Uncoupling Protein 2: A Possible Link Between Fatty Acid Excess and Impaired Glucose-Induced Insulin Secretion?

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The mechanism by which long-term exposure of the β-cell to elevated concentrations of fatty acid alters glucose-induced insulin secretion has been examined. Exposure of INS-1 β-cells to 0.4 mmol/l oleate for 72 h increased basal insulin secretion and decreased insulin release in response to high glucose, but not in response to agents acting at the level of the K_{ATP} channel (tolbutamide) or beyond (elevated KCl). This also suppressed the glucose-induced increase in the cellular ATP-to-ADP ratio. The depolarization of the plasma membrane promoted by glucose was decreased after oleate exposure, whereas the response to KCl was unchanged. Cells exposed to free fatty acids displayed a lower mitochondrial membrane potential and a decreased glucose-induced hyperpolarization. The possible implication of uncoupling protein (UCP)-2 in the altered secretory response was examined by measuring UCP2 gene expression after chronic exposure of the cells to fatty acids. UCP2 mRNA and protein were increased twofold by oleate. Palmitate and the nonoxidizable fatty acid bromopalmitate had similar effects on UCP2 mRNA, suggesting that UCP2 gene induction by fatty acids does not require their metabolism. The data are compatible with a role of UCP2 and partial mitochondrial uncoupling in the decreased secretory response to glucose observed after chronic exposure of the β-cell to elevated fatty acids, and suggest that the expression and/or activity of the protein may modulate insulin secretion in response to glucose. *Diabetes* 50:803–809, 2001

Type 2 diabetes is considered a polygenic disease aggravated by environmental factors, such as low physical activity or a hypercaloric lipid-rich diet (1). Obese type 2 diabetic patients show insulin resistance of skeletal muscle, enhanced hepatic glucose production, and decreased glucose-induced insulin secretion, the molecular nature of which is still unknown. Several studies have proposed that free fatty acids (FFAs) could be the common factor producing gradually in a tissue-specific manner the alterations observed in type 2 diabetes (2–4). At the level of the β-cell, long-term exposure to fatty acids could alter the coupling of glucose metabolism to insulin secretion by acting on the expression of specific genes or by acting directly on the activity of some enzymes. In vitro, long-term exposure of β-cells to FFAs increases basal insulin release, but strongly decreases secretion in response to glucose (4). Thus, long-term exposure to FFAs increases the expression of carnitine palmitoyl transferase 1 (CPT-1), which is considered the rate-limiting step in fatty acid oxidation (5). Increased fatty acid oxidation could reduce glucose metabolism through operation of the glucose–fatty acid cycle (6), as suggested in some studies (7,8). In contrast, other work has shown long-term effects of FFAs on glucose-induced insulin secretion without major changes in glucose oxidation or any evidence of an operative glucose–fatty acid cycle (9–11). This suggests that other factors account for the long-term effects of FFAs. Glucose metabolism in β-cells is linked to insulin secretion, at least in part, through changes in the cytosolic ATP-to-ADP ratio leading to the closure of K_{ATP} channels (12). This results in depolarization of the plasma membrane and leads to calcium entry through voltage-gated Ca^{2+} channels and an increase in cytosolic Ca^{2+} (12). Among other effects, the increase in long-chain acyl-CoA esters, resulting from exposure to FFAs, may open K_{ATP} channels and hyperpolarize the β-cell; this could prevent glucose-induced closure of the channel and the consequent depolarization (13). Chronic FFA exposure also alters the energy metabolism of the β-cell, elevates the redox state, and increases basal oxygen consumption; this suggests an effect at the level of ATP generation (11), or beyond.

Proteins of the uncoupling protein (UCP) family are located in the inner mitochondrial membrane and act as proton channels or transporters (14). They uncouple the electrochemical gradient produced by the respiratory chain from ATP synthesis. The UPC family consists of four main isoforms with different tissue distribution. Among them, UCP2 shows a quasi-ubiquitous expression. Fatty acids or dietary fats increase UCP2 mRNA and protein levels in several tissues (14,15). Leptin and troglitazone increase its expression in isolated islets from control and Zucker diabetic fatty (ZDF) rats (16,17).

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AU, arbitrary units; BSA, bovine serum albumin; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CPT-1, carnitine palmitoyl transferase 1; ECL, enhanced chemiluminescence; FACS, fluorescence-associated cell sorting; FCS, fetal calf serum; FFA, free fatty acid; PPAR, peroxysome proliferator–activated receptor; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; UCP, uncoupling protein.

In the pancreatic β-cells, an increase in UCP2 could decrease glucose-induced ATP production by uncoupling the mitochondrial oxidative phosphorylation, thereby decreasing glucose-stimulated insulin secretion without an associated change in glucose metabolism. Long-term exposure to fatty acids could increase UCP2 expression and/or activity and thereby specifically modify insulin secretion in response to glucose and other fuel stimuli. This hypothesis is supported by the observation that adenovirus-mediated overexpression of UCP2 in pancreatic islets abolished glucose-stimulated insulin secretion (18). In contrast, another study reported that overexpressing UCP2 in islets from ZDF rats partly restored glucose-stimulated insulin secretion (19).

Because chronic exposure of the β-cell to FFAs alters glucose-induced insulin secretion without major changes in glucose oxidation (9–11), the aim of the present study was to investigate the possible role of UCP2 in the long-term effect of FFAs on glucose-induced insulin release. The results show that chronic exposure of the β-cell to FFAs increases the expression level of the UCP2 gene and decreases the effects of glucose on the ATP-to-ADP ratio and on the plasma membrane and mitochondrial membrane potentials. Therefore, these results are compatible with the hypothesis that a long-term effect of fatty acids on UCP2 expression and/or activity leads to a decrease in glucose-induced insulin secretion.

RESEARCH DESIGN AND METHODS

Cell culture, incubation condition, and islet isolation. INS-1 cells were grown in monolayer cultures, as previously described (20), in RPMI 1640 medium containing 11 mmol/l glucose supplemented with 10 mmol/l HEPES, 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin in an humidified atmosphere (5% CO_2 and 95% air). After 6–7 days (60–80% confluence), cells were incubated in the same medium at 5 mmol/l glucose with 0.5% bovine serum albumin (BSA) or 0.4 mmol/l of various fatty acids bound to BSA. Albumin-bound fatty acids were prepared by stirring the fatty acid sodium salt at 50°C with defated BSA (Sigma, St. Louis, MO). After adjustment of the pH to 7.4, the solution was filtered through a 0.22-μm filter, and the fatty acid concentration was measured using a commercial kit (NEFA PAP, bioMérieux, Lyon, France).

