Alteration of the Malonyl-CoA/Carnitine Palmitoyltransferase I Interaction in the β-Cell Impairs Glucose-Induced Insulin Secretion

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Carnitine palmitoyltransferase I, which is expressed in the pancreas as the liver isoform (LCPTI), catalyzes the rate-limiting step in the transport of fatty acids into the mitochondria for their oxidation. Malonyl-CoA derived from glucose metabolism regulates fatty acid oxidation by inhibiting LCPTI. To examine directly whether the availability of long-chain fatty acyl-CoA (LC-CoA) affects the regulation of insulin secretion in the β-cell and whether malonyl-CoA may act as a metabolic coupling factor in the β-cell, we infected INS(832/13) cells and rat islets with an adenovirus encoding a mutant form of LCPTI (Ad-LCPTI M593S) that is insensitive to malonyl-CoA. In Ad-LCPTI M593S–infected INS(832/13) cells, LCPTI activity increased sixfold. This was associated with enhanced fatty acid oxidation, at any glucose concentration, and a 60% suppression of glucose-stimulated insulin secretion (GSIS). In isolated rat islets in which LCPTI M593S was overexpressed, GSIS decreased 40%. The impairment of GSIS in Ad-LCPTI M593S–infected INS(832/13) cells was not recovered when cells were incubated with 0.25 mmol/l palmitate, indicating the deep metabolic influence of a nonregulated fatty acid oxidation system. At high glucose concentration, overexpression of a malonyl-CoA–insensitive form of LCPTI reduced partitioning of exogenous palmitate into lipid esterification products and decreased protein kinase C activation. Moreover, LCPTI M593S expression impaired K_{ATP} channel–independent GSIS in INS(832/13) cells. The LCPTI M593S mutant caused more pronounced alterations in GSIS and lipid partitioning (fat oxidation, esterification, and the level of nonesterified palmitate) than LCPTI wt in INS(832/13) cells that were transduced with these constructs. The results provide direct support for the hypothesis that the malonyl-CoA/CPTI interaction is a component of a metabolic signaling network that controls insulin secretion. *Diabetes* 54:462–471, 2005

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

Stimulation of insulin secretion by glucose alters CoA derivative levels in clonal pancreatic β-cells, in particular malonyl-CoA and LC-CoAs. The malonyl-CoA/LC-CoA model of GSIS holds that during glucose stimulation, anaplerosis increases citrate (7), which is exported and finally converted to malonyl-CoA, resulting in inhibition of carnitine palmitoyltransferase I (CPTI) (8) and fatty acid oxidation (6,9). Therefore, the increase in malonyl-CoA may be responsible for the accumulation of LC-CoAs in the cytosol (10). Moreover, the β-cell LC-CoA content increases with the supplement of exogenous NEFA. In addition, glucose metabolism in the β-cell raises cytosolic LC-CoA levels, which in combination with α-glycerophosphate may increase the levels of triglycerides, phosphatidic acid, and diacylglycerol (1). LC-CoA may act as coupling factors in insulin secretion by stimulating several isoforms of protein kinase C (PKC) (11,12), through acylation of exocytotic proteins, or by generating complex lipid signaling molecules such as diacylglycerol and phosphatidate (1).

Both malonyl-CoA and LC-CoA are thought to participate in the signal transduction for insulin secretion: the former as a regulator and the latter as an effector (13). Systems that regulate both malonyl-CoA and LC-CoA seem to be involved in insulin secretion. Accordingly, acetyl-CoA carboxylase (ACC), which controls the synthesis of malonyl-CoA; malonyl-CoA decarboxylase (MCD), which catalyzes malonyl-CoA degradation; and CPTI, which is regulated by malonyl-CoA, are components of a metabolic signaling network that senses the level of fuel stimuli (1,8). The physiological role of malonyl-CoA in the endocrine pancreas, unlike many other tissues, is not de novo synthesis of fatty acids but rather the regulation of CPTI activity, because the level of fatty acid synthetase in

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ACC, acetyl-CoA carboxylase; ASP, acid-soluble product; CE, cholesterol ester; CPTI, carnitine palmitoyltransferase I; DAG, diacylglycerol; ECF, enhanced chemifluorescence; FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; KRBH, Krebs-Ringer bicarbonate HEPES; LC-CoA, long-chain fatty acyl-CoA; LCPTI, liver carnitine palmitoyltransferase I; MCD, malonyl-CoA decarboxylase; MCDc, MCD in the cytosol; NEFA, nonesterified fatty acid; NE palm, nonesterified labeled palmitate; PKC, protein kinase C.

normal β-cells is very low (14). The metabolism of several nutrients that converge to form malonyl-CoA and increase LC-CoA esters (carbohydrate, amino acids, and ketoacids) might play a key role in fuel-regulated insulin secretion in the β-cell (2). Thus, for example, stable expression of an ACC-antisense construct in INS1 cells (15) or overexpression of MCD in rat insulinoma INS(832/13) cells (16) decreased malonyl-CoA levels, increased LC-CoA oxidation, and decreased insulin secretion.

The overexpression of native LCPTI in clonal INS-1E β-cells increased β oxidation of fatty acids and decreased insulin secretion at high glucose (17). The effect of LCPTI was reverted by etomoxir, an irreversible inhibitor of CPTI, and by the exogenous addition of fatty acids. However, using this approach, glucose-derived malonyl-CoA is still able to inhibit LCPTI in cells overexpressing the enzyme; consequently, fat oxidation is moderately altered (17). To directly test the hypothesis that the CPTI/malonyl-CoA interaction is involved in GSIS, we chose to overexpress LCPTI M593S, a mutant enzyme that is insensitive to malonyl-CoA (18). The LCPTI mutant and the native LCPTI were overexpressed in INS(832/13) cells and rat islets using recombinant adenoviruses.

INS(832/13) cells that were transduced with the M593S mutant had increased CPTI activity and protein levels, a markedly increased palmitate oxidation rate, and a more impaired GSIS than cells that were transduced with LCPTI wt. At high glucose, esterification products and PKC activation were decreased in cells expressing the mutated CPTI. Overall, the data provide direct support for the view that the malonyl-CoA/CPTI interaction is involved in glucose-regulated insulin secretion.

RESEARCH DESIGN AND METHODS

The collagenase used to isolate rat pancreatic islets was from Serva Electrophoresis (Heidelberg, Germany). Protran nitrocellulose membranes for protein analysis were from Schleicher & Schuell (Keene, NH), and the Bradford solution for protein assay was from Bio-Rad Laboratories (Hercules, CA). The enhanced chemifluorescence (ECF) reagent pack from Amersham Biosciences was used for Western blot analysis. TLC plates were purchased from Merck (Rahway, NJ). Defatted BSA; palmitic acid (sodium salt); and the migration standards phosphatidyl-serine, dipalmitoyl-glycerol, glyceryl tripalmitate, and cholesteryl palmitate were from Sigma-Aldrich. Radioactive compounds [1-^{14}C]palmitic acid, [U-^{14}C]glucose, [1-^{14}C]acetyl-CoA, and L-[methyl-^{3}H]carnitine were from Amersham Biosciences.

