Mitochondrial Functional State in Clonal Pancreatic β-Cells Exposed to Free Fatty Acids*

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Excessive free fatty acid (FFA) exposure represents a potentially important diabetogenic condition that can impair insulin secretion from pancreatic β-cells. Because mitochondrial oxidative phosphorylation is a main link between glucose metabolism and insulin secretion, in the present work we investigated the effects of the FFA oleate (OE) on mitochondrial function in the clonal pancreatic β-cell line, MIN6. Both the long term (72 h) and short term (immediately after application) impact of OE exposure on β-cells was investigated. After 72 h of exposure to OE (0.4 mM, 0.5% bovine serum albumin) cells were washed and permeabilized, and mitochondrial function (respiration, phosphorylation, membrane potential formation, production of reactive oxygen species) was measured in the absence or presence of OE. MIN6 cells exposed to OE for 72 h showed impaired glucose-stimulated insulin secretion and decreased cellular ATP. Mitochondria in OE-exposed cells retained normal functional characteristics in FFA-free medium; however, they were significantly more sensitive to the acute uncoupling effect of OE treatment. The mitochondria of OE-exposed cells displayed increased depolarization caused by acute OE treatment, which is attributable to the elevation in the FFA-transporting function of uncoupling protein 2 and the dicarboxylate carrier. These cells also had an increased production of reactive oxygen species in complex I of the mitochondrial respiratory chain that could be activated by FFA. A high level of reduction of respiratory complex I augmented acute FFA-induced uncoupling in a way compatible with activation of mitochondrial uncoupling protein by intramitochondrial superoxide. A stronger augmentation was observed in OE-exposed cells. Together, these events may underlie FFA-induced depression of the ATP/ADP ratio in β-cells, which accounts for the defective glucose-stimulated insulin secretion associated with lipotoxicity.

Chronic elevation of circulating free fatty acids (FFAs)[1] is associated with obesity and type 2 diabetes. Long term exposure of insulin-secreting pancreatic β-cells to elevated concentrations of FFAs alters glucose-induced insulin secretion and is considered as an important factor in the pathogenesis of diabetes (1–3). In pancreatic β-cells, mitochondrial oxidative phosphorylation is a crucial intermediate between glucose metabolism and insulin secretion (4, 5), and thus the effect of fatty acids on the functional state of mitochondria in this cell type is an important area of investigation.

Previous work demonstrated that fatty acids applied for 48–72 h cause partial uncoupling of oxidative phosphorylation (decreased mitochondrial membrane potential and ATP content and increased respiration) (1, 6). Although fatty acids are natural weak uncouplers of oxidative phosphorylation, a direct uncoupling effect of fatty acids on β-cell mitochondria was considered as unlikely since these effects were observed after long term (48–72 h) but not short term (15 min–2 h) application (1, 6). Instead, alteration in activity and/or expression of certain mitochondrial enzymes and transporters was suggested as the most likely cause of the fatty acid effect on oxidative phosphorylation in β-cells (7). In particular, it was suggested that the lowered mitochondrial membrane potential and impaired glucose-induced rise in the ATP/ADP ratio in FFA-exposed β-cells are caused by induction of uncoupling protein 2 (UCP2) in mitochondria (1). Indeed, FFA exposure leads to elevated levels of UCP2 mRNA and protein in β-cell mitochondria (1, 8) due to transcriptional regulation in which sterol regulatory element-binding protein-1 plays a major role (8). However, it is not clear if this can account entirely for alterations in mitochondrial function. In fact, the uncoupling (protonophoric) function of UCP2 and UCP3 is still a subject of debate (9–11). There is considerable evidence to suggest that the two functions of UCPs are to regulate reactive oxygen species (ROS) production and to export FFA anions from mitochondria, rather than to provide basal proton conductance of the mitochondrial membrane (12, 13).

To gain further insight into the mechanism of the effect of fatty acid in the present study we applied permeabilized cell techniques to assess mitochondrial function in the β-cell and the effects of the long term (72 h) and short term (immediately after FFA addition to the assay medium) application of FFA. Studying intact cells provides only limited information about mitochondrial function because it is difficult to control the extramitochondrial medium and the mitochondrial functional

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[1] The abbreviations used are: FFA, free fatty acid; OE, oleate; UCP, uncoupling protein; DIC, dicarboxylate carrier; SOD, superoxide dismutase; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; ROS, reactive oxygen species; BSA, bovine serum albumin; KRB, Krebs-Ringer buffer; TBS, Tris-buffered saline; TBS-T, TBS-Tween.

state (14, 15). Permeabilization of the plasma membrane for low molecular weight solutes allows the investigation of mitochondrial processes under precisely controlled conditions *in situ*, where mitochondrial interaction with intracellular structures is largely preserved. To obtain sufficient amounts of uniform material, which is required for this kind of study, we used the pancreatic β-cell line, MIN6. This cell line is one of the few that retains insulin-secretory responses to glucose and other secretagogues and has been used extensively in studies on the mechanisms controlling insulin secretion (16–18). In the present study, it was found that long term exposure of MIN6 cells to oleate (OE) sensitizes mitochondria to its direct uncoupling (acute) action. Another result of the long term OE application was stimulation of mitochondrial ROS production, mostly accounted for by the activity of complex I of the respiratory chain. In turn, the high level of reduction of complex I was accompanied by an additional uncoupling effect (mitochondrial depolarization), which was more pronounced in the OE-exposed cells. Dependence of this depolarization on the presence of FFA in the assay medium, GDP, inhibitors of complex I and a superoxide dismutase (SOD) mimetic suggests that it was caused by activation of UCP2 (the only UCP found in pancreatic β-cells) with intramitochondrial superoxide. These findings are of interest in light of continuing debate on UCPs potential functions (9, 10, 19, 20). In addition, OE-induced mitochondrial depolarization was sensitive to the dicarboxylate carrier (DIC) substrate malonate and OE-exposed cells demonstrated an elevated level of this carrier. Together these observations suggest that an increase in OE uncoupling efficiency caused by the long term incubation with OE is associated with a FFA-transporting function of the mitochondrial inner membrane proteins UCP2 and DIC.

