Channels formed by the gap junction protein connexin36 (Cx36) contribute to the proper control of insulin secretion. We investigated the impact of chronic hyperlipidemia on Cx36 expression in pancreatic β-cells. Prolonged exposure to the saturated free fatty acid palmitate reduced the expression of Cx36 in several insulin-secreting cell lines and isolated mouse islets. The effect of palmitate was fully blocked upon protein kinase A (PKA) inhibition by H89 and (Rp)-cAMP, indicating that the cAMP/PKA pathway is involved in the control of Cx36 expression. Palmitate treatment led to overexpression of the inducible cAMP early repressor (ICER-1-γ), which bound to a functional cAMP-response element located in the promoter of the Cx36 gene. Inhibition of ICER-1-γ overexpression prevented the Cx36 decrease, as well as the palmitate-induced β-cell secretory dysfunction. Finally, freshly isolated islets from mice undergoing a long term high fat diet expressed reduced Cx36 levels and increased ICER-1-γ levels. Taken together, these data demonstrate that chronic exposure to palmitate inhibits the Cx36 expression through PKA-mediated ICER-1-γ overexpression. This Cx36 down-regulation may contribute to the reduced glucose sensitivity and altered insulin secretion observed during the pre-diabetic stage and in the metabolic syndrome.

The fine-tuning of insulin secretion in response to nutrient stimulation relies on a closely coordinated functioning of pancreatic β-cells. The cell-to-cell communication mediated by gap junction channels contributes to synchronization of β-cell clusters and has been demonstrated to be essential for the proper regulation of storage and release of insulin, both in vitro and in vivo (for review see Ref. 1). Gap junctions are specific membrane structures consisting of aggregates of intercellular channels interconnecting the cytoplasms of neighboring cells and providing them with a direct pathway for sharing ions, nutrients, and intracellular messengers. We and others (2–5) have demonstrated that only one connexin, the constitutive unit of these channels, is expressed in insulin-secreting cells, connexin36 (Cx36 2 for 36 kDa), and that this connexin plays a critical role in β-cell function. In addition, modulation of the Cx36 levels results in impaired glucose-induced insulin secretion, indicating that Cx36 must be expressed at a very precise level to maintain a normal insulin secretion (2, 6). Thus, changes in the Cx36 expression levels might impair β-cell function and hence be involved in the pathophysiology of diabetes mellitus.

Type 2 diabetes partly ensues from β-cell failure to compensate for peripheral insulin resistance (for review see Ref. 7). Abnormalities in both glucose and lipid metabolism contribute to the pathogenesis of this condition and in particular to the inexorable decline of β-cell function (8, 9). Recently, we have demonstrated that long term exposure to a high concentration of glucose resulted in a reduced expression of Cx36 in insulin-secreting cells (10). Although hyperglycemia is undoubtedly a major contributor to the onset of β-cell failure, chronic elevation in circulating free fatty acids (FFAs) also accompany the progression to type 2 diabetes (11–14). Indeed, prolonged exposure of β-cells to elevated concentrations of FFAs, both in vivo and in vitro, leads to elevated basal secretion and blunted response to glucose, both defects being reminiscent of the diabetic state (14–20).

Here we report the effects of a prolonged exposure of insulin-secreting cells to various FFAs on Cx36 expression, and we document the generation of a selective decrease of Cx36 expression by saturated FFAs. This decrease was concomitant with increased ICER-1-γ (inducible cAMP early repressor) expression, and CX36 promoter studies revealed that ICER-1-γ mediates the inhibitory effect of palmitate on Cx36 expression. We demonstrated that ICER-1-γ blockade prevented the deleterious effect of palmitate on β-cell secretion, providing a mechanistic explanation for the altered β-cell function observed after a prolonged exposure to FFA. Finally, we further observed in vivo that mice fed a high fat diet for 15 weeks displayed a marked decrease in Cx36 levels that correlates with increased levels of circulating FFAs.

The abbreviations used are: Cx36, connexin36; FFA, free fatty acid; PKA, cAMP-dependent protein kinase A; ChIP, chromatin immunoprecipitation; hGH, human growth hormone; RT, reverse transcription; CREM, cAMP-response element modulator; CRE, cAMP-response element; NC, normal chow; HF, high fat; ICER-1-γ, inducible cAMP early repressor 1; AS, antisense.
EXPERIMENTAL PROCEDURES

Materials—Glucose, palmitate, methyl ester palmitate, stearate, oleate, linoleate, and (R)-3′-cAMP were purchased from Sigma, and compound C and H89 were from Calbiochem. All experiments using FFAs were performed in the absence of serum at 5 mM glucose for INS-1E cells and islets, and 11 mM glucose for MIN6-B1 at a final concentration of 1% bovine serum albumin. The pSV-ICER expression plasmid (21) was provided by Dr. Regazzi, University of Lausanne, Switzerland. The plasmid encoding ICER antisense (ICER AS) was constructed by inserting PCR-amplified fragment of ICER from pSVICER. Primers used were as follows: 5′-AGAAGTCT-AGACATGGCTGAACGGAG-3′ (sense) and 5′-ACTGTGCAAGATCTGGAAGCGACG-3′ (antisense). The PCR fragment was inserted between the BamHI and XbaI cloning sites of the pcDNA3 vector (22).