Mitochondria were prepared from INS-1 cells after homogenization in 250 mmol/l sucrose, 0.5 mmol/l EGTA, and 5 mmol/l HEPES, with 1 μl/ml of a protease inhibitor cocktail (cat no. P 8340, Sigma). After a low-speed centrifugation (800*g* for 3 min), the supernatant was centrifuged for 10 min at 10,000*g* and the pellet used as a crude mitochondrial preparation. The 10,000*g* supernatant was centrifuged for 2 h at 180,000*g* and the pellet used as a crude membrane preparation. Pancreatic islets from 250-g male Wistar rats were isolated by collagenase digestion (21) using Histopaque (Sigma) centrifugation. The islets were either used directly for total RNA extraction or were homogenized in 250 mmol/l sucrose, 0.5 mmol/l EGTA, and 5 mmol/l HEPES, with 1 μl/ml protease inhibitor cocktail for Western blotting. Islets were also digested with trypsin, and β-cells were separated from non–β-cells by fluorescence-associated cell sorting (FACS). For Western blotting, β-cells were homogenized in the same buffer as the islets. Protein measurements were carried out using the Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany), with BSA as standard. DNA was measured according to Labarca and Paigen (22).

mRNA analysis. Cells or tissues were washed with cold phosphate-buffered saline, and total RNA was extracted by the guanidium isothiocyanate method (23). RNA samples were denatured with formaldehyde, subjected to electrophoresis in 1% agarose gels, transferred to a nylon membrane, and fixed by UV exposure. mRNA analysis was carried out by the Northern blotting hybridization method, using human UCP2 ^{32}P-labeled cDNA probe. The human UCP2 cDNA probe of 772 bp was obtained by polymerase chain reaction from cloned human UCP2 cDNA (24). The membranes were also hybridized with a β-actin probe and a [γ^{32}P]ATP-labeled synthetic oligonucleotide specific for the 18S rRNA subunit to ensure that equivalent amounts of RNA were loaded on the gel. Membranes were exposed for autoradiography, and the autoradiograms were analyzed by laser densitometry scanning.

Insulin secretion. INS-1 cells were plated (10^5 cells/well) into 24-well plates and cultured for 6 days in RPMI medium, then cultured for 72 h in the same medium containing 5 mmol/l glucose and 0.5% BSA or BSA-bound fatty acids (0.4 mmol/l). They were then washed with Krebs-Ringer bicarbonate HEPES (KRBH) buffer medium containing 10 mmol/l HEPES and 0.07% BSA and preincubated for 60 min in KRBH medium containing 2.8 mmol/l glucose and 0.5% BSA. The cells were then incubated for 30 min with the additions mentioned, and the insulin concentration in the medium was determined. Total cellular insulin content measured after acid/ethanol extraction (1.5% HCl and 75% ethanol) and insulin secretion were quantified by radioimmunoassay using rat insulin as standard (25).

ATP and ADP measurements. After a 15-min incubation, the medium was rapidly removed, and 0.75 ml of 0.6N perchloric acid was added to the cells. Precipitated proteins were removed by centrifugation at 10,000*g* and the supernatants neutralized by addition of 2.7 mol/l K_2CO_3. The potassium perchlorate was eliminated by centrifugation. ATP and ADP were assayed in the supernatants by luciferase measurements (Sigma). ATP was assayed directly. For ADP measurements, ATP was first hydrolyzed to AMP using ATP sulfurylase (26). After inactivation of the sulfurylase, ADP was converted to ATP with pyruvate kinase and phosphoenolpyruvate and was measured by luciferase (26).

Measurements of plasma and mitochondrial membrane potential. After a 72-h culture in the absence or presence of fatty acids, the cells were then detached by incubation in the presence of EDTA without trypsin, washed, and resuspended in KRBH buffer containing 0.1% BSA and 2.8 mmol/l glucose. The cells were loaded with 20 nmol/l tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Eugene, OR) for 30 min at 37°C, and flow cytometry was performed with a FACStar^{Plus} cell sorter (Beckton Dickinson, Bedford, MA) on 10,000 cells per sample using the 514-nm line of an argon laser to assess the mitochondrial membrane potential. Fluorescent light was directed to a photomultiplier tube equipped with a filter selecting >590 nm. The data were analyzed with Lysis II software. Each determination was carried out in the absence or presence of 75 μmol/l carbonylcyanide *m*-chlorophenylhydrazone (CCCP).

Glucose- and CCCP-induced changes in mitochondrial membrane potential were also measured in cell suspension after loading the cells with 10 μg/ml rhodamine 123 (Molecular Probes) in KRBH buffer for 10 min at 37°C (27). After centrifugation, the cells were resuspended in the same medium without rhodamine and transferred to a fluorimeter. Fluorescence was excited at 490 nm and measured at 530 nm. For plasma membrane potential measurements, 2.5×10^6 cells were pelletted and resuspended in 2 ml KRBH buffer containing 2.8 mmol/l glucose and 100 μmol/l bisoxonol (Molecular Probes) and transferred to the thermostatic cuvette. Cells were excited at 540 nmol/l, and emission was recorded at 580 nmol/l (20). All fluorescence measurements were performed in a LS-50B fluorimeter (Perkin Elmer, Bucks, U.K.) at 37°C with gentle stirring.

Western blotting. Cell proteins (30 μg) were subjected to electrophoresis on a 12% polyacrylamide gel then electrotransfered to an Immobilon P membrane (Millipore), blocked with 0.5% nonfat dry milk in Tris-buffered saline with 0.1% Tween, and incubated overnight at 4°C in the same buffer containing goat polyclonal antibody to the NH_2-terminal domain of UCP2 (Research Diagnostics, Flanders, NJ) or rabbit polyclonal antibody to the COOH-terminal domain of the protein (Alpha Diagnostic International, San Antonio, TX) anti-UCP2 antibody. Membranes were then rinsed in Tris-buffered saline with Tween and incubated with a horseradish peroxidase–coupled goat or rabbit IgG antibody (Amersham, U.K.) for 1 h at room temperature. Cytochrome oxidase and prohibitin were detected using a monoclonal antibody against subunit IV of cytochrome oxidase (Molecular Probes) or against prohibitin (Research Diagnostics) and horseradish peroxidase–coupled anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibody, respectively. The resolved bands were visualized by enhanced chemiluminescence (ECL) detection reagents (Amersham) and exposed to Hyperfilm ECL (Amersham) for 1–10 min.

