

A Role for the Malonyl-CoA/Long-Chain Acyl-CoA Pathway of Lipid Signaling in the Regulation of Insulin Secretion in Response to Both Fuel and Nonfuel Stimuli

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The malonyl-CoA/long-chain acyl-CoA (LC-CoA) model of glucose-induced insulin secretion (GIIS) predicts that malonyl-CoA derived from glucose metabolism inhibits fatty acid oxidation, thereby increasing the availability of LC-CoA for lipid signaling to cellular processes involved in exocytosis. For directly testing the model, INSr3 cell clones overexpressing malonyl-CoA decarboxylase in the cytosol (MCDc) in a tetracycline regulatable manner were generated, and INS(832/13) and rat islets were infected with MCDc-expressing adenoviruses. MCD activity was increased more than fivefold, and the malonyl-CoA content was markedly diminished. This was associated with enhanced fat oxidation at high glucose, a suppression of the glucose-induced increase in cellular free fatty acid (FFA) content, and reduced partitioning at elevated glucose of exogenous palmitate into lipid esterification products. MCDc overexpression, in the presence of exogenous FFAs but not in their absence, reduced GIIS in all β -cell lines and in rat islets. It also markedly curtailed the stimulation of insulin secretion by other fuel and nonfuel secretagogues. In the absence of MCDc overexpression, the secretory responses to all types of secretagogues were amplified by the provision of exogenous fatty acids. In the presence of exogenous FFAs, the fatty acyl-CoA synthetase inhibitor triacsin C reduced secretion in response to glucose and nonfuel stimuli. The data show the existence of important links

between the metabolic coupling factor malonyl-CoA, the partitioning of fatty acids, and the stimulation of insulin secretion to both fuel and nonfuel stimuli. *Diabetes* 53: 1007–1019, 2004

Although glucose is the most potent nutrient secretagogue for the islet β -cell, its effectiveness as such can be markedly modulated by other fuel stimuli, hormones, and neurotransmitters (1–3). Of the nutrient modulators, fatty acids are particularly important as, at one extreme, islet fatty acid deprivation essentially abolishes glucose-induced insulin secretion (GIIS) (4–7), whereas at the other extreme, acutely elevated fatty acid levels augment GIIS (6,8,9). Elevated circulating free fatty acids (FFAs) most probably contribute to islet β -cell compensation in insulin-resistant states (10–13) but if chronically elevated, particularly in association with high glucose (14), cause β -cell failure via diverse mechanisms including induction of β -cell apoptosis, reduced GIIS, and insulin biosynthesis resulting in type 2 diabetes (10–16). The mechanisms by which fatty acids acutely modulate GIIS are poorly understood, although there is evidence to support a role for their intracellular metabolism with the production of lipid signaling molecules such as the C-kinase activator diacylglycerol (DAG) (17,18).

It is widely accepted that glucose metabolism is essential for coupling to occur between glucose sensing by the β -cell and insulin release (3,19,20). It is also widely accepted that an increase in the cytosolic ATP-to-ADP ratio, as a result of glucose metabolism, is an important event in this process (3,21,22). An increase in this ratio closes ATP-sensitive K^+ (K^+_{ATP}) channels, causing depolarization of the plasma membrane. This in turn opens voltage-dependent Ca^{2+} channels, causing Ca^{2+} influx and Ca^{2+} -induced exocytosis of insulin secretory granules. An abundance of pharmacological evidence also indicates the existence of K^+_{ATP} -independent pathways in nutrient-induced secretion (23–25), but less understood are the metabolism-derived coupling factors involved in this process (3,20,22,26).

We have proposed a model of β -cell nutrient sensing and insulin secretion in which, in addition to the K^+_{ATP} -dependent pathway, a parallel anaplerotic/lipid signaling

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ACS, acyl-CoA synthetase; ASP, acid-soluble products; CPT-1, carnitine-palmitoyltransferase 1; DAG, diacylglycerol; FFA, free fatty acid; GIIS, glucose-induced insulin secretion; GLP-1, glucagon-like peptide 1; K^+_{ATP} , ATP-dependent K^+ channels; LC-CoA, long-chain acyl-CoA; MCD, malonyl-CoA decarboxylase; MCDc, cytosolic MCD; NEpal, nonesterified-labeled palmitate; PKC, protein kinase C; PMA, phorbol-5-myristate 13-acetate.

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pathway exists wherein malonyl-CoA acts as a coupling factor (3,13,26). A glucose-induced rise in β -cell cytosolic malonyl-CoA, acting via inhibition of carnitine-palmitoyl-transferase 1 (CPT-1), reduces fatty acid oxidation, thereby increasing cytosolic long-chain acyl-CoA (LC-CoA) levels, which signal directly or indirectly via fatty acid esterification and/or protein acylation processes to augment insulin release (26). In this model, the effectiveness of malonyl-CoA in causing insulin release would depend both on a cytosolic Ca^{2+} rise caused by the K^{+} -ATP-dependent pathway and the prevailing availability of fatty acids to the β -cell (3,13,25,27).

Many predictions that are based on this "malonyl-CoA/LC-CoA" model have been verified by us and others. For instance, glucose, via anaplerosis, elevates citrate, the precursor for malonyl-CoA production (28–30). Citrate oscillation in response to glucose has also been reported in INS-1 cells (31). Importantly, malonyl-CoA levels increase rapidly in response to glucose, and this precedes glucose-stimulated insulin secretion in β -cells (26,27,32). Elevated glucose causes an inhibition of fatty acid oxidation (33–35) and an increase in esterified lipids such as DAG (26,27,36,37). Studies in which pharmacological and molecular tools have been used to investigate this model, however, have been inconsistent in their conclusions, as has been reviewed (12). Whereas the majority of these studies are supportive (26,34,38–43), at variance with the hypothesis are two studies (44,45). In one of these, to lower malonyl-CoA levels, malonyl-CoA decarboxylase (MCD) was overexpressed in the cytoplasm (MCDc) of INS 832/13 cells (45) and no effect on GIIS was found. Furthermore, triacsin C, a pharmacological inhibitor of acyl-CoA synthetase (ACS), which lowers LC-CoA levels, also failed to alter insulin secretion (44,45). Of note, these two studies (44,45) were performed mainly in the absence of exogenous FFAs, as is also true for the majority of the previous supportive studies.

As already alluded to, the malonyl-CoA/LC-CoA model predicts that the effectiveness of malonyl-CoA to couple nutrient sensing to insulin secretion will be dependent on the availability of FFAs to the β -cell. This very important component of the model, however, has not yet been well addressed. Thus, we further examined the malonyl-CoA/LC-CoA hypothesis with particular emphasis on the role of FFA supply. To this end, we overexpressed MCDc (to lower malonyl-CoA levels) and used triacsin C (to pharmacologically lower LC-CoA levels) in various INS cell clones and isolated rat islets. Overexpression of MCDc in INS cells and isolated islets markedly reduced malonyl-CoA content and changed lipid partitioning in terms of increasing fatty acid oxidation and reducing glucose-induced lipid esterification processes. Triacsin C also markedly altered lipid partitioning with reductions in glucose-induced fatty acid esterification. Importantly, in the presence of exogenous FFAs but not in their absence, MCDc overexpression and triacsin C reduced both nutrient- and non-nutrient-stimulated insulin secretion. These results support the hypothesis proposing a role for malonyl-CoA and lipid partitioning in GIIS and reconcile the previous results that were not in favor of the model (44,45) by showing the importance of the provision of exogenous FFAs for metabolic signal transduction in the β -cell. They

also shed new light on the role of lipid signaling in insulin exocytosis. Thus, as altered lipid partitioning affected insulin secretion to all secretagogues tested, this suggests the existence of at least one lipid-derived factor with an important regulatory role, not only a permissive role, at a distal step in the coupling mechanisms of both fuel and nonfuel stimuli.

RESEARCH DESIGN AND METHODS

Materials. Pancreatic islets were isolated using collagenase (type IV) from Worthington (Lakewood, NJ), and Ficoll DL-400 from Sigma (St. Louis, MO). Protran nitrocellulose membranes for protein analysis were from Scheicher & Schuell (Dassel, Germany), and the bicinchoninic acid protein assay was from Pierce (Rockford, IL). The enhanced chemiluminescence detection kit from Amersham (Uppsala, Sweden) was used for Western blot analysis. The horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody and ζ -probe membranes were obtained from Bio-Rad (Hercules, CA). Forskolin, human glucagon-like peptide 1 fragment 6-36 amide (GLP-1), triacsin C, BSA (fraction V), and defatted BSA were purchased from Sigma. Phorbol-5-myristate 13-acetate (PMA), palmitate, and oleate were from ν -Check Prep (Elysian, MN). Carbachol was from Calbiochem (San Diego, CA). Radioactive compounds [^{14}C]palmitate, [^{14}C]oleate, and [^{14}C]acetate were from ICN (Irvine, CA). [^{14}C]glucose was from NEN (Boston, MA), and [^{14}C]citrate and [^{14}C]aspartate were from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Fatty acid synthase was isolated from rat liver according to the previously published protocol (46). Thin-layer chromatography plates were purchased from Whatman (Fairfield, NJ).

