Adenovirus-mediated overexpression of liver carnitine palmitoyltransferase I in INS1E cells: effects on cell metabolism and insulin secretion

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Lipid metabolism in the β-cell is critical for the regulation of insulin secretion. Pancreatic β-cells chronically exposed to fatty acids show higher carnitine palmitoyltransferase I (CPT I) protein levels, higher palmitate oxidation rates and an altered insulin response to glucose. We examined the effect of increasing CPT I levels on insulin secretion in cultured β-cells. We prepared a recombinant adenovirus containing the cDNA for the rat liver isoform of CPT I. The overexpression of CPT I in INS1E cells caused a more than a 5-fold increase in the levels of CPT I protein (detected by Western blotting), a 6-fold increase in the CPT activity, and an increase in fatty acid oxidation at 2.5 mM glucose (1.7-fold) and 15 mM glucose (3.1-fold). Insulin secretion was stimulated in control cells by 15 mM glucose or 30 mM KCl. INS1E cells overexpressing CPT I showed lower insulin secretion on stimulation with 15 mM glucose (-40%; $P < 0.05$). This decrease depended on CPT I activity, since the presence of etomoxir, a specific inhibitor of CPT I, in the preincubation medium normalized the CPT I activity, the fatty-acid oxidation rate and the insulin secretion in response to glucose. Exogenous palmitate (0.25 mM) rescued glucose-stimulated insulin secretion (GSIS) in CPT I-overexpressing cells, indicating that the mechanism of impaired GSIS was through the depletion of a critical lipid. Depolarizing the cells with KCl or intermediary glucose concentrations (7.5 mM) elicited similar insulin secretion in control cells and cells overexpressing CPT I. Glucose-induced ATP increase, glucose metabolism and the triacylglycerol content remained unchanged. These results provide further evidence that CPT I activity regulates insulin secretion in the β-cell. They also indicate that up-regulation of CPT I contributes to the loss of response to high glucose in β-cells exposed to fatty acids.

Key words: beta cells, etomoxir, fatty acid oxidation, glucose-stimulated insulin secretion, pancreas.

INTRODUCTION

Lipid metabolism in the β-cell is critical for the normal regulation of insulin secretion [1,2]. Depletion of lipid stores together with deprivation of non-esterified fatty acids (NEFA) alters glucose-stimulated insulin secretion (GSIS) in rats and humans [3–5]. NEFAs, presumably via long-chain acyl-CoA (LC-CoA), generate signals for insulin secretion [6].

Stimulation of insulin secretion by glucose alters the CoA derivatives in clonal pancreatic β-cells, especially malonyl-CoA and fatty acyl-CoA. The LC-CoA model of GSIS holds that during glucose stimulation, anaplerosis in the mitochondria increases citrate [7], which is exported and converted into malonyl-CoA, resulting in inhibition of carnitine palmitoyltransferase I (CPT I) [8] and fatty acid oxidation [2,6,9]. Therefore the increase in malonyl-CoA may be responsible for the accumulation of fatty acyl-CoAs in the cytosol [10]. Moreover cytosolic acyl-CoA content increases with the addition of exogenous fatty acids. In addition, glucose metabolism in the β-cell raises acyl-CoA levels, which, in combination with α-glycerophosphate, may increase the levels of triacylglycerols, phosphatidic acid and diacylglycerol [2]. Fatty acyl-CoAs may act as coupling factors in insulin secretion by stimulating several isoforms of protein kinase C [11,12], by overcoming malonyl-CoA inhibition of CPT I [13], by stimulating the ATP-sensitive K^+ channel [14], and by acetylating proteins to target them to appropriate membrane sites [15].

Both malonyl-CoA and LC-CoA participate in the signal transduction for insulin secretion: malonyl-CoA as a regulator and LC-CoA as an effector signal [16]. Systems that regulate both malonyl-CoA and LC-CoA appear to be involved in insulin secretion. Accordingly, acetyl-CoA carboxylase, which controls the synthesis of malonyl-CoA, and CPT I, which is regulated by it, are considered to integrate the circulating fuel stimuli. The fate of malonyl-CoA in the pancreatic β-cell, in contrast with other tissues, is not *de novo* synthesis of fatty acids, but the regulation of CPT I activity, due to the very low levels of fatty acid synthase in the β-cell [17]. The metabolism of several nutrients that converge to form malonyl-CoA and increase LC-CoA esters (carbohydrate, amino acids and ketoacids) might act as fuel sensors in the β-cell. Therefore stable expression of an acetyl-CoA carboxylase antisense construct in INS1 cells decreased malonyl-CoA levels and insulin secretion [18]. Moreover, hydroxycitrate, which inhibits ATP-citrate lyase, and consequently malonyl-CoA formation, inhibited GSIS from the perifused rat pancreas [19]. Inhibition of fatty acid oxidation by a CPT I-specific irreversible inhibitor, etomoxir [19], or non-metabolizable fatty acid analogues [20] stimulates GSIS in perfused pancreas and isolated islets.