Statistical analysis. All results are expressed as means ± SE of the indicated number of experiments. Statistical significance was calculated by Student's *t* test for paired or unpaired data.

RESULTS

Long-term exposure of INS-1 cells to oleate specifically reduces glucose-induced insulin release in association with alterations in the ATP-to-ADP ratio as well as plasma and mitochondrial membrane potential. Exposure of INS-1 β-cells to 0.4 mmol/l oleate for 72 h

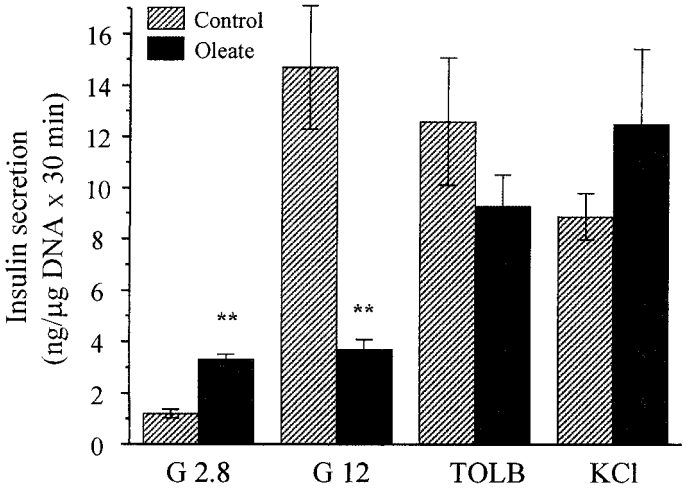

FIG. 1. Effects of a long-term exposure of INS-1 cells to oleate on insulin secretion in response to glucose, tolbutamide, and KCl. Cells were cultured in RPMI medium containing 10% FCS with 0.5% BSA for 72 h at 5 mmol/l glucose, in the absence or presence of 0.4 mmol/l oleate. They were then incubated for 30 min in KRBH buffer at 2.8 mmol/l glucose (G 2.8), 12 mmol/l glucose (G 12), 2.8 mmol/l glucose + 100 μmol/l tolbutamide (TOLB), or 30 mmol/l KCl (KC1). The insulin contents in cells cultured in the absence or presence of oleate were 231 ± 43 and 190 ± 8 ng/μg DNA, respectively ($P < 0.05$ by paired t test). Data are means ± SE of 3–5 experiments. **$P < 0.02$.

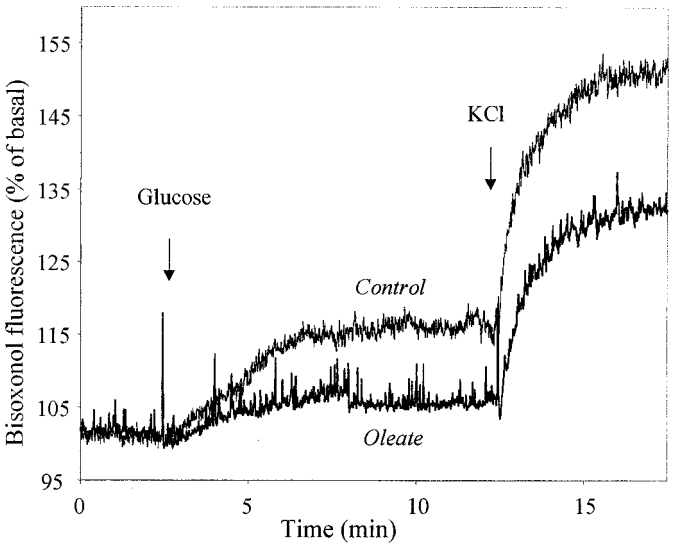

FIG. 2. Effect of a long-term exposure to oleate on glucose-induced changes in plasma membrane potential. INS-1 cells were cultured in RPMI medium containing 10% FCS with 0.5% BSA for 72 h at 5 mmol/l glucose, in the absence or presence of 0.4 mmol/l oleate. Cells were detached and incubated in KRBH buffer containing 2.8 mmol/l glucose and bisoxonol (100 μmol/l), and fluorescence was used to measure plasma membrane potential. At the indicated time, glucose was added at a final concentration of 12.8 mmol/l and then at 30 mmol/l KCl. Data are from one of four representative experiments.

results in an altered insulin secretion in response to glucose, with an elevated secretion at low glucose that is not further enhanced by increasing the concentration of the secretagogue. This observation has already been made in isolated islets (7–9), INS-1 (11), and HC9 cells (10). In contrast, agents that stimulate insulin secretion by acting at the level of the K_{ATP} channel (tolbutamide), or trigger an increase in Ca^{2+} as a consequence of membrane depolarization (elevated KCl), elicited comparable levels of insulin secretion in control and oleate-treated cells (Fig. 1).

Because a large part of the generation of signals coupling glucose metabolism to insulin secretion involves changes in the ATP-to-ADP ratio elicited by glucose metabolism, ATP and ADP were quantified in control and oleate-treated INS-1 cells subsequently incubated at low and high glucose. Table 1 shows that the increase in ATP and in the ATP-to-ADP ratio measured after glucose administration in control cells was not observed in cells chronically exposed to oleate. In contrast, short-term (i.e., during the 15-min incubation period) fatty acid exposure did not affect the action of glucose. Glucose-induced changes in the ATP-to-ADP ratio of the β-cell is known to be followed by a depolarization of the plasma membrane. Figure 2 shows that the addition of glucose to a final concentration of 12.8 mmol/l produced a clear depolarization of the plasma membrane in control cells and a minor change of plasma membrane potential in cells chronically exposed to oleate (47 ± 7% of control cells, $n = 4$, $P < 0.02$). Fatty acid exposure did not alter the depolarization in response to KCl (fatty acid exposed cells 92 ± 8% of control cells, $n = 4$, NS).