EXPERIMENTAL PROCEDURES

Growth, Fatty Acid Treatment, and Permeabilization of Cells—MIN6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.05 mM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin for 3–4 days. For fatty acid exposure, growth medium was changed to Roswell Park Memorial Institute (RPMI 1640) medium containing the ingredients listed above in addition to 0.4 mM OE bound to bovine serum albumin (BSA) (0.5% BSA for control experiments). After 72 h of incubation, with daily medium change, trypsinized cells were washed in Ca^{2+}-free Krebs-Ringer buffer (KRB buffer, 120 mM NaCl, 1.0 mM $MgCl_2$, 24 mM $NaHCO_3$, and 10 mM HEPES, pH 7.3) and permeabilized essentially as described by Civelek *et al.* (21). Briefly, cells from two 10-cm dishes were suspended in 0.7 ml of KRB buffer containing 80 μg/ml saponin. After incubation at room temperature for 5 min, the cells were pelleted by centrifugation at 4 °C, washed in cold buffer, and suspended in cold 0.25 M sucrose containing 10 mM HEPES, pH 7.3. This produced stable cell preparations that retained permeable plasma membranes and coupled mitochondria when stored overnight at 4 °C or for at least 2 months at −80 °C.

Insulin Secretion Assay—MIN6 cells cultured in 12- or 24-well plates after OE exposure as described above were washed and preincubated twice for 30 min in a modified KRB (115 mM NaCl, 5.0 mM KCl, 2.5 mM $MCaCl_2$, 1.0 mM $MgCl_2$, 24 mM $NaHCO_3$, 1.25 mM glucose, and 10 mM HEPES, pH 7.3) with 0.1% BSA, then incubated for 1 h in the same buffer containing 1.25–25 mM glucose similar to a previous report (22). Insulin secretion in response to glucose was quantified using a radioimmunoassay (Linco Research, St. Charles, MO) according to manufacturer's instruction and normalized by cellular protein content.

Respiration Measurements—Oxygen consumption by permeabilized MIN6 cells was measured using a Clark-type electrode connected to an Oxygraph unit (Hansatech, Pentney, England). Cells were suspended at a concentration of 0.6–0.9 mg of protein/ml in incubation medium containing 0.25 M sucrose, 10 mM HEPES, 10 mM P_i, 2 mM $MgCl_2$, 1 mM EGTA, and 0.2% BSA. 10 mM succinate, 5 mM glutamate/5 mM malate or 7.5 mM glycerol-3-phosphate were added as respiratory substrates. Oxygen kinetic traces were treated as described by Estabrook (23), and respiration rates were converted into molar oxygen units using O_2 solubility in sucrose medium, as reported by Reynafarje *et al.* (24).

Mitochondrial Membrane Potential Monitoring—Mitochondrial membrane potential was monitored by following safranin O fluorescence in suspensions of permeabilized cells (25) and rhodamine 123 fluorescence in intact cells (1). Different methods for measurements were due to a superior responsiveness to K^+/valinomycin titration by safranin in permeabilized cells and higher loading ability of rhodamine 123 in intact cells. Measurements were performed using a FluoroCount plate reader (Packard Instrument Company, Meriden, CT) with excitation/emission wavelengths of 530/590 nm for safranin and 485/530 nm for rhodamine 123, respectively. A decrease in fluorescence corresponded to an increase in mitochondrial membrane potential. Incubation medium for permeabilized cells was identical to that used for respiratory assays but was supplemented with 2.5 μM safranin. Intact cells were incubated in KRB buffer. The magnitude of the fatty acid-induced depolarization in permeabilized cells was estimated by titration with K^+/valinomycin by supplementing the assay medium with 0.7 μM valinomycin and the addition of 0.05–7.4 mM K^+. Intact cells were loaded with rhodamine-123 at 10 μg/ml as previously described (22, 26) and the fluorescence response to the addition of glucose and the uncoupler carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) was monitored.

ATP Assay—To monitor synthesis and hydrolysis of ATP, samples were withdrawn from the cell suspension at appropriate time points and fixed with dimethyl sulfoxide (Me_2SO) (27). ATP was measured using the luciferin-luciferase kit from Sigma according to the manufacturer's instructions.

Fluorometric Determination of Hydrogen Peroxide Production—Generation of ROS was estimated as hydrogen peroxide formation by monitoring catalase-sensitive appearance of dichlorofluorescein (excitation 490 nm, emission 530 nm) from non-fluorescent dichlorofluorescein in the presence of horseradish peroxidase (28–30). High concentrations (1.55 units/ml) and the low K_m of peroxidase helps to circumvent interference from endogenous hydrogen peroxide-metabolizing enzymes (31). Dichlorofluorescein was obtained from the stable compound dichlorofluorescin diacetate by alkaline hydrolysis.

