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

Nathalie Lameloise,1 Patrick Muzzin,1 Marc Prentki,2,3 and Françoise Assimacopoulos-Jeannet1

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 increased 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 UCP 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).
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 μmol/l penicillin, and 100 μg/ml streptomycin in an humidified atmosphere (5% CO₂ 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 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™ 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 carboxylcyanide 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 KRBB 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 × 10⁶ cells were pelleted and resuspended in 2 ml KRBB 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 nm, and emission was recorded at 580 nm (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 electrotransferred 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-terminal domain of UCP2 (Research Diagnostics, Flanders, NJ) or rabbit polyclonal antibody (1:2,000) 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. Statistic 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...
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 KATP 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

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<th>Glucose (mmol/l)</th>
<th>ATP (nmole/μg DNA)</th>
<th>ATP-to-ADP ratio</th>
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<tr>
<td></td>
<td>2.8</td>
<td>12</td>
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<tr>
<td>Long-term</td>
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<tr>
<td>BSA 72 h</td>
<td>0.420 ± 0.048</td>
<td>4.9 ± 0.1</td>
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<tr>
<td>Oleate 0.4 mmol/l 72 h</td>
<td>0.500 ± 0.048</td>
<td>6.4 ± 0.2</td>
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<tr>
<td>Short-term</td>
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<tr>
<td>BSA 15 min</td>
<td>0.296 ± 0.033</td>
<td>ND</td>
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<tr>
<td>Oleate 0.4 mmol/l 15 min</td>
<td>0.216 ± 0.021</td>
<td>ND</td>
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Data are means ± SE of four independent experiments. *P ≤ 0.02 vs. 2.8 mmol/l glucose; †P ≤ 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.
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 ($\Delta = A - B$) 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 displayed a smaller hyperpolarization in response to 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 $\mu$mol/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; $\Delta$, $A - B$. Data are means of four 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<sub>ATP</sub> 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. 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.

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 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, 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-<sup>γ</sup>) 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...
The absence of change in the ATP-to-ADP ratio after long-term FFA exposure should prevent the closure of $K_{\text{ATP}}$ 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 $\beta$-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 $\beta$-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, $\beta$-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 $\beta$-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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