In the present study the CPT I liver isoform, predominantly expressed in rat pancreatic islets, was overexpressed in rat insulinoma INS1E cells by a recombinant adenovirus vector. The transduced cells had increased CPT I protein levels, an increased palmitate oxidation rate and impaired GSIS, and these effects

Abbreviations used: AdCA, adeno-chicken actin promoter; CPT I, carnitine palmitoyltransferase I; DMEM, Dulbecco's modified Eagle's medium; DNA-TPC, DNA-terminal protein complex; FBS, fetal bovine serum; GSIS, glucose-stimulated insulin secretion; LC-CoA, long-chain acyl-CoA; NEFA, non-esterified fatty acids; TBS, Tris-buffered saline; KRBH buffer, Krebs–Ringer bicarbonate Hepes buffer; UCP2, uncoupling protein 2.

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were overcome by incubation with etomoxir. Incubation of INS1E cells with KCl had no effect on insulin secretion in CPT I overexpressed cells. CPT I overexpression did not change glucose-induced ATP generation, glucose oxidation, or the triacylglycerol levels. These results show that an increase in fatty acid oxidation rate in the β-cell impairs GSIS, confirming data of other authors on the role of fatty acyl-CoA. They are also compatible with the hypothesis that an increase in CPT I [1], together with an increase in uncoupling protein 2 (UCP2) [21,22], could contribute to the altered insulin response to glucose in β-cells after fatty acid exposure.

MATERIAL AND METHODS

Cell culture and materials

INS1E cells [23] were chosen since they secrete more insulin in response to glucose in the range 5–20 mM than the parental INS-1 cells [24]. INS1E cells (passages 46–70) were cultured in a humidified atmosphere containing 5% CO_2 in a medium composed of RPMI 1640 supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol as originally described [24]. An antibody against rat insulin was purchased from LINCO (St. Louis, MO, U.S.A.). RPMI 1640, Dulbecco's modified Eagle's medium (DMEM) and FBS were obtained from Life Technologies, Inc. Reagents commonly used for the experiments were from Sigma Chemical Co. and Fluka Chemie AG. Secondary antibody and radioactively labelled compounds were obtained from Amersham Biosciences. Chemiluminescent reagent (Supersignal West Pico Chemiluminescent substrate) was obtained from Pierce.

Adenovirus construction

Recombinant adenovirus was constructed as described previously [25]. Briefly, the expression unit to be introduced into the recombinant adenovirus was first inserted into the unique *Swa*I site of the E1-substitution type full-length adenovirus genome cloned in a cassette cosmid. The cassette bearing the expression unit was then co-transfected into HEK-293 cells together with the adenovirus DNA-terminal protein complex (DNA-TPC) digested at several sites with *Eco*T22I.

The CPT I cDNA, containing the whole coding sequence, was obtained by reverse-transcriptase PCR from rat liver mRNA, subcloned in BlueScript and sequenced, revealing no change with the previously reported rat liver CPT I [26]. The CPT I cDNA was then digested, blunt-ended and subcloned into the cosmid pAdCA (where AdCA corresponds to adeno-chicken actin promoter) previously cut with *Swa*I and dephosphorylated. The CPT I insert (nt 58–2700 of the previously reported mRNA) contained the ATG codon and the stop codon. The presence of the insert was checked with the restriction enzyme *Cla*I and its correct orientation was checked with *Bgl*II. The cosmid containing the full coding sequence for rat liver CPT I was called pAdCA-CPT I. The adenovirus DNA-TPC of the parent adenovirus was prepared through a buoyant CsCl density gradient with 4 M guanidine hydrochloride [25]. Cassette cosmid pAdCA-CPT I (8 μg) and 1 μg of the *Eco*T22I-digested DNA-TPC were co-transfected in a 6-cm dish with calcium phosphate (Cellfect Transphection Kit; Pharmacia) in HEK-293 cells and 1 day later the cells were distributed in 96-well plates [27]. The desired recombinant adenovirus was generated by overlapping recombination. At 10 days after transfection, the cell lysate from the selected viral clones was used to infect 24-well dishes, the adenoviral DNA was extracted from these cells and the DNA was analysed by digestion with *Cla*I to check the CPT I insert.

To amplify and purify the selected virus the cell lysate containing the virus with full-length rat liver CPT I (AdCA-CPT I) was used to infect two 138-mm dishes of HEK-293 cells. The AdCA-CPT I and AdCA-LacZ viruses, which express the bacterial β-galactosidase [28] were amplified and purified by CsCl ultracentrifugation. Adenovirus amplification was performed in HEK-293 cells cultured in DMEM containing 5 or 10% (v/v) FBS.

Viral treatment of INS1E cells

INS1E cells (500000) were seeded in 12-well dishes (Falcon) and cultured for 48 h prior to infection. For infection, cells were incubated with 500 μl of medium containing 20 plaque-forming units of the recombinant adenovirus/cell for 90 min, washed once in the medium, and cultured in RPMI medium for 20 h before experiments to allow the transgenes to be expressed, before initiating metabolic studies or measurements of insulin secretion.

Immunoblot analysis

For detection of rat liver CPT I protein, infected INS1E cells were collected and dissolved in SDS sample buffer. Proteins were subjected to SDS/PAGE (10% gels) and transferred on to nitrocellulose membranes. The membranes were blocked for 1 h with Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) non-fat dried milk. The membrane was washed three times in TBS/Tween at 22 °C. Membranes were probed with the CPT I-specific antibody [29] by overnight incubation, and then incubated for 1 h with anti-sheep IgG conjugated to horseradish peroxidase (1:5000 dilution). The signal was detected by chemiluminescence.