Creation of stable clones of INS cells expressing MCDc. Recombinant DNA molecules were constructed by standard cloning procedures and propagated in *Escherichia coli* DH5. The coding region of rat MCD, without its mitochondrial and peroxisomal targeting sequences, named MCDc for cytosolic MCD, was amplified using upstream (5'-GCT AGA TCT ATG GAC GAG CTG CTA-3') and downstream (5'-TCC CTG TTT AAA CTA GTT GCT CTG GA-3') primers. The PCR product was digested with *Bgl*III/PmeI and subcloned in the AdTR5-K7-GFP_Q vector (47) to obtain AdTR5-MCDc-K7-GFP_Q. INSr3 cells expressing the rTA protein (48) were transfected by a calcium phosphate method with AdTR5-MCDc-K7-GFP_Q. INSr3 clones expressing GFP were isolated by a Quixell technique (49). Briefly, a pool of GFP-positive cells were obtained by a fluorescence-activated cell sorter and cultured for 1 week in regular RPMI medium in 100-mm Petri dishes. Individual GFP-positive cells were picked up with a micromanipulator and transferred to 96-well plates. Each clone was cultured for 2 weeks for amplification and GFP-positive clones were tested for MCDc expression by Western blot analysis. Approximately 20 positive clones were analyzed for MCDc expression. Two clones expressing MCDc at particularly high levels in the presence of 1 $\mu\text{g}/\text{ml}$ doxycycline (INSr3-MCDc#5 and INSr3-MCDc#14) were further used in this study.

Construction of recombinant adenoviruses expressing MCDc in a tetracycline-regulatable manner and constitutively. Recombinant adenovirus expressing MCDc in a regulatable manner was made by cotransfecting 293A cells with the FseI-restricted vector AdTR5-MCDc-K7-GFP_Q and the large right-end fragment of the Ad5/DE1DE3 viral genome (50) digested with *Clal*. Positive plaques were purified and expanded in 293 cells following the protocol of Jani et al. (51) to minimize the occurrence of replication-competent adenoviruses. Large-scale viral stocks were prepared by infecting 3×10^9 293 cells in suspension cultures as described previously (50). Viral titers were determined by a plaque assay on 293A cells (47). For adenovirus expressing MCDc constitutively, the cDNA encoding rat MCDc was introduced into the pAdTrack-CMV shuttle vector and the resulting construct was transformed into BJ5183 cells, in which homologous recombination was carried out. Positive recombinants were confirmed by restriction enzyme digest and were transformed into DH5 α cells for amplification and isolation. Recombinant adenoviral DNA from single DH5 α colonies was transfected into HEK293 cells to generate clonal recombinant adenovirus. The resultant adenovirus was then purified from HEK293 by density gradient centrifugation and then titered.

INS cell culture and adenovirus infection. INSr3-MCDc (#5 and #14) and INS(832/13) (52) cells were seeded in 60-mm tissue culture dishes (3×10^6 cells/dish), 25-cm² flasks (2×10^6 cells/flask), or six-well plates (0.5×10^6 cells/well) as indicated and cultured in regular RPMI medium containing 11 mmol/l glucose and 10% FCS (tetracycline-free; Clontech, Palo Alto, CA) in addition to 10 mmol/l HEPES (pH 7.4), 1 mmol/l sodium pyruvate, and 50 $\mu\text{mol}/\text{l}$ β -mercaptoethanol (complete RPMI). INSr3-MCDc cells (seeded in 60-mm dishes for all studies) were cultured the next day (24 h) in complete

RPMI containing 11 mmol/l glucose with or without doxycycline (1 $\mu\text{g/ml}$) to induce MCDc protein expression followed by an additional 18 h in complete RPMI containing 3 mmol/l glucose in the presence or absence of doxycycline. INS(832/13) cells for tetracycline-regulatable MCDc overexpression (25-cm² flasks for fatty acid oxidation and esterification studies and six-well plates for insulin secretion studies) were dually infected after 2 days of culture for 4 h in complete RPMI containing 3 mmol/l glucose in the presence or absence of doxycycline (10 $\mu\text{g/ml}$) with adenoviruses expressing the tTA protein (Ad-CMV-tTA) and MCDc (Ad-TR5-MCDc) (5 pfu/cell for both viruses). They were then washed and further cultured for 16 h in the same media without virus. INS(832/13) cells for constitutively overexpressed MCDc (seeded in 60-mm dishes) were infected after 1 day of culture with 50 pfu/cell of the AdCMV-MCDc or AdCMV- β Gal (control) viruses for 18 h in complete RPMI containing 11 mmol/l glucose. The cells were then washed with PBS and further cultured for 18 h in complete RPMI at 3 mmol/l glucose. INS(832/13) cells for nonviral studies were prepared by culturing in complete RPMI for 48 h at 11 mmol/l glucose followed by 18 h at 3 mmol/l glucose.

Pancreatic islet isolation and adenovirus infection. Islets were isolated from Wistar rats (Charles River, St. Constant, Québec, Canada) that weighed ~200 g by collagenase digestion of the total pancreas and subsequent separation on discontinuous Ficoll DL-400 gradients as described by Gotoh et al. (53). At the end of the isolation step, islets were maintained in culture at 15–20 islets/ml in regular RPMI 1640 medium containing 11 mmol/l glucose supplemented with 10% FCS, 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. For adenovirus infection, batches of 100–200 islets were infected for 18 h with AdCMV-MCDc or AdCMV-Luc (control infection) with 10⁸ pfu/islet. The islets were then washed with PBS and further cultured before the experiments as indicated.

Western blot analysis. After sedimentation by centrifugation, islets or INS cell proteins were extracted by sonication in a Tris-HCl (80 mmol/l), EDTA (5 mmol/l), and SDS (5%) buffer containing protease inhibitors. Proteins obtained from cell extracts were analyzed on 10% SDS-containing polyacrylamide gels. Proteins were then transferred to nitrocellulose filters, and MCD was detected using an IgG fraction (3 $\mu\text{g/ml}$) of a rabbit polyclonal antibody raised against the COOH-terminal part of the protein (H-Q-V-L-S-L-V-A-Q-F-Q-K-N-S-K-L) (54) and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (dilution 1:8,000). Detection was carried out with the enhanced chemiluminescence detection technique.

MCD activity. MCD enzymatic activity in INSr3-MCDc#5 cells was measured using a radiometric method according to the protocol described by Kudo et al. (55). Briefly, after a 42-h incubation period with or without doxycycline (1 $\mu\text{g/ml}$), INSr3-MCDc#5 cells were sonicated in ice-cold buffer (10 mmol/l HEPES [pH 7.4], 75 mmol/l KCl, 20 mmol/l sucrose, 1 mmol/l EGTA, 0.5 mmol/l dithiothreitol, and protease inhibitors), and MCD activity was measured in the supernatants after centrifugation of extracts at 14,000g for 10 min. Supernatant extracts (~50 μg of protein) were incubated for 10 min in assay buffer in the presence of 1 mmol/l malonyl-CoA as described (56). The reactions were then stopped with perchloric acid, and the precipitated proteins were sedimented by centrifugation. The conversion of the acetyl-CoA formed by MCD to [¹⁴C]citrate was obtained by coupled enzymatic reactions, in which [¹⁴C]aspartate (203 mCi/mmol) is first transformed to [¹⁴C]oxaloacetate with glutamic-oxaloacetate transaminase and then the [¹⁴C]oxaloacetate is reacted with acetyl-CoA to form [¹⁴C]citrate in the presence of citrate synthase. In the final step of the process, unreacted [¹⁴C]aspartate and [¹⁴C]citrate are separated by stirring the solution into a 1:2 (wt/vol) suspension of Dowex 50W-8X9 (100–200 mesh) and centrifugation at 400g for 10 min. The unreacted [¹⁴C]aspartate is contained in the pellet; the radioactivity in supernatants, which contains [¹⁴C]citrate, was estimated by scintillation spectroscopy. MCD activity, expressed as nanomoles of acetyl-CoA formed per minute per milligram of protein, was quantified by comparison with acetyl-CoA standards. MCD activity in isolated islet lysates obtained after sonication was determined by a carnitine acetyltransferase-linked assay that measures acetyl-CoA produced from malonyl-CoA decarboxylation, as detailed elsewhere (44) with minor modification for islets. Briefly, the rate of acetyl-CoA production was monitored by colorimetric assay of CoA-SH produced by cleavage of the thioester bond of acetyl-CoA by carnitine acetyltransferase. The thiol group of the CoA-SH was detected with the colorimetric compound 5,5'-dithiobis(nitrobenzoic acid) (Ellman's reagent). The reaction mixture (0.2 ml) contained 10 mmol/l Tris (pH 7.1), 0.2 mmol/l of 5,5'-dithiobis(nitrobenzoic acid), 1 mmol/l L-carnitine, 10 mmol/l malonyl-CoA, and 3 units of carnitine acetyltransferase (Sigma). Islets (250–300) were lysed in 30 μl of 50 mmol/l HEPES and 1% Triton X-100. For initiating the reaction, 10 μl of the islet homogenates was added to the reaction mixture, and absorbance was measured continuously at 412 nm for 10 min, during which time the rate of product accumulation was linear.