Preparation of FFAs—FFAs were prepared as described previously (23). Briefly, stock palmitic acid or methyl ester palmitic acid solution in methanol (80 mmol/liter) was conjugated to fatty acid-free bovine serum albumin in a 3:1 molar ratio at 37 °C for at least 1 h prior to treatment. The oleic and linoleic acid used were purchased pre-conjugated to albumin (Fluka Chemie). The FFAs concentrations were measured using the NEFA C kit (Wako). The FFAs concentrations were measured using the NEFA C kit (Wako).

Cell Lines—The rat insulinoma cell line INS-1E (kindly provided by Dr. Pierre Maechler, CMU, University of Geneva) was maintained in the complete RPMI 1640 medium as described previously (2, 24, 25). MIN6-B1 (kindly provided by Dr. Philippe Halban, CMU, University of Geneva) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, 25 mM glucose, 71 μM 2-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/liter streptomycin (26). Cells were kept at 37 °C in a humidified incubator under 5% CO2.

Mouse Models and Mouse Islets Isolation—Our institutional review committee for animal experiments approved all the procedures for mouse care, surgery and euthanasia. Adult male C57BL6 mice were submitted to a 15-week high fat diet (72% fat, 28% protein, and <1% carbohydrate, low nitrates, 30% lard, 20% corn oil from Unité d’Alimentation Rationnelle, Epinay-sur-Orge, France) (27, 28). Mice fed normal chow or a high fat diet were anesthetized by inhalation of 5% halothane (Arovet-Orge, France) (27, 28). Mice fed normal chow or a high fat diet were anesthetized by inhalation of 5% halothane (Arovet-Orge, France) (27, 28). Mice fed normal chow or a high fat diet were anesthetized by inhalation of 5% halothane (Arovet-Orge, France) (27, 28).

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Blood Chemistry—Plasma concentrations of glucose were measured in the fed state in conscious mice using the Ascencia DEX blood glucose meter (Bayer). The NEFA (nonesterified fatty acids) and cholesterol concentrations were measured using the NEFA C kit (Wako) and the cholesterol CHOD-PAP method (Axonlab), according to manufacturer’s instructions. Plasma insulin levels were determined using the ultrasensitive mouse insulin enzyme-linked immunosorbent assay (Mercodia).

RNA Isolation and Quantitative RT-PCR (Lightcycler®)—Cells were homogenized in the Tripure isolation reagent (Roche Diagnostics), and total RNA was extracted using the kit procedure. Mouse islets mRNA was isolated using nucleospin RNA II columns (Macherey-Nagel). Transcripts (1 μg) were reverse-transcribed using ImProm-2 Reverse transcription System (Promega). Quantitative PCR was performed using the SYBR® Premix ExTaQ™ (Takara) in a Lightcycler Instrument (Roche Diagnostics). cDNAs were amplified using the following primers: rat Cx36, 5′-ATACAGGTTGATTAGGGAGG-ATG-3′ (sense) and 5′-TGGAGGGTGTACAGTGAAGAAGAG-AGG-3′ (antisense); and rat ribosomal protein L-27, 5′-GATCCAGATCAAGTCTTGTGACACCC-3′ (sense) and 5′-CTGGGTCTCTTG-TAAACACTCT-3′ (antisense); insulin 5′-TGGTTCTTCTACACACC-3′ (sense) and 5′-TCTAGTTGCGATGTCTTCT-3′ (antisense); ICER-1γ, 5′-CTGGGTCTCTGAAACACTCTT-3′ (sense) 5′-CACCTTTGTGGCAGAAGCAGTA-3′ (antisense); and acetyl-CoA carboxylase, 5′-CAGTTTCAAGGCGAGAGT-3′ (sense) and 5′-ATGATGGCTTCCAGTGAAGAA-3′ (antisense).

Western Blotting—INS-1E cells were solubilized by sonication in SDS-buffer (62.5 mM Tris-EDTA, pH 6.8, SDS 5%). Protein content was measured using a detergent-compatible BCA protein assay kit (Pierce). Western blots were carried out as described previously (10). Membranes were saturated for 1 h in TBS containing 5% milk and 0.05% Tween 20 prior to overnight hybridization at 4 °C with rabbit polyclonal antibodies against Cx36 (2, 29), diluted 1:200, monoclonal anti α-tubulin antibodies (Fluka Chemie; diluted 1:2,000), and rabbit polyclonal anti CREM-1 sc440 (Santa Cruz Biotechnology; diluted 1:500). After incubation at room temperature (1 h) with the convenient secondary antibody conjugated to horseradish peroxidase (Fluka Chemie; diluted 1:20,000), membranes were revealed by enhanced chemiluminescence (ECL, Amersham Biosciences). Densitometric analyses of immunolabeled proteins (Western blots films) were performed using the ImageQuant software (Amersham Biosciences).