Determination of CPT I activity in INS1E cells

INS1E cells (12 million) were seeded in 15-cm dishes and cultured for 48 h prior to infection. For infection, cells were incubated with 10 ml of medium containing 20 plaque-forming units of the recombinant adenovirus (AdCA-LacZ and AdCA-CPT I)/cell for 90 min, washed once in the medium, and cultured in RPMI medium for 20 h. Prior to obtaining the mitochondria-enriched cell fraction, cells were preincubated for 30 min in Krebs–Ringer bicarbonate Hepes buffer (KRBH buffer; 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$ and 10 mM Hepes) + 1% (w/v) BSA without glucose in the absence or the presence of etomoxir at 50 and 200 μM. After preincubation, the cells were washed with 10 ml of PBS and resuspended in 5 ml of the same solution. The cells were pooled at this point and sedimented by centrifugation at 1200 ***g*** for 5 min at 4 °C. After resuspension in 1 ml of 5 mM Tris/HCl (pH 7.2)/150 mM KCl (buffer A), they were broken with 10 cycles of a glass homogenizer fitted with a tight pestle. Further centrifugation was performed in a microfuge for 5 min at 4 °C at 16000 ***g***, and the final pellet was resuspended in the desired volume of buffer A. CPT I was assayed in these preparations where the mitochondria remain largely intact [30]. Carnitine acyltransferase activity of 10 μg of protein was determined by the radiometric method as previously described [31].

[1-^{14}C]Palmitate oxidation

At 20 h after infection, INS1E cells seeded in 12-well dishes were placed in a water bath at 37 °C, washed once in 1 ml of solution A, which contained KRBH + 0.1% BSA (essentially fatty acid

free). The cells were then preincubated for 30 min with 500 μl of KRBH containing 1 % (w/v) BSA (solution B), with or without 200 μM etomoxir and washed in 1 ml of KRBH.

The rate of fatty acid oxidation was measured essentially as described by Chen et al. [19]. Briefly, the cells were washed in 1 ml of solution A and harvested by resuspension in 300 μl of KRBH. Cell suspension (160 μl) was added to a centre well containing 20 μl of 2.5 mM palmitate complexed to 10 % (w/v) BSA, with 0.5 μCi of [1-^{14}C]palmitic acid as a tracer and 20 μl of a solution containing 25 or 150 mM D-(+)-glucose plus 8 mM carnitine, and the centre well was immediately placed in a sealed vial. After 2 h at 37 °C, the reaction was stopped by the addition of 100 μl of 7 % (w/v) perchloric acid. The rate of [1-^{14}C]palmitate oxidation was measured as released $^{14}CO_2$, which was trapped by 300 μl of benzethonium chloride, added to the bottom of the sealed vials. After 5 h, the centre wells and the rubber stoppers were discarded and 10 ml of scintillation mixture was added. Control incubations without INS1E cells were run with each series. Following overnight incubation at 22 °C, $^{14}CO_2$ production was counted in an LS6500 liquid scintillation counter (Beckman Instruments Inc.). Palmitate oxidation was expressed as nmol of palmitate oxidized/h per mg of cell protein.

ATP determinations

ATP was generated in 6-well plates following 30 min of preincubation in KRBH. Cells were incubated for 10 min in KRBH with 2.5 or 15 mM glucose before the stimulation was arrested on ice by washing with ice-cold KRBH and the addition of 0.4 M perchloric acid. ATP levels were determined using a bioluminescence assay kit (Roche, Rotkreuz, Switzerland).

Measurements of cellular insulin secretion and insulin content

Insulin was measured essentially as described previously [32]. INS1E cells (500000) were seeded in 12 wells. Two days after seeding the cells were treated with the recombinant adenovirus and 20 h later INS1E infected cells were placed in a water bath at 37°C, washed once in 1 ml of solution A, preincubated for 30 min with 500 μl of solution B, with or without 200 μM etomoxir, and washed again in solution A.

Insulin secretion was measured for 30 min. Briefly, 1 ml of solution A containing 2.5, 7.5 or 15 mM glucose, or 2.5 mM glucose plus 30 mM KCl, was added. After 30 min the solution was collected and centrifuged at 10000 rev./min in a microcentrifuge for 1 min to remove cell debris. To measure insulin content, INS1E cells remaining in the wells were extracted with 1 ml of 75 % ethanol/0.2 M HCl added to each well. The supernatant and cell insulin samples were immediately stored at −20 °C until insulin determination by RIA, using rat insulin as a standard and an antibody specific for rat insulin. The insulin secretion was expressed as a percentage of the cell insulin content.

[U-^{14}C]Glucose oxidation

Glucose oxidation was measured as $^{14}CO_2$ production from [U-^{14}C]glucose with the same experimental design as for the measurement of palmitate oxidation, except that cells were incubated for 1 h with [U-^{14}C]glucose in the absence or in the presence of 0.25 mM palmitate [32].

INS1E cells infected with recombinant adenovirus as described above were washed with 1 ml of solution A and harvested by light resuspension in 600 μl of KRBH; 180 μl of the cell suspension was added to a centre well containing 20 μl of 25 or 150 mM D-(+)-glucose with 0.5 μCi of [U-^{14}C]glucose as a tracer, and the centre well was placed immediately in a sealed vial. After 1 h at 37 °C, the reaction was stopped by the addition of 100 μl of 7 % (w/v) perchloric acid. The rate of [U-^{14}C]glucose oxidation was measured as released $^{14}CO_2$, which was trapped by 300 μl of benzethonium chloride at the bottom of the sealed vials to bind the liberated $^{14}CO_2$. Labelled CO_2 was measured as described above. The data were expressed as nmol of glucose oxidized/h per mg of cell protein.