Western Blot for DIC—Whole cell protein extracts from MIN6 cells (40 μg of protein/lane) were separated on 14% polyacrylamide gels and electroblotted to nitrocellulose membrane (Millipore Corp., Bedford, MA). Blots were blocked with 5% (w/v) nonfat dry skimmed milk in Tris-buffered saline with 0.1% (v/v) Tween (TBS-T) for 1 h and incubated with DIC antibody (polyclonal rabbit antibody against mitochondrial dicarboxylate carrier, 1:1000 dilution in TBS-T with 0.5% BSA) (32) for 2 h. Blots were washed with TBS-T and exposed to horseradish peroxidase-coupled anti-rabbit IgG (Amersham Biosciences) for 1 h at room temperature. Blots were washed again with TBS-T and developed by enhanced chemiluminescence using a standard kit (ECL, Amersham Biosciences). Band intensity was measured by densitometry, analyzed using image analysis software (Scion Image v.4.02; Scion Corporation, Frederick, MD) and normalized by cytochrome *c* content in parallel samples.

Statistics—In all cases a Student's *t* test was used to analyze data between groups. A $p \leq 0.05$ was considered statistically significant.

RESULTS

Effect of Oleate Exposure on Insulin Secretion—Incubation with 0.4 mM OE for 72 h caused an increase in the basal level of insulin secretion in MIN6 cells, while inhibiting glucose-stimulated insulin secretion (Fig. 1). These alterations are very similar to those observed in β-cell lines and pancreatic islets by us and others (6, 7, 22, 33).

Effect of Oleate on Mitochondrial Function—Permeabilization of the plasma membrane with saponin allows observation of metabolic transitions accompanying oxidative phosphorylation in β-cell mitochondria *in situ*. Representative oxygen kinetic traces in control and OE-exposed cells (Fig. 2) demonstrate that oxygen consumption in permeabilized cells responds to respiratory substrate, ADP, oligomycin, and FCCP in a way typical for isolated mitochondria (15, 25). Occurrence of the state 3-state 4 transition is of interest since in permeabilized cells it is usually prevented by cytosolic ATPases (21, 27). Therefore, we measured the rates of ATP synthesis after the addition of ADP. We also measured ATP hydrolysis after the completion of ADP phosphorylation and the inhibition of mitochondrial ATP synthase with oligomycin. For these measurements, samples were taken from cell suspensions in the
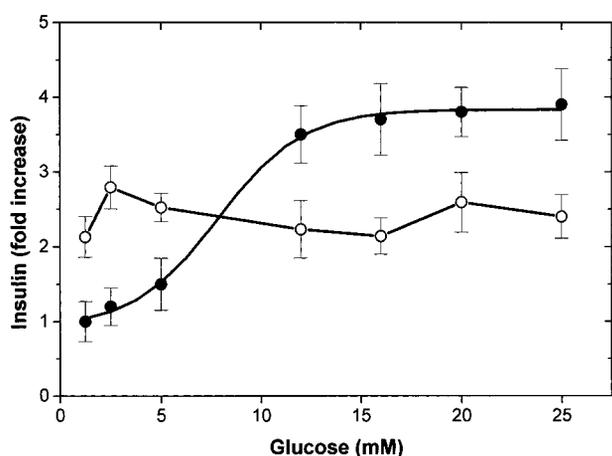
FIG. 1. **Effect of oleate on glucose-stimulated insulin secretion in MIN6 cells.** Insulin secretion measured in control cells (●) and those exposed to 0.4 mM oleate for 72 h (○) are shown. Insulin secretion is represented as fold-over basal secretion in control cells (n = six independent experiments). Insulin secretion between OE and control is significantly different at each concentration tested, $p \leq 0.05$.
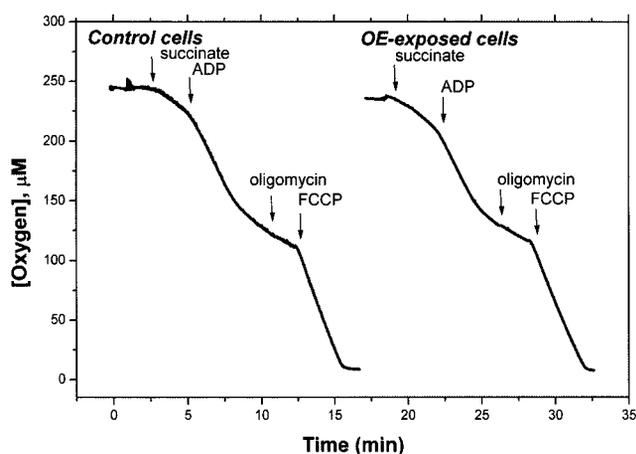

FIG. 2. **Respiratory kinetics during oxidative phosphorylation in permeabilized MIN6 cells.** Permeabilized MIN6 cells were placed into respiratory incubation medium (see "Experimental Procedures") at a concentration of 0.8 mg of protein/ml. Other additions indicated by *arrows* include: 10 mM succinate, 0.2 mM ADP, 1.3 μM oligomycin, and 4.7 μM FCCP. Kinetic traces are representative of six independent experiments.

course of the reaction, fixed with Me$_2$SO, and analyzed for ATP with luciferin-luciferase. It was found that succinate-driven ATP synthesis in state 3 in permeabilized MIN6 cells is faster than cytosolic ATP hydrolysis in state 4 by more than an order of magnitude (56.5 ± 4.8 and 3.3 ± 0.6 nmol ATP/min per mg of protein, n = 3). Thus the present experimental conditions allow an estimation of mitochondrial ATP synthesis despite interference of cytosolic enzymes. Mitochondrial respiratory and coupling parameters derived from respiratory kinetic traces suggest apparently normal functional performance of mitochondria in OE-exposed cells (Table I). This is consistent with the observation that mitochondrial membrane potential kinetics in OE-treated cells closely parallels that of control cells (Fig. 3A). Fluorescent kinetics show that quenching of safranin fluorescence is entirely dependent on the addition of succinate, suggesting a mitochondrial origin of the safranin response and low levels of endogenous mitochondrial substrates in permeabilized washed cells. Succinate (or FCCP)-dependent safranin fluorescence quenching $\Delta F_{quench}/\Delta F_{total}$ of 0.647 ± 0.024 and 0.648 ± 0.032 was found for the control and OE-exposed cells, respectively (n = 4).