Transient Transfection and Luciferase Assays—INS-1E cells plated in 24-well dishes were transiently transfected using Lipofectamine 2000 reagent (Invitrogen) at a DNA/lipid ratio of 1:1 according to manufacturer’s instructions. Transfection assay consisted of a mixture of the luciferase reporter plasmid containing the mouse luciferase gene under control of different fragments of the human CX36 promoter, together with an empty vector (pCDNA3), or a plasmid allowing constitutive expression of ICER-1 (21) or an ICER antisense plasmid (22), and the internal control plasmid pRL-SV40 (Promega). 24 h after transfection, the cells were incubated in presence or absence of 0.2 mM palmitate. 24 h later, PLB (passive lysis buffer, Promega) cell extracts were prepared for dual luciferase reporter assays (Promega) in a Turner TD-20/20 luminometer. Promoter activity was normalized by the Renilla activity of the pRLSV40, as described previously (10).

Chromatin Immunoprecipitation (ChIP) Assay—INS-1E cells (106 cells) were cross-linked with 1% formaldehyde at room temperature for 10 min. The cross-linking reaction was stopped by adding glycine to a final concentration of 125 nmol/liter. Cells were collected and resuspended in SDS lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1. Cell
Palmitate Down-regulates Cx36 Expression

pellets were lysed and sonicated to obtain the desired chromatin length (~500 bp). Protein concentration of the cell supernatants was determined by BCA protein assay (Pierce), and the samples were diluted to 1 mg/ml in ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1, and protease inhibitors). Samples were precleared by incubation with blocked protein A-Sepharose (Amersham Biosciences) for at least 1 h at 4 °C. The precleared chromatin lysates were immunoprecipitated overnight at 4 °C with either polyclonal rabbit antibodies specific to CREM-1, c-Myc (9E10, Santa Cruz Biotechnology), or human REST (29). The DNA-protein antibody complexes were collected by addition of protein A-Sepharose for 2 h at 4 °C. Then the DNA-protein complexes were washed twice in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), and once in high salt final wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl). The complexes were eluted from the Sepharose beads by incubation at room temperature for 15 min in 1% SDS and 0.1 M NaHCO3. The pellets were diluted in TE, RNase A- and proteinase K-treated, and purified using the PCR product cleanup kit (Roche Diagnostics) according to manufacturer’s instructions. DNA was submitted to classic PCR amplification or LightCycler PCR (1 cycle at 95 °C for 3 min, followed by 32 cycles at 94 °C for 25 s, 58 °C for 25 s, 72 °C for 25 s, and finally 1 cycle at 72 °C for 3 min), using specific primers 5’-CACCAGCTGTCTGTCTTCTTCC-3’ (sense) and 5’-ATCTTGCGGTCTGAGGGAG-3’ (antisense).

**Human Growth Hormone (hGH) Secretion**—INS-1E cells plated in 24-wells dishes were transiently cotransfected using Lipofectamine 2000 (Invitrogen) with a construct encoding the hGH (Nicholls), together with an empty vector (pCDNA3), a plasmid permitting constitutive expression of ICER-1 (21), an antisense ICER construct (22), or a Cx36 coding plasmid (2). 24 h later, the cells were cultured in presence or absence of 0.2 mM palmitate for 48 h. Then the cells were washed and preincubated for 1 h in KRBH (Kreb’s-Ringer/bicarbonate/HEPES) buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM Na2HPO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES, and 0.1% bovine serum albumin). The medium was then removed, and the cells were incubated for 30 min in KRBH added with 2 mM glucose (basal condition) or in KRBH containing 20 mM glucose, 10 μM forskolin, and 100 μM isobutylmethylxanthine (stimulatory condition). The total amount of hGH produced by transfected cells and the fraction released into the medium during the incubation period were determined by enzyme-linked immunosorbent assay (Roche Diagnostics).

**Statistical Analyses**—Data were expressed as mean ± S.E. Difference between means were assessed by Student’s t test.

In experiments involving the comparison of more than two experimental conditions, analyses of variance were performed. All computations were carried out with the JMP software, version 3.2.2 (SAS Institute, Cary NC). The α level of all tests was set at 0.05. Statistical significance was defined as a value of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

**RESULTS**

Palmitate, but Not Oleate or Linoleate, Represses Cx36 Expression in Insulin-secreting Cells—Pancreatic β-cells were cultured at 6 mM glucose without serum in the absence or presence of saturated palmitate (C 16:0), monounsaturated oleate (C 18:1), or polyunsaturated linoleate (C 18:2) for 48 h.

Palmitate (0.2 mM), but not oleate (0.2 mM) or oleate (0.2 mM), induced a 40% reduction in Cx36 mRNA expression levels in the rat β-cell line INS-1E (Fig. 1A). This effect was more pronounced in the mouse β-cell line MIN6-B1 (50%) and in isolated mouse islets (70%). As a positive control, acetyl-CoA carboxylase (ACC) mRNA expression levels were evaluated. Data represent mean ± S.E. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control.