Construction of recombinant adenoviruses. Ad-LCPTI wt encoding LCPTI wt was constructed as previously described (17). Ad-LacZ, which expresses bacterial β-galactosidase, was used as a control adenovirus. Ad-LCPTI M593S, encoding the malonyl-CoA–insensitive LCPTI M593S cDNA under the chicken actin (CA) promoter, was constructed using the Adenovirus Expression Vector Kit (Takara Biomedicals). Briefly, blunt-ended LCPTI M593S cDNA (18) was subcloned into the cosmid pAdCA previously cut with *Swa*I and dephosphorylated. The resulting cosmid was packaged using the Gigapack III Plus Packaging Extract (Stratagene, La Jolla, CA). The presence and right orientation of the insert were checked by restriction enzyme digestions using *Cla*I and *Bgl*II, and the presence of the mutation was checked by sequencing. The cosmid was cotransfected with the adenovirus DNA of terminal protein complex in a 10-cm dish with calcium phosphate (CellPhect; Amersham Pharmacia Biotech) in human embryonic kidney (HEK 293) cells (17). Cell lysate from selected viral clones was analyzed by Western blot and probed with a LCPTI-specific antibody. The adenovirus was amplified, purified by CsCl ultracentrifugation, and carefully titrated using the Adeno-X Rapid Titer kit (Clontech Laboratories, Palo Alto, CA).

INS cell culture. The clonal β-cell line INS(832/13) (19), derived and selected from the parental rat insulinoma INS-1 (20), was cultured (passages 48–60) in a 5% CO_2 humidified atmosphere in complete medium that was composed of RPMI 1640 (Life Technologies, Grand Island, NY) that contained 11 mmol/l glucose and was supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Wisent), 10 mmol/l HEPES (pH 7.4), 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, 50 μmol/l 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. The maintenance culture was passaged once a week by gentle trypsinization, cells were seeded in Falcon dishes, and medium was changed every 2–3 days.

Pancreatic islet isolation. Islets were prepared from male Wistar rats that weighed 250–350 g by collagenase digestion and handpicking (21). At the end of the isolation step, islets were maintained in culture in regular RPMI 1640 medium that contained 11 mmol/l glucose supplemented with 10% (vol/vol) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere that contained 5% CO_2.

Viral treatment. INS(832/13) cells were seeded in 12-well plates (0.5×10^6 cells/well), 25-cm^2 flasks (2×10^6 cells), 10-cm dishes (7×10^6 cells), or 15-cm dishes (10×10^6 cells) and cultured 48 h before infection. For infection, cells were incubated for 90 min with complete RPMI medium that contained 4.1 pfu/cell Ad-LacZ, 1.7 pfu/cell Ad-LCPTI wt, or 4.1 pfu/cell Ad-LCPTI M953S. Cells were cultured for 24 h before experiments to allow the transgenes to be expressed before initiating metabolic studies or measurements of insulin secretion. One day after the isolation, batches of 100–200 islets were infected in 1 ml of RPMI 1640 medium for 1 h with $10–150 \times 10^4$ pfu/islet (22) of the recombinant adenoviruses and further cultured for 24 h before experiments were performed. At these incubation times, no toxicity effects were seen.

Western blot analysis. For detection of LCPTI protein, infected INS(832/13) cells or islets were resuspended directly in SDS sample buffer and sonicated. Proteins obtained from cell extracts were analyzed by SDS/PAGE (8% gels) and transferred onto nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Cell according to the manufacturer's instructions (Bio-Rad). Immunoblots were developed by incubation with the LCPTI-specific polyclonal antibody against amino acids 317–430 of the rat liver CPTI (23) (1:6,000 dilution) and the antirabbit IgG alkaline phosphatase goat antibody (1:10,000 dilution). Detection was carried out with the ECF immunoblotting detection system (Amersham Biosciences). For detection of PKC protein, cytosolic or membrane proteins of infected INS(832/13) cells were separated on an 8% SDS/PAGE gel and transferred to a nitrocellulose membrane as described above. Membranes were incubated with mouse anti-PKC monoclonal antibody (MC5, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with an antimouse IgG + IgM alkaline phosphatase goat antibody (1:10,000 dilution). Antibody binding was visualized using the ECF system as described above, and band intensities were quantified using a Storm 840 Laser scanning system (Molecular Dynamics, Amersham Pharmacia Biotech). Intensities of the spots were expressed as arbitrary optical units.

CPTI activity assay. INS(832/13) cells (10×10^6) were seeded in 15-cm dishes and cultured for 48 h before infection with the different adenoviruses. Twenty-four hours later, cells were pretreated as follows: cells were washed in Krebs-Ringer bicarbonate HEPES buffer (KRBH buffer; 135 mmol/l NaCl, 3.6 mmol/l KCl, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgSO_4$, 1.5 mmol/l $CaCl_2$, 2 mmol/l $NaHCO_3$, and 10 mmol/l HEPES [pH 7.4]) that contained 0.1% (wt/vol) defatted BSA, preincubated at 37°C for 30 min in KRBH plus 1% BSA without glucose in the absence or presence of etomoxir at 200 μmol/l, and washed again in KRBH 0.1% BSA. Mitochondrion-enriched cell fractions were obtained as previously described (17). LCPTI was assayed in preparations in which the mitochondria remained largely intact. CPTI activity in 8 μg of protein was determined by the radiometric method as previously described (24) with minor modifications. The substrates were L-[methyl-^{3}H]carnitine and palmitoyl-CoA. Enzyme activity was assayed for 5 min at 30°C in a total volume of 200 μl. For malonyl-CoA inhibition assays, 8 μg of mitochondrion-enriched cell fractions was preincubated for 1 min at 30°C with different amounts of malonyl-CoA before CPTI activity assay.

Fatty acid oxidation. Fatty acid oxidation to CO_2 and acid-soluble products (ASPs), essentially ketone bodies (25), were measured in INS(832/13) cells that were cultured in 25-cm^2 flasks. Cells were pretreated as described above and incubated for 2 h at 37°C with fresh KRBH that contained 2.5, 7.5, or 15 mmol/l glucose in the presence of 0.8 mmol/l carnitine plus 0.25 palmitate and 1 μCi/ml [1-^{14}C]palmitic acid complexed to 1% (wt/vol) BSA. Oxidation to CO_2 and ASPs were measured as previously described (16).

Fatty acid esterification. Fatty acid esterification to complex lipids was measured in INS(832/13) cells that were cultured in 12-well plates and pretreated as described above. Cells were incubated for 2 h at 37°C with fresh KRBH that contained 2.5 or 15 mmol/l glucose in the presence of 0.8 mmol/l carnitine plus 0.25 palmitate and 1 μCi/ml [1-^{14}C]palmitic acid complexed to 1% (wt/vol) BSA. Cells were washed in cold PBS, and lipids were extracted as described previously (17). Total lipids that were dissolved in 30 μl of chloroform were separated by thin-layer chromatography to measure the incorporation of labeled fatty acid into phospholipids, diacylglycerol (DAG), triacylglycerides, nonesterified labeled palmitate (NE palm), and cholesterol esters (CEs) described before (16). Phosphatidyl-serine, dipalmitoyl-glycerol, glyceryl tripalmitate, cholesteryl palmitate, and labeled control palmitate were

used as migration references. Plates were developed with hexane:diethyl-ether:acetic acid (70:30:1, vol/vol/vol) as described before (26) and quantified with a Storm 840 Laser scanning system (Molecular Dynamics; Amersham Pharmacia Biotech).

Triglyceride content. For the cellular triglyceride content measurement, INS(832/13) cells were cultured in 12-well plates and pretreated as described above. Total lipids were extracted as described previously (17) and dissolved in 30 μl of chloroform. Ten microliters of Thesit (Sigma-Aldrich) 20% (vol/vol) in chloroform was added, samples were air dried and dissolved in 50 μl of water, and triglyceride content was measured with triolein as a standard using the Sigma 337 triacylglycerol kit.