The apparent normal oxidative phosphorylation in the fatty acid-exposed cells is in contradiction with the earlier reported uncoupling effect of fatty acids on β-cell mitochondria (1, 6). For further verification of our findings, mitochondrial membrane potential was tested in the same cells before permeabilization, as shown in Fig. 3B. Typical kinetic curves demonstrate that FCCP causes a significantly smaller fluorescent response in OE-exposed cells (48 ± 8% of the fluorescent response in control cells, $p < 0.05$) indicating that mitochondria in OE-treated cells, in the presence of endogenous substrates or external glucose, are partially depolarized. This confirms results of previous work and indicates that mitochondria in β-cells exposed to fatty acids are partly uncoupled when functioning in their native intracellular environment but show normal functional capacity when placed into standard incubation medium (see "Experimental Procedures"). This suggests that mitochondrial uncoupling in β-cells exposed to OE is caused primarily by cytosolic mediators that appear in the cells treated with fatty acid. A natural candidate for such a role would be OE itself, which is well known as a weak mitochondrial uncoupler (34). To test this hypothesis, we titrated mitochondrial membrane potential in the control and OE-exposed permeabilized MIN6 cells with OE (35, 36). Typical titration kinetics (Fig. 4A) and analysis of the data (Fig. 4B) indicate that the mitochondrial potential in OE-exposed cells is more sensitive to the uncoupling effect of OE. Qualitatively similar results were observed when the cells were exposed to an oleate/palmitate mixture (2:1) of the same sum molarity (Fig. 4B). It is known that oxidative phosphorylation is characterized by a steep dependence on the mitochondrial membrane potential, which is confined within a 20–30 mV potential range (37, 38). Calibration of mitochondrial depolarization shown in Fig. 4A demonstrates that fatty acid-induced alterations in membrane depolarization are located in this physiologically important interval.

Formation of ROS in Mitochondria—Oxidative stress is considered an important component of disorders related to high levels of FFA (39). In particular, ROS are thought to contribute significantly to defective β-cell function associated with type 2 diabetes (40); however, this notion is based on the effects of exogenous effectors such as alloxan (41) and oxygen radical scavengers (42), while endogenous ROS production in β-cells remains poorly understood. Recently, it was shown that palmitate and cytokines induce the formation of ROS in pancreatic β-cells (6, 43), but the metabolic source of these radicals was not clearly identified. Because the mitochondrial respiratory chain is considered to be the most significant generator of oxygen radicals in mammalian cells, we tested ROS formation in MIN6 cell mitochondria and its dependence on long or short term OE exposure. ROS generation in MIN6 cells is induced by the mitochondrial respiratory substrate succinate, inhibited by ADP and the uncoupler FCCP, and stimulated by oligomycin (Fig. 5). All of these features are typical of mitochondrial oxygen radical formation, indicating its strong dependence on the magnitude of mitochondrial membrane potential. Respiratory inhibitors, rotenone and antimycin A, were used as a tool to determine maximal ROS-producing capacity as well as the location of ROS-producing centers in the respiratory chain of MIN6 cells. Respiratory complexes I and III are both potential generators of oxygen radicals in the respiratory chain with their relative contributions dependent on the type of tissue (44, 45). In β-cell mitochondria we found that inhibitors of complexes I and III, rotenone and antimycin A, respectively, both suppress ROS generation driven by succinate. The rate of ROS production amounted to 66 ± 4% of the uninhibited state with rotenone (n = 5, $p < 0.05$) and 62 ± 10% with antimycin A (n = 4, $p < 0.05$). This suggests that complex I is the main radical

Effect of Oleate on β-Cell Mitochondrial Function

TABLE I

Respiratory rates and coupling parameters in control and oleate-treated permeabilized MIN6 cells (n = 4)

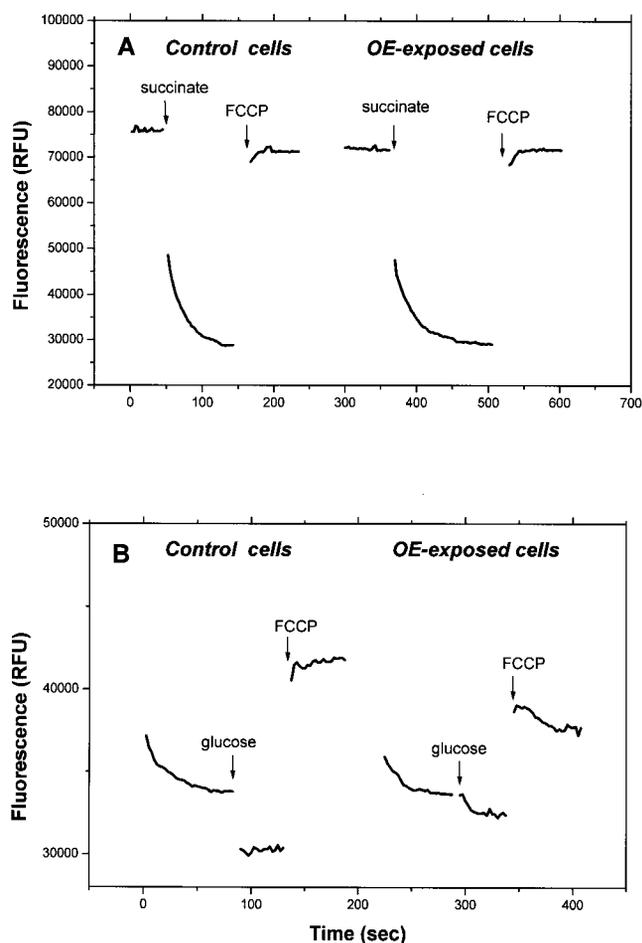|	State 3 respiration $V_{3,ADP}$	ADP/O	Respiratory control ratio
	nmol O_2/min mg of protein		
Control cells	20.1 ± 2.0	1.33 ± 0.07	3.06 ± 0.49
Oleate-treated cells	18.8 ± 1.4	1.38 ± 0.04	3.33 ± 0.39