Preparation of BSA-bound fatty acids. Stock solutions of fatty acids (palmitate and oleate) bound to BSA were prepared as follows. Briefly, a saturating quantity of the corresponding sodium salt fatty acid was dissolved at 37°C for 16 h under a nitrogen atmosphere in Krebs-Ringer bicarbonate buffer containing 10 mmol/l HEPES (pH 7.4; KRBH) and 5% (wt/vol) fatty acid-free BSA. Solutions were adjusted to pH 7.4 and then filtered through a 0.2- μm filter. BSA-bound FFA was quantified using the NEFA C kit (Wako Chemical, Neuss, Germany), and stock solutions were finally adjusted to 4 mmol/l FFA using 5% fatty acid-free BSA in KRBH and stored at -20°C under nitrogen. For fatty acid oxidation and esterification, labeled FFA ([1-¹⁴C]palmitate or [1-¹⁴C]oleate) was equilibrated for 2 h before the experiments at 37°C with the KRBH incubation medium containing the unlabeled FFA complexed to 0.5% BSA.

Fatty acid metabolism and triglyceride measurements. Fatty acid oxidation to CO₂ and acid-soluble products (ASP; essentially ketone bodies) (57), and fatty acid esterification into complex lipids were measured in INS(832/13) cells cultured in 25-cm² flasks. In some experiments, fatty acid esterification was measured in INSr3-MCDc#5 cells prepared in 60-mm dishes. For the INS(832/13) experiments, cells were washed and preincubated at 37°C either for 30 min in KRBH (pH 7.4) containing 0.5% defatted BSA at 2 mmol/l glucose (MCDc overexpression studies) or for 2 h in the same medium with or without triacsin C (10 $\mu\text{mol/l}$). Cells were then incubated for 1 h at 37°C in 2 ml of fresh KRBH containing 0.5% defatted BSA at 2 or 16 mmol/l glucose in the presence of 1 mmol/l carnitine plus cold and labeled FFAs. For the MCDc overexpression studies, cells were incubated with 0.25 mmol/l palmitate and 0.25 Ci/ml [1-¹⁴C]palmitate (55 mCi/mmol). For the triacsin C experiments, cells were incubated with 0.25 mmol/l FFA (palmitate/oleate, 1:1 ratio) and in parallel wells with 0.25 $\mu\text{Ci/ml}$ of either [1-¹⁴C]palmitate or [1-¹⁴C]oleate (60 mCi/mmol). For the INSr3-MCDc cell experiments, cells were washed with PBS and preincubated at 37°C for 30 min in KRBH containing 0.07% BSA at 3 mmol/l glucose. Cells were then incubated for 1 h at 37°C in 2 ml of fresh KRBH containing 0.5% defatted BSA at 3 and 20 mmol/l glucose in the presence of 1 mmol/l carnitine, 0.1 mmol/l palmitate, and 0.5 $\mu\text{Ci/ml}$ [1-¹⁴C]palmitate. For oxidation measurements, the 25-cm² flasks were sealed at the beginning of the incubation with a stopper supporting a 3-cm length of PVC tubing (internal diameter 4.7 mm) containing a piece of Whatman GF/B paper (one-third of a 25-mm-diameter circle) soaked in 5% KOH. At the end of the incubation period, 0.2 ml of perchloric acid (40% vol/vol) was injected into each flask via a needle through the cap to acidify the medium and liberate the CO₂. After overnight isotopic equilibration at room temperature, papers were removed and the trapped ¹⁴CO₂ was measured by liquid scintillation counting after overnight equilibration in the scintillation fluid. Fatty acid oxidation into ASP was measured from the perchloric acid-treated medium and cell extracts after centrifugation (14,000g for 10 min). The supernatants containing the labeled ASP were collected and counted by liquid scintillation (57). The discarded pellets contained proteins and lipids, including the labeled fatty acids. For FFA esterification determinations, cells were washed and then scraped in cold PBS, pelleted by centrifugation, and resuspended in 3 ml of Folch reagent as described previously (35). Total lipids were extracted and separated by thin-layer chromatography to measure the incorporation of labeled fatty acid into phospholipids, DAG, triacylglycerols, and cholesterol esters, as described (58). The cellular accumulation of nonesterified-labeled palmitate (NEpal) was also assessed after thin-layer chromatography separation of lipids, using labeled control palmitate as migration reference. The calculations of FFA oxidation and esterification determinations with the palmitate/oleate 1:1 mixture were made by adding the palmitate and oleate results from the parallel wells containing only labeled palmitate or oleate. The cellular triglyceride content of INSr3-MCDc#5 cells was measured according to a published procedure (35).

Malonyl-CoA measurements. After INSr3-MCDc#5 cell culture in 150-mm Petri dishes in complete RPMI medium with or without 1 $\mu\text{g/ml}$ doxycycline at 11 mmol/l glucose for 24 h and 3 mmol/l glucose for 18 h, the cells were washed with PBS and preincubated for 30 min at 37°C in KRBH (pH 7.4) containing 0.07% BSA (fraction V) and 3 mmol/l glucose. Cells were then incubated for 30 min at 37°C in fresh KRBH containing 0.5% defatted BSA and 0.1 mmol/l palmitate at 3 or 20 mmol/l glucose. Incubation media were discarded, and malonyl-CoA was extracted from cells with 10% trichloroacetic acid (46). After centrifugation of precipitated proteins, cell extracts were brought to pH 5–6 by successive ether extractions. Samples were lyophilized and stored at -70°C. Malonyl-CoA was assayed with a radioactive method using fatty acid synthase (59).

Insulin secretion. For insulin secretion determinations, INSr3-MCDc#5 and INSr3-MCDc#14 clone cells and INS(832/13) cells infected with AdCMV-MCDc (AdCMV- β Gal control) were plated (3 \times 10⁶ cells) into 60-mm Petri dishes. Cells were cultured and treated with doxycycline or the viral constructs as described above. They were subsequently washed and preincubated at 37°C in

KRBH (pH 7.4) containing 0.07% BSA at 3 mmol/l glucose and then incubated in fresh KRBH containing 0.5% defatted BSA in the presence of 3 mmol/l glucose, 20 mmol/l glucose, and 3 mmol/l glucose plus various secretagogues, in the absence or presence of the indicated concentrations of either palmitate or oleate. The cellular protein content was measured using the bicinchoninic acid assay. INS(832/13) cells either dually infected with AdTR5-MCDc and AdCMV-tTA viruses or uninfected were seeded in six-well plates, prepared as described above. Cells were washed and preincubated for 30 min (2 h for triacsin C experiments) at 37°C in KRBH containing 0.5% defatted BSA at 2 mmol/l glucose with or without FFAs and 10 μ mol/l triacsin C. Insulin secretion was then measured during a 45-min incubation at 37°C in KRBH containing 0.5% defatted BSA at 2, 6, or 16 mmol/l glucose or at 2 mmol/l glucose with various secretagogues, in the absence or presence of palmitate and oleate, as indicated. For secretion studies in adenovirus-treated islets, batches of 10 islets were washed twice in KRBH containing 0.1% BSA and 2.8 mmol/l glucose and preincubated for 20 min at 37°C in 2 ml of the same medium. Islets were then incubated for 60 min in KRBH containing 0.5% defatted BSA at 2.8 or 16.7 mmol/l glucose in the absence or presence of various concentrations of palmitate. For islets acutely exposed to triacsin C, islets were washed in KRBH containing 0.5% BSA at 3 mmol/l glucose and were preincubated for 2 h in the same medium at 37°C with or without 10 μ mol/l triacsin C. The islets were then incubated for 45 min in fresh KRBH containing 0.5% BSA at 3 and 16 mmol/l glucose with or without 10 μ mol/l triacsin C and 0.25 mmol/l FFA (palmitate/oleate, 1:1 ratio). The total insulin content of the various INS cell clones and islets was measured after acid-ethanol (1.5% HCl, 75% ethanol) extraction. The insulin concentration in the medium was determined by radioimmunoassay using rat insulin standards (35).

Statistical analysis. All results are expressed as means \pm SE. Statistical significance was calculated with the *t* test or, for multiple comparisons, one-way ANOVA with Bonferroni post hoc testing and, where indicated, two-way ANOVA.