Malonyl-CoA assay. INS(832/13) cells were cultured in 10-cm dishes and pretreated as described above. Cells then were incubated for 30 min at 37°C with fresh KRBH that contained 2.5 or 15 mmol/l glucose. Malonyl-CoA was extracted as described previously (27) and assayed with a radioenzymatic method (28) using [1-^{14}C]acetyl-CoA. The fatty acid synthetase required for the assay was isolated from rat liver as described previously (29).

Insulin secretion. Insulin secretion was measured as previously described with minor changes (30). INS(832/13) cells (0.5×10^6) seeded in 12-well plates were pretreated as described above. Insulin secretion was measured during a 1-h incubation at 37°C in 1 ml of KRBH 0.1% BSA in the presence of 2.5 mmol/l glucose, 15 mmol/l glucose, 15 mmol/l glucose plus 0.25 mmol/l palmitate complexed to 1% (wt/vol) BSA, or 2.5 mmol/l glucose plus 30 mmol/l KCl. After this time, insulin content, obtained as described previously (17), was determined by radioimmunoassay using rat insulin standards with the Coated Tube Insulin RIA kit (Insulin-CT; Schering).

For studies of K_{ATP} channel–independent insulin secretion, assays were performed as described above except that 35 mmol/l KCl (depolarizing K^+) was included; consequently, the Na^+ concentration in the KRBH was reduced from 135 to 89.9 mmol/l to maintain osmolarity, and 250 μmol/l diazoxide was added.

Isolated rat islets were transduced with Ad-LCPTI wt, Ad-LCPTI M593S, or Ad-LacZ and used after 24 h for insulin secretion experiments. Batches of 10 islets each were incubated for 30 min in KRBH 0.1% BSA that contained 2.8 or 16.7 mmol/l glucose. Insulin was determined by radioimmunoassay (30).

PKC translocation assay. INS(832/13) cells (7×10^6) seeded in 10-cm dishes were infected and pretreated as described above. For promoting PKC translocation, cells were incubated for 30 min at 37°C in 2 ml of KRBH 0.1% BSA in the presence of 15 mmol/l glucose. For the control situation, cells were incubated at low (2.5 mmol/l) glucose. Then, cells were washed in cold PBS and used directly for preparation of membrane and cytosol fractions as described previously (12). Western blot of membrane and cytosol proteins was performed using a specific antibody (MC5; Santa Cruz Biotechnology) against the pancreatic β-cell isoforms of PKC (α, β, and γ) as described above. Band intensities were assessed using a Storm 840 Laser scanning system.

Glucose oxidation. Glucose oxidation to CO_2 was measured in INS(832/13) cells that were cultured in 25-cm^2 flasks, infected, and pretreated as described above. Cells were incubated for 2 h at 37°C with fresh KRBH 1% BSA that contained 2.5 or 15 mmol/l glucose in the presence of 0.5 μCi/ml [U-^{14}C]glucose. Oxidation to CO_2 was measured as previously described (16).

Statistical analysis. Data are expressed as means ± SE for at least four independent experiments performed in triplicate. Different experimental groups were compared with one-way ANOVA followed by Bonferroni's test for comparisons post hoc. A probability level of $P < 0.05$ was considered to be statistically significant.

RESULTS

CPTI activity is not inhibited by malonyl-CoA in INS(832/13) cells expressing LCPTI M593S. In a previous study, we documented a LCPTI M593S mutant that was insensitive to malonyl-CoA (18). The capacity of LCPTI M593S to show enzyme activity despite the presence of malonyl-CoA was evaluated in pancreatic β-cells. INS(832/13) cells were infected with Ad-LCPTI wt and Ad-LCPTI M593S. Mitochondrion-enriched fractions of cells were incubated with different amounts of malonyl-CoA, and CPTI activity assay was performed. In the presence of malonyl-CoA (100 μmol/l), mitochondrion-enriched fractions of cells that were infected with Ad-LCPTI M593S retained 80% of their activity, whereas that of the LCPTI wt was almost completely inhibited (Fig. 1).

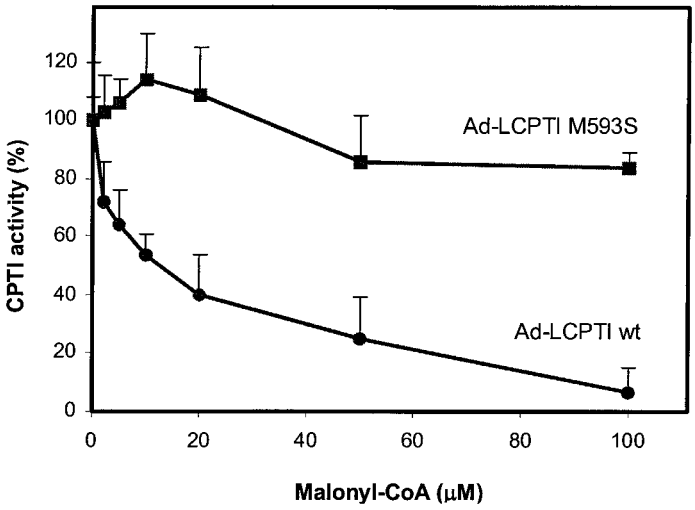

FIG. 1. CPTI activity of INS(832/13) cells that were infected with Ad-LCPTI M593S is insensitive to malonyl-CoA. INS(832/13) cells were infected with Ad-LCPTI wt or Ad-LCPTI M593S, and 24 h later, CPTI activity assay was performed with 8 μg of mitochondrion-enriched cell fractions that were incubated with different amounts of malonyl-CoA. Data are the mean ± SE of six experiments.

CPTI protein and activity in INS(832/13) cells that were infected with Ad-LCPTI wt and Ad-LCPTI M593S. INS(832/13) cells were infected with different amounts of Ad-LCPTI wt (Fig. 2*A*) and Ad-LCPTI M593S (Fig. 2*B*). CPTI activity assay was performed with mitochondrion-enriched cell fractions. In both cases, CPTI activity increased to a plateau of 9- to 10-fold compared with the endogenous LCPTI, calculated from Ad-LacZ–infected cells (5.0 ± 1.8 nmol · mg protein^{-1} · min^{-1}, mean ± SE of six experiments). For subsequent experiments, we used the amount of adenovirus LCPTI wt (1.7 pfu/cell) and LCPTI M593S (4.1 pfu/cell) that increased CPTI activity sixfold (30 nmol · mg protein^{-1} · min^{-1}) with respect to the control Ad-LacZ. In an additional experiment, as a control, cells were also incubated for 30 min with or without 200 μmol/l etomoxir. This irreversible CPTI inhibitor blocked CPTI activity in Ad-LacZ– and Ad-LCPTI wt–infected cells but left 65% CPTI activity in Ad-LCPTI M593S–infected cells (Fig. 2*C*). LCPTI protein expression was measured by Western blot. Total protein was isolated from INS(832/13) cells that were infected with the amounts of adenoviruses that gave a sixfold increase in CPTI activity. Consistent with the activity assays, Western blots showed similar amounts of protein in Ad-LCPTI wt– and Ad-LCPTI M593S–infected cells (Fig. 2*D*) and fold increase over the control situation (Ad-LacZ). Similar results were obtained in Western blots that were performed with infected rat islets (Fig. 2*E*). Thus, expression of LCPTI wt and LCPTI M593S in the β-cell was successful in markedly increasing the protein and enzymatic activity of CPTI.