![FIGURE 1. Effects of FFAs on Cx36 mRNA expression in insulin-secreting cells. IN5-1E cells, MIN6-B1 cells, or primary mouse islets were cultured for 48 h in the presence or absence of 0.2 mM oleate, palmitate, or linoleate. A, Cx36 mRNA expression was analyzed by quantitative RT-PCR and normalized to the levels of the housekeeping gene L27. B, as a positive control, acetyl-CoA carboxylase (ACC) mRNA expression levels were evaluated. Data represent mean ± S.E. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control.](image-url)}
The effect of palmitate was reversible, as INS-1E cells preincubated for 24 h in presence of 0.2 mM palmitate and incubated for another 24 h in absence of palmitate showed control levels of Cx36 (Fig. 2D). To determine whether this regulation process was specific to saturated FFAs, we tested the effect of stearate and observed that this saturated FFA decreased the Cx36 levels to the same extent as did palmitate (Fig. 2, A and B). Metabolism of palmitate has been shown to be required to elicit acetyl-CoA carboxylase down-regulation (30) and β-cell apoptosis (23). To test whether palmitate metabolism is mandatory to the regulation of Cx36 expression, INS-1E cells were treated with palmitic acid methyl ester, a nonmetabolizable analogue of palmitate that is not activated into a fatty acyl-CoA in the cytosol (31–33). As shown in Fig. 2C, 0.2 mM methyl palmitate induced a significant 25% decrease in Cx36 levels, suggesting that palmitate oxidation is not required for the Cx36 decrease.

**Mechanism(s) of Palmitate-mediated Cx36 Down-regulation**

We have recently demonstrated that glucose inhibits Cx36 expression through activation of the cAMP/PKA pathway (10). INS-1E cells were exposed to palmitate, in the presence of the PKA inhibitor H89 or the specific membrane-permeable inhibitor of PKA activation (Rp)-cAMP. As shown in Fig. 3A, both compounds prevented the Cx36 decrease elicited by palmitate, suggesting that the cAMP/PKA pathway mediates the palmitate effect on Cx36 expression.

Recent findings established that ICER-1γ is overexpressed after prolonged exposure to FFAs (34). Here palmitate, neither oleate nor linoleate induced a 3-fold increase in ICER-1γ mRNA levels in INS-1E cells and isolated mouse islets (Fig. 3B). Western blot analyses using an antibody directed against CREM-1 detected the two major repressive isoforms of CREM expressed in β-cells, ICER-1 and ICER-1γ (35), immunolocalized at 17 and 14 kDa, respectively, and confirmed that ICER-1γ is the major isoform expressed in INS-1E cells. Palmitate-treated INS-1E cells displayed twice as much ICER-1 and ICER-1γ protein after 48 h, in comparison with untreated cells (Fig. 3C). As a positive control, 20 mM glucose treatment resulted in a 4-fold induction of ICER-1 and ICER-1γ content. The palmitate-mediated overexpression of ICER-1γ and ICER-1γ was fully blocked in the presence of H89 (Fig. 3C). Of note, glucose or FFAs had no effect on CREM-1 expression (around 45 kDa).

We also studied the potential additive effects of glucose and palmitate on Cx36 and ICER-1γ expression. Glucose and palmitate did not synergize in reducing Cx36 expression or inducing ICER-1γ expression at high glucose concentration (20 mM) (Fig. 4A). However, at 10 mM glucose, palmitate further decreased Cx36 mRNA levels in INS-1E cells. These data were clearly inversely correlated with the levels of ICER-1γ mRNA, although the palmitate-mediated increase in ICER-1γ levels in the presence of 10 or 20 mM glucose did not reach statistical significance, as compared with untreated cells (Fig. 4B). Oleate treatment, which had no effect on Cx36 and ICER-1γ expression at low glucose, remained ineffective in presence of higher glucose concentrations.

We have previously demonstrated that the human and rodent cx36 promoters contain a highly conserved cAMP-response element (CRE) located between bases −566 and −556.

**FIGURE 2. Cx36 down-regulation by palmitate is dose-dependent and reversible.** A, INS-1E cells were cultured for 48 h in the presence of increasing concentrations of palmitate (left panel) or stearate (right panel), as indicated. The blots are representative of three independent experiments. Quantitative assessment of Western blot analyses on INS-1E cells cultured in the presence of increasing concentrations of palmitate (black square), stearate (empty square), oleate (black circle), or linoleate (empty circle). Results are means ± S.E. of four independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 versus INS-1E control. B, INS-1E cells were cultured for 48 h in presence of 0.2 mM palmitate or methyl ester palmitate (0.2 mM), and Cx36 levels were assessed by Western blot. Upper panel, representative Western blot; lower panel, quantitative assessment of Western blot analyses. Results are means ± S.E. of four independent experiments. *p < 0.05; **p < 0.01 versus INS-1E control. C, INS-1E cells were further incubated for 24 h in absence of palmitate (palmitate reversibility). Upper panel, representative Western blot; lower panel, quantitative assessment of Western blot analyses. Results are means ± S.E. of three independent experiments. **p < 0.01 versus INS-1E control.
Palmitate Down-regulates Cx36 Expression

A, INS-1E cells were cultured for 48 h in the presence or absence of 0.2 mM palmitate, with or without H89 (10 μM) or (R)-y-CAMP (20 μM). Cx36 levels were evaluated by Western blot analyses. B, primary mouse islets or INS-1E cells were cultured for 48 h in the presence or absence of oleate (0.2 mM), palmitate (0.2 mM), or linoleate (0.2 mM). ICER-1 mRNA levels were analyzed by quantitative RT-PCR. Results are mean ± S.E. of five independent experiments. **, p < 0.01; ***, p < 0.001 versus control.