RESULTS

Overexpression of MCDc in a tetracycline-regulatable manner in INS cell clones and in a constitutive manner in isolated rat islets. Total protein was isolated from stable clones of INS (INSr3-MCDc) cells treated with doxycycline to overexpress MCDc (tet on system; controls were untreated), INS(832/13) dually infected with AdCMV-tTA and AdTR5-MCDc treated without doxycycline to overexpress MCDc (tet off system; controls were treated with doxycycline), and islets infected with AdCMV-MCDc (controls infected with AdCMV-Luc). MCDc protein expression was measured by Western blot analysis. Figure 1A shows doxycycline-inducible expression of MCDc in one of the stable INSr3-MCDc clones (#5). A similar increase in MCDc expression was observed in the INSr3-MCDc#14 stable clone (data not shown). MCDc protein expression was doxycycline dose-dependent, becoming detectable after treatment with 0.1 μ g/ml and maximally expressed after treatment with 1 μ g/ml doxycycline. In INS(832/13) cells infected with AdCMV-tTA and AdTR5-MCDc (Fig. 1D), MCDc was strongly expressed in the absence of doxycycline and minimally expressed in the presence of 10 μ g/ml doxycycline, suggesting minor leakage in the tet off system. MCDc was not detectable in uninfected INS(832/13) cells (not shown). In islets (Fig. 1E), MCDc protein was strongly expressed after infection with the AdCMV-MCDc adenovirus but was undetectable after infection with the AdCMV-LUC control virus. Cellular MCD activity was also measured in the INSr3-MCDc and islet protein extracts (Fig. 1B and F). The level of MCD activity was very low in control INSr3-MCDc#5 clone cells (not treated with doxycycline) compared with MCDc overexpressing cells (Fig. 1B) but was similar to what we (56) and others (44,45) have already published. Consistent with the Western blot results, doxycycline-dependent

MCD activity was observed with a maximal 13-fold increase in cells treated with 1 μ g/ml doxycycline (Fig. 1B). The MCD activity was also sevenfold increased in islets infected with AdCMV-MCDc compared with control islets infected with AdCMV-Luc (Fig. 1F). To determine the effect of MCDc overexpression on malonyl-CoA levels, we measured malonyl-CoA content in the stable INSr3-MCDc#5 clone at both low and elevated glucose concentrations. Consistent with our previous findings in INS-1 cells (26), high glucose acutely increased the malonyl-CoA content in control INSr3-MCDc#5 cells (Fig. 1C). Doxycycline-induced overexpression of rat MCDc markedly decreased malonyl-CoA content at both low (3 mmol/l) and high (20 mmol/l) glucose concentrations (Fig. 1C). The level of malonyl-CoA was not detectable at low glucose and was reduced by \sim 75% at high glucose. Thus, overexpression of rat MCDc in both clonal INS cells and islets was successful in markedly increasing the activity of MCD and, as shown in the INSr3-MCDc cells, lowering malonyl-CoA levels.

Impact of cytosolic MCDc overexpression with reduction of malonyl-CoA content on lipid partitioning. To test the metabolic relevance of lowering malonyl-CoA levels by MCDc overexpression, we assessed fatty acid partitioning into the oxidation and esterification pathways in control and MCDc-overexpressing cells. Figure 2 shows that fatty acid oxidation at 2 mmol/l glucose was unchanged by MCDc overexpression in INS(832/13) cells. At elevated glucose (16 mmol/l), however, fatty acid oxidation to CO₂, ASP, and the sum of both was 38% higher (for each of the measures) in the MCDc-overexpressing cells compared with the control (doxycycline-treated) adenovirus-infected cells. Fatty acid oxidation at 16 mmol/l glucose was increased by 2- to 2.5-fold in MCDc-overexpressing cells and, to a lesser extent, control infected cells when compared with uninfected cells (Fig. 2), consistent with some MCDc expression leakage in the tet off system as was observed by Western blotting (Fig. 1D). These findings are consistent with the prediction that MCDc overexpression, by reducing malonyl-CoA levels and therefore the inhibition of CPT-1 at elevated glucose, would increase fatty acid oxidation.

Of further interest was the finding that fatty acid oxidation to ASP (57) accounted for 65 and 67% of the total fatty acid oxidation at 2 mmol/l and 16 mmol/l glucose, respectively, in uninfected cells (Fig. 2). Oxidation to ASP is clearly an important component of total fatty acid oxidation in INS β -cells. This measure, to our knowledge, has not previously been taken into account. Hence, total fatty acid oxidation in islet tissue and β -cell lines has most likely been previously underestimated.

It was also predicted that MCDc overexpression, by increasing fatty acid oxidation, would reduce the availability of LC-CoA for lipid signaling in the β -cell. In accordance, MCDc overexpression markedly decreased glucose-induced esterification processes (Fig. 3). In MCDc-overexpressing INSr3-MCDc#5 cells (Fig. 3A-C), glucose-induced palmitate esterification into the various complex lipid species (expressed as the delta between high and low glucose [Δ 3/20G]) was decreased for phospholipids, DAG, and triacylglycerol by \sim 45, 60, and 80%, respectively. Esterification processes at low glucose were

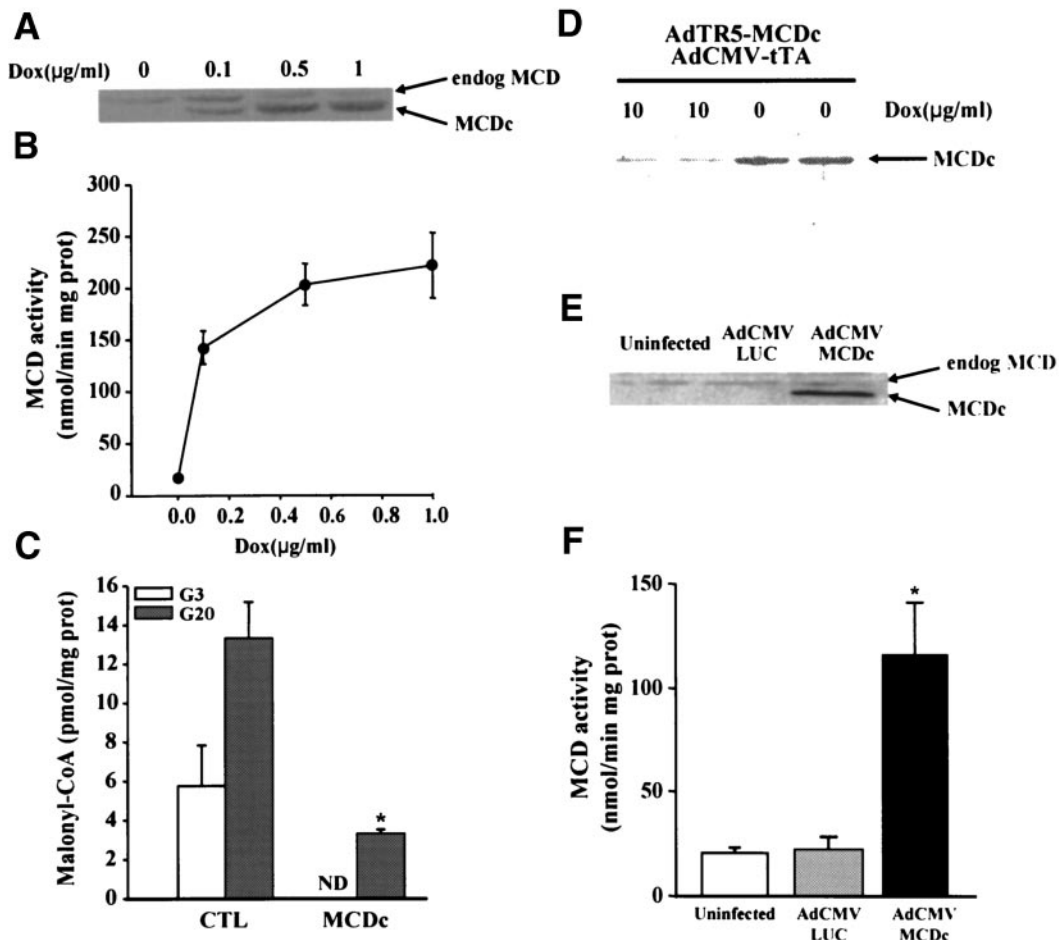


FIG. 1. Overexpression of MCDc in a tetracycline-regulatable manner in the INSR3-MCDc (tet on) and INS(832/13) (tet off) cell clones and in a constitutive manner in isolated rat islets. *A–C:* Stable clones of INS cells, which overexpress MCDc in the presence of doxycycline (tet on), were generated using the INSR3 clone, which constitutively expresses the tetracycline transactivator. INSR3-MCDc (clone #5) cells were cultured for 42 h with the indicated concentrations of doxycycline to induce MCDc protein expression in complete RPMI medium initially containing 11 mmol/l glucose (24 h) followed by 3 mmol/l glucose (18 h). The cells were then processed for analysis. *A:* Endogenous MCD and MCDc protein expression as measured by Western blot. *B:* MCD activity. Data are means \pm SE of three experiments. *C:* Cellular malonyl-CoA content measured in MCDc-overexpressing cells (MCDc) cultured using doxycycline (1 μ g/ml) and control cells (CTL; cultured without doxycycline) after preincubation for 30 min in KRHB medium containing 0.07% BSA at 3 mmol/l glucose followed by incubation for 1 h at 3 (G3) and 20 mmol/l (G20) glucose. Data are means \pm SE of three experiments. * P < 0.03 vs. CTL. ND, not detectable. *D:* INS(832/13) cells were dually infected for 4 h in complete RPMI containing 3 mmol/l glucose in the presence or absence of doxycycline (10 μ g/ml) with adenoviruses expressing the tTA protein (AdCMV-tTA) and MCDc (Ad-TR5-MCDc; 5 pfu/cell for both viruses). They were then cultured for an additional 16 h in the same media without virus, after which MCDc protein expression was determined by Western blot. *E* and *F:* Islets (batches of 100–200) were infected (10^8 pfu/islet) for 18 h with an adenovirus expressing MCDc under the control of a CMV promoter (AdCMV-MCDc) or with an Ad-luciferase control virus (AdCMV-LUC). They were subsequently incubated for 24 h in regular RPMI medium containing 11 mmol/l glucose before being processed for analysis. *E:* Endogenous MCD and MCDc protein expression as determined by Western blot. *F:* MCDc activity. Data are means \pm SE of three experiments. * P < 0.03.

not different in comparison with control cells. Cellular triacylglycerol content in INSR3-MCDc#5 cells after culture in the conditions as described in Fig. 1A–C was not altered by overexpression of MCDc (Fig. 3D), consistent with the results shown that reduced malonyl-CoA in the β -cell affected lipid esterification processes only upon a glucose challenge (Fig. 3A–C). Esterification processes into triacylglycerol were also affected at elevated glucose by MCDc overexpression in the INS(832/13) cells (Fig. 3E). Palmitate esterification into cholesterol esters was reduced by 33% at low glucose and by 75% at elevated glucose by MCDc overexpression (Fig. 3F).