Triacylglycerol content measurements

At 20 h after infection, INS1E cells seeded in 12-well dishes were placed in a water bath at 37 °C, washed once with 1 ml of solution A, preincubated for 30 min with 500 μl of solution B and washed with solution A.

Lipids were extracted as described elsewhere [33]. Briefly, 1 ml of methanol/PBS (2:3, v/v) was added to each well and the cells were gently collected in a pipette, centrifuged at 700 ***g*** for 5 min and washed with PBS; 200 μl of 0.2 M NaCl was then added to the pellet and the mixture was immediately frozen in liquid N_2. To separate aqueous and lipid phases, 750 μl of chloroform/methanol (2:1, v/v) and 50 μl of 0.1 M KOH were added and, after vigorous vortex-mixing, the phases were separated by 15 min of centrifugation at 2000 ***g***. The top aqueous phase was removed and the lipid phase was washed with 200 μl of methanol/water/chloroform (48:47:3, by vol.). After vortex-mixing and centrifugation, 400 μl of the lower phase was taken and dried. Triacylglycerols were measured in each sample using the Sigma 334 triacylglycerol kit. The method involves the hydrolysis of triacylglycerols by lipoprotein lipase and measures 'true triacylglycerols'.

Statistical analysis

Unless otherwise indicated data are represented as the means ± S.E.M. for at least three independent experiments performed in triplicate. Differences between groups are assessed by the Student's *t* test for unpaired data.

RESULTS

Assessment of adenovirus-mediated CPT I overexpression in INS1E cells

The capacity of AdCA-CPT I to direct expression of liver CPT I in insulinoma INS1E cells was evaluated by Western-blot analysis of extracts from cells infected with AdCA-CPT I and AdCA-LacZ as a control. The conditions of the infection are described in the Materials and methods section. Immunoblotting revealed a band of the expected size of 88 kDa and the signal increased according to the viral amount from 0 to 40 plaque-forming units/cell (Figure 1A). An infection titre of 20 plaque-forming units/cell was used for the rest of the experiments. There was a 6-fold increase in the CPT activity in the mitochondria-enriched fractions of the cells infected with AdCA-CPT I compared with the cells infected with AdCA-LacZ. In contrast, preincubation of the AdCA-CPT I-infected INS1E cells with increasing amounts of etomoxir diminished the CPT activity (60 and 9 % of the control with 50 and 200 μM etomoxir respectively) (Figure 1B).

Impact of recombinant CPT I adenovirus on palmitate oxidation in INS1E cells

We evaluated the metabolic effects of the AdCA-CPT I virus. [1-^{14}C]Palmitate oxidation was measured in adenovirus-treated INS1E cells for 2 h, after a 30 min preincubation in KRBH with no glucose and no fatty acids, in the absence or in the presence

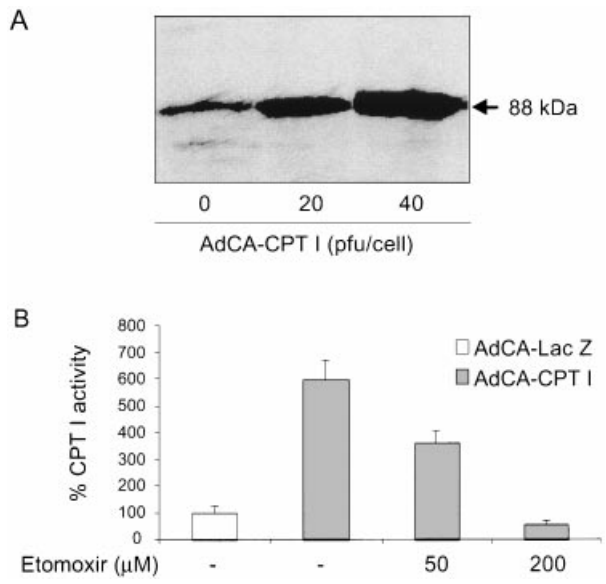

Figure 1 Immunoblot analysis of CPT I expressed in INS1E infected cells and CPT activity in mitochondria-enriched INS1E fractions

(**A**) Samples were prepared and analysed as described in the Materials and methods section. At 20 h after the infection of INS1E cells with 20 and 40 plaque-forming units (pfu) of recombinant adenovirus/cell, cells were collected and protein extracts (100 μg) were separated by SDS/PAGE (10% gels) and subjected to immunoblotting by using specific antibodies for CPT I from rat liver. A unique band corresponding to a protein of approx. 88 kDa was seen in control and AdCA-CPT I-infected cells. (**B**) CPT I activity was measured in mitochondrial-enriched fractions of LacZ and CPT I-overexpressing cells, before and after treatment with etomoxir. Results are the means of three experiments performed in duplicate.

