FIG. 2. Plasma membrane potentials ($\Delta\psi_p$) and mitochondrial membrane potentials ($\Delta\psi_m$) in INS-1 and INS-1 ρ^0 cells. Suspensions of cells were loaded either with the fluorescent probe bis-oxonol (*A*, *B*, *E*, and *F*) to monitor $\Delta\psi_p$ in INS-1 (*A* and *E*) or INS-1 ρ^0 cells (*B* and *F*) or with Rh123 (*C*, *D*, *G*, and *H*) to monitor $\Delta\psi_m$ in INS-1 (*C* and *G*) or INS-1 ρ^0 cells (*D* and *H*).

in comparison to control cells that had not been exposed to EtBr (2.6 ± 0.3 nmol · min^{-1} · mg^{-1} protein for EtBr-treated cells vs. 140.8 ± 57.4 nmol · min^{-1} · mg^{-1} protein for control cells, $n = 4$; $P < 0.03$). Moreover, as shown in Fig. 1, the ability of INS-1 ρ^0 cells to produce ATP in response to stimulation with glucose is completely abolished. This scenario would be expected in a situation where respiration is compromised and where the cells survive solely by glycolysis. In comparison with control cells, in which 10 mmol/l glucose produces a time-dependent increase in the ATP concentration with an approximate twofold increase at 10 min, the curve for the corresponding ATP production by INS-1 ρ^0 cells tends to decrease rather than increase following exposure to glucose.

To examine the impact of a loss of oxidative phosphorylation in INS-1 ρ^0 cells, we measured mitochondrial and plasma membrane potentials. As expected, 10 mmol/l glucose hyperpolarizes the mitochondrial membrane potential ($\Delta\psi_m$) and depolarizes the plasma membrane potential ($\Delta\psi_p$) of control INS-1 cells (Fig. 2*A* and *C*). In contrast, glucose fails to raise the $\Delta\psi_m$ while actually slightly hyperpolarizing the $\Delta\psi_p$ in INS-1 ρ^0 cells (Fig. 2*B*). This hyperpolarization of $\Delta\psi_p$ may reflect ATP consumption due to glucose phosphorylation, which is not compensated sufficiently by ATP production in the absence of functional mitochondria. The absence of any effect of the protonophore FCCP (1 μmol/l) on $\Delta\psi_m$ indicates that the mitochondrial membranes are completely depolarized