FIGURE 3. Cx36 down-regulation by palmitate requires the activation of the cAMP-PKA pathway. A, INS-1E cells were transfected with the CREM-1 wild type (CREM-1) or CREM-1 mutant (CREM-1m). Western blot analyses revealed the expression of two ICER-1 isoforms, ICER-1 and ICER-1γ. Quantitative assessment showed that ICER-1γ levels were increased in presence of glucose or palmitate, as compared with the CREM-1 levels. Results are mean ± S.E. of five independent experiments. *, p < 0.05; **, p < 0.01; ***., p < 0.001 versus control.

FIGURE 4. The effects of glucose and palmitate are partly additive. INS-1E cells were cultured at 5, 10, or 20 mM glucose for 48 h in presence or absence of 0.2 mM oleate or palmitate. A, Cx36 mRNA expression was analyzed by quantitative RT-PCR and normalized to the levels of the housekeeping gene L27. B, ICER-1γ mRNA levels were analyzed by quantitative RT-PCR. Results are mean ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.01; ***., p < 0.001 versus the respective control (at the same glucose concentration).

upstream of the transcription start site of the cx36 gene (10). To assess the role of this CRE in the palmitate-induced Cx36 down-regulation, a plasmid expressing the reporter gene luciferase under the control of a fragment of the human Cx36 promoter containing the CRE (pGL3–1079) was transiently transfected in INS-1E cells incubated in presence, or absence, of 0.2 mM palmitate for 24 h. Palmitate induced a 30% decrease in the luciferase activity driven by the Cx36 promoter fragment (Fig. 5). In contrast, palmitate did not reduce the activity of a similar plasmid containing a mutated Cx36 CRE (pGL3–1079m). To investigate the involvement of ICER-1γ in the control of the Cx36 gene expression, INS-1E cells were cotransfected with the pGL3–1079 plasmid, together with either an empty vector (pCDNA3), a plasmid coding for ICER-1γ, or an antisense ICER construct (ICER AS) reducing the endogenous ICER-1 and ICER-1γ content (22). Overexpression of ICER-1γ induced a 40% reduction in the activity of the native Cx36 promoter fragment (pGL3–1079) but had no effect when the CRE was mutated (pGL3–1079m) (Fig. 5A). Palmitate did not reduce the Cx36 promoter activity in cells cotransfected with the antisense ICER plasmid (ICER AS), indicating that the palmitate effect requires the presence of ICER-1γ. Quantitative assessment of ICER-1γ and Cx36 levels in INS-1E cells transiently transfected with the ICER-1γ coding plasmid or the antisense ICER construct (ICER AS) confirmed the efficiency of these plasmids (Fig. 5B). Indeed, although transient transfection efficiency was only about 50% (data not shown), ICER-1γ overexpression in transfected cells resulted in a 2-fold increased in the total ICER-1γ mRNA levels and a 40% decrease in the Cx36 mRNA levels. Conversely, transfection of the antisense construct blocked the effects of palmitate on ICER-1γ and Cx36 mRNA expression levels.

The ability of ICER-1γ to bind to the Cx36 CRE in situ was then evaluated by ChIP experiments. Using chromatin from unstimulated or palmitate-treated INS-1E cells, ICER-1γ was immunoprecipitated with the anti-CREM-1 antibodies recognizing ICER-1 and ICER-1γ (Fig. 3C). The expected 385-bp PCR product corresponding to bases –796 to –411 of the Cx36 promoter was detected in anti-CREM immunoprecipitates. As a negative control, antibodies against c-Myc, which has no putative binding site in the Cx36 promoter, or against REST, a repressor that is not expressed in ß-cells (29), did not immunoprecipitate the Cx36 promoter fragment (Fig. 6, upper panel). Anti-CREM antibodies immunoprecipitated the Cx36 promoter fragment in both unstimulated and palmitate-treated cells, but there was a clear enrichment in palmitate-treated cells. Real time PCR analyses further revealed a significantly increased binding of CREM isoforms to the Cx36 promoter in the presence of palmitate (Fig. 6, lower panel). H89 treatment, which prevented the palmitate-mediated ICER-1γ overexpression (Fig. 3C), also prevented the increased binding
**Palmitate Down-regulates Cx36 Expression**

![Graph](image)