Effect of LCPTI M593S expression on palmitate oxidation in INS(832/13) cells. To evaluate the metabolic effects of the Ad-LCPTI wt and Ad-LCPTI M593S constructs, we measured fatty acid oxidation in pancreatic β-cells. [1-^{14}C]palmitate oxidation was determined in adenovirus-treated INS(832/13) cells. Fatty acid oxidation was highest in cells that were infected with Ad-LCPTI M593S at all tested glucose concentrations (Fig. 3). At high glucose

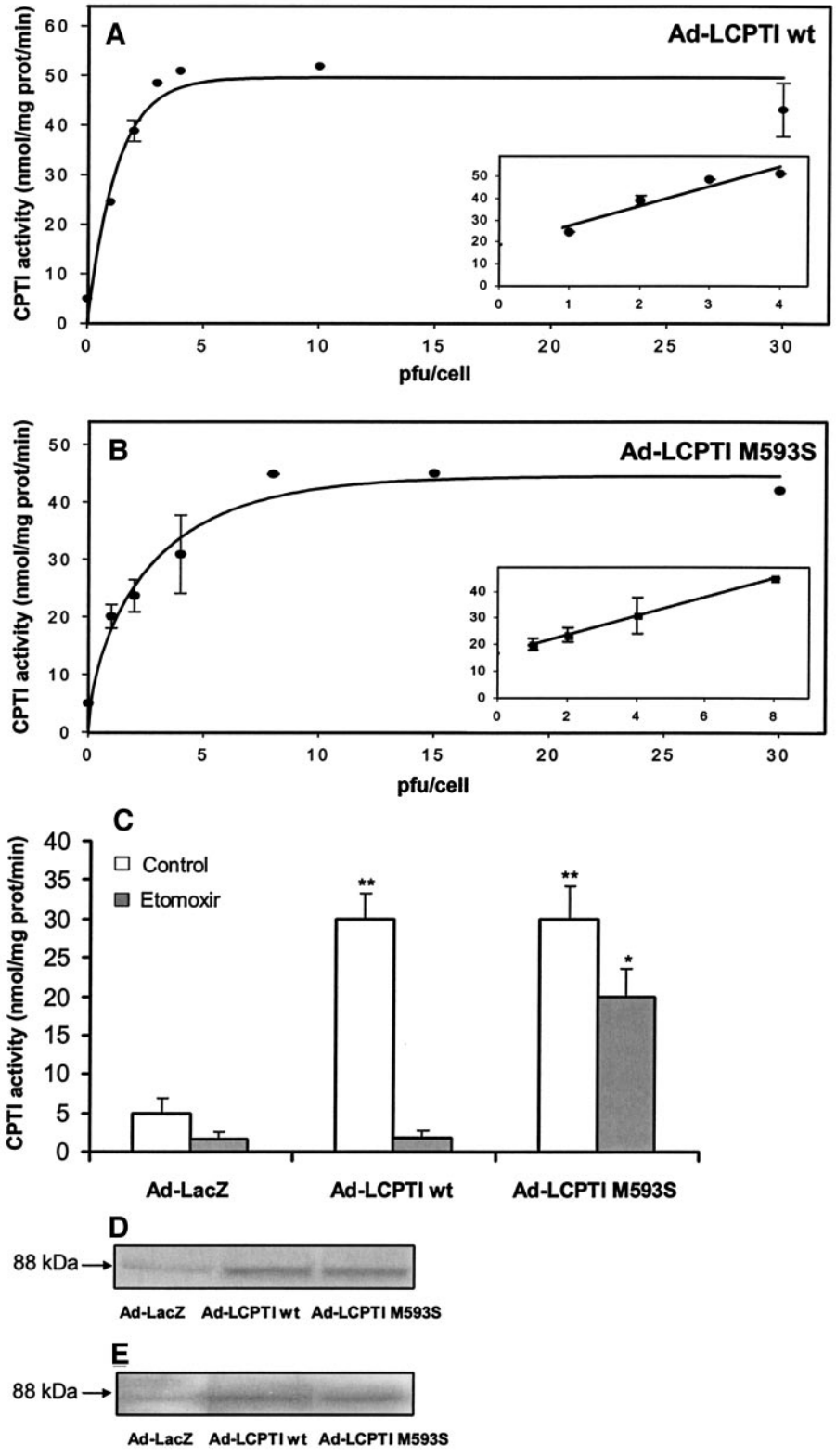

FIG. 2. CPTI activity and immunoblot analysis of LCPTI expressed in infected INS(832/13) cells and rat islets. INS(832/13) cells were infected with different pfu/cell of Ad-LCPTI wt (*A*) or Ad-LCPTI M593S (*B*); 24 h later, mitochondrion-enriched cell fractions were obtained and 8 μg of protein was used for the CPTI activity assay. The amount of both viruses that increased CPTI activity sixfold, compared with Ad-LacZ (0 pfu/cell), were chosen for further experiments (1.7 pfu/cell for Ad-LCPTI wt and 4.1 pfu/cell for Ad-LCPTI M593S). *Insert*: Expanded dose-response curve. *C*: INS(832/13) cells that were infected with Ad-LacZ, Ad-LCPTI wt, or Ad-LCPTI M593S were incubated for 30 min in KRBH 1% BSA with or without etomoxir (200 μmol/l). After that, mitochondrion-enriched cell fractions were obtained and CPTI activity assay was performed. *D*: INS(832/13) cells that were infected with Ad-LacZ, Ad-LCPTI wt, or Ad-LCPTI M593S were collected, and protein extracts were separated by SDS/PAGE (8% gels) and subjected to immunoblotting by using specific antibodies for CPTI from liver. A unique band corresponding to a protein of ~88 kDa was seen in control and in Ad-LCPTI wt– and Ad-LCPTI M593S–infected cells. *E*: Rat islets (batches of 100–200) were infected with the different adenoviruses as described in RESEARCH DESIGN AND METHODS, and 24 h later, LCPTI protein expression was determined by Western blot. Data in *A*, *B*, and *C* are the mean ± SE of four experiments. **P* < 0.05 vs. Ad-LCPTI M593S without etomoxir; ***P* < 0.001 vs. Ad-LacZ.