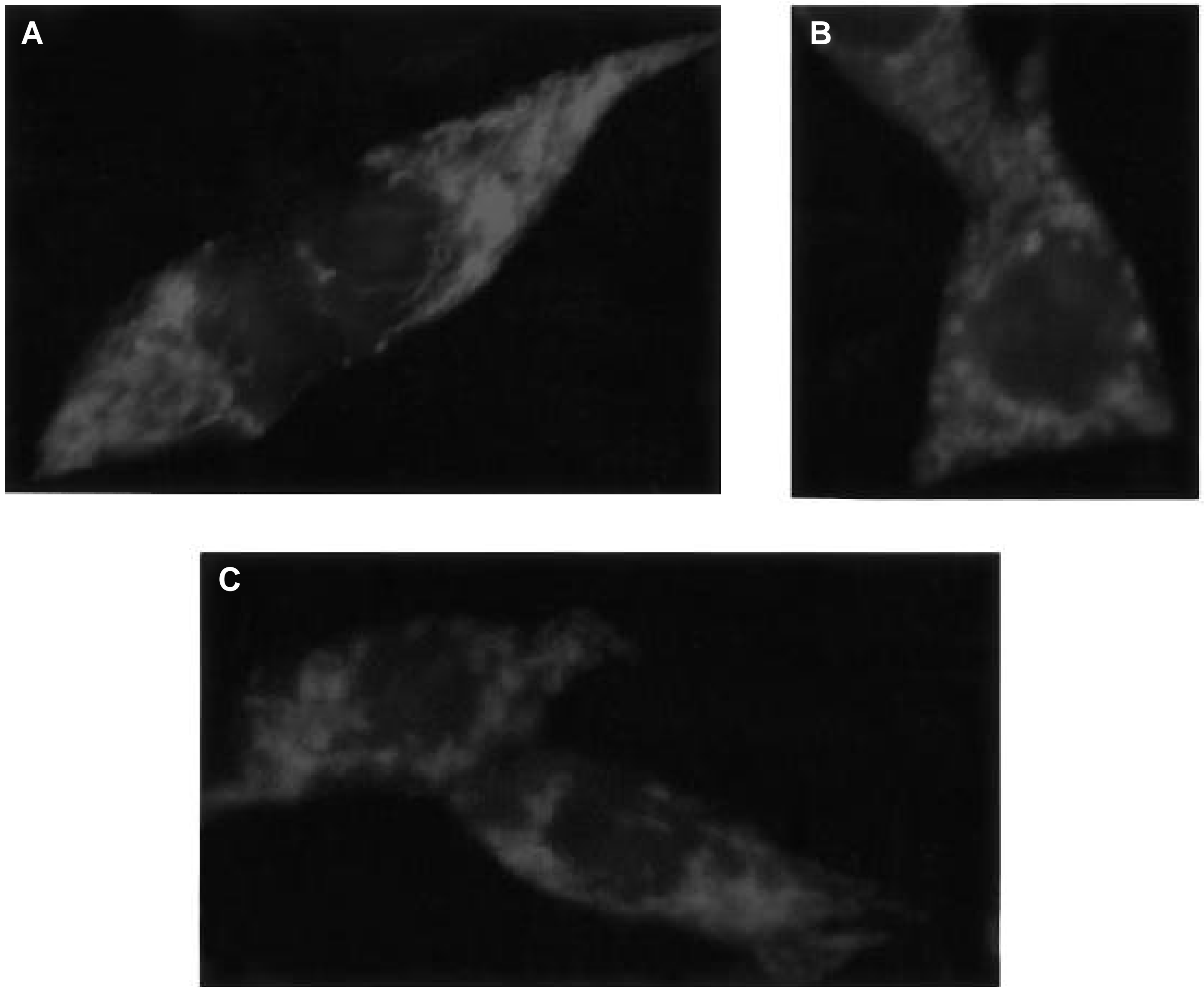

FIG. 3. Mitochondrial morphology in INS-1 and INS-1 ρ^0 cells. *A:* INS-1 cell mitochondria stained with MitoTracker. *B:* INS-1 ρ^0 cell mitochondria stained with MitoTracker. *C*: INS-1 cell mitochondria stained with MitoTracker in the presence of 1 µmol/l FCCP for 30 min. Images were taken with a Zeiss laserscan confocal 410 microscope.

in INS-1 ρ^0 cells (Fig. 2*D*), which contrasts with a marked depolarization caused by FCCP in the control cells (Fig. 2*C*). Nonetheless, the $\Delta\psi_p$ is still controlled in INS-1 ρ^0 cells, as 30 mmol/l KCl caused a similar depolarization in both cell lines (Fig. 2*A* and *B*). Succinate, the TCA cycle intermediate, is known to mimic the effects of glucose on insulin secretion (38) and on cytosolic Ca^{2+} increases (5) when applied as its membrane permeant derivative, MeS. This agent allows the direct assessment of signal generation by the mitochondria as it bypasses the steps of glucose uptake and glycolysis. MeS depolarizes $\Delta\psi_p$ (Fig. 2*E*) and hyperpolarizes $\Delta\psi_m$ (Fig. 2*G*) in control INS-1 cells as previously reported (5). Again, there is no change in $\Delta\psi_m$ and, in contrast to glucose, MeS does not hyperpolarize $\Delta\psi_p$ in INS-1 ρ^0 cells (Fig. 2*F* and *H*).