**FIGURE 5. Palmitate decreases the transcriptional activity of the Cx36 promoter. A, INS-1E cells were cotransfected with a plasmid expressing the reporter gene luciferase under the control of a 1-kb fragment of the Cx36 promoter (pGL3–1079) or a fragment containing a mutated CRE (pGL3–1079m), with or without a plasmid allowing the overexpression of ICER-1 γ, or a plasmid expressing an antisense ICER blocking the ICER-1 and ICER-1 γ expression (ICER AS). After transfection, cells were cultured for 24 h in the presence or absence of 0.2 mM palmitate and assessed for luciferase activity. *, p < 0.05; **, p < 0.01 versus untreated cells. B, quantitative RT-PCR analyses of Cx36 and ICER-1 mRNA expression in transiently transfected INS-1E cells. Results are means ± S.E. of four independent experiments. *, p < 0.05; **, p < 0.01 versus Cx36 mRNA levels in control.

on the Cx36 promoter fragment. Taken together, ChIPs and luciferase assays indicate that ICER-1 γ inhibits Cx36 expression in response to palmitate via a CRE located in the Cx36 promoter in INS-1E cells.

Functional Significance of ICER-1 γ Overexpression and Cx36 Down-regulation on β-Cell Function—To elucidate the functional significance of the palmitate-mediated overexpression of ICER-1 and subsequent Cx36 down-regulation, we evaluated the secretory function of untreated and palmitate-treated INS-1E cells with modified ICER-1 and Cx36 levels. Palmitate is known to alter insulin content (19, 36). To assess the secretory activity of cells independently of their capacity to produce insulin, INS-1E cells were transiently transfected with a plasmid encoding the hGH, which is secreted in the same granules as insulin (22, 37). The transfected cells were treated or not with palmitate for 48 h prior to measuring their secretory capacity under basal (2 mM glucose) and stimulated conditions (20 mM glucose, 100 μM isobutylmethylxanthine, and 10 μM forskolin). As expected, basal secretion was increased in palmitate-treated cells, whereas stimulated secretion was impaired (Table 1). INS-1E cells cotransfected with the ICER-1 coding plasmid displayed a significantly reduced stimulated secretion, whereas basal secretion was not significantly changed. In the presence of palmitate, ICER-1 overexpression had no significant impact on secretory function. Conversely, the cells cotransfected with the plasmid encoding the antisense ICER (ICER AS) were protected against the deleterious effects of palmitate on both basal and stimulated secretion.

To prevent the palmitate-mediated Cx36 down-regulation, INS-1E cells were cotransfected with a plasmid encoding the Cx36 protein. Ectopic Cx36 expression restored stimulated secretion at control level in the presence of palmitate. On the other hand, basal secretion was significantly increased during these experiments, either in the absence or in presence of palmitate.

**In Vivo Experiments**—To ascertain the relevance of the effects of the saturated FFA observed on both insulin-secreting cells and isolated mouse islets, the impact of hyperlipidemia was studied in vivo. C57BL6 mice were fed either a normal chow (NC) or a high fat (HF) diet (lard, corn oil) for 15 weeks, as described previously (27, 28). Body weight was significantly higher in the HF group after 15 weeks of diet versus the NC group (Table 2). Blood glucose levels in the fed state were significantly higher in the HF group, whereas the plasma insulin levels were only slightly increased in the HF group, in comparison with the NC group. As expected, the total plasma FFAs and cholesterol levels were significantly increased in the HF group, as compared with the NC group (Table 2). The impact of hyperlipidemia on Cx36 and ICER-1 γ mRNA levels was assessed in freshly isolated Langerhans islets. Cx36 expression was decreased by 70% in the HF group, whereas the ICER-1 γ expression was increased by 2-fold (Fig. 7A). These results were further confirmed by Western blot analyses of freshly isolated islets of NC and HF mice demonstrating a 60% decrease in the Cx36 protein levels (Fig. 7B).

**DISCUSSION**

Chronic exposure to FFAs is known to impair β-cell function and to be linked to the development of type 2 diabetes and metabolic syndrome (13, 17, 38). FFAs regulate the expression of a number of genes in β-cells, including insulin, acetyl-CoA carboxylase, fatty-acid synthase, and carnitine palmitoyltransferase 1 (CPT1) (30, 39–42). Here, we identify Cx36 as a new target gene regulated following chronic exposure to FFAs. Among the FFAs tested, only saturated FFAs palmitate and stearate were effective, suggesting that the Cx36 gene regula-
Palmitate Down-regulates Cx36 Expression

INS-1E cells were transiently cotransfected with an hGH coding plasmid, together with either an empty vector, a plasmid leading to the overexpression of ICER-1γ, an antisense ICER plasmid (ICER AS), or a Cx36-expressing vector. 24 hours after transfection, the cells were incubated for 48 h in the presence or absence of 0.2 mM palmitate, and the secretory capacity was assessed. Basal, 2 mM glucose; stimulated, 20 mM glucose, 10 μM forskolin, and 100 μM isobutylmethylxanthine. Results are means ± S.E. of two independent experiments performed in duplicate. Statistical analyses were performed using a one-way analysis of variance test and modified unpaired Student’s t test between the two groups.