Of major additional interest were the findings related to measurements of NEpal in adenovirus-infected INS(832/13) cells (Fig. 3G). First, in the control (doxycycline-treated) cells, there was a fourfold higher accumulation of

NEpal after incubation at high (16 mmol/l) compared with low (2 mmol/l) glucose (Fig. 3G). Second, this increase in NEpal accumulation at elevated glucose was essentially abolished by MCDc overexpression (Fig. 3G). Furthermore, the autoradiographs from the thin-layer chromatography plates of the INSR3-MCDc#5 cell palmitate esterification experiments, analyzed by scanning densitometry, showed similar changes in NEpal accumulation (densitometric units of 377 ± 68 vs. 339 ± 57 at 3 mmol/l glucose and $1,020 \pm 150$ vs. 417 ± 52 at 20 mmol/l glucose in control compared with MCDc-overexpressing INSR3-MCDc cells, respectively; P < 0.001 at 20 mmol/l glucose). Considering that NEpal accumulation is expected to parallel cytosolic LC-CoA levels (because upon entry to cells FFAs are first metabolized to LC-CoA), these findings are

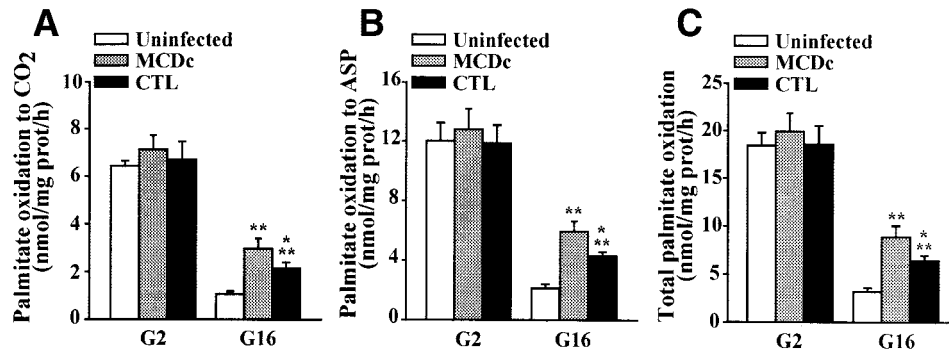


FIG. 2. Fatty acid oxidation is increased at elevated glucose in INS(832/13) cells overexpressing MCDc. INS(832/13) cells were uninfected or infected as described in the legend to Fig. 1D with AdCMV-tTA and AdTR5-MCDc viruses in the presence of doxycycline (10 μ g/ml; CTL) or in its absence (MCDc). Cells were then preincubated for 30 min at 37°C in KRBH medium containing 0.5% BSA at 2 mmol/l glucose and then incubated for 1 h at 2 (G2) and 16 mmol/l (G16) glucose in the presence of 1 mmol/l carnitine, 0.25 μ Ci/ml [14 C]palmitate, 0.25 mmol/l unlabeled palmitate, and 0.5% BSA. Palmitate oxidation to CO₂ (A), ASP (B), and total palmitate oxidation (CO₂ + ASP) (C) was measured as described in RESEARCH DESIGN AND METHODS. Data are means \pm SE of six determinations in two separate experiments. * P < 0.05 vs. MCDc; ** P < 0.001 vs. uninfected cells.

consistent with the prediction that MCDc overexpression, by reducing malonyl-CoA levels and consequently inhibition of CPT-1, would prevent glucose-induced increases in cytosolic LC-CoA.

GIIS is reduced in INS cells and rat islets overexpressing MCDc when measured in the presence of exogenous FFA. GIIS in the presence of exogenous FFA was examined in INS cells overexpressing MCDc using three different expression systems: tet on in the stable INSR3-MCDc cell clones #5 and #14, tet off in INS(832/13) cells dually infected with AdCMV-tTA and AdTR5-MCDc,

and in a constitutive manner in INS(832/13) cells infected with AdCMV-MCDc. Figure 4A–C shows that GIIS was similarly impaired (45–51%) in MCDc-overexpressing cells compared with control cells, irrespective of the expression system used. A 43% reduction in GIIS (P < 0.05 vs. control cells) was also seen in INSR3-MCDc#14 cells (results not shown). Secretion was measured in the presence of 0.1 mmol/l exogenous palmitate (or oleate; see Fig. 5A) in INSR3-MCDc cells (tet on system) and 0.25 mmol/l exogenous palmitate in INS(832/13) cells (tet off system), the experimental conditions that were used to assess lipid

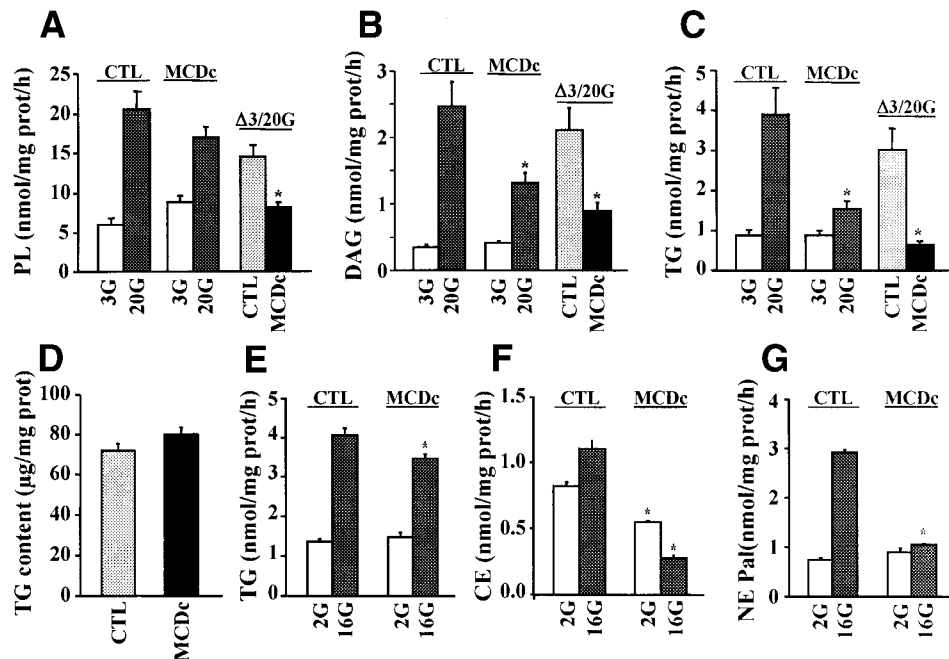


FIG. 3. Glucose-induced fatty acid esterification processes are reduced in INS cells overexpressing MCDc. A–C: INSR3-MCDc (clone #5) cells prepared in the absence (CTL) or presence (MCDc) of doxycycline (1 μ g/ml) as described in the legend to Fig. 1A–C were preincubated for 30 min at 37°C in KRBH medium containing 0.07% BSA at 3 mmol/l glucose and then incubated for 1 h at 3 (G3) and 20 mmol/l (G20) glucose in the presence of 1 mmol/l carnitine, 0.5 μ Ci/ml [14 C]palmitate, 0.1 mmol/l unlabeled palmitate, and 0.5% BSA. Fatty acid esterification into phospholipids (A), DAG (B), and triglyceride (C) were assessed using thin-layer chromatography after lipid extraction. Δ 3/20G is the difference between the incorporation of palmitate in the particular lipid class at 20 (G20) versus 3 mmol/l (G3) glucose. D: The total triglyceride content of CTL and MCDc-overexpressing INSR3-MCDc cells prepared as described in Fig. 1A–C. E–G: INS(832/13) cells were infected as described in the legend to Fig. 1D with AdCMV-tTA and AdTR5-MCDc viruses in the presence of doxycycline (10 μ g/ml; CTL) or absence of doxycycline (MCDc). Cells were incubated in the presence of labeled and cold palmitate as described in the legend to Fig. 2. Fatty acid esterification into triglyceride (E), cholesterol esters (F), and NEpal (G) was assessed as described above. Data are means \pm SE of nine determinations in three separate experiments (A–D) or three determinations (E–G). * P < 0.05 at least.