Figure 3 (*A*–*C*) shows confocal images obtained from control cells and mtDNA-less cells in which the mitochondria have been stained with MitoTracker. Figure 3*A* shows normal mitochondrial staining in the presence of the dye, which accumulates specifically in the mitochondria. In contrast, in INS-1 ρ^0 cells, the mitochondria have a much more rounded appearance (Fig. 3*B*). This pattern is very similar to that seen in cells exposed to FCCP (Fig. 3*C*). Taken together, the data from Figs. 2 and 3 indicate that the mitochondria in INS-1 ρ^0 cells exhibit a completely depolarized state.

Mitochondrial metabolism generates coupling factors that promote exocytosis (1–5). As can be seen in Fig. 4*A*, glucose-stimulated insulin secretion is completely abolished in INS-1 ρ^0 cells. This contrasts with the large insulin secretion evoked by KCl depolarization, which is similar to the response of control cells (Fig. 4*B*). These findings indicate that mitochondrial function, not the exocytotic process, is compromised under these experimental conditions. The difference in the observed secretion cannot be explained by the insulin content of the two cell lines (control 1,554.8 ± 134.6 ng/ 1 ×

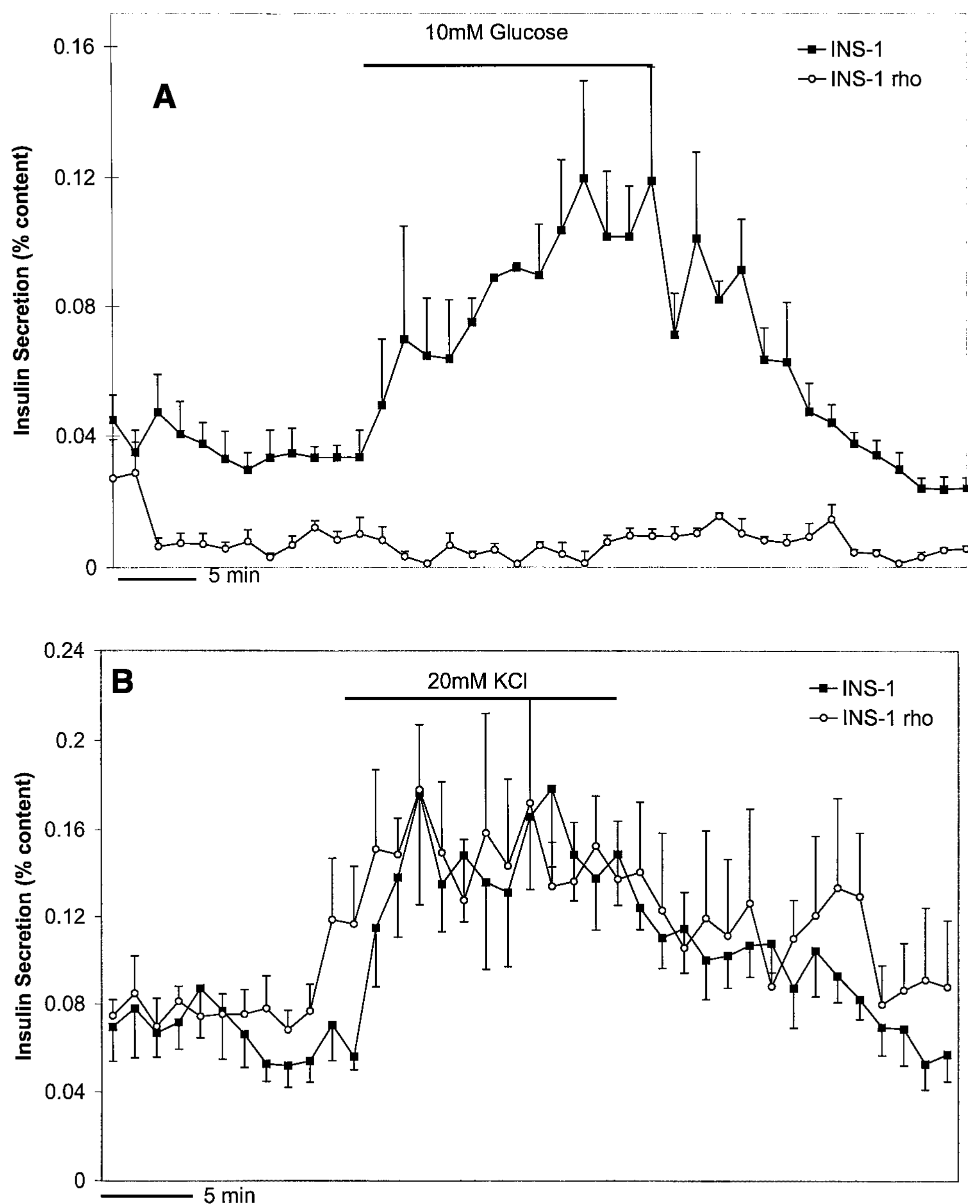

FIG. 4. Insulin secretion profiles from INS-1 and INS-1 ρ^0 cells in perifusion. *A*: Effect of 10 mmol/l glucose on insulin secretion in INS-1 (■) and INS-1 ρ^0 (□) cells; means ± SE, *n* = 3. *B*: Effect of 20 mmol/l KCl on insulin secretion in INS-1 (■) and INS-1 ρ^0 (□) cells; means ± SE, *n* = 3.