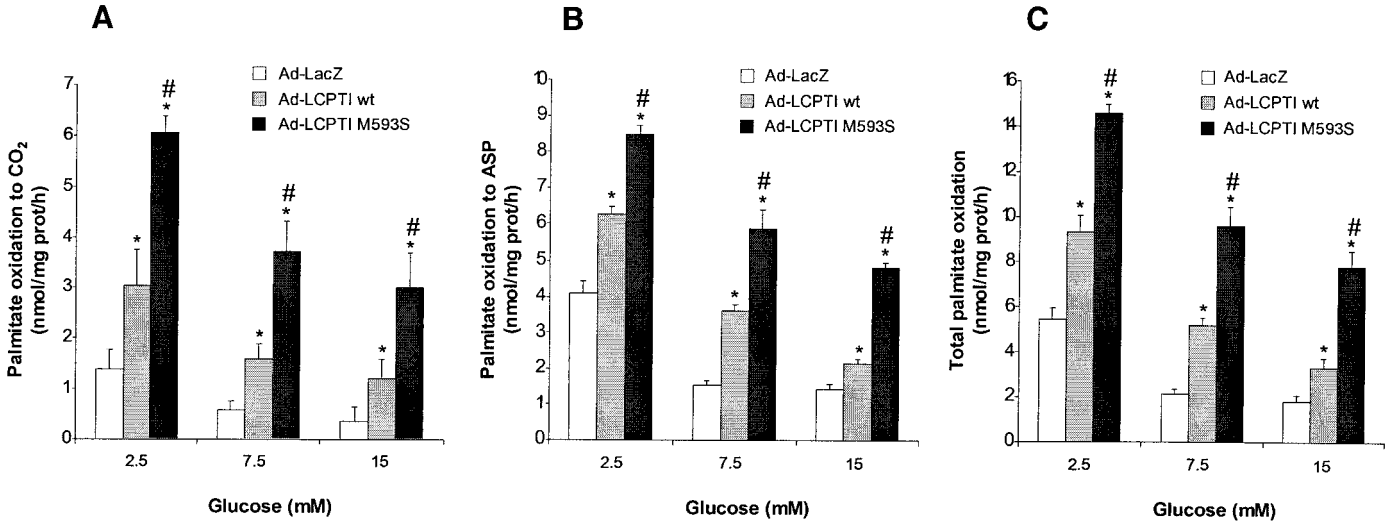

FIG. 3. Fatty acid oxidation is increased in INS(832/13) cells expressing LCPTI M593S. INS(832/13) were infected with Ad-LacZ, Ad-LCPTI wt, or Ad-LCPTI M593S. Twenty-four hours after viral treatment, cells were preincubated for 30 min at 37°C in KRBH medium that contained 1% BSA and then incubated for 2 h at 2.5, 7.5, or 15 mmol/l glucose in the presence of 0.8 mmol/l carnitine, 1 μCi/ml [1-^{14}C]palmitic acid, and 0.25 mmol/l unlabeled palmitate complexed to 1% (wt/vol) BSA. Palmitate oxidation to CO_2 (*A*), ASP (*B*), and total palmitate oxidation (CO_2 + ASP; *C*) was measured as described in RESEARCH DESIGN AND METHODS. Data are the mean ± SE of five experiments. *$P < 0.05$ vs. Ad-LacZ; #$P < 0.05$ vs. Ad-LCPTI wt.

(15 mmol/l), fatty acid oxidation to CO_2, ASP, and their sum was increased by 8.3-, 3.4-, and 4.4-fold, respectively, compared with Ad-LacZ–infected cells. These oxidation rates were higher than those obtained when overexpression was performed with Ad-LCPTI wt (3.3-, 1.5-, and 1.8-fold, respectively). Malonyl-CoA levels were not modified after LCPTI wt or LCPTI M593S overexpression, compared with LacZ control cells, either at 2.5 or 15 mmol/l glucose (Fig. 4*A*). These findings confirm that even at high malonyl-CoA concentrations (high glucose), fatty acid oxidation in LCPTI M593S–overexpressing cells is much higher than in the control Ad-LacZ adenovirus–infected cells and still higher than in cells that were infected with Ad-LCPTI wt. Because LCPTI M593 is resistant to malonyl-CoA, β oxidation to CO_2 and ketone bodies (acid-soluble compounds) is accelerated in cells overexpressing the mutated enzyme. Glucose oxidation at low (2.5 mmol/l) and high (15 mmol/l) glucose remained unaltered in cells expressing both the wt and mutated LCPTI constructs (Fig. 4*B*). This is in accordance with the view that a Randle cycle is inoperative in the β-cell (31).

GSIS is reduced in INS(832/13) cells and rat islets expressing LCPTI M593S. GSIS was reduced by 60% in INS(832/13) cells that were infected with Ad-LCPTI M593S and by 40% in Ad-LCPTI wt–infected cells, both with respect to the control Ad-LacZ (Fig. 5*A*). GSIS was recovered completely in the presence of 0.25 mmol/l palmitate only in the case of LCPTI wt, suggesting that at high glucose, the provision of exogenous free fatty acid (FFA) was not matched with a rise in intracellular FFA (NE palm) in cells overexpressing the mutated enzyme as a result of the dramatically enhanced fatty acid oxidation. The results in Fig. 6*D* are in total accordance with this view.

As a control of the insulin secretion mechanism, incubation at low glucose in the presence of 30 mmol/l KCl was performed and insulin release was similar in the three cases of adenovirus-infected cells, indicating that the exocytotic machinery is preserved in cells that were transduced with the various constructs. Insulin secretion was also studied in the presence of 35 mmol/l K^+ plus 250 μmol/l diazoxide, a condition revealing the K_{ATP} channel–independent pathway of glucose sensing (32). The expression of LCPTI wt and LCPTI M593S led to a decrease in insulin release (the difference between 15 and 2.5 mmol/l glucose) of 55 ± 12% and 85 ± 17% ($n = 3$), respectively, compared with Ad-LacZ–infected cells. This is consistent with the view that K_{ATP}-independent signaling (also referred to as the amplification pathway) was affected in cells overexpressing the LCPTI construct (Fig. 5*B*).

Isolated rat islets that were infected with Ad-LCPTI wt or Ad-LCPTI M593S showed increased levels of LCPTI protein in Western blot analysis compared with Ad-LacZ–infected islets (Fig. 2*C*). In control islets, raising glucose from 2.8 to 16.7 mmol/l resulted in a 6.8-fold insulin secretion. In islets overexpressing LCPTI M593S, GSIS was decreased by 40% compared with the Ad-LacZ control, whereas no difference was seen with Ad-LCPTI wt–infected islets (Fig. 5*C*). To explain the difference in the results between INS(832/13) cells and islets, we hypothesize that normal β-cells are more protected than INS cells against lipid depletion as a result of enhanced fat oxidation, such that only the more efficient LCPTI M593S construct is active in rat islets.

Effect of LCPTI M593S on glucose-induced changes in lipid partitioning. To examine whether the increase in fatty acid oxidation could reduce the availability of LC-CoA for lipid signaling, we measured esterification processes in INS(832/13) cells that were infected with Ad-LCPTI wt and Ad-LCPTI M593S. The levels of [1-^{14}C] palmitate incorporated to phospholipids, DAG, triacylglycerides, CEs, and the levels of NE palm were measured. Glucose-induced palmitate esterification into the different complex lipid species was expressed as the differences (ΔG) between high (15 mmol/l) and low (2.5 mmol/l) glucose (Fig. 6). The glucose-induced rise in