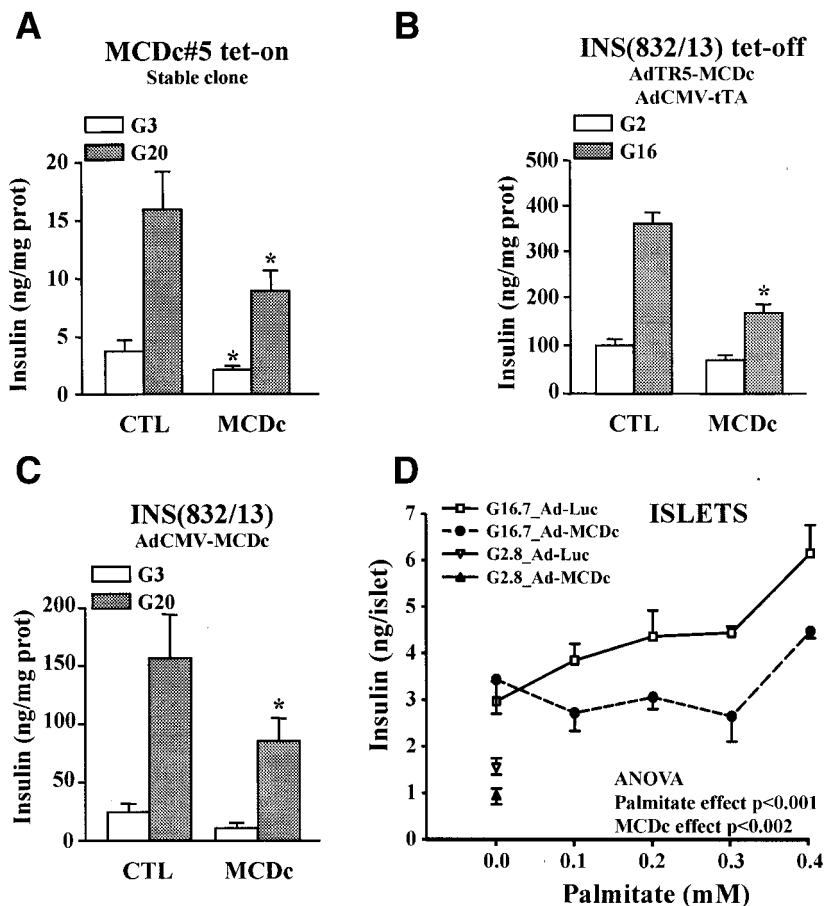


FIG. 4. Glucose-induced insulin release is impaired in various INS cells and isolated rat islets overexpressing MCDc when secretion is measured in the presence of exogenous FFA. **A:** INSr3-MCDc cells prepared in the absence (CTL) or presence of 1 $\mu\text{g}/\text{ml}$ doxycycline (MCDc) as described in Fig. 1A–C. **B:** INS(832/13) cells infected with AdCMV-tTA/AdTR5-MCDc prepared in the absence (MCDc) or presence of 10 $\mu\text{g}/\text{ml}$ doxycycline (CTL) as described in Fig. 1D. **C:** INS(832/13) cells infected with AdCMV-MCDc (MCDc) or AdCMV- βGal (CTL) prepared as described in RESEARCH DESIGN AND METHODS. Cells were preincubated for 30 min in KRBH containing 3 mmol/l glucose and 0.07% BSA (A and C) or 2 mmol/l glucose and 0.5% BSA (B). Cells were then incubated in KRBH for 1 h in the presence of 0.1 mmol/l palmitate complexed with 0.5% BSA at 3 (G3) and 20 mmol/l (G20) glucose (A and C) or for 45 min in the presence of 0.25 mmol/l palmitate complexed with 0.5% BSA at two (G2) or 16 mmol/l (G16) glucose (B). Data are means \pm SE of nine determinations in three separate experiments (A and C) or six determinations in two separate experiments (B). * $P < 0.02$ compared with CTL for same condition. **D:** Isolated rat islets were infected as detailed in RESEARCH DESIGN AND METHODS with AdCMV-luciferase (Ad-Luc) as control or AdCMV-MCDc (Ad-MCDc) to overexpress MCDc. Batches of 10 islets each were washed twice in KRBH containing 0.1% BSA and 2.8 mmol/l glucose and preincubated for 20 min at 37°C. Islets were then incubated for 1 h in fresh KRBH medium containing 0.5% BSA at 2.8 or 16.7 mmol/l glucose in the presence of the concentrations of palmitate as indicated. Data are means \pm SE of three separate experiments. Two-way ANOVA is indicated.

metabolism. Different levels of insulin secretion were observed in the various cell lines as a result of clonal difference in insulin content; however, elevated glucose caused a robust increase in insulin secretion in all tested cells (Fig. 4A–C). No significant difference in insulin content was observed in INSr3-MCDc#5 cells overexpressing MCDc (0.130 ± 0.004 ng/ μg protein) compared with control cells (0.148 ± 0.007 ng/ μg protein) or in INS(832/13) cells infected with AdCMV-tTA- and AdTR5-MCDc-overexpressing MCDc (8.0 ± 0.7 ng/ μg protein) compared with control cells (8.9 ± 0.5 ng/ μg protein).

Insulin secretion was also assessed in isolated rat islets infected with AdCMV-MCDc and AdCMV-Luc (control islets) in the absence and presence of palmitate at concentrations from 0.1 to 0.4 mmol/l. Whereas overexpression of MCDc did not reduce GIIS in the absence of exogenous fatty acids (Fig. 4D), insulin release was reduced in the AdCMV-MCDc-infected islets by 27 to 40% in the presence of the various concentrations of palmitate that caused a dose-dependent enhancement of secretion (Fig. 4D). MCDc overexpression in isolated rat islets also reduced KCl-induced secretion by 52% when measured in the presence of 2.8 mmol/l glucose and 0.4 mmol/l oleate bound to 0.5% BSA (1.66 ± 0.19 vs. $0.80 \pm 0.05\%$ of insulin content secreted per hour, AdCMV-Luc vs. AdCMV-MCDc; $P < 0.05$), with basal secretion at 2.8 mmol/l glucose with oleate equal to 0.03 ± 0.01 and $0.03 \pm 0.02\%$ of insulin content secreted per hour for the AdCMV-Luc and AdCMV-MCDc islets, respectively. Overexpression of MCDc in islets did not change proinsulin biosynthesis measured

using ^{35}S -methionine (60) at either low (2.8 mmol/l) or high (16.7 mmol/l) glucose, whether in the presence or absence of exogenous FFA. High glucose increased insulin biosynthesis by approximately sixfold in both control (5.8 ± 0.5 and 6.3 ± 0.5 -fold; $n = 4$ –5) and MCDc-infected islets (6.2 ± 0.6 and 5.5 ± 0.7 -fold, with and without exogenous FFA, respectively; $n = 4$ –5). Islet total ATP content was assessed as luciferase activity in islets expressing firefly (*Photinus pyralis*) luciferase cDNA (AdCMV-LUC) as we have done before (60). Islets were dually infected with AdCMV-MCDc and AdCMV-LUC (MCDc) or AdCMV-GFP and AdCMV-LUC (control). Basal ATP levels at 2.8 mmol/l glucose in the presence of oleate (0.3 mmol/l) were not lowered in islets infected with MCDc. Furthermore, high glucose increased ATP similarly in the control and MCDc islets (1.3 ± 0.07 and 1.40 ± 0.05 -fold increase above basal, respectively; $n = 4$ –5). MCDc overexpression had no effect on islet glucose oxidation measured by quantifying $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glucose}$ at 2.8 mmol/l (10.5 ± 3.6 and 11.8 ± 4.3 pmol \cdot islet $^{-1} \cdot \text{h}^{-1}$) or 16.7 mmol/l glucose (28.7 ± 6.0 and 27.9 ± 5.3 pmol \cdot islet $^{-1} \cdot \text{h}^{-1}$, AdCMV-Luc and AdCMV-MCDc islets, respectively).

Insulin secretory responses to both fuel and nonfuel stimuli are markedly reduced by MCDc overexpression in β -cells only in the presence of exogenous fatty acids. In the presence of exogenous fatty acid, the insulin responses in MCDc-overexpressing INSr3-MCDc#5 cells were markedly curtailed for all secretagogues tested (Fig. 5A). These included high glucose,