FIG. 3. **Mitochondrial membrane potential formation and breakdown in permeabilized and intact MIN6 cells.** *A*, permeabilized cells (0.7 mg of protein/ml) were placed into respiratory incubation medium (see "Experimental Procedures") supplemented with 2.5 μM safranin. Other additions are indicated by *arrows*: 10 mM succinate and 4.7 μM FCCP. *B*, intact cells were loaded with 10 μg/ml rhodamine 123 for 10 min, preincubated in glucose-free medium for 20 min, and washed and placed into KRB buffer supplemented with 0.1% (w/v) BSA. Additions are indicated by *arrows*: 15 mM glucose and 4.7 μM FCCP. Kinetic traces are representative of seven (*A*) and three (*B*) independent experiments.
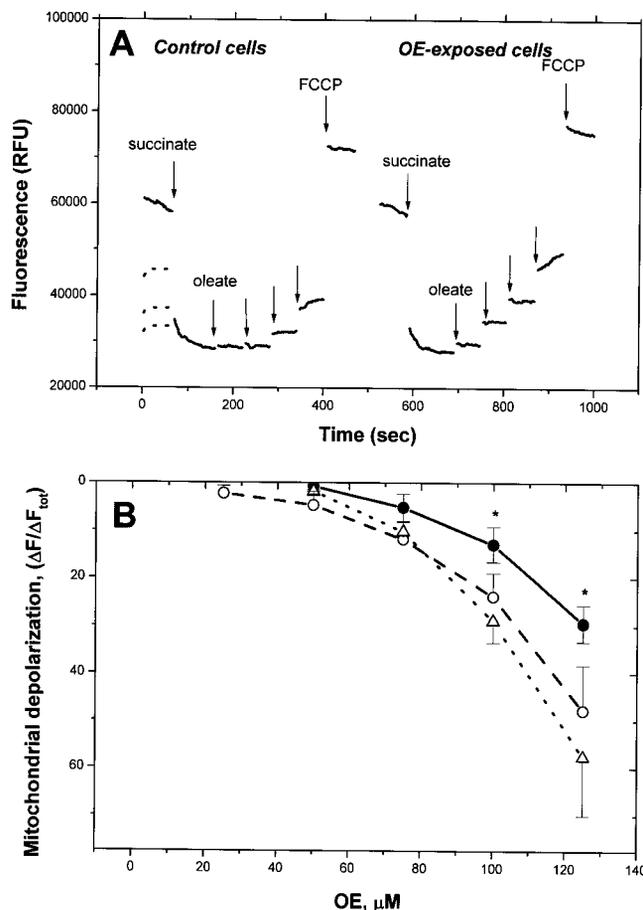

FIG. 4. **Mitochondrial membrane depolarization in permeabilized MIN6 cells.** *A*, typical kinetics with control and oleate-exposed cells. The cells at 0.7 mg of protein/ml were placed in respiratory incubation medium containing 0.1% (w/v) BSA and supplemented with 1.3 μM oligomycin. Additions are indicated by *arrows*: 10 mM succinate, 25 μM OE (nominal concentration) at each addition, and 4.7 μM FCCP. *Dotted lines* show calibrating marks for 15, 30, and 50 mV depolarization. *B*, statistical treatment of acute depolarization caused by short term OE administration in control cells ($\bullet$), those exposed to 0.4 mM oleate for 72 h ($\bigcirc$) (n = 4), and those exposed to 0.4 mM oleate/palmitate mixture for 72 h ($\triangle$) (n = 3).

source accepting electrons from succinate through reverse transport (inhibited by rotenone) driven by energy released in the descending part of the respiratory chain (inhibited by antimycin A). In accordance with this conclusion, ROS generation supported by NAD-linked substrates glutamate/malate was stimulated by both these inhibitors with a similar efficiency, up to $266 \pm 15\%$ with rotenone ($n = 8$, $p < 0.05$) and $237 \pm 15\%$ for antimycin A ($n = 7$, $p < 0.05$). Thus with respect to the formation of ROS, mitochondria from clonal β-cells resemble those from brain, which produce oxygen radicals mainly in the NADH dehydrogenase segment of the respiratory chain and differ from heart mitochondria where the Q cycle contributes principally to ROS production (44, 45).

Another distinctive property of pancreatic β-cells is the high activity of glycerol phosphate dehydrogenase, which exceeds that in other tissues by more than an order of magnitude (46, 47) and ensures transfer of cytosolic reducing equivalents into mitochondria by the glycerophosphate shuttle. In MIN6 cells, glycerophosphate dehydrogenase exhibited the highest activity among primary dehydrogenases feeding the respiratory chain (glycerophosphate oxidase activity exceeded succinate oxidase by ~2-fold). According to this, we found that glycerol phosphate-driven respiration in state 4 supports active H_2O_2 production in β-cell mitochondria, while in heart, brain, and kidney mitochondria this substrate is found to be the poorest source of H_2O_2 (45). This suggests that in pancreatic β-cells, in contrast to other tissues, functioning of the glycerol phosphate shuttle may be the significant source of ROS, provided that mitochondrial membrane potential is sufficiently high.