10^6 cells vs. ρ^0 = 1,252.6 ± 110.9 ng/1 × 10^6 cells, n = 11). Furthermore, the data in Fig. 4 are expressed as percents of cellular hormone content. Additional secretion experiments were performed using static incubations that reemphasized the insulin exocytosis profiles observed in the perifusion analyses. Under basal conditions in 2.8 mmol/l glucose, INS-1 cells secrete 2.15 ± 0.51 ng insulin/5 × 10^5 cells (n = 3), which increases to 4.45 ± 0.12 ng/ 5 × 10^5 cells (n = 3) and 4.47 ± 1.04/5 × 10^5 cells (n = 3) in the presence of 10 mmol/l glucose and 10 mmol/l MeS, respectively. In contrast, the basal secretion in INS-1 ρ^0 cells (3.02 ± 0.89 ng/5 × 10^5 cells, n = 3) is virtually unaltered by stimulation with 10 mmol/l glucose (3.78 ± 0.07 ng/ml, n = 3) or 10 mmol/l MeS (3.78 ± 1.18 ng/ 5 × 10^5 cells, n = 3). Again, the secretion in response to 20 mmol/l KCl is largely unaltered in both the control and ρ^0 cells (8.5 ± 0.6 ng/5 × 10^5 cells vs. 6.5 ± 0.9 ng/5 × 10^5 cells, n = 3, control and ρ^0 cells, respectively).

To ensure, however, that what was being observed was truly a mitochondrial phenomenon, we performed additional insulin secretion experiments in permeabilized cells, imposing conditions in which mitochondrial substrates are still capable of eliciting insulin secretion (5). Fig. 5 shows the effects of succinate and methylpyruvate on insulin secretion from *Staphylococcus aureus* α-toxin permeabilized control and ρ^0 cells at permissive [Ca^{2+}] (500 nmol/l). Both succinate (5 mmol/l) and methylpyruvate (5 mmol/l) elicit insulin secretion. In comparison, neither of the mitochondrial substrates has this effect in permeabilized INS-1 ρ^0 cells. There is no difference in basal secretion rates.