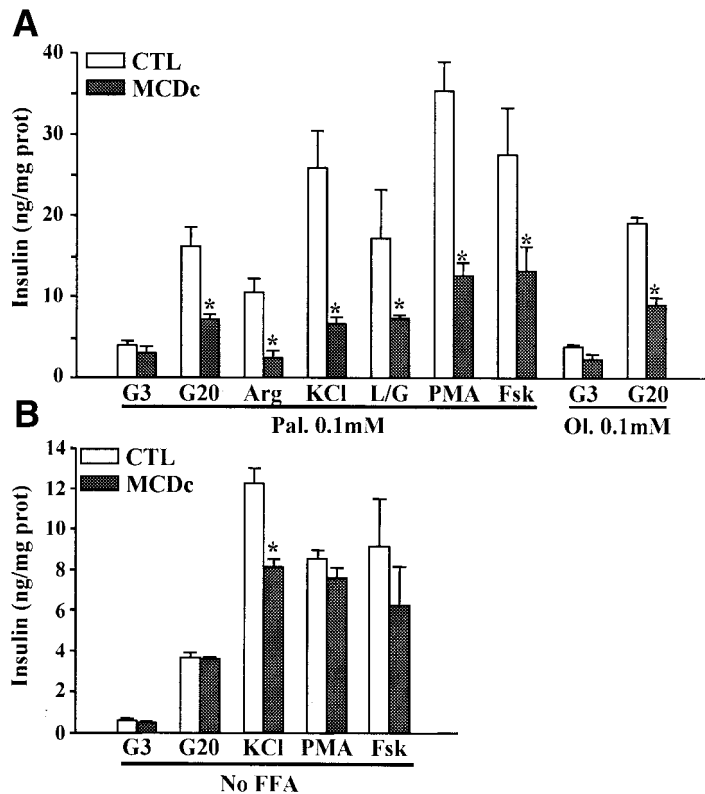


FIG. 5. The secretory response to both fuel and nonfuel stimuli is markedly reduced in INSR3-MCDc#5 cells overexpressing MCDc only in the presence of exogenous FFA. **A:** INSR3-MCDc#5 cells were prepared and preincubated as described in the legends to Fig. 1A–C and Fig. 4A. The cells were then incubated in KRBH containing 0.5% BSA at 37°C for 1 h in the presence of 0.1 mmol/l palmitate (Pal) or oleate (Ol) in the presence of 3 mmol/l glucose (G3) without or with 10 mmol/l L-arginine (Arg), 35 mmol/l KCl, 5 mmol/l leucine plus 5 mmol/l glutamine (L/G), 0.1 μ mol/l PMA, and 5 μ mol/l forskolin (Fsk) or at 20 mmol/l glucose (G20). **B:** Same experimental conditions as in A except that cells were incubated for 1 h in the absence of exogenous fatty acids. Data are means \pm SE of nine determinations in three separate experiments (A) or six determinations in two separate experiments (B). * $P < 0.02$ at least.

L-arginine, KCl, leucine plus glutamine, forskolin, and PMA (Fig. 5A). The same effect was obtained in the presence of oleate. In the absence of exogenous FFAs, the secretory rate of control cells under all conditions was lower (Fig. 5A compared with B). In contrast, the secretion of insulin measured in the absence of FFAs remained unaffected by MCDc overexpression when cells were challenged with high glucose, PMA, and forskolin (Fig. 5B). KCl was the only tested secretagogue applied to MCDc-overexpressing cells, in the absence of fatty acid, which had an impaired secretory response, although the reduction was modest in comparison with the studies observed in the presence of exogenous FFAs. The results of Fig. 5 suggest that exogenous fatty acids augment insulin secretion not only to glucose but also to other secretagogues and that it is this component that is affected by MCDc overexpression.

Triacsin C reduces fatty acid partitioning toward β -oxidation and esterification products and impairs FFA augmentation of insulin secretion in INS(832/13) cells and isolated rat islets. The ACS inhibitor triacsin C markedly reduced fatty acid partitioning in INS(832/13) cells toward both oxidation (80, 95, and 100% reduced at 2, 6, and 16 mmol/l glucose, respectively; Fig. 6A) and esterification products (60 and 67% reduced at 6 and 16 mmol/l glucose, respectively; Fig. 6B). Total fatty acid esterification at 2 mmol/l glucose was not affected by triacsin C treatment. In response to 16 compared with 2 mmol/l glucose, esterification into phospholipids, DAG, and triacylglycerol was 128, 245, and 14% higher, respectively, in triacsin C-treated cells compared with 452, 629, and 219% higher, respectively, in untreated cells (means of three determinations).

In INS(832/13) cells, triacsin C abolished the FFA augmentation of GIIS at 6 mmol/l glucose but failed to do so at 16 mmol/l glucose (Fig. 6C). In islets, exogenous FFAs augmented GIIS at 16 mmol/l glucose by 137%, and this augmentation was completely abolished by triacsin C (Fig. 6D). As triacsin C failed to reduce GIIS in the INS(832/13) cells at 16 mmol/l glucose (with or without FFA present) or in islets at 16 mmol/l glucose (without FFA present), it is highly unlikely that it had nonspecific toxic effects on secretion. Therefore, the inhibitory effects of triacsin C on exogenous FFA augmentation of GIIS at intermediate glucose in cells and high glucose in islets were almost certainly due to its effects on altering lipid partitioning. FFA esterification into phospholipids and DAG did increase in triacsin C-treated cells at 16 mmol/l glucose, as described above, albeit to a much lesser extent than in untreated cells. Therefore, it is possible that a critical threshold for lipid-signaling molecules for insulin secretion was met at 16 mmol/l glucose in the triacsin C-treated INS(832/13) cells (but not in islets) and that this is the reason for the discrepancy between the effects of this inhibitor at 6 compared with 16 mmol/l glucose (Fig. 6C).

Exogenous FFAs augment insulin secretion in response to glucose and nonfuel stimuli, and this augmentation is blocked by triacsin C. Figure 7A shows that the secretory responses to various secretagogues tested (16 mmol/l glucose, KCl, forskolin, GLP-1, and carbachol) were considerably greater when the experiments were performed in the presence of exogenous fatty acid in INS(832/13) cells that were preincubated for 30 min only in KRBH before the experiment. The secretion to nonfuel secretagogues in cells preincubated in KRBH for 2 h (to allow optimal triacsin C action; Fig. 7B) were

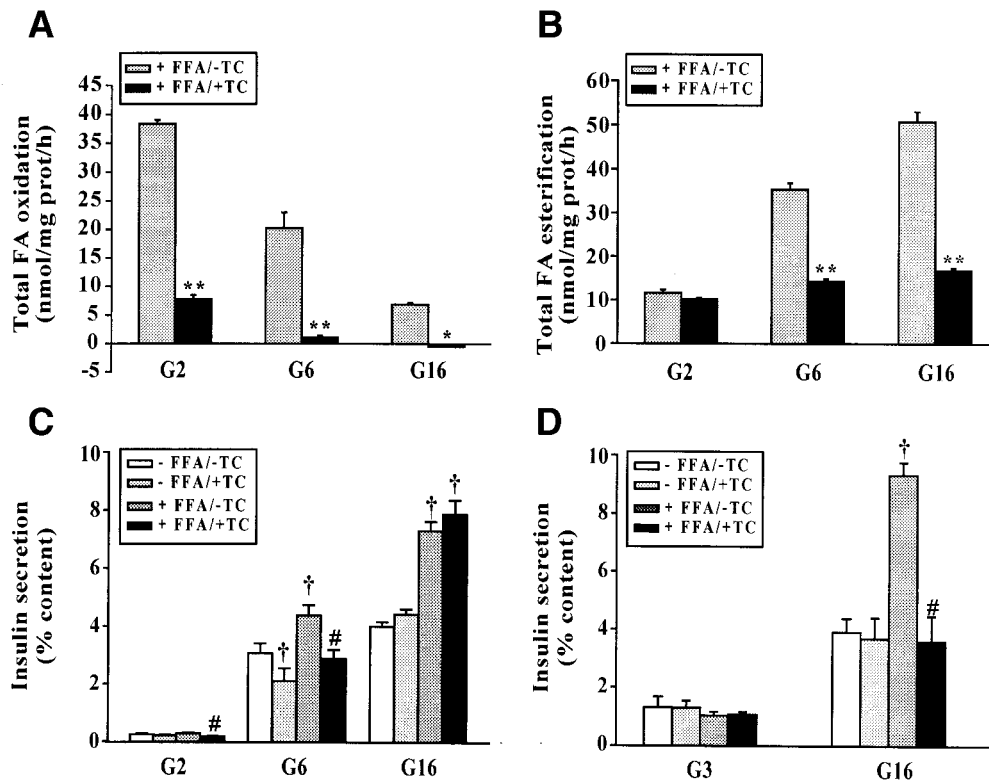


FIG. 6. The ACS inhibitor triacsin C markedly alters fatty acid partitioning in INS(832/13) cells and abolishes FFA augmentation of insulin secretion to intermediate glucose in this INS cell clone and to high glucose in isolated rat islets. Total FFA oxidation (from palmitate and oleate to CO₂ and ASP) (A) and total esterification (from palmitate and oleate) into complex lipids (B) were measured in INS(832/13) cells as described in RESEARCH DESIGN AND METHODS. Cells were preincubated for 2 h in KRBH at 2 mmol/l glucose containing 0.5% BSA with or without 10 μ mol/l triacsin C (TC) and incubated in the same media with the labeled and cold fatty acid mix for 1 h at 2 (G2), 6 (G6), and 16 mmol/l (G16) glucose. Data are means \pm SE of three determinations. * P < 0.01 and ** P < 0.001 vs. same condition without triacsin C. INS(832/13) cells (C) and isolated rat islets (D) were preincubated for 2 h in KRBH at 2 mmol/l (cells) or 3 mmol/l (islets) glucose containing 0.5% BSA with or without 10 μ mol/l triacsin C and incubated in the same media with or without 0.25 mmol/l palmitate/oleate (1:1) at 2, 6, and 16 mmol/l glucose (cells) or 3 (G3) and 16 mmol/l glucose (islets). Data are means \pm SE of nine determinations in three separate experiments (cells) and four determinations (islets). † P < 0.05, at least, for same condition without FFA, without triacsin C; # P < 0.001 vs. same condition with FFA, without triacsin C.

considerably lower than after preincubation for 30 min (Fig. 7A), particularly for secretion to forskolin, and this was shown in a separate experiment, in fact, to be due to the longer preincubation time (results not shown). Triacsin C abolished fatty acid augmentation of secretion to the nonfuel secretagogues forskolin, GLP-1, and PMA in INS(832/13) cells.