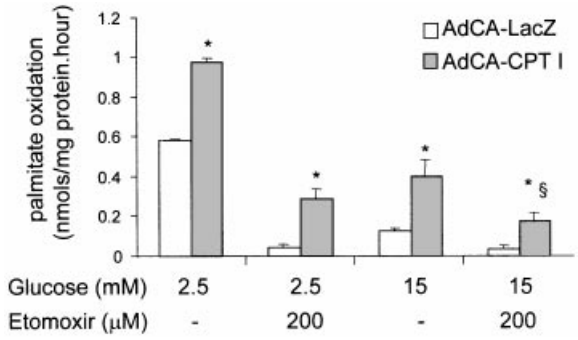

Figure 2 [1-^{14}C]Palmitate oxidation in INS1E infected cells

INS1E cells were treated with AdCA-LacZ or AdCA-CPT I. At 20 h after viral treatment, [1-^{14}C]palmitate oxidation was measured after 30 min of preincubation with either 2.5 or 15 mM glucose with or without etomoxir. Results are expressed as means $\pm$ S.E.M. for three independent experiments, which were performed in triplicate. *$P < 0.01$ compared with AdCA-LacZ; §$P < 0.05$ compared with AdCA-CPT I without etomoxir.

of 200 μM etomoxir. Incubation with AdCA-LacZ at 15 mM glucose reduced the rate of palmitate oxidation to 20% in comparison with cells incubated with 2.5 mM glucose (Figure 2). This decrease in fatty acid oxidation rate has been reported previously and in similar fashion for rat islets [19] and INS1 cells [34]. According to these results [19,34], when glucose is raised from non-stimulatory to stimulatory concentration, INS1E cells (like INS1 cells and islet β-cells) increase glycolytic flux and decrease the rate of palmitate oxidation.

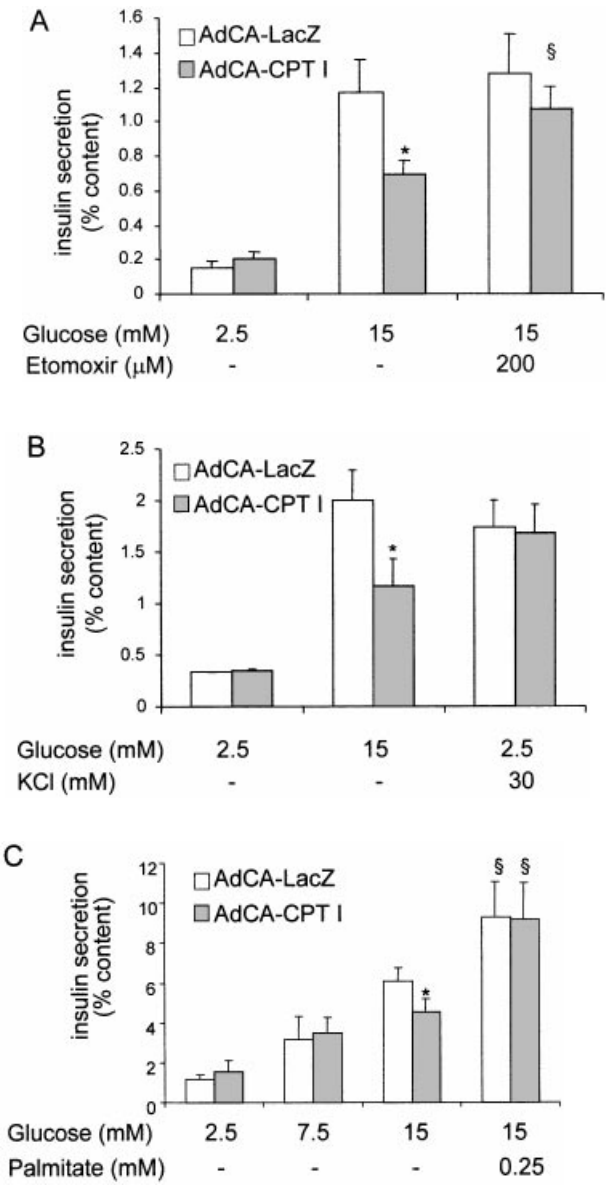

Figure 3 Effect of CPT I overexpression on GSIS in INS1E infected cells

(**A**) INS1E cells were treated with the indicated adenovirus and cultured for 20 h in regular medium. After a 30 min preincubation in KRBH solution without glucose and with or without 200 μM etomoxir, cells were washed and incubated in KRBH solution containing either 2.5 or 15 mM glucose for 30 min and insulin release was determined. Results represent the means $\pm$ S.E.M. for three independent experiments performed in triplicate. (**B**) A similar set of experiments was performed, in the absence of etomoxir preincubation, testing 2.5, 15 mM glucose and 2.5 mM glucose plus 30 mM KCl. Results are the means $\pm$ S.E.M. of one representative experiment out of three, performed in triplicate. *$P < 0.05$ compared with AdCA-LacZ. (**C**) To test the effect of intermediate glucose concentrations and of 0.25 mM palmitate, cells were seeded on 24 wells and insulin secretion was measured in a 1 h period and expressed as a percentage of the content. Results are the means $\pm$ S.E.M. for three experiments performed in triplicate. *$P < 0.05$ compared with AdCA-LacZ; §$P < 0.05$ compared with 15 mM glucose.