The effects of long-term exposure to fatty acid on mitochondrial membrane potential was measured by flow cytometry using TMRE, a fluorescent dye sensitive to the mitochondrial membrane potential. Cells exposed to oleate for 72 h displayed a significant decrease in the level of

TABLE 1
Effects of a long-term or short-term exposure of INS-1 cells to oleate on the ATP content and ATP-to-ADP ratio at low and high glucose

	Culture conditions			
	ATP (nmole/μg DNA)		ATP-to-ADP ratio	
Glucose (mmol/l)	2.8	12	2.8	12
Long-term				
BSA 72 h	0.420 ± 0.048	0.480 ± 0.043*	4.9 ± 0.1	8.3 ± 0.9†
Oleate 0.4 mmol/l 72 h	0.500 ± 0.048	0.510 ± 0.056	6.4 ± 0.2	6.7 ± 0.5
Short-term				
BSA 15 min	0.296 ± 0.033	0.355 ± 0.029‡	ND	ND
Oleate 0.4 mmol/l 15 min	0.216 ± 0.021	0.329 ± 0.029§	ND	ND

Data are means ± SE of four independent experiments. *$P \leq 0.02$ vs. 2.8 mmol/l glucose; †$P \leq 0.05$ vs. 2.8 mmol/l glucose; ‡$P < 0.02$ vs. 2.8 mmol/l glucose by paired analysis; and §$P < 0.01$ vs. 2.8 mmol/l glucose + oleate by paired analysis. ND, not determined.

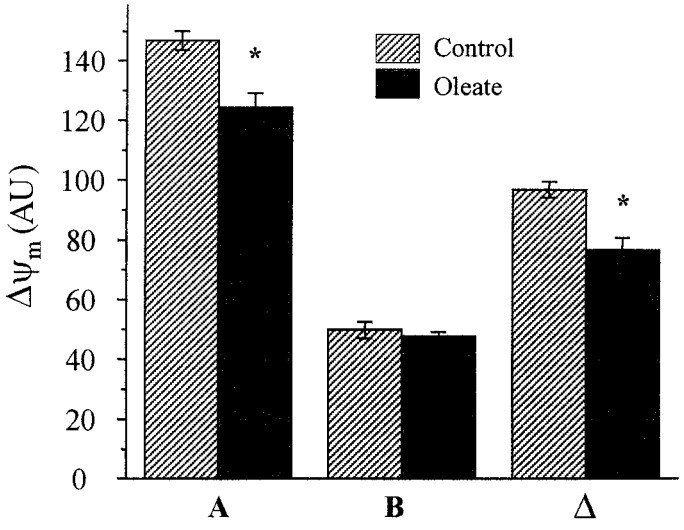

FIG. 3. Effect of a long-term exposure to oleate on the mitochondrial membrane potential ($\Delta\Psi_m$) of INS-1 cells. Cells were cultured in RPMI medium containing 10% FCS with 0.5% BSA for 72 h at 5 mmol/l glucose, in the absence or presence of 0.4 mmol/l oleate, then detached and incubated 30 min in KRBH buffer containing 0.1% BSA and 2.8 mmol/l glucose with the mitochondrial potential sensitive dye TMRE, and analyzed by flow cytometry. CCCP (75 μmol/l) was added to uncouple the cells. A, $\Delta\Psi_m$ in the absence of CCCP; B, $\Delta\Psi_m$ in the presence of CCCP; Δ, A – B. Data are means of four experiments. *P < 0.05.

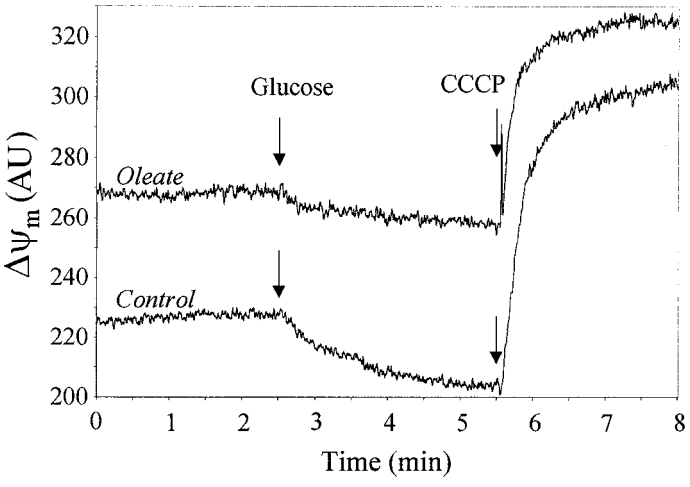

FIG. 4. Effect of a long-term exposure to oleate on glucose-induced changes in mitochondrial membrane potential ($\Delta\psi_m$). INS-1 cells were cultured at 5 mmol/l glucose, in the absence or presence of 0.4 mmol/l oleate, then detached and incubated in KRBH buffer containing 0.1% BSA and 2.8 mmol/l glucose with the mitochondrial membrane potential sensitive dye rhodamine 123. At the indicated times, glucose was added at a final concentration of 12.8 mmol/l. At the end of each trace, CCCP (75 μmol/l) was added to uncouple the cells. Data are from one of four representative experiments.

fluorescence per cell. The mean values of fluorescence in arbitrary units (AU) are presented in Fig. 3. The addition of the protonophore CCCP uncoupled both cell populations to a similar level of final fluorescence. The values obtained after uncoupling were subtracted from those obtained without CCCP. Figure 3 shows that the effect of CCCP (Δ = A–B) was significantly less in cells exposed to oleate, indicating a reduced mitochondrial membrane potential at a low concentration of glucose. This action of oleate was not observed when the fatty acid was added 30 min before the fluorescence measurement (data not shown).

The increased supply of reducing equivalents resulting from glucose metabolism increases the rate of electron transport and H^+ efflux from the mitochondria, resulting in a hyperpolarization of the mitochondrial membrane. Therefore, the action of glucose on the mitochondrial membrane potential was also tested in control and oleate-exposed cells by monitoring rhodamine 123 fluorescence. In control cells, raising glucose from 2.8 to 12.8 mmol/l hyperpolarized the mitochondria (Fig. 4), and CCCP depolarized it. In contrast, cells previously exposed to oleate for 72 h displayed a smaller hyperpolarization in response to high glucose (51 ± 15% of control cells, $n = 4$, $P < 0.05$). In agreement with the flow cytometry measurements, depolarization induced by CCCP was of smaller amplitude in oleate-treated cells; this suggests that the mitochondria were already partly depolarized in cells chronically exposed to FFAs (55 ± 10% of control cells, $n = 4$, $P < 0.05$).