Effect of Oleate on ROS Formation—To estimate the effect of OE on ROS production in MIN6 cells, two parameters were
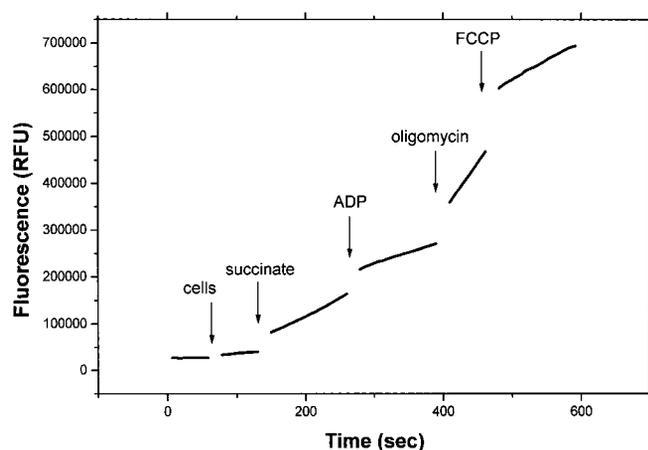
FIG. 5. **Modulation of ROS production in MIN6 cells with effectors of mitochondrial metabolism.** Permeabilized cells were placed at 0.8 mg of protein/ml into respiratory incubation medium supplemented with 2.75 μM 2′,7′-dichlorofluorescin and 5 μg/ml horseradish peroxidase. Additions indicated by *arrows* include: 10 mM succinate, 0.2 mM ADP, 1.3 μM oligomycin, and 4.7 μM FCCP. Typical kinetics represented by three independent experiments.

TABLE II
Rates of ROS production (nmol/min × mg of protein) in control and oleate-treated permeabilized MIN6 cells (n = 5 for succinate-supported production, n = 6 for glutamate/malate + antimycin A supported production)

	ROS production with succinate	ROS production with glutamate/malate + antimycin A
Control cells	0.24 ± 0.04	0.43 ± 0.15
Oleate-treated cells	0.40 ± 0.08[a]	0.67 ± 0.15[a]

[a] Significant increase at $p < 0.05$ vs control cells.

TABLE III
Maximal levels of stimulation of ROS production caused by acute application of oleate onto control and oleate-exposed (for 72 h) permeabilized MIN6 cells (expressed as percentage in relation to ROS production in oleate-free medium)

	ROS production with succinate	ROS production with glutamate/malate
Control cells	568%	545%
Oleate-exposed cells	501%	508%

tested. First, succinate-supported H_2O_2 generation that reflects membrane potential-dependent ROS production was quantified. Second, H_2O_2 generation supported by glutamate/malate in the presence of antimycin A, reflecting maximal ROS-producing mitochondrial capacity was measured. Both parameters were increased in the cells exposed to OE for 72 h (Table II). With regard to the short term FFA effect, an even greater (~5-fold) acceleration of mitochondrial formation of ROS was observed immediately after the addition of 25–75 μM OE to permeabilized β-cells (Table III). This acute stimulation of mitochondrial reactive oxygen generation occurred in both control cells and those exposed to OE for 72 h.

ROS and FFA-induced Uncoupling—Induction of UCP2 in FFA-exposed β-cells is well documented and thought to cause less efficient mitochondrial coupling (1, 8, 48). However, UCPs themselves cannot exert such an effect because their protonophoric function requires cofactors (FFAs) and, according to a recent report (20), superoxide radicals. This demonstration of superoxide necessity for UCP function prompted us to test how conditions favoring intramitochondrial superoxide production would affect fatty acid-induced uncoupling. Succinate oxidation was used to build up and maintain mitochondrial membrane potential, and glutamate/malate (in the presence of rotenone, which inhibits succinate-dependent but stimulates glutamate/malate-dependent ROS formation) were applied as an additional superoxide-producing factor. It was found that this factor applied in the presence of fatty acid causes additional mitochondrial depolarization (Fig. 6A). This depolarization was more pronounced in OE-exposed cells compared with control cells (16.2 ± 3.3 and 3.06 ± 1.54% of the total fluorescence signal, respectively, in the presence of 75 μM OE, n = 3). This effect could be reversed by BSA, prevented by GDP (Fig. 6A), and did not occur in the absence of FFA in the medium (not shown). Preincubation of MIN6 cells with the SOD-mimetic Mn(III) tetrakis (4-benzoic acid) porphyrin (100 μM, 30 min) decreases depolarization by 40–50%. It was not possible to construct a dose response for the SOD mimetic because higher concentrations of Mn(III) tetrakis (4-benzoic acid) porphyrin interfere strongly with the membrane potential assay.

DIC Involvement in OE-induced Uncoupling—Transport of fatty acid anions across the mitochondrial inner membrane and consequently the uncoupling effect of fatty acids is mediated by several mitochondrial-uncoupling proteins and anion transporters. Using specific modulators of their function we found that not only UCP2 (1, 8, 48) but also DIC can be responsible for the high sensitivity of OE-exposed β-cells to acute FFA. If malonate (which competes with FFA for the carrier) is used as a substrate of the DIC, it is expected to cause recoupling (membrane potential repolarization) if the DIC participates in FFA-induced uncoupling (membrane potential depolarization) (49). To avoid the effect of malonate on succinate dehydrogenase, α-glycerophosphate was used as respiratory substrate in these experiments. Typical responses to malonate (Fig. 6B) demonstrate that the involvement of the DIC in FFA-induced uncoupling is minor in control cells but increases significantly after 72 h of exposure to OE. Expression levels of the DIC in control and OE-exposed cells were analyzed by Western blotting (Fig. 7) and demonstrate a 2-fold increase of DIC in OE-treated cells.