| TABLE 1

Blocking the palmitate-induced ICER-1γ overexpression prevents the palmitate-mediated alteration of secretory function of the INS-1E cells |

| Empty vector | ICER-1γ |
|---------------|---------|
| Control | Palmitate | Control | Palmitate |
| Basal | 0.98 ± 0.03 | 2.01 ± 0.32 | 1.29 ± 0.27 | 1.54 ± 0.21 |
| Stimulated | 7.21 ± 1.45 | 3.23 ± 0.71 | 4.26 ± 0.45 | 3.88 ± 0.36 |

| ICER AS | Cx36 |
|---------|------|
| Control | Palmitate | Control | Palmitate |
| Basal | 0.90 ± 0.13 | 0.91 ± 0.17 | 2.20 ± 0.57 | 3.15 ± 0.74 |
| Stimulated | 7.30 ± 1.74 | 6.63 ± 0.94 | 6.55 ± 1.05 | 6.57 ± 1.31 |

* p < 0.05 versus control basal secretion (empty vector).
* p < 0.05 versus control stimulated secretion (empty vector).
* p < 0.01 versus the respective basal secretion.
* p < 0.05 versus palmitate-treated stimulated secretion (empty vector).

FIGURE 6. An isoform of CREM binds to the Cx36 promoter. INS-1E cells were cultured for 48 h in presence or absence of 0.2 mM palmitate, with or without the PKA inhibitor H89 (10 μM) . Fixed chromatin was immunoprecipitated using anti-CREM antibodies, and the Cx36 promoter fragment containing the CRE was amplified by PCR. Upper panel, representative gel showing a chromatin immunoprecipitation experiment. Lower panel, quantitative RT-PCR of the immunoprecipitated promoter fragment demonstrated that palmitate enhanced the binding of a CREM isoform to the Cx36 promoter fragment containing the CRE in INS-1E cells. Results are means ± S.E. of four independent experiments. *** p < 0.001 versus control; #, p < 0.05 palmitate-treated cells versus untreated cells.

FIGURE 7. Mice fed a high fat diet display decreased Cx36 levels in freshly isolated islets. Adult male C57BL6 mice were fed NC or an HF diet (30% lard, 20% corn oil) for 15 weeks. mRNA or protein extractions were performed on freshly isolated mouse islets. A, Cx36, ICER-1γ, and insulin mRNA expression were analyzed by quantitative RT-PCR and normalized to the expression of the housekeeping gene L27. Results are means ± S.E. of nine animals in each group. B, Western blot analyses of Cx36 levels of mouse islets revealed that the Cx36 levels were decreased in HF diet-fed mice. Each lane corresponds to ~120 islets pooled from two mice. Results are means ± S.E. of three samples in each group, corresponding to six mice. * p < 0.05; **, p < 0.01 versus NC group.

TABLE 2

Blood chemical analyses of mice undergoing either a normal or a high fat diet

|          | Normal Chow | High Fat |
|----------|-------------|----------|
| Weight (g) | 28.9 ± 0.61 | 33.6 ± 1.40 |
| Glucose (mM) | 6.12 ± 0.24 | 7.05 ± 0.29 |
| Insulin (pmol/liter) | 156.1 ± 16.9 | 195.8 ± 15.9 |
| FFAs (mM) | 0.23 ± 0.03 | 0.42 ± 0.02 |
| Cholesterol (mM) | 2.27 ± 0.22 | 3.52 ± 0.41 |

* p < 0.05.
* p < 0.001 versus normal Chow group.
* p < 0.001.

In a recent paper (10), we demonstrated that glucose down-regulates the expression of Cx36 through cAMP-dependent activation of the protein kinase A. Interestingly, the palmitate effect on Cx36 expression was also mediated by activation of the PKA, suggesting that glucose and palmitate down-regulate the expression of Cx36 through a similar mechanism. This hypothesis is supported by the fact that the palmitate effect requires the presence of the same DNA-binding motif on the Cx36 gene promoter as the one involved in the effect of glucose (10), namely a highly conserved and functional CRE.
Zhou et al. (34) have shown, using mRNA microarrays, that a 48–96-h exposure to a mixture of palmitate and oleate (1:1) stimulates the expression of ICER-1γ in isolated rat islets. Here, we further demonstrated that palmitate specifically overexpresses ICER-1γ expression in insulin-secreting cells through PKA activation. Using an antisense ICER strategy, we further demonstrated that ICER-1γ mediates the effect of palmitate on Cx36 expression. Overall, the combination of both mRNA and protein expression data, together with promoter-activity studies and ChIP experiments, indicates that palmitate transcriptionally represses Cx36 expression through PKA-dependent overexpression of ICER-1γ, which binds to the CX36 CRE. The respective impacts of hyperlipidemia and hyperglycemia on β-cell function are considered to converge under a process referred to as glucolipotoxicity (7–9). Study of the potential additive effects of glucose and palmitate revealed that, although palmitate does not further decrease Cx36 expression in the presence of high glucose concentration (20 mM), the effects of glucose and palmitate are slightly additive at 10 mM glucose. These results suggest that relatively modest (and clinically relevant) increases in palmitate and glucose concentrations may have a cumulative action on ICER-1γ expression, and hence on Cx36 down-regulation. However, the maximal effect is rapidly reached, as a further increase in the ICER-1γ levels does not decrease the Cx36 levels, suggesting a saturation process of ICER-1γ-mediated Cx36 down-regulation.