UCP2 is present in the β-cells, and fatty acids increased UCP2 gene expression. The observations that long-term FFA exposure modified glucose-induced but not tolbutamide-induced insulin secretion prevented glucose-induced changes in the ATP-to-ADP ratio and reduced the mitochondrial membrane potential as well as the amplitude of the glucose-induced hyperpolarization ($\Delta\Psi_m$) suggest that the mitochondria are partly uncoupled. This could be because of the presence and/or the activation of an uncoupling protein. Therefore, the presence of UCP2 mRNA in β-INS-1 cells and in pancreatic islets was first examined by Northern blot analysis and compared with other tissues known to express the protein. Figure 5*A* shows that INS-1 cells and isolated rat islets contain UCP2 mRNA at levels comparable with that of white adipose tissue, but less than that of the spleen. Similarly, UCP2 protein was found in INS-1 cells, pancreatic islets, and purified β-cells (Fig. 5*B*).

Fractionation of INS-1 cells indicates that the protein concentrates with cytochrome *c* oxidase, confirming its mitochondrial localization (Fig. 5*C*).

The effect of various fatty acids on UCP2 mRNA expression level was examined in INS-1 cells. Figure 6 shows that an 18-h exposure of the cells to oleate or palmitate increased UCP2 mRNA expression approximately twofold. Similar data were obtained with 2-bromopalmitate, suggesting that fatty acid oxidation is not required. Glucose was without effect after 18 h, whereas the cAMP agonist forskolin decreased UCP2 mRNA. The possible role of peroxisome proliferator–activated receptors (PPARs) in the effects of fatty acids was examined by using several ligands: clofibrate (200 μmol/l) for PPAR-α, Ly 171–883 (20 μmol/l) for PPAR α + β, and ciglitazone (10 μmol/l) for PPAR-γ. Only clofibrate increased UCP2 mRNA by 38% at the concentration used (data not shown). Long-term (72-h) fatty acid exposure also doubled the amount of UCP2 protein (Fig. 7). The effect corresponds to a specific increase in the UCP2 protein, because two other mitochondrial proteins, subunit IV of cytochrome *c* oxidase and prohibitin, were unchanged by fatty acid exposure (149 ± 10 and 99 ± 10% of control for subunit IV of cytochrome oxidase and prohibitin, respectively, $n = 3$, NS).

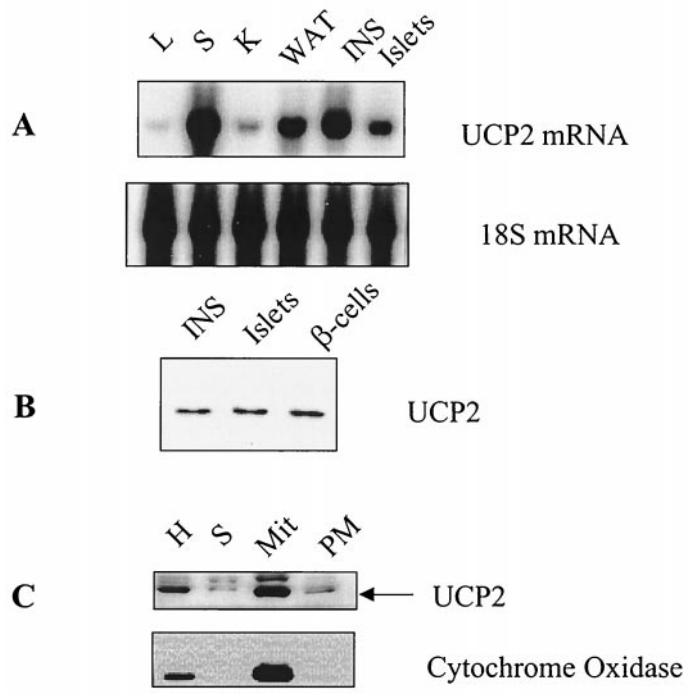

FIG. 5. Distribution and expression of UCP2 in rat tissues and INS-1 cells. *A*: Comparison of UCP2 mRNA expression in rat tissues and INS-1 cells. UCP2 and 18S mRNA were detected on Northern blots containing 8 μg total RNA per lane and hybridized with ^{32}P-labeled rat UCP2 or 18 S probes. L, liver; S, spleen; K, kidney; WAT, white adipose tissue. *B*: Comparison of UCP2 protein expression in INS-1 cells, rat pancreatic islets, and purified β-cells. Western blotting was performed with 30 μg protein from INS-1 cells, pancreatic islets, and β-cells using a polyclonal antibody to the COOH-terminal domain of UCP2. *C*: UCP2 is found in the same cellular fraction as the mitochondrial enzyme cytochrome oxidase. H, homogenate; S, postnuclear supernatant; mit, mitochondrial fraction; PM, crude plasma membrane fraction. Western blotting was performed with 30 μg protein from homogenate and subcellular fractions of INS-1 cells using a polyclonal antibody to the NH_2-terminal domain of UCP2.