DISCUSSION

The continuous culture of insulin-secreting cells in low concentrations of EtBr has been established in order to delineate the effects of the loss of mtDNA on exocytosis. Using several different parameters, it has been shown that the mitochondria of cells exposed to EtBr become respiration deficient as assayed both by the cytochrome C oxidase activity and impaired polarization of the mitochondrial membrane poten-

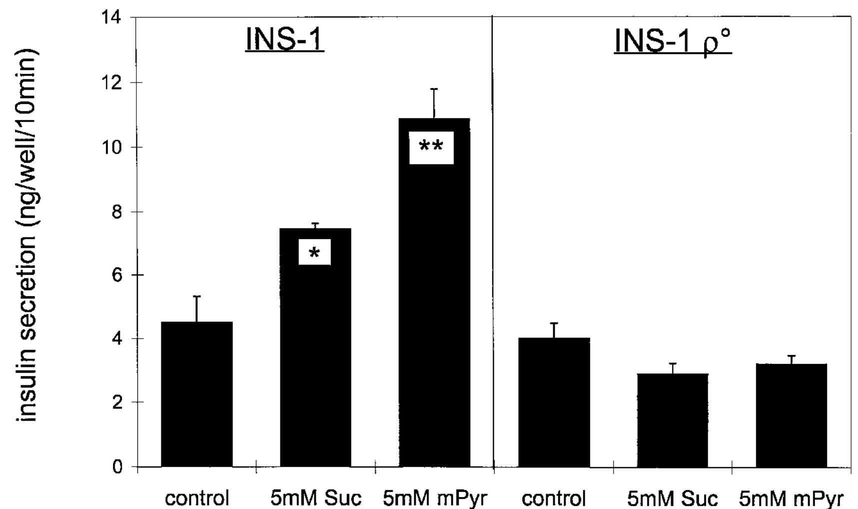

FIG. 5. Insulin secretion from permeabilized preparations of INS-1 and INS-1 ρ^0 cells. Cells were permeabilized for 8 min with *Staphlococcus aureus* α-toxin in an intracellular buffer and stimulated as described (see METHODS). Data are means ± SE of two experiments performed in triplicate. *$P < 0.05$, **$P < 0.02$.

tial, with consequent failure to increase ATP production in response to glucose. This failure to increase ATP generation reflects ATP consumption by glucose phosphorylation and by the synthesis of proteins, including insulin, which is not counterbalanced by the limited ATP production of glycolysis (39). The higher basal ATP levels observed in the INS-1 ρ^0 cell line may reflect a compensatory mechanism of adaptation to the reduced oxidative phosphorylation. In this context, it has recently been shown that a patient with diabetes associated with a mutation in position 3243 of the mtDNA displayed a low level of cytochrome C oxidase while succinate dehydrogenase activity in the β-cell was increased, suggesting an adaptation in cellular metabolism (40). EtBr treatment of another insulin-secreting cell line, βHC9, has recently been shown in a preliminary report to decrease the mtDNA content within 7 days as measured by Northern analyses (41).

The dramatic effects of EtBr treatment on the mitochondrial membrane potential are underscored both by the complete lack of effect of FCCP, which normally collapses the $\Delta\psi_m$, and by the altered morphology of the mitochondria in the INS-1 ρ^0 cell line. This mitochondrial aspect in the presence of EtBr is identical to that observed in HeLa cells exposed to FCCP (42) and confirmed here in INS-1 cells (Fig. 3*C*). The protonophore FCCP acts by dissipating the $\Delta\psi_m$ and thus compromises oxidative metabolism. The destruction of the $\Delta\psi_m$ caused by the presence of EtBr has a profound effect on the structure of the mitochondria, eliciting major rearrangements (such as condensation of the organelle), and highlights the view that the energy state of the mitochondria is fundamental in the ultimate determination of their shape (43). The morphology of the mitochondria in control INS-1 cells stained with MitoTracker is identical to that observed using either a polyclonal antibody raised against cytochrome C oxidase or the membrane potential dye JC-1 (13).