This hypothesis may explain our in vivo data indicating that mice fed a high fat diet, and presenting only a 2-fold increase in the FFAs levels, display drastically decreased Cx36 levels. Indeed, although the modest hyperglycemia of the HF mice group (7.04 versus 6.12 mmol in the NC group) is unlikely to be responsible for this strong effect, we cannot exclude that increased levels of glucose and palmitate converge to further decrease Cx36 expression in vivo. Still, this stronger decrease probably reflects the discrepancies between a severe but relatively acute in vitro model, and a more physiological but chronic in vitro model. One cannot exclude either that the 2-fold increase in the total plasma cholesterol level in the HF group may also be involved in the Cx36 decrease as prolonged exposure to elevated levels of low density lipoproteins have been shown to severely impact β-cell function (43, 44).

ICER-1γ expression is increased in pancreatic islets of type 2 diabetic rats (35). Here we demonstrated that ICER-1γ is overexpressed in pre-diabetic mice, in the presence of hyperlipidemia and mild hyperglycemia. This study also represents the first evidence that Cx36 content is decreased in vivo in conditions reminiscent of those involved in the development of diabetes.

It has been reported that oscillations in plasma insulin level (45, 46) are disorganized in patients with type 2 diabetes mellitus or obesity (47–49). Moreover, the glucose-induced Ca2+ oscillations are perturbed in islets of ob/ob mice (50), and Cx36 is required to maintain the regular Ca2+ oscillation and causal pulsatile insulin secretion waves, both in vitro (51) and in vivo (52, 53). Thus, the Cx36 down-regulation elicited by hyperlipidemia and/or hyperglycemia might be involved in the alterations of both basal and stimulated pulsatile insulin secretion observed in obese and diabetic patients. Whether Cx36 expression is decreased and contributes to the development of a pre-diabetic state in these animal models, as well as in diabetic or obese patients, remains to be assessed.

Prolonged exposure to palmitate is known to alter both basal and stimulated insulin secretion (14, 30, 41, 54–56). Here, we confirmed the negative impact of palmitate on the secretory function and further demonstrated that ICER-1 overexpression, which is known to alter β-cell function (22, 34), mimics the deleterious effect of long term exposure to palmitate on stimulated secretion, although it seems not to have any effect on basal secretion, in the presence or in absence of palmitate. On the other hand, blockade of the palmitate-mediated ICER-1γ overexpression was sufficient to protect β-cell from the deleterious effects of prolonged exposure to palmitate on both basal and secreted secretory function, suggesting that ICER-1γ blockade is sufficient to prevent the palmitate-mediated alteration of secretion.

This work supports the hypothesis that ICER-1γ overexpression could be involved in glucolipotoxicity (34), through sustained repression of several critical genes of β-cell function, including insulin (35), several genes involved in exocytosis (22), and CX36 (present study). Further studies remain to be carried out to ascertain this result in other models, as it could point to a potential therapeutic target for the protection of β-cell from the harmful impact of FFAs.

In this study, we also attempted to restore the secretory function of β-cells exposed to palmitate by overexpressing Cx36 in cells treated with the FFA. We observed that ectopic Cx36 expression restored stimulated secretion. However, basal secretion was also increased, both in untreated and palmitate-treated cells. Therefore, we cannot conclude that Cx36 overexpression prevents the deleterious effect of palmitate on secretion. However, these data are compatible with previous studies indicating that the levels of Cx36 have to be maintained within a narrow range of native values to maintain proper insulin secretion (2, 6). Hence, our data may be explained by the fact that the plasmid-driven exogenous expression of Cx36 was too high to preserve or restore the β-cell secretory function. One could also argue that restoring Cx36 levels might not be sufficient to restore fully the secretory function altered by palmitate.

In conclusion, long term exposure to palmitate caused a sustained dose-dependent transcriptional down-regulation of Cx36 in β-cell lines and isolated mouse islets. These in vivo data correlate with in vivo evidence that hyperlipidemia decreases Cx36 mRNA and protein levels in Langherans islets. The transcriptional regulation of Cx36 expression involves the PKA-dependent overexpression of the repressor ICER-1γ, which subsequently binds to a CRE located in the CX36 gene promoter. Together with our previous study documenting the glucose-induced Cx36 down-regulation (10), we provide a common mechanistic explanation for the deleterious effects of both glucose and palmitate on Cx36 expression. Given that Cx36 expression levels have to be maintained in a narrow range to ensure an optimal secretory response and although other proteins are involved in glucolipotoxicity (22, 57–59), Cx36 down-regulation and, more prominently, ICER-1γ overexpression might contribute to β-cell failure in relation to hyperlipidemia at the pre-diabetic stage.
Palmitate Down-regulates Cx36 Expression

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