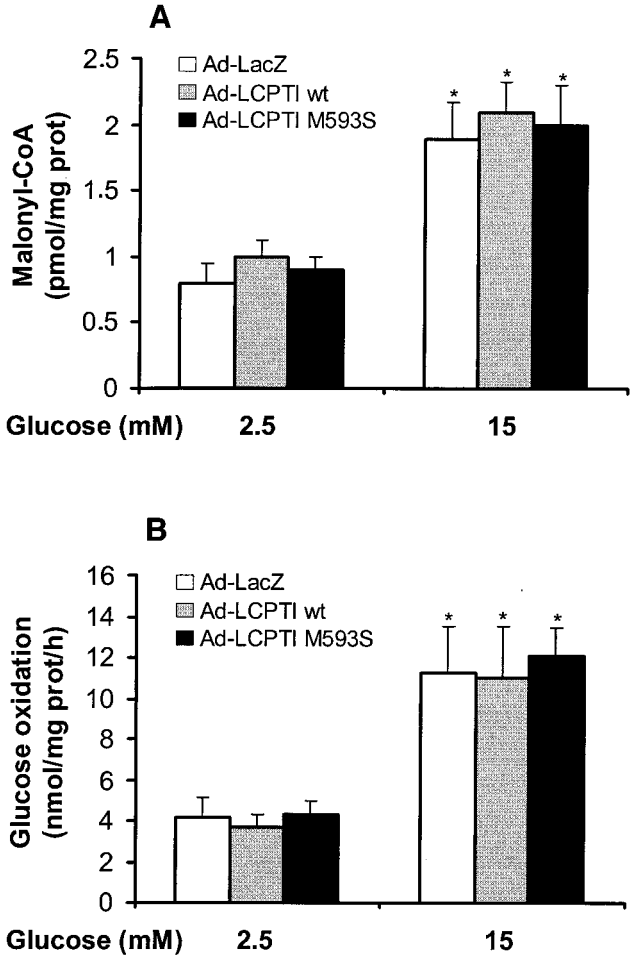

FIG. 4. Malonyl-CoA content and glucose oxidation are unaltered in INS(832/13) cells expressing LCPTI M593S. INS(832/13) cells were infected with Ad-LacZ, Ad-LCPTI wt, or Ad-LCPTI M593S, and 24 h later, they were preincubated for 30 min at 37°C in KRBH medium that contained 1% BSA. *A*: For the malonyl-CoA content measurements, cells were incubated for 30 min with KRBH that contained 2.5 or 15 mmol/l glucose and malonyl-CoA was extracted and assayed with a radioactive method using purified fatty acid synthetase as detailed in RESEARCH DESIGN AND METHODS. *B*: Glucose oxidation to CO_2 was measured after a 2-h incubation of the cells at 2.5 or 15 mmol/l glucose in the presence of 0.5 μCi/ml [U-^{14}C]glucose as described in RESEARCH DESIGN AND METHODS. Data are the mean ± SE of four experiments. *P < 0.05 vs. 2.5 mmol/l glucose.

phospholipids, DAG, triacylglycerides, and NE palm was decreased by 20, 25, 35, and 38%, respectively, for Ad-LCPTI wt–infected cells and by 37, 58, 59, and 71%, respectively, for Ad-LCPTI M593S–infected cells (Fig. 6*A*–*D*), both compared with Ad-LacZ–infected cells. Palmitate esterification to CEs was reduced by 23 and 50% for Ad-LCPTI wt and Ad-LCPTI M593S, respectively, at low glucose and by 42 and 60% for Ad-LCPTI wt and Ad-LCPTI M593S, respectively, at high glucose, compared with the Ad-LacZ control (Fig. 6*E*). Figure 6*D* also shows that the glucose-induced rise in NE palm, an indirect measurement of cytosolic LC-CoA (16), was markedly curtailed in cells expressing the LCPTI construct and that the CPTI mutant was most effective in this respect. Changes in the cellular triacylglyceride content were not observed by the expression of LCPTI wt or LCPTI M593S (Fig. 6*F*). This is probably because differences in the incorporation of LC-CoA into triacylglycerides are not reflected in the large and stable total triacylglyceride pool.

PKC activation is impaired in INS(832/13) cells expressing LCPTI M593S. DAG and LC-CoA activate many PKC enzymes (11,12). To assess whether the decrease of the glucose-induced rise in DAG and NE palm seen in Ad-LCPTI wt– and Ad-LCPTI M593S–infected cells is associated with alteration in PKC activity in the β-cell, we measured PKC activation as estimated by its translocation from cytosol to membranes. Western blot carried out on membrane and cytosol proteins of INS(832/13) cells that were incubated at high (15 mmol/l) glucose showed that PKC translocation of Ad-LCPTI wt– and Ad-LCPTI M593S–infected cells was decreased by 63 and 78%, respectively, in comparison with Ad-LacZ–infected cells. No difference in the partitioning of PKC enzyme between the cytosolic and membrane compartments was noted in Ad-LacZ–, Ad-LCPTI wt–, and Ad-LCPTI M593S–infected cells that were incubated at low glucose (2.5 mmol/l; Fig. 7).

DISCUSSION

Many studies implicate NEFAs in type 2 diabetes (33,34). Long-term exposure of β-cells to NEFAs in vitro has several effects: *1*) it increases basal insulin release and decreases secretion in response to glucose (35); *2*) it alters the coupling of glucose metabolism to insulin secretion by acting on the expression of specific genes, such as UCP2 (36,37); and *3*) it increases the expression of CPTI, which is considered the rate-limiting step in fatty acid oxidation (38). CPTI upregulation after chronic NEFA exposure of the β-cell may contribute to reduced GSIS. In an earlier study, we evaluated the capacity of LCPTI wt overexpression to alter the insulin response to glucose in β-cells (17). The results showed that overexpression of a cDNA encoding LCPTI using an adenovirus not only increased β-oxidation in INS-1E cells but also decreased GSIS by 40%. In the search for a malonyl-CoA–insensitive LCPTI, we found that the M593S LCPTI mutant was almost completely refractory to malonyl-CoA (18). In view of the interest in the malonyl-CoA/LC-CoA model of GSIS, which is still under discussion (27,39), we directly tested the hypothesis that the malonyl-CoA/CPTI interaction is implicated in GSIS using a β-cell–derived cell line overexpressing the malonyl-CoA–insensitive LCPTI. Because liver CPTI is the only isoform present in the β-cell, we used the rat liver isoform of CPTI to construct the adenovirus.

Overexpression of the malonyl-CoA–insensitive form of LCPTI increased fatty acid oxidation rates in INS(832/13) at all glucose concentrations. Fat oxidation rates were much higher than in control Ad-LacZ–infected cells or in cells overexpressing malonyl-CoA–sensitive LCPTI wt. INS(832/13) cells overexpressing LCPTI M593S secreted less insulin in response to high glucose concentration (~60% reduction) but not in response to a depolarizing concentration of KCl. Thus, exocytosis per se was preserved in cells overexpressing the mutated LCPTI, because the effect of the Ca^{2+}-raising agent (30 mmol/l KCl) was unaltered. The use of elevated K^+ and diazoxide to discern between the K_{ATP} channel–dependent and –independent pathways of glucose sensing showed that LCPTI M593S overexpression affected the K_{ATP}-independent pathway of GSIS, thus providing direct support for the view that the malonyl-CoA/CPTI interaction is involved in the amplification arm of secretion. In isolated rat islets,