The overexpression of CPT I in INS1E cells increased palmitate oxidation at low (1.7-fold) and high (3.1-fold) glucose concentrations ($P < 0.01$). An increase in palmitate oxidation at high glucose was also described for INS1 and INS1-derived (832/13) β-cells overexpressing malonyl-CoA decarboxylase, the enzyme which eliminates malonyl-CoA, the CPT I physiological inhibitor [34,35]. Nevertheless the increase in fatty acid oxidation at low

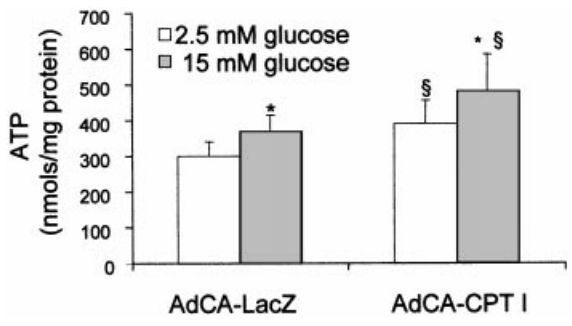

Figure 4 Glucose-induced ATP generation

Cytosolic ATP levels were measured in INS1E cells overexpressing LacZ or CPT I, preincubated for 30 min without glucose and exposed to 2.5 and 15 mM glucose for 10 min. Results represent the means ± S.E.M. *$P < 0.05$ compared with 2.5 mM glucose; §$P < 0.05$ AdCA-CPT I compared with AdCA-LacZ.

glucose concentration was not observed by these authors, since at low glucose the malonyl-CoA levels are low.

The stimulatory glucose concentration was more effective in suppressing fatty acid oxidation in control cells (20 % residual palmitate oxidation) than in CPT I-overexpressing cells, which showed a 40 % residual palmitate oxidation after the switch from low glucose to high glucose.

Control and CPT I-overexpressing INS1E cells treated with etomoxir showed a lower fatty acid oxidation rate, as expected from its capacity to inhibit CPT I irreversibly. The presence of etomoxir decreased the high oxidation rate in CPT I-overexpressing cells from 0.4 to 0.17 nmol/h per mg of protein ($P < 0.05$). The fatty acid oxidation rate in etomoxir-treated CPT I-overexpressing cells was similar to the control cells without etomoxir and high concentrations of glucose (0.176 compared with 0.128). This indicates that the treatment with etomoxir reversed the effect of CPT I overexpression.

Effect of CPT I overexpression on INS1E insulin secretion

Having established that AdCA-CPT I increases the rate of fatty acid oxidation in all the conditions studied we evaluated the effect of CPT I on insulin secretion. INS1E cells were maintained in culture for 2 days, infected with AdCA-LacZ and AdCA-CPT I and cultured for 20 h prior to the experiments. For insulin secretion experiments, cells were preincubated for 30 min in solution B with or without 200 μM etomoxir. After this treatment cells were washed to eliminate etomoxir, and insulin secretion was determined for 30 min with KRBH plus 2.5 mM glucose and 15 mM glucose.

In INS1E cells treated with the control virus AdCA-LacZ, 15 mM glucose caused an 8-fold increase in insulin secretion relative to secretion at 2.5 mM glucose, while cells treated with AdCA-CPT I exhibited only a 3.5-fold increase, indicating that the increase in lipid metabolism caused by the overexpression of CPT I affects GSIS (Figure 3A). In these conditions insulin secretion was reduced by 40 % ($P < 0.01$) in overexpressed CPT I cells in relation to control cells.

Control cells treated with 200 μM etomoxir gave the same response to glucose as non-treated control cells (8.5-fold compared with 8-fold) (Figure 3A). Moreover, AdCA-CPT I-infected cells pre-treated with etomoxir showed a significantly higher increase in insulin secretion (5.5-fold compared with 3.5-fold, $P < 0.05$). The treatment of the AdCA-CPT I-infected cells with etomoxir suggests a specific effect of CPT I on insulin secretion, since etomoxir partially restores the normal insulin secretion in these cells. Insulin secretion was also stimulated at basal 2.5 mM glucose with 30 mM KCl, as a Ca^{2+}-raising agent. As shown in Figure 3(B), the effects of 30 mM KCl on insulin exocytosis were similar in control cells and cells overexpressing CPT I. These results distinguish between the K^+–Ca^{2+} axis and the anaplerotic effects of glucose and fatty acid oxidation.

To test the effect of intermediate glucose concentrations and long-chain fatty acids in insulin secretion in INS1E cells, insulin secretion was performed over a 1 h period in infected INS1E cells seeded in 24 wells. In control cells (AdCA-LacZ-infected), raising glucose concentrations from a basal concentration of 2.5 mM to 7.5 and 15 mM stimulated insulin secretion 2.8-fold ($P < 0.05$) and 5.4-fold ($P < 0.01$) respectively (Figure 3C). Insulin release at basal (2.5 mM) or intermediate (7.5 mM) glucose concentration was not affected in cells overexpressing CPT I. In contrast, insulin secretion stimulated by 15 mM glucose (3-fold versus basal glucose, $P < 0.05$) was inhibited by 27 % ($P < 0.05$) compared with the corresponding control at high glucose. In the same experiments palmitate potentiated GSIS in control and CPT I-overexpressing cells (9.3 compared with 6.1 in control cells, and 9.3 compared with 4.5 in CPT I-overexpressing cells) (Figure 3C), emphasizing the role of fatty acids in the signal transduction of insulin secretion.

Effect of CPT I overexpression on glucose-induced ATP generation and ATP levels

Incubation of INS1E cells with glucose stimulated the synthesis of ATP by activation of the electron transport chain. The levels of ATP/mg of protein were measured following a 10 min incubation period, at low and high glucose. ATP levels at 15 mM glucose compared with 2.5 mM glucose were elevated by 23.1 % (367 ± 49 compared with 298 ± 42 respectively) in AdCA-LacZ control cells and by 23.3 % (481 ± 101 compared with 390 ± 66 respectively) ($P < 0.05$) in AdCA-CPT I cells (Figure 4). Therefore CPT I overexpression did not modify the glucose-induced ATP increase. However, there was a 31 % elevation in ATP levels in CPT I- versus Lac Z-overexpressing cells ($P < 0.05$) at low and high glucose concentrations.