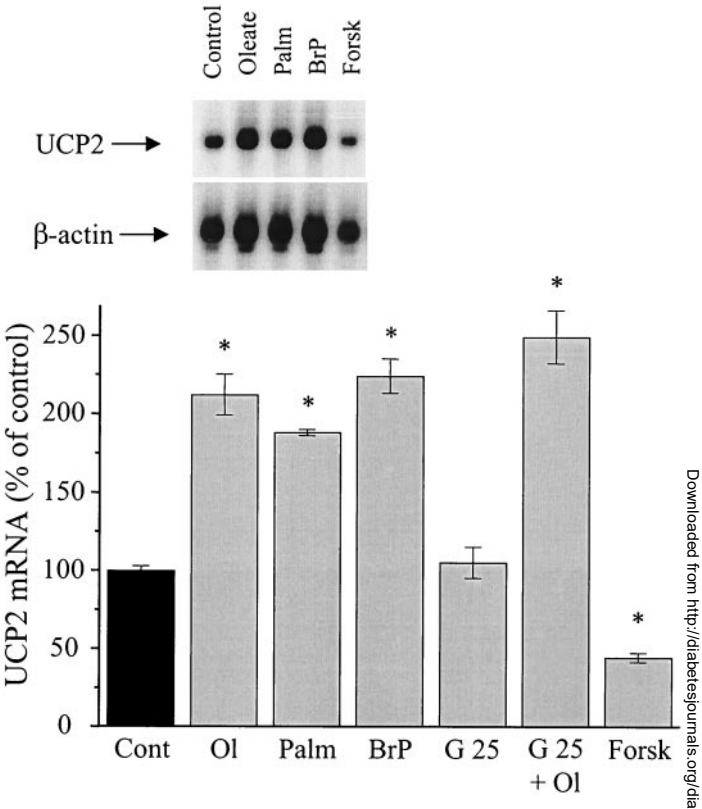

FIG. 6. Effects of oleate, palmitate, bromopalmitate, glucose, and forskolin on UCP2 mRNA expression. INS-1 cells were cultured for 18 h with 0.5% BSA, fatty acid bound to BSA (0.4 mmol/l), 25 mmol/l glucose, or 50 μmol/l forskolin. UCP2 transcript was measured by Northern blotting hybridization on 8 μg total RNA. Cont, control; OL, oleate; Palm, palmitate; BrP, 2-bromopalmitate; G25, 25 mmol/l glucose; Forsk, forskolin. The inset shows the signal of a representative experiment. Data are means ± SE of four to five separate experiments. *$P < 0.05$.

DISCUSSION

Long-term exposure of INS cells to fatty acids alters insulin secretion in response to glucose, but not insulin secretion caused by agents acting at the level of or beyond K_{ATP} channels. Fatty acid exposure also suppresses the glucose-induced increase in the ATP-to-ADP ratio, without major changes in glucose metabolism and oxidation (11). These two observations allow us to localize, at least in part, the long-term effects of fatty acids at any step between glucose metabolism/oxidation and ATP generation; this suggests an uncoupling between NADH oxidation and ATP synthesis. The observation of a lower mitochondrial membrane potential and a smaller hyperpolarization induced by glucose in FFA-exposed cells is compatible with an uncoupling effect of FFA that could be explained by an increased level of UCP2. Similarly, evidence of uncoupling, enhanced respiration, lower mitochondrial potential, and increased mitochondrial volume has recently been observed in rat pancreatic islets cultured for 1–2 days with palmitate (28).

What are the mechanisms by which FFA may uncouple mitochondria in the β-cell? FFAs are known to act as weak uncouplers of mitochondrial respiration in several cell types (29). However, their mechanism of action is still uncertain. Studies show that long-chain fatty acyl-CoA exerts an inhibitory effect on the adenine nucleotide translocase in hepatocytes (30). A similar action in INS-1 cells could explain the lack of effect of glucose on changes in ATP-to-ADP ratio after long-term FFA exposure. However, the action of FFAs on the translocase takes place within minutes in hepatocytes (30), and therefore should also be observed in our experiments after a short-term exposure (15 min) of the cells to FFAs. Thus, an effect of FFAs on adenine nucleotide translocase is unlikely to explain the long-term effect of fatty acids on ATP production. FFAs can also uncouple the mitochondria by acting on UCP activity. Recent studies on UCP2 reconstituted in proteoliposomes show that minimal amounts of FFAs are necessary to measure uncoupling activity (31). A direct effect of FFAs on UCP2 is unlikely, because it should also be seen in short-term experiments with FFAs.

Increased expression of UCP2 by fatty acids and by ligand of PPARs has been measured in several tissues in vivo and in vitro (32). UCP2 mRNA is increased by BRL 49654 (a ligand of PPAR-γ) and by bromopalmitate in cultured rat and human adipose tissues (33,34). Troglitazone increases UCP2 mRNA in isolated pancreatic islets from ZDF rats (17). Lipids also induce UCP2 in hepatocytes, which normally do not express this protein (35). UCP2 expression is associated with hepatic ATP depletion (36). In the present study, long-term exposure to FFAs increased UCP2 mRNA and protein, but did not decrease ATP levels. The uncoupling effect may be compensated for

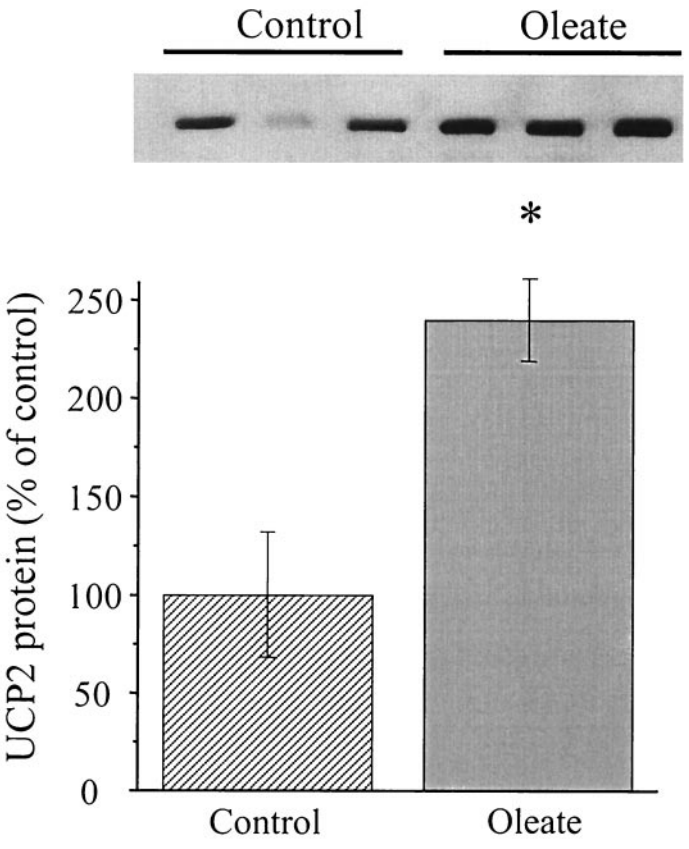

FIG. 7. Effect of oleate on UCP2 protein expression. INS-1 cells were cultured for 72 h with normal medium containing 0.5% BSA of BSA-oleate (0.4 mmol/l), and UCP2 was measured by Western blotting using 30 μg cell homogenate. Data are means of triplicate determinations. *$P < 0.05$.

by the twofold increase in oxygen consumption and accelerated fat oxidation measured after long-term FFA exposure (11).