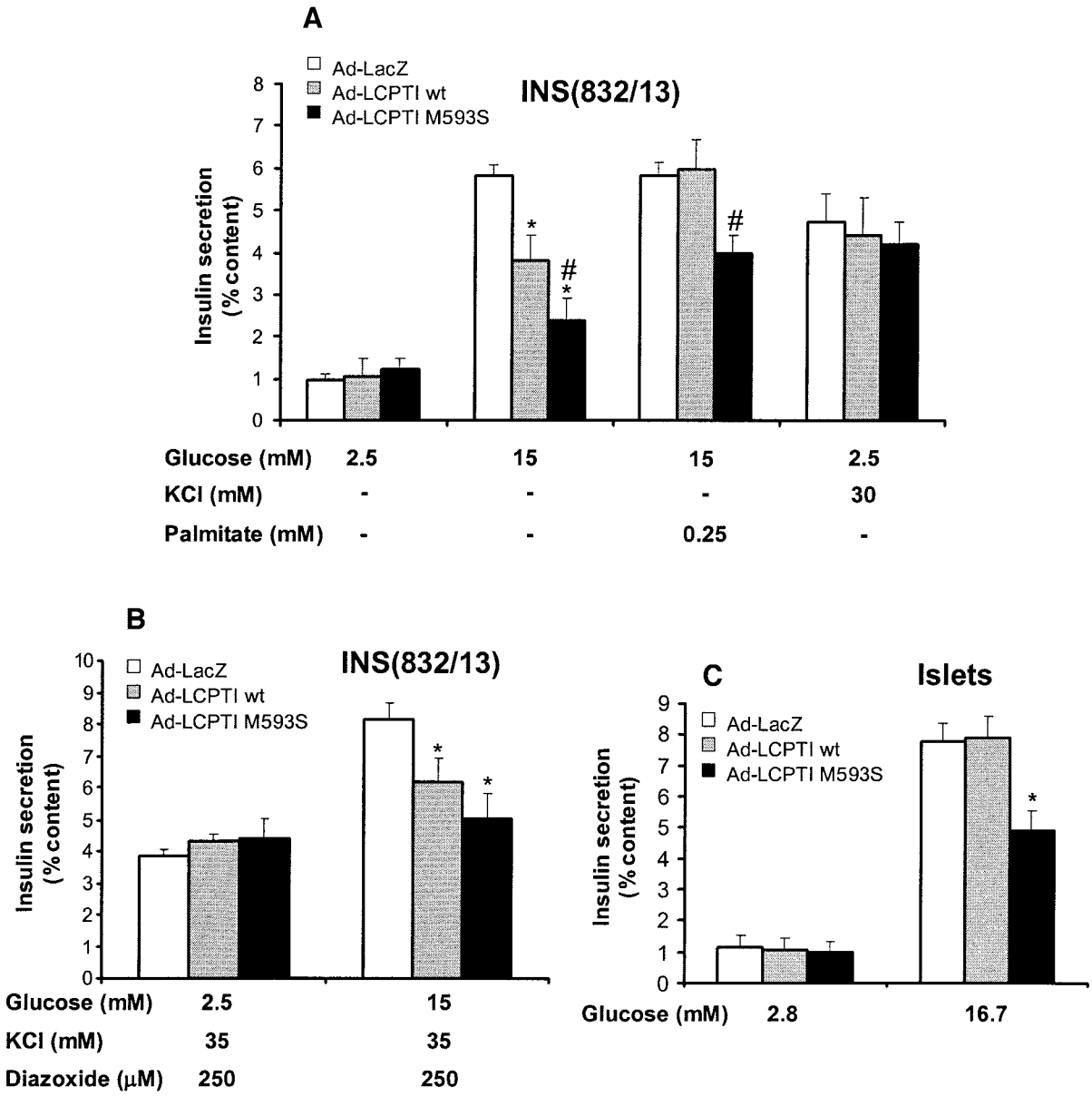

FIG. 5. Glucose-induced insulin release is impaired in INS(832/13) cells and in isolated rat islets expressing LCPTI M593S. INS(832/13) cells or rat islets were infected with Ad-LacZ, Ad-LCPTI wt, or Ad-LCPTI M593S as detailed in RESEARCH DESIGN AND METHODS. *A*: After a 30-min preincubation in KRBH 1% BSA without glucose, INS(832/13) cells were washed and incubated for 1 h in KRBH 0.1% BSA that contained 2.5 mmol/l glucose, 15 mmol/l glucose, 15 mmol/l glucose plus 0.25 mmol/l palmitate complexed to 1% (wt/vol) BSA, or 2.5 mmol/l glucose plus 30 mmol/l KCl. Insulin release was determined by radioimmunoassay. *B*: Experiments conducted in the presence of depolarizing K^+ (35 mmol/l) and 250 μmol/l diazoxide to measure K_{ATP} channel–independent glucose sensing. *C*: Batches of 10 islets each were washed and incubated for 30 min in KRBH 0.1% BSA that contained 2.8 or 16.7 mmol/l glucose, and insulin release was determined by radioimmunoassay. Data are the mean ± SE of four experiments. *$P < 0.05$ vs. Ad-LacZ; #$P < 0.05$ vs. Ad-LCPTI wt.

similar results were obtained, i.e., reduced secretory responses to high glucose upon LCPTI M593S expression. It is interesting that overexpression of LCPTI wt did not affect GSIS in islets, suggesting preserved lipid signaling above a critical threshold of cytosolic LC-CoA.

Incubation of LCPTI M593S–transduced cells with 0.25 mmol/l palmitate did not completely restore GSIS, showing the strong metabolic influence of LCPTI M593S on fatty acid oxidation and insulin secretion in the pancreatic β-cell. This points to a possible mechanism by which increased metabolic flux through LCPTI diminishes insulin secretion via the depletion of a critical lipid synthesized at or near the mitochondrial outer membrane that would act as a signal molecule.

It is interesting to compare the present results with those of Roduit et al. (16), who overexpressed MCD in the cytosol (MCDc) by infecting INS(832/13) cells and rat islets with an adenovirus that contained the cDNA for MCD devoid of its mitochondrial and peroxisomal targeting sequences. The authors found that MCDc overexpression in the absence of exogenous FFAs had no effect on GSIS and that MCDc overexpression suppressed the additional secretion in response to glucose provided by the presence of exogenous FFAs (16). In the present study, exogenous fatty acids had no effect on GSIS at 15 mmol/l glucose, perhaps because we used a relatively low concentration of fatty acids (0.25 mmol/l bound to 1% BSA) or possibly because lipolysis was higher in the current study.