DISCUSSION

In obese individuals who go on to develop type 2 diabetes, it is speculated that years of hyperlipacidemia has a deleterious or "lipotoxic" effect on pancreatic β-cell function that contributes to the diabetic pathogenesis. Studies in animals and tissue culture models support this hypothesis (3). Although the characteristic increase in basal insulin secretion with reduced glucose-stimulated secretion is well documented in multiple laboratories, it is still unclear how long term exposure to elevated FFAs causes dysregulation of β-cell function.

The novel observations in the present study include detailed characterization of oxidative phosphorylation and mitochondrial ROS production in the presence and absence of FFAs. In addition the interrelationship between endogenous ROS and the regulation of mitochondrial coupling in the pancreatic β-cell line MIN6 was investigated. Oxidative phosphorylation in mitochondria of MIN6 cells was studied after cell exposure to free OE, which impairs glucose-stimulated insulin secretion (Fig. 1). Mitochondria in these cells retain normal functional capabilities in the virtual absence of FFAs. However, mitochondria in OE-treated cells are more sensitive to uncoupling caused by direct application of FFA at physiological levels compared with control cells. These data confirm earlier reports (1, 6) suggesting a less coupled state of mitochondria in FFA-exposed β-cells and presents the following significant findings. First, partial uncoupling caused by exposure to OE is revealed only in the presence of FFA. Second, it is accompanied by the induction of not only UCP2 (1, 8) but also DIC. Third, uncoupling is further augmented by the highly reduced state of respiratory complex I. The latter conclusion is based upon the
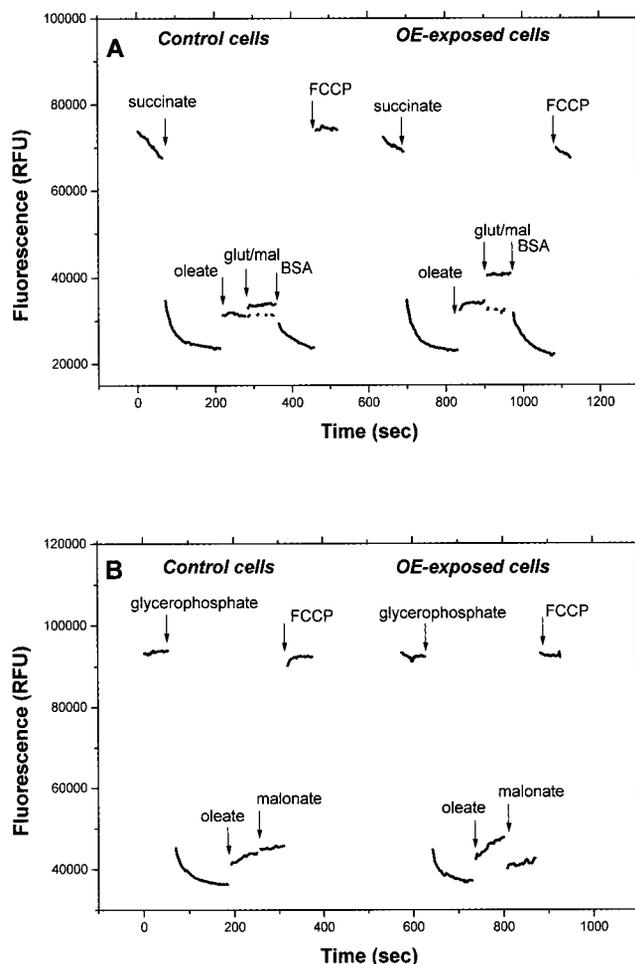
FIG. 6. **Effects of glutamate/malate and malonate on OE-induced mitochondrial depolarization.** Permeabilized cells (0.7 mg of protein/ml) were placed into respiratory incubation medium containing 0.1% BSA and 1 μM rotenone. Additions are indicated by *arrows. A,* 10 mM succinate, 75 μM OE, 5 mM glutamate + 5 mM malate, 0.1% (w/v) BSA, and 4.7 μM FCCP. *Dotted segments* in *A* are membrane potential kinetics in the presence of 1 mM GDP demonstrating that GDP in Mg^{2+}-free medium abolishes the caused glutamate/malate-induced depolarization. *B,* additions include 7.5 mM glycerophosphate, 50 μM OE, 7.5 mM malonate, and 4.7 μM FCCP. Kinetic traces are representative of five (*A*) and three (*B*) independent experiments.

FIG. 7. **Western blot analysis of DIC expression in MIN6 cells.** Total protein lysates from control (BSA only) and OE-exposed MIN6 cells were analyzed by SDS/PAGE and Western blotting for the presence of DIC. Two representative lysates (one experiment) are shown for control and OE-exposed cells (*A*). Densitometric analysis of DIC content in control and OE-exposed cells (*B, n = 3; *, p < 0.05*).

additional mitochondrial depolarization caused by complex I substrates in the presence of rotenone. Uncoupling caused by complex I substrates may be due to the stimulation of uncoupling function of UCP2 by superoxide radicals (20) produced in complex I. This is supported by the following observations: (i) complex I is the active source of oxygen radicals in MIN6 mitochondria; (ii) depolarization is observed only in the presence of FFA, which are required for UCP function; (iii) depolarization is prevented by addition of GDP (Fig. 6*A*), which inhibits the native uncoupling function of UCPs (9, 20); (iv) preincubation of MIN6 cells with the SOD-mimetic decreases depolarization by 40–50%. Recently activation of UCP function by superoxide originating from the quinone moiety reduced by the mitochondrial respiratory chain was demonstrated (50). Our present results suggest that UCP can be activated by oxygen radicals formed in respiratory complex I, which is thought to be the major physiologically and pathologically relevant ROS generator in mitochondria (51, 52).