In the presence of elevated concentrations of glucose or MeS, INS-1 ρ^0 cells are incapable of eliciting insulin secretion. Signal generation by these nutrient stimuli is no longer feasible, as reflected by the absence of mitochondrial membrane hyperpolarization, ATP generation, and plasma membrane depolarization. Soejima et al. (31) found that in mtDNA-depleted cells, glucose could not raise Ca^{2+}. Considering that it is known that glucose-induced increases in $[Ca^{2+}]_c$ are relayed to the mitochondria (13), it would be expected that $[Ca^{2+}]_m$ no longer changes in INS-1 ρ^0 cells. This would further diminish the production of coupling factors of mitochondrial origin generated by increased TCA cycle activity (5). In contrast to the proposal by Mertz et al. (44), the failure of glucose to stimulate insulin secretion in both mtDNA-deficient MIN6 cells (31) and in INS-1 ρ^0 cells (Fig. 4*A*) makes it unlikely that the generation of factors through glycolysis per se is sufficient to trigger insulin secretion. To highlight that the detrimental effects of mtDNA depletion are restricted to signal generation by the mitochondria, it is noteworthy that KCl depolarization can elicit insulin secretion in INS-1 ρ^0 cells in a similar manner to control cells, because its action does not primarily depend on metabolic coupling factors. These findings, together with the preserved insulin content of the INS-1 ρ^0 cells, suggest that insulin biosynthesis, storage, and exocytosis are apparently unaffected by EtBr treatment.

The INS-1 ρ^0 cells were also useful to pinpoint further the requirement for functional mitochondrial metabolism in insulin exocytosis stimulated by succinate or pyruvate (5). Accordingly, in contrast to control cells, the effects of these agents were abolished in permeabilized INS-1 ρ^0 cells, suggesting that their action is not exerted directly on the exocytotic process.

In conclusion, the data presented here provide evidence that mitochondrial dysfunction artificially precipitated by the presence of EtBr has profound effects on exocytosis induced by nutrient secretagogues. This provides a model of genetic alterations in the mtDNA as being implicated in the development of certain forms of diabetes (26–29). As suggested by mitochondrial replenishment experiments performed in MIN6 ρ^0 cells (31), it should be possible to test fibroblast mitochondria from patients with defined mitochondrial disorders for their capacity to restore glucose-stimulated insulin secretion. One obvious obstacle to the success of such an approach is the need for a human β-cell line. Human mitochondria cannot survive in rodent cells because of the known species incompatibility between mitochondrial and nuclear gene products (45). Nonetheless, these rodent β-cell ρ^0 lines can be exploited to examine the mitochondrial function of various animal models of diabetes, including the

BDECDB rat (4), which carries a mutation in subunit 6 of the F_1-F_0-ATPase (46).