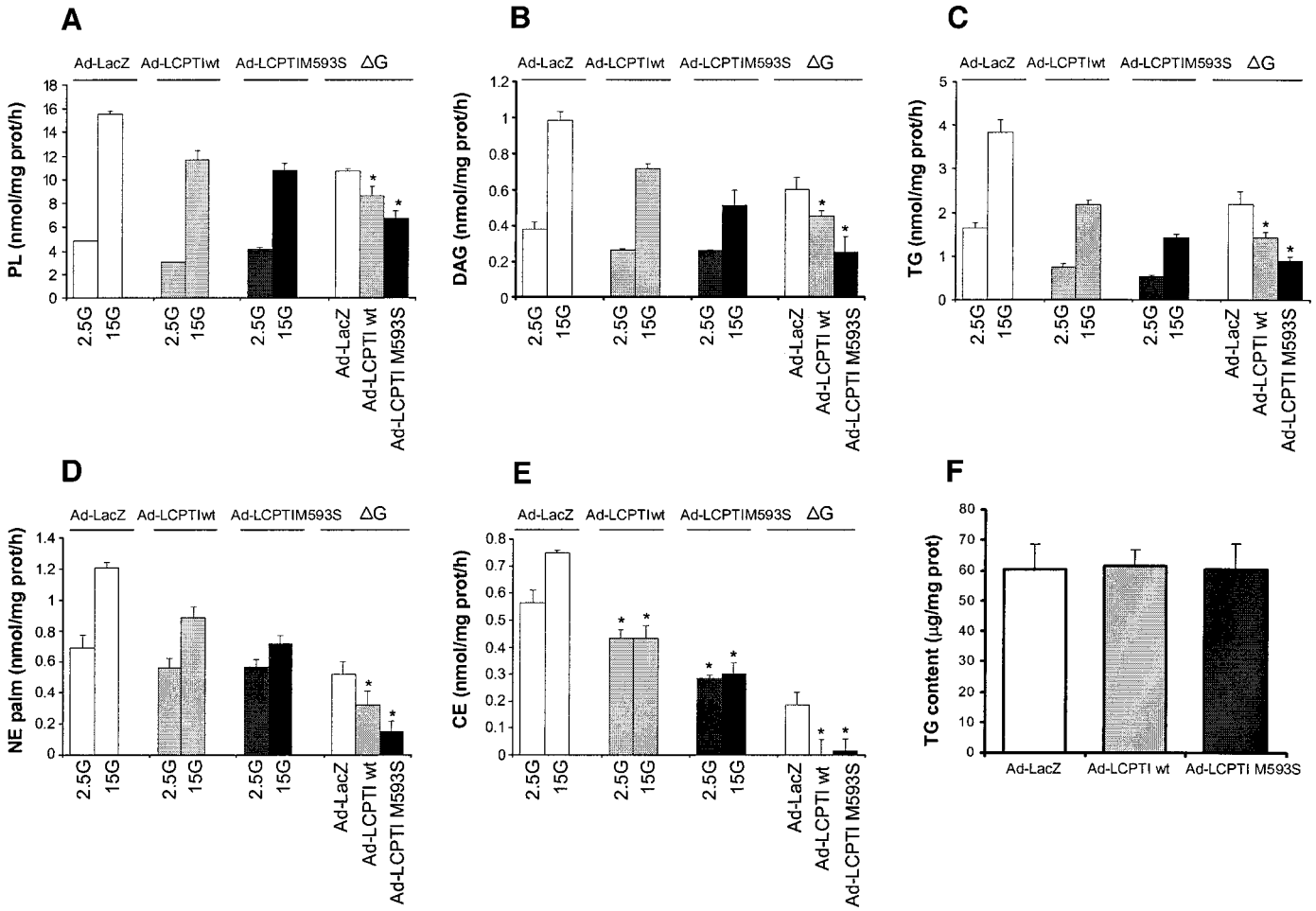

FIG. 6. Glucose-induced fatty acid esterification processes are reduced in INS(832/13) cells expressing LCPTI. INS(832/13) cells that were infected with Ad-LacZ, Ad-LCPTI wt, or Ad-LCPTI M593S were preincubated for 30 min at 37°C in KRBH medium that contained 1% BSA and then incubated for 2 h at 2.5 or 15 mmol/l glucose in the presence of 0.8 mmol/l carnitine, 1 μCi/ml [1-^{14}C]palmitic acid, and 0.25 mmol/l unlabeled palmitate complexed to 1% (wt/vol) BSA. Fatty acid esterification into phospholipids (*A*), DAG (*B*), triacylglycerides (*C*), NE palm (*D*), and CEs (*E*) was assessed using thin-layer chromatography after lipid extraction. ΔG is the difference between the incorporation of palmitate in the particular lipid classes at 15 vs. 2.5 mmol/l glucose. *F*: Total triglyceride content. Data are the mean ± SE of four experiments. **P* < 0.05 vs. Ad-LacZ.

Nonetheless, it is interesting to note that altering β-cell lipid partitioning with the malonyl-CoA–insensitive mutant of LCPTI had a more profound consequence than with the overexpression of cytosolic MCD on both GSIS and lipid partitioning with more enhanced fat oxidation and reduced esterification processes at both low and elevated glucose. In other studies (27), the lack of correlation between malonyl-CoA levels and β-oxidation has been remarked on, suggesting that glucose might regulate cytosolic LC-CoA in a manner not entirely dependent of malonyl-CoA. Whatever is the explanation for the differences in alterations in GSIS in the absence or presence of exogenous FFAs when MCDc (16) or CPTI (present study) are overexpressed, both studies show a reduction of GSIS when the malonyl-CoA/CPTI interaction is perturbed.

The data of the current study in cells overexpressing a malonyl-CoA–insensitive CPTI together with previous results obtained in INS cells or islets overexpressing MCDc in the cytoplasm provide evidence that malonyl-CoA acts as a glucose-driven coupling factor that regulates the partitioning of fatty acids into effector molecules in the insulin secretory pathway. The nature of these molecules (NE palm, LC-CoA, phospholipids, and/or DAG) and their mechanisms of action on insulin secretion are poorly understood. Overexpression of LCPTI M593S altered lipid partitioning of exogenous palmitate from oxidation into esterification products at high glucose. The incorporation of palmitate into phospholipids, DAG, triacylglycerides, NE palm, and CEs was reduced compared with controls. Determination of PKC translocation from cytosol to membrane showed that PKC activation in Ad-LCPTI wt– and Ad-LCPTI M593S–infected cells was decreased compared with Ad-LacZ–infected cells. This suggests that enhanced β oxidation of fatty acids by the LCPTI M593S mutant reduces GSIS as a result of a decrease in PKC activation. The lower values of DAG and NE palm (an indirect determination of LC-CoA levels) observed in the LCPTI M593S–overexpressing cells support this view because both DAG and LC-CoA activate a number of PKC enzymes (11,12).

In conclusion, this study shows directly for the first time that when the malonyl-CoA/CPTI interaction is altered, glucose-induced insulin release is impaired. Together with the results obtained in previous studies in which MCDc

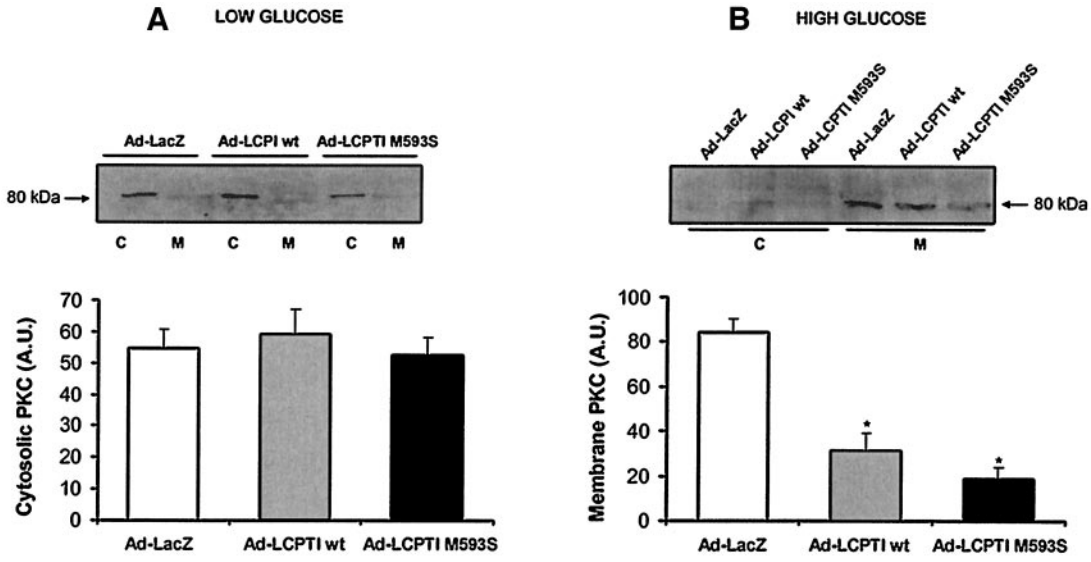

FIG. 7. PKC activation is impaired in Ad-LCPTI wt– and Ad-LCPTI M593S–infected cells. Ad-LacZ–, Ad-LCPTI wt–, and Ad-LCPTI M593S INS(832/13)–infected cells were incubated for 30 min at low (2.5 mmol/l; *A*) or high (15 mmol/l; *B*) glucose. Western blot of membrane (M) and cytosolic (C) fractions was performed using a specific antibody against the PKC isoforms expressed in pancreatic β-cells (α, β, and γ), giving a unique band of 80 kDa. The intensity of the PKC bands was quantified using a Laser scanning system. The lower panels in *A* and *B* show mean results ± SE of three experiments. A.U., arbitrary optical units. **P* < 0.05 vs. Ad-LacZ.

was overexpressed (16) or ACC expression was reduced (15), the data point to a critical role of the malonyl-CoA/CPTI metabolic signaling network in the coupling mechanism of insulin secretion. These results also favor the hypothesis that upregulation of CPTI (33) contributes to the early loss of glucose responsiveness seen in β-cells that are chronically exposed to high concentrations of fatty acids and provide direct support for the malonyl-CoA/LC-CoA model of fuel-induced insulin secretion.

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