Another mitochondrial protein that is up-regulated in OE-exposed MIN6 cells and contributes to their increased sensitivity to FFA uncoupling is DIC. Interestingly, its FFA-induced
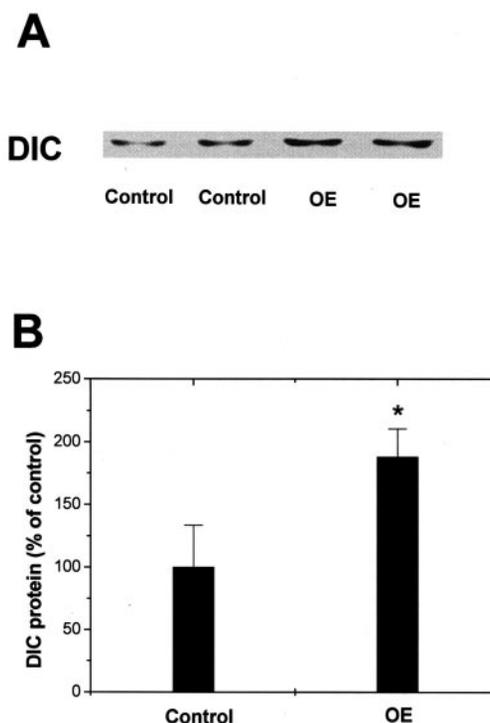
overexpression was also found in white adipocytes (32) and may, therefore, be a rather general phenomenon. Regarding the physiological significance of a DIC increase in response to FFA, its role in reesterification of FFA was considered (32). Earlier published data (49) and our results suggest that the DIC can facilitate the transport of FFA anions across mitochondrial membrane.

The observed stimulatory effect of the long term OE exposure on ROS production in MIN6 cells corroborates recently reported effects in pancreatic β-cells (6). In addition, it demonstrates a mitochondrial origin of elevated ROS induced by FFA in insulin-secreting cells. Considering a possible basis for this effect, two recent findings may provide some insight. First, significant up-regulation of carnitine palmitoyltransferase-1 (CPT-1) in MIN6 cells exposed to OE (as well as palmitate) was reported (33, 53) and we have demonstrated large increases in CPT-1 transcripts in pancreatic islets of mice placed on a long term high fat diet (54). CPT-1 catalyzes the rate-limiting step of FFA translocation into the mitochondrial matrix and is a key regulatory site of FFA oxidation (55). The latter process is considered as a potential source of oxygen radicals in mitochondria (56). Second, the same treatment with FFA causes up-regulation of the genes involved in the inflammatory response, most of which are absent in MIN6 cells under control conditions (33). Inflammatory responses are often accompanied by an increased level of ROS (39), whose generation is initiated at the mitochondrial level by ceramide (29). In turn, the ceramide regulatory pathway is reported to be activated by prolonged exposure of pancreatic islets to FFA (57). All of the above pieces of evidence suggest that the activation of proinflammatory genes may be responsible for the increased production of ROS in MIN6 cells after long term OE treatment.

The acute application of OE caused stimulation of ROS production that was even more pronounced than that seen after 72 h exposure. This is an interesting effect because FFAs can

act as natural "mild uncouplers" and are thought to prevent mitochondrial ROS production by lowering mitochondrial membrane potential and the degree of reduction of superoxide-producing complexes in the respiratory chain (34). Indeed, this was demonstrated on animal and plant mitochondria (58–60). However, a recent report demonstrating the opposite effect of arachidonate, palmitate, and oleate on heart mitochondria suggests that FFA can play a dual role in ROS regulation (28). This was ascribed to the fact that besides a protonophoric effect that slows down ROS production, FFAs also exert a direct inhibiting effect on the mitochondrial respiratory chain (28, 34). This increases its reduction and hence ROS production. Our results confirm the ability of FFAs to stimulate mitochondrial formation of ROS and suggest that it can contribute significantly to FFA-induced cell damage.

In conclusion, long term exposure to OE impairs oxidative phosphorylation in β-cells by causing: (i) the sensitization of mitochondria to the acute uncoupling action of fatty acids associated with the FFA-transporting function of UCP2 and the DIC, and (ii) indirect (after 72 h of OE exposure) and direct (immediately after OE application) stimulation of mitochondrial ROS production, mostly in respiratory complex I. In addition, OE-exposed cells are more sensitive to additional uncoupling caused by reduction of respiratory complex I, which is dependent on FFA and is likely to be due to activation of UCP2 with intramitochondrially produced superoxide. These OE-induced mitochondrial metabolic alterations may be responsible for impaired glucose-induced insulin secretion. The metabolic basis for elevated basal insulin secretion in OE-exposed cells is also likely to be complex. It may be related to the observation that FFAs induce uncoupling activity only at sufficiently high membrane potential (61). The absence of glucose is expected to lower the mitochondrial membrane potential, and under these conditions FFAs may serve as a fuel rather than an uncoupler. In a broad perspective, FFAs are apparently the main natural uncoupling agent in the cell, and therefore the mechanism of their action is highly relevant. Experimental work on isolated mitochondria disclosed that fatty acids exert uncoupling action on oxidative phosphorylation through a protonophoric mechanism mediated by several mitochondrial transporters. Our present results, along with the previous work performed on whole cells (1, 32), show that this protonophoric mechanism is preceded and strengthened by FFA-induced up-regulation of some of the mitochondrial transporters (UCP2 and DIC). Apparently this represents an additional amplification step in fatty acid-induced uncoupling on the cellular level.

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Mitochondrial Functional State in Clonal Pancreatic β-Cells Exposed to Free Fatty Acids

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