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REFERENCES

1. Pralong W-F, Bartley C, Wollheim CB: Single islet β-cell stimulation by nutrients: relationship between pyridine nucleotide, cytosolic Ca^{2+} and secretion. *EMBO J* 9:53–60, 1990
2. Newgard CB, McGarry JD: Metabolic coupling factors in pancreatic β-cell signal transduction. *Annu Rev Biochem* 64:689–719, 1995
3. Prentki M: New insights into pancreatic β-cell metabolic signaling in insulin secretion. *Eur J Endocrinol* 134:272–286, 1996
4. Matschinsky FM: Banting Lecture 1995: a lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 45:223–241, 1996
5. Maechler P, Kennedy ED, Pozzan T, Wollheim CB: Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic β-cells. *EMBO J* 16:3833–3841, 1997
6. Polonsky KS, Sturis J, Bell GI: Non-insulin dependent diabetes mellitus: a genetically programmed failure of the beta cell to compensate for insulin resistance *N Engl J Med* 334:777–783, 1996
7. Ashcroft FM, Rorsman P: Electrophysiology of the pancreatic β-cell frog. *Biophys Molec Biol* 54:87–143, 1989
8. Detimary P, Jonas J-C, Henquin J-C: Possible links between glucose-induced changes in the energy state of pancreatic β-cells and insulin release. *J Clin Invest* 96:1738–1745, 1995
9. Wollheim CB, Lang J, Regazzi R: The exocytotic process of insulin secretion and its regulation by Ca^{2+} and G-proteins. *Diabetes Rev* 4:276–297, 1996
10. Rizzuto R, Simpson AWM, Brini M, Pozzan T: Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature* 358:325–327, 1992
11. Rizzuto R, Bastianutto C, Brini M, Murgia M, Pozzan T: Mitochondrial Ca^{2+} homeostasis in intact cells. *J Cell Biol* 126:1183–1194, 1994
12. Rutter GA, Theler JM, Murgia M, Wollheim CB, Pozzan T, Rizzuto R: Stimulated Ca^{2+} influx raises mitochondrial free Ca^{2+} to supramicromolar levels in a pancreatic β-cell line: possible role in glucose and agonist induced insulin secretion. *J Biol Chem* 268:22385–22390, 1993
13. Kennedy ED, Rizzuto R, Theler JM, Pralong WF, Bastianutto C, Pozzan T, Wollheim CB: Glucose-stimulated insulin secretion correlates with changes in mitochondrial and cytosolic Ca^{2+} in aequorin expressing INS-1 cells. *J Clin Invest* 98:2524–2538, 1996
14. McCormack JG, Halestrap AP, Denton RM: Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 70:391–425, 1990
15. Hansford RG: Physiological role of mitochondrial Ca^{2+} transport. *J Bioenerg Biomemb* 26:495–508, 1994
16. Pralong W-F, Spat A, Wollheim CB: Dynamic pacing of cell metabolism by intracellular Ca^{2+} transients. *J Biol Chem* 269:27310–27314, 1994
17. Civalek VN, Deeney JT, Shalosky NJ, Tornheim K, Hansford RG, Prentki M, Corkey BE: Regulation of pancreatic β-cell mitochondrial metabolism: influence of Ca^{2+}, substrate and ADP. *Biochem J* 318:615–621, 1996
18. Schuit F, De Vos A, Farfari S, Moens K, Pipeleers D, Brun T, Prentki M: Metabolic fate of glucose in purified islet cells. *J Biol Chem* 272:18572–18579, 1997
19. Wallace DC: Diseases of the mitochondrial DNA. *Annul Rev Biochem* 61:1175–1212, 1992
20. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG: Sequence and organization of the human mitochondrial genome. *Nature* 290:457–465, 1981
21. Wallace DC: Structure and evolution of organelle genomes. *Microbiol Rev* 46:208–240, 1982
22. Shoffner JM, Wallace DC: Oxidative phosphorylation diseases: disorders of two genomes. *Adv Hum Genet* 19:267–330, 1990
23. Wallace DC, Shoffner JM, Trounce I, Brown MD, Ballinger SW, Corral-Debrinski M, Horton T, Jun AS, Lott MT: Mitochondrial DNA mutations in human degenerative diseases and aging. *Biochim Biophys Acta* 1271:141–151, 1995