Effect of CPT I expression on glucose utilization

To investigate whether the impaired GSIS in INS1E cells overexpressing CPT I could be due to a decrease in glucose utilization in INS1E cells we measured the release of $^{14}CO_2$ at the same glucose concentrations used for the insulin secretion experiments in INS1E cells overexpressing LacZ or CPT I and using [U-^{14}C]glucose. The use of [U-^{14}C]glucose allows the measurement of the CO_2 released not only at the steps in the tricarboxylic acid cycle but at the pyruvate dehydrogenase step.

The release of $^{14}CO_2$ was determined for 1 h in the presence of 2.5 and 15 mM glucose after a 30 min preincubation with or without 200 μM etomoxir. The presence of a stimulatory glucose concentration increased the rate of $^{14}CO_2$ release from 2.5 to 15 mM glucose by 5.2-fold both in cells overexpressing LacZ and in cells overexpressing CPT I (Figure 5). The treatment of the cells with etomoxir did not change the CO_2 formation from glucose in either LacZ- or CPT I- overexpressing cells. This result shows that, in the absence of external fatty acids, there is no difference in the rate of glucose utilization between control and CPT I-overexpressing cells. Etomoxir had no effect on glucose utilization in either control or CPT I-overexpressing cells. This result suggests that the mechanism by which the overexpression of CPT I decreases GSIS in INS1E cells is not a decrease in glucose oxidation. Glucose oxidation was determined in LacZ-

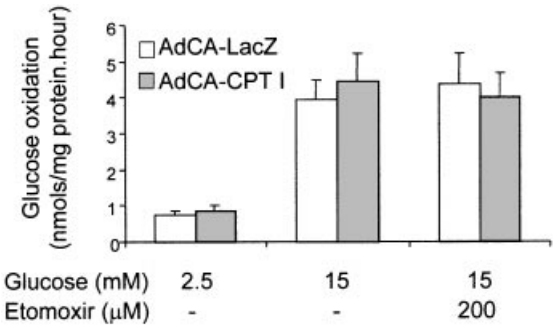

Figure 5 Glucose oxidation in INS1E infected cells

[U-^{14}C]Glucose oxidation was measured in INS1E cells overexpressing LacZ or CPT I. After a 30 min preincubation in KRBH solution without glucose and with or without 200 μM etomoxir, cells were washed and resuspended in KRBH with [U-^{14}C]glucose at the indicated concentrations for 1 h and the CO_2 released was measured as described in the Materials and methods section. Data are the means $\pm$ S.E.M. for three independent experiments performed in triplicate and are expressed as nmol of oxidized glucose/h per mg of protein.

and CPT I-overexpressing cells and the effect of 0.25 mM palmitate was tested. The addition of 0.25 mM palmitate had no significant effect on glucose oxidation in control or CPTI-overexpressing cells over a 1 h period (results not shown). Comparison of Figures 2 and 5 shows that at high glucose concentration, glucose oxidation in INS1E cells is much higher (32 times) than fatty acid oxidation, these results agreeing with those described in INS1 cells [1] and in islets [36,37]; therefore it appears unlikely that accelerated fatty acid oxidation influences glucose metabolism.

Effect of CPT I overexpression on triacylglycerol levels

It has been shown that a depletion of β-cell lipids, measured as triacylglycerols [5], blocks insulin secretion in response to glucose and other secretagogues, and that perifusion with a mixture of fatty acids restored insulin responses to supranormal levels. To address the question of whether the impaired insulin response to glucose seen in INS1E cells overexpressing CPT I was due to a decrease in triacylglycerol levels, we measured triacylglycerol content in INS1E cells infected with either the control virus AdCA-LacZ or AdCA-CPT I. The cells were infected and 20 h later incubated in KRBH without glucose for 30 min as previously described. After this incubation cells were washed and collected for the triacylglycerol measurement. The triacylglycerol content was not significantly different between LacZ- and CPT I-overexpressing cells (14.8 ± 1.2 compared with 13.6 ± 1.3 ng of triacylglycerols/mg of protein). Thus a decrease in triacylglycerols does not seem to account for the lower insulin secretion at stimulatory glucose concentrations seen in CPT I-overexpressing cells.

DISCUSSION

Many studies implicate NEFAs in type II diabetes [2,38,39]. Long term exposure of β-cells to NEFAs *in vitro* has several effects: (1) it increases basal insulin release and strongly decreases secretion in response to glucose [2]; (2) it alters the coupling of glucose metabolism to insulin secretion by acting on the expression of specific genes, such as that for UCP2 [21,22]; and (3) it increases the expression of CPT I, which is considered to be the rate-limiting step in fatty acid oxidation [1]. CPT I up-regulation may contribute to the change in insulin secretion. The aim of the present study was to evaluate the capacity of CPT I overexpression to alter the insulin response to glucose in β-cells. In view of the interest in the LC-CoA model of GSIS, which is still under discussion [40], we examined this effect in a β-cell-derived cell line, which is more sensitive at physiological glucose concentrations than the parenteral INS1 cell line.