The UCP2 promoter contains a PPAR response element (37). The observation that FFAs increase UCP2 mRNA independently of their oxidation suggests an implication of PPAR in the induction of UCP2 by FFAs. However, among the PPAR ligand tested, only clofibrate had a modest effect on UCP2 mRNA, suggesting that FFAs may act, at least in part, through transcription factors other than PPAR. Long-chain fatty acyl-CoA may be a ligand of hepatic nuclear factor-4α and could conceivably act on the UCP2 gene via this transcription factor (38).

The long-term effects of fatty acid on β-cell function with respect to insulin secretion may require cellular accumulation of long-chain fatty acyl-CoA esters or other fatty acid derivatives acting on UCP2 expression and/or activity (31,34), the opening of K_{ATP} channels (13), and the expression of metabolic genes implicated in the β-cell fuel-sensing process. Thus, fatty acids alter the expression of several enzymes of lipid metabolism; they decrease acetyl CoA carboxylase (39) and increase CPT-1 (5), resulting in an increased capacity for fatty acid oxidation. Long-term fatty acid exposure also results in an accumulation of triglycerides (11,16) that could behave as an endogenous source of FFA for oxidation and ATP generation, explaining the high rate of oxygen consumption, the elevated redox state, and the insulin release measured even at low glucose concentrations (11).

The absence of change in the ATP-to-ADP ratio after long-term FFA exposure should prevent the closure of K_{ATP} channels, the plasma membrane depolarization, and, therefore, the increase in cytosolic Ca^{2+}. Hyperpolarization of the mitochondria contributes to relay the increase in cytosolic Ca^{2+} into the mitochondria. Increase in mitochondrial Ca^{2+} activates Ca^{2+}-sensitive dehydrogenases (40) and generates factors that potentiate or maintain glucose-induced insulin secretion (41). Our studies show that basal mitochondrial membrane potential as well as hyperpolarization produced by glucose addition are lower in fatty acid–exposed cells compared with controls. This could also contribute to the loss of glucose-induced insulin secretion by impairing the production of mitochondrial signals originating directly or indirectly from the Krebs cycle, such as glutamate (42), malate (43), or citrate (44).

The exact role of UCP2 in cell physiology is unclear. Increase in its expression is often associated with high fatty acid supply and suggests that this protein may protect the cell from the consequences of a high rate of β-oxidation (32). Formation of reactive oxygen species (ROS) by the mitochondria is correlated to the mitochondrial potential (45). This raises the possibility that some uncoupling could prevent excessive increase in mitochondrial potential, particularly when fatty acid oxidation is high, thereby limiting the production of ROS (46). These products are particularly deleterious for the β-cells, because their content in ROS-inactivating enzymes is low (47,48). Thus, to avoid lipotoxicity and death through ROS upon chronic exposure to elevated FFAs, β-cells might be subject to partial mitochondrial uncoupling, resulting in an altered ATP generation at high glucose and impaired insulin secretion.

All of the present data do not entirely correspond with the traditional idea of uncoupling; however, they can be explained by a partial uncoupling of cells chronically exposed to oleate in the presence of high glucose. Whether this is attributable to an activation of the endogenous UCP2 by some fatty acid derivatives and/or an increase in UCP2 and/or a direct effect of fatty acid on the mitochondria cannot be determined by the present experiments.

In summary, our data show that INS-1 and pancreatic β-cells express UCP2 and that long-term fatty acid exposure uncouples the mitochondria in association with increased UCP2 mRNA and protein expression. Therefore, UCP2 could be a link between the abundance of fatty acids and the loss of glucose-induced insulin secretion as observed in obesity-associated type 2 diabetes.