24. Wallace DC, Zheng X, Lott MT, Shoffner JM, Hodge JA, Kelley RI, Epstein CM, Hopkins LC: Familial mitochondrial encephalomyopathy (MERFF): genetic, pathophysiological and biochemical characterisation of a mitochondrial DNA disease. *Cell* 55:601–610, 1988
25. Shoffner JM, Lott MT, Lezza AMS, Seibel P, Ballinger SW, Wallace DC: Myoclonic epilipsy and ragged-red fiber disease (MERFF) is associated with a mitochondrial DNA $tRNA^{Lys}$ mutation. *Cell* 61:931–937, 1990
26. Luft R, Landau BR: Mitochondrial medicine. *J Int Med* 238:405–421, 1995
27. Maassen JA, Kadowaki T: Maternally inherited diabetes and deafness: a new diabetes subtype. *Diabetologia* 39:375–382, 1996
28. Johns DR: The other human genome: mitochondrial DNA and disease. *Nature Med* 2:1065–1068, 1996
29. Gerbitz KD, Gempel K, Brdiczka D: Genetic, biochemical and clinical implications of the cellular energy circuit. *Diabetes* 45:113–126, 1996
30. King MP, Attardi G: Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 246:500–503, 1989
31. Soejima A, Inoue K, Takai D, Kaneko M, Ishihara H, Oka Y, Hayashi JI: Mitochondrial DNA is required for regulation of glucose-stimulated insulin secretion in a mouse pancreatic β-cells line, MIN6. *J Biol Chem* 271:26194–26199, 1996
32. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol dependent differentiated insulin secreting cell lines. *Endocrinology* 130:167–178, 1992
33. King MP, Attardi G: Isolation of human cell lines lacking mitochondrial. *DNA Meth Enzymol* 264:305–313, 1996
34. Appelmans F, Wattiaux R, de Duve C: Tissue fractionation studies. V. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem J* 59:438–445, 1955
35. Stanley PE, Williams SG: Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal Biochem* 29:381–392, 1969
36. Wayner EA, Gil SG, Murphy GF, Wilke MS, Carter WG: Epiligrin, a component of epithelial basement membranes, is an adhesive ligand for α3β1 positive T lymphocytes. *J Cell Biol* 121:1141–1152, 1993
37. Weitzman JB, Pasquallini R, Takada Y, Hemler ME: The function and distinctive regulation of the integrin VLA-3 in cell adhesion, spreading and homotypic cell aggregation. *J Biol Chem* 268:8651–8657, 1993
38. McDonald MJ, Fahien L: Glyceraldehyde phosphate and methyl esters of succinic acid. *Diabetes* 37:997–999, 1988
39. Skelly RH, Schuppin GT, Ishihara H, Oka Y, Rhodes CJ: Glucose regulated translational control of proinsulin biosynthesis with that of the proinsulin endopeptidases PC2 and PC3 in the insulin-producing MIN6 cell line. *Diabetes* 45:37–43, 1996
40. Kobayashi T, Nakanishi K, Nakase H, Kajio H, Okubo M, Murase T, Kosaka K: In situ characterization of islets in diabetes with a mitochondrial DNA mutation at nucleotide position 3243. *Diabetes* 46:1567–1571, 1997
41. Noda M, Shen L, Sharp GWG: Secretory characteristics of βHC9 insulin secreting cells with dysfunctional mitochondria. *J Gen Physiol* 110:14a–15a, 1997
42. Rizzuto R, Brini M, Pizzo P, Murgia M, Pozzan T: Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. *Curr Biol* 5:635–642, 1996
43. Bereiter-Hahn J: Behaviour of mitochondria in the living cell. *Int Rev Cytol* 122:1–63, 1990
44. Mertz RJ, Worley JF III, Spencer B, Johnson JH, Dukes ID: Activation of stimulus-secretion coupling in pancreatic β-cells by specific products of glucose metabolism: evidence for privileged signaling by glycolysis. *J Biol Chem* 271:4838–4845, 1996
45. Inoue K, Ito S, Takai D, Soejima A, Shisa H, LePecq J-B, Segal-Bendirdjian E, Kagawa Y, Hayashi J-I: Isolation of mitochondrial DNA-less mouse cell lines and their application for trapping mouse synaptosomal mitochondrial DNA with deletion mutations. *J Biol Chem* 272:15510–15515, 1997
46. Mathews CE, McGraw RA, Berdanier CD: A point mutation in the mitochondrial DNA of diabetes-prone $BHE/_{CDB}$ rats. *FASEB J* 9:1638–1642, 1995