Recent advances in adenoviral vector technology have facilitated the transfer of multiple foreign genes into well-differentiated cell lines. The chicken actin promoter [25,41] allows the use of low adenoviral infection titres, with high levels of protein expression. The rat liver CPT I is the only isoform present in both the β-cell and INS1E cells. Therefore, since the muscle CPT I isoform has different malonyl-CoA inhibition properties, we used the rat liver isoform to construct the adenovirus.

The overexpression of CPT I increased fatty acid oxidation rates in the INS1E cells, as expected. However, the percentage increase in ATP (23 %) in CPT I-overexpressing cells and in controls was identical when glucose was increased from 2.5 to 15 mM, showing that CPT I overexpression had no effect on the electron transport chain. Stimulation of fatty acid oxidation by CPT I was higher than that induced by malonyl-CoA decarboxylase or acetyl-CoA carboxylase [18,34]. These enzymes modulate CPT I activity indirectly through changes in malonyl-CoA, whereas CPT I controls the fatty acid oxidation flux directly. Interestingly, the overexpression of CPT I increased the rate of fatty acid oxidation not only at high glucose, but also at low glucose concentrations. INS1E cells overexpressing CPT I secreted less insulin in response to high glucose concentration, but not in response to intermediate glucose concentrations or non-nutrient secretagogues, such as KCl. Thus exocytosis was preserved, since the effect of the cytosolic [Ca^{2+}]-raising agent (30 mM KCl) was not altered by CPT I overexpression. The increase in fatty acid oxidation may be responsible for the change in the insulin response. This indicates that lipid homoeostasis is necessary for the correct insulin response to glucose.

The specificity of the CPT I effect on insulin secretion was tested by the use of etomoxir, an irreversible inhibitor of CPT I activity. Etomoxir reversed the CPT I effect on fatty acid oxidation and insulin secretion, which indicates that the effect on insulin response is due to the increase in fatty acid oxidation [42]. Only preincubation of the INS1E cells with 200 μM etomoxir abolished the higher CPT I activity after infection with AdCA-CPT I (Figure 1B). To avoid any non-specific effect on the secretory machinery, etomoxir was eliminated after the preincubation and the cells were washed prior to the experiments. Under these conditions we consider that the only effect of etomoxir was the inhibition of CPT I through direct and irreversible covalent binding of CPT I and not as a fatty acid analogue. Under these conditions our control INS1E cells did not show any enhancement of GSIS, in contrast with what has been shown previously [19]. In the work of Chen et al. [19], 200 μM etomoxir, added with the glucose in rat perfusion experiments stimulated GSIS. This discrepancy could be explained by the presence of etomoxir throughout all the secretion experiments. Palmitate, which potentiates insulin secretion in HIT cells [6] and islets [43], increased insulin secretion to the same extent in control and CPT I-overexpressing INS1E cells. This could indicate the mechanism by which CPT I diminishes insulin secretion, that is, the depletion of a critical lipid which could act as a signal molecule. In fact, palmitate has been shown to increase the long chain acyl-CoA ester content in HIT cells [6].

Some authors did not find a correlation of cell malonyl-CoA levels with insulin secretion since the reduction of malonyl-

CoA levels by overexpression of malonyl-CoA decarboxylase in an INS1 cell-derived clone had no effect on insulin secretion [34, 35]. However, malonyl-CoA depletion incompletely reverses the glucose-induced suppression of fatty acid oxidation. The authors attribute this to the regulation of CPT I activity by factors derived from glucose metabolism other than malonyl-CoA in the β-cell [35]. Interestingly, a regulation of CPT I that is independent of malonyl-CoA has been demonstrated in rat hepatocytes [44]. Another explanation for this lack of correlation between malonyl-CoA levels and fatty acid oxidation could be the presence of different pools of malonyl-CoA in the cell. There are two acetyl-CoA carboxylases, one in the cytosol the other in the mitochondria, the latter directly modulating the malonyl-CoA levels accessible to CPT I [45].

INS1 cells overexpressing CPT I show higher rates of palmitate oxidation at low and high glucose concentrations (Figure 2) than control cells, yet the rates of glucose oxidation were nearly identical in the CPT I-non-expressing and expressing cells. This would mean that the excess of CPT I activity would increase fatty acid oxidation, without affecting the oxidation of glucose. This excess of fatty acid oxidation under CPT I overexpression would explain the 31 % increase in ATP generation observed in these cells, which therefore would be exclusively produced by the overactivity of CPT I under the conditions of the assay. The increased CPT I, in addition, slightly decreased the triacylglycerol content, indicating that the pool of fatty acyl-CoA present in the cell was responsible for generation of ATP, without the need to hydrolyse triacylglycerols.

In conclusion, the present study shows that the overexpression of CPT I increases the fatty acid oxidation rate at high and low glucose concentrations. The increase in fatty acid oxidation affected insulin response to high glucose but not to intermediate glucose concentrations and non-metabolizable secretagogues, like KCl, while other cell parameters remained unchanged. The effect of CPT I was reverted by the use of etomoxir and by the exogenous addition of fatty acids. These results show that CPT I activity regulates insulin secretion, probably allowing a certain level of fatty acids to be available for exocytosis of insulin [46]. These results also favour the hypothesis that up-regulation of CPT I [1] contributes to the early loss of glucose responsiveness seen in β-cells chronically exposed to high concentrations of fatty acids.

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