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REFERENCES

1. Kahn CR: Insulin action, diabetogenes, and the cause of type II diabetes. *Diabetes* 43:1066–1084, 1994
2. McGarry JD: What if Minkowski had been ageusic? An alternative angle on diabetes. *Science* 258:766–770, 1992
3. Unger RH: Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. *Diabetes* 44:863–870, 1995
4. Prentki M, Corkey BE: Are the β-cell signaling molecules malonyl-CoA and cytosolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? *Diabetes* 45:273–283, 1996
5. Assimacopoulos-Jeannet F, Thumelin S, Roche E, Esser V, McGarry JD, Prentki M: Fatty acids rapidly induce the carnitine palmitoyltransferase I gene in the pancreatic β-cell line INS-1. *J Biol Chem* 272:1659–1664, 1997
6. Randle PJ, Hales CN, Garland, Newsholme EA: The glucose-fatty acid cycle: its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1:785–789, 1963
7. Sako Y, Grill VE: A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127:1580–1589, 1990
8. Zhou YP, Grill VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J Clin Invest* 93:870–876, 1994
9. Liu Y, Tornheim K, Leahy J: Glucose-fatty acid cycle to inhibit glucose utilization and oxidation is not operative in fatty acid–cultured islets. *Diabetes* 48:1747–1753, 1999
10. Liang Y, Buettger C, Berner DK, Matschinsky FM: Chronic effect of fatty acids on insulin release is not through the alteration of glucose metabolism in a pancreatic β-cell line (β HC9). *Diabetologia* 40:1018–1027, 1997
11. Segall L, Lameloise N, Assimacopoulos-Jeannet F, Roche E, Corkey P, Thumelin S, Corkey B, Prentki M: Lipid rather than glucose metabolism is implicated in altered insulin secretion caused by oleate in INS-1 cells. *Am J Physiol* 40: E521–E528, 1999
12. Ashcroft FM, Proks P, Smith PA, Ammala C, Bokvist K, Rorsman P: Stimulus-secretion coupling in pancreatic β-cells. *J Cell Biochem* 55:54–65, 1994
13. Larsson O, Deeney JT, Branstrom R, Berggren PO, Corkey BE: Activation of the ATP-sensitive K^+ channel by long chain acyl-CoA: a role in modulation of pancreatic β-cell glucose sensitivity. *J Biol Chem* 271: 10623–10626, 1996
14. Boss O, Muzzin P, Giacobino JP: The uncoupling proteins: a review. *Eur J Endocrinol* 139:1–9, 1998
15. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, Bouillaud F, Seldin MF, Surwit RS, Ricquier D, Warden CH: Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet* 15:269–272, 1997
16. Zhou YT, Shimabukuro M, Koyama K, Lee Y, Wang MY, Trieu F, Newgard CB, Unger R: Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation. *Proc Natl Acad Sci U S A* 94:6386–6390, 1997
17. Shimabukuro M, Zhou YT, Lee Y, Unger RH: Induction of uncoupling protein-2 mRNA by troglitazone in the pancreatic islets of Zucker diabetic fatty rats. *Biochem Biophys Res Commun* 18:359–361, 1997
18. Chan CB, MacDonald PE, Saleh MC, Johns DC, Marbàn, Wheeler MB: Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. *Diabetes* 48:1482–1486, 1999
19. Wang MY, Shimabukuro M, Lee Y, Trinh KY, Chen JL, Newgard CB, Unger RH: Adenovirus-mediated overexpression of uncoupling protein-2 in pancreatic islets of Zucker diabetic rats increases oxidative activity and improves β-cell function. *Diabetes* 48:1020–1025, 1999
20. 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
21. Lacy PE, Kostianovski M: Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16:35–39, 1967
22. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* 102:344–352, 1980
23. Chomczinsky P, Sacchi N: Single step method of RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. *Anal Biochem* 162: 156–160, 1987
24. Boss O, Samec S, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Tissue-dependent upregulation of rat uncoupling protein-2 expression in response to fasting and cold. *FEBS Lett* 412:111–114, 1997
25. Herbert V, Lau KS, Gottlieb CW, Bleicher SJ: Coated charcoal immunoassay of insulin. *J Clin Endocrinol Metab* 25:1375–1384, 1965
26. Schultz V, Sussman I, Bokwist K, Tornheim K: Bioluminometric assay of ADP and ATP at high ATP/ADP ratios: assay of ADP after enzymatic removal of ATP. *Anal Biochem* 215:302–304, 1993
27. Duchen MR, Smith PA, Ashcroft FM: Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β-cells. *Biochem J* 294:35–42, 1993
28. Carlsson C, Borg LA, Welsh N: Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology* 140:3422–3428, 1999
29. Soboll S, Seitz HJ, Sies H, Ziegler B, Scholz R: Effect of long chain acyl-CoA on mitochondrial and cytosolic ATP/ADP ratios in intact liver cells. *Biochem J* 220:371–376, 1984
30. Soboll S, Grundel S, Schwabe U, Scholz R: Influence of fatty acids on energy metabolism. II. Kinetics of changes in metabolic rates and changes in subcellular adenine nucleotide contents and pH gradients following addition of octanoate and oleate in perfused rat liver. *Eur J Biochem* 141:231–236, 1984
31. Jaburek M, Varecha M, Gimeno RE, Dembski M, Jezek P, Zhang M, Burn P, Tartaglia LA, Garlid KD: Transport functions and regulation of mitochondrial uncoupling proteins 2 and 3. *J Biol Chem* 274:26002–26007, 1999
32. Boss O, Hagen T, Lowell BB: Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. *Diabetes* 49:143–156, 2000
33. Camirand A, Marie V, Rabelo R, Silva JE: Thiazolidinediones stimulate uncoupling protein-2 expression in cell lines representing white and brown adipose tissues and skeletal muscle. *Endocrinology* 139:428–431, 1998
34. Viguerie-Bascands N, Saulnier-Blache JS, Dandine M, Dauzats M, Daviaud D, Langin D: Increase in uncoupling protein-2 mRNA expression by BRL49653 and bromopalmitate in human adipocytes. *Biochem Biophys Res Commun* 5:138–141, 1999
35. Cortez-Pinto H, Lin HZ, Yang SQ, Odwin Da Costa S, Diehl AM: Lipids up-regulate uncoupling protein 2 expression in rat hepatocytes. *Gastroenterology* 116:1184–1193, 1999
36. Chavin KD, Yand S, Lin HZ, Chatham J, Chacko VP, Hoek JB, Walajtys-Rode E, Rashid A, Chen CH, Huang CC, Wu TC, Lane MD, Diehl AM: Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. *J Biol Chem* 274:5692–5700, 1999
37. Kelly LJ, Vicario PP, Thompson GM, Candelore MR, Doebber TW, Ventre J, Wu MS, Meurer J, Forrest MJ, Conner MW, Cascieri MA, Moller DE: Peroxysome proliferator-activated receptors γ and α mediate in vivo regulation of uncoupling protein (UCP-1, UCP-2, UCP-3) gene expression. *Endocrinology* 139:4920–4927, 1998
38. Herz R, Magenheim J, Berman I, Bar-Tana J: Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4α. *Nature* 392:512–515, 1998
39. Brun T, Assimacopoulos-Jeannet F, Corkey BE, Prentki M: Long-chain fatty acids inhibit acetyl-CoA carboxylase gene expression in the pancreatic β-cell line INS-1. *Diabetes* 46:393–400, 1997
40. McCormack JG, Halestrap AP, Denton RM: Role of calcium ions in the regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 70:391–425, 1990
41. 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
42. Maechler P, Wollheim CB: Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* 402:685–689, 1999
43. MacDonald MJ: Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets: further implication of cytosolic NADPH in insulin secretion. *J Biol Chem* 270:20051–20058, 1995
44. Farfari S, Schulz V, Corkey B, Prentki M: Glucose-regulated anaplerosis and cataplerosis in pancreatic β-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. *Diabetes* 49:718–726, 2000
45. Skulachev VP: Uncoupling: new approaches to an old problem of bioenergetics. *Biochim Biophys Acta* 1363:100–124, 1998
46. Negre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, Pénicaud L, Casteilla L: A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxyde generation. *FASEB J* 11:809–815, 1997
47. Benhamou PY, Moriscot C, Richard MJ, Batrix O, Badet L, Pattou F, Kerr-Conte J, Chroboczek J, Lemarchand P, Halimi S: Adenovirus-mediated catalase gene transfer reduces oxidant stress in human, porcine and rat pancreatic islets. *Diabetologia* 41:1093–1100, 1998
48. Tiedge M, Lortz S, Drinkgern J, Lenzen S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733–1742, 1997