DISCUSSION

The results of this study, in which β -cell malonyl-CoA levels were lowered by MCDc overexpression in two different INS cell lines (INSr3 and INS[832/13]) and in rat islets are supportive of the malonyl-CoA/LC-CoA hypothesis proposing a role for malonyl-CoA as a coupling factor between glucose metabolism and insulin secretion that involves changes in lipid partitioning. The study also clearly demonstrates the importance of FFA availability for the lipid-signaling arm of secretion in the β -cell. MCDc overexpression markedly increased MCD activity 6- to 13-fold; dramatically lowered malonyl-CoA levels under both low and high glucose conditions; altered partitioning of exogenous palmitate from oxidation into esterification products at elevated glucose; and, in the presence of exogenous fatty acids, reduced GIIS. In the absence of exogenous fatty acids, an unphysiological situation, MCDc overexpression had no effect on GIIS. The effect of lower-

ing malonyl-CoA content on GIIS was essentially on the fatty acid-augmented component that was abolished at FFA concentrations between 0.1 and 0.3 mmol/l. An additional important finding was that MCDc overexpression in β -cells, as well as inhibition of LC-CoA formation with triacsin C, impaired not only GIIS but also secretion to the combined fuel stimuli leucine and glutamine and various nonfuel stimuli, again, predominantly only in the presence of exogenous FFA. This latter finding is indicative of fatty acid signaling having a major regulatory role at a common distal step in the insulin secretion pathway.

It is important to discuss our results, in which MCDc overexpression under certain conditions did alter GIIS, and two earlier studies (44,45), in which MCD overexpression using adenovirus technology did not alter GIIS. In the first of the earlier studies (44), there is doubt concerning the conclusion as the goose MCD cDNA used still had an intact COOH-terminal SKL peroxisomal targeting motif. The MCD overexpression, therefore, was not specific to the cytoplasm. In the second study (45), human MCD devoid of both its mitochondrial and peroxisomal target sequences (MCDc) was used, thus targeting expression to the cytosol in INS(832/13) cells. In this study (45), GIIS was investigated predominantly without fatty acids present but in a limited experiment with fatty acids. Irrespective of whether fatty acids were present, however,

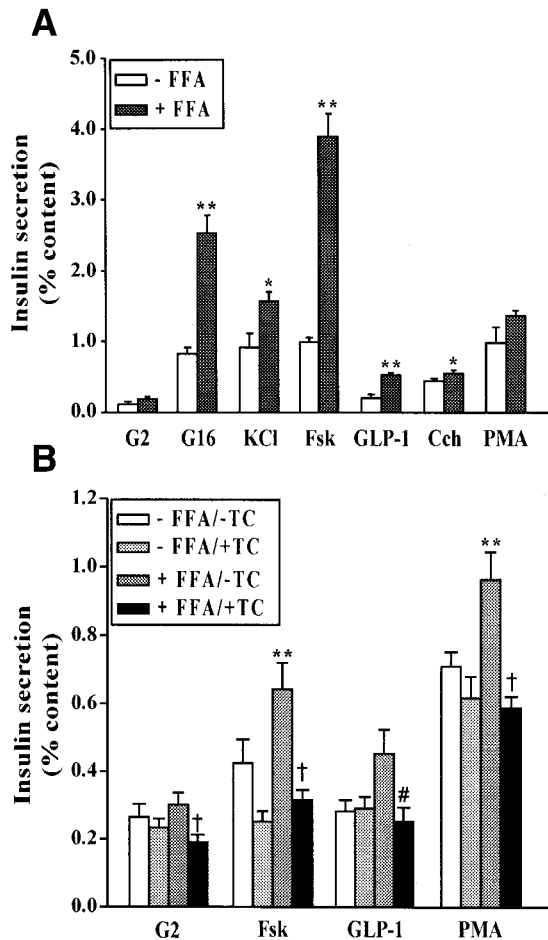


FIG. 7. Insulin secretion to glucose and nonfuel stimuli is augmented by exogenous FFAs, and this augmentation is blocked by triacsin C. **A:** INS(832/13) cells were preincubated for 30 min in KRBH at 2 mmol/l glucose containing 0.5% BSA with or without 0.25 mmol/l palmitate/oleate (1:1) and then incubated for 45 min in KRBH containing 0.5% BSA at 2 mmol/l glucose (G2), without or with the nonfuel stimuli 25 mmol/l KCl, 5 μ mol/l forskolin (Fsk), 10 nmol/l GLP-1, 200 μ mol/l carbachol (Cch), and 0.2 μ mol/l PMA or at 16 mmol/l glucose (G16), all in the absence (-FFA) or presence (+FFA) of 0.25 mmol/l palmitate/oleate (1:1). **B:** INS(832/13) cells were incubated for 2 h in KRBH at 2 mmol/l glucose containing 0.5% BSA with or without 10 μ mol/l triacsin C (TC) and incubated in the same media for 45 min with or without 0.25 mmol/l FFA (palmitate:oleate, 1:1) at 2 mmol/l glucose with or without the nonfuel stimuli Fsk, GLP-1, or PMA. Data are means \pm SE of nine determinations in three separate experiments. * $P < 0.05$ and ** $P < 0.001$ for same condition without FFA; # $P < 0.05$ and † $P < 0.001$ vs. same condition with FFA, without triacsin C.

MCDc overexpression did not alter GIIS. Importantly, there is a very consistent finding between this study (45) and the present study, which is that lowering of malonyl-CoA by MCDc overexpression in the absence of exogenous FFAs has no effect on GIIS. Variance in the results exists only for GIIS assessment in the presence of exogenous fatty acid. The reason for this variance might simply relate to differences in the concentrations of fatty acid and BSA complexing used. In all of the INS cell line experiments in the current study, exogenous fatty acid (mostly palmitate) was used at physiological concentrations of 0.1–0.25 mmol/l complexed to 0.5% BSA compared with the high concentration of 1.0 mmol/l oleate/palmitate (BSA concentration not stated) used in the other study (45). This latter high concentration of FFAs (45) might have overridden the consequences of reducing malonyl-CoA

and LC-CoA in the cytoplasm. As can be seen in the current islet experiments (Fig. 4D), the effect of MCDc overexpression on abolishing the augmentation of fatty acids on GIIS was in part overridden at high concentrations of fatty acid. An additional possibility for explaining the difference in insulin secretion between the studies is lower enhancement of MCD activity in the previous study (45), as MCDc overexpression in that study, unlike the current study, did not effect basal malonyl-CoA levels. Triacsin C did not alter GIIS in INS-1 (44) or INS(832/13) cells (45), and the authors concluded that this was evidence against the malonyl-CoA/LC-CoA hypothesis. However, in neither of the studies was the effect of triacsin C on GIIS measured in the presence of exogenous fatty acid, whereas the present results show that only the FFA augmentation component of GIIS is reduced by the drug. Thus, overall, the current study reconciles the contradiction raised by the two mentioned reports (44,45) by showing that the malonyl-CoA/LC-CoA lipid-signaling arm in GIIS is mostly observed only in the presence of exogenous FFA, a physiological situation.

With MCDc overexpression or exposure of cells to triacsin C, exogenous fatty acids essentially lost their capacity to augment insulin secretion to all tested secretagogues. Therefore, the results provide evidence that malonyl-CoA, as a nutrient-derived coupling factor, regulates the partitioning of fatty acids into effector molecules of the insulin secretory pathway important for all classes of secretagogues. The precise nature of these effector molecules (possibly LC-CoA, phospholipids, and/or DAG) and their mechanisms of action on insulin secretion are poorly understood. At least one fatty acid moiety must act at a distal point in the insulin secretion pathway, as altered lipid partitioning as a result of MCDc overexpression or exposure to triacsin C altered insulin secretion to KCl or arginine, PMA, and forskolin or GLP-1, indicative of an effect distal to depolarization of the membrane, activation of protein kinase C (PKC; class c), and cAMP-dependent protein kinase, respectively. Insulin exocytosis is a complex process involving many steps, including vesicle movement, docking, priming, and ultimately fusion with the plasma membrane (61). This is a likely site for regulation by fatty acid moieties, acting directly on signal transducing effectors of secretion or indirectly via activation of some kinases, in particular the PKC enzymes by DAG and LC-CoA (62). Possibilities of more direct distal effects include 1) LC-CoA acylation of proteins, such as the exocytotic proteins synaptosomal-associated protein-25 (63) and synaptogamin (64), which can enhance their association with target membranes, and 2) DAG modulation of exocytotic machinery proteins such as the synaptic vesicle priming protein Munc-13 (65). Of potential importance, Munc-13 has a C_1 domain binding site for DAG, and it is direct binding to this site, rather than PKC activation, by which β -phorbol esters and DAG mediate augmentation of neurotransmitter release from neurons (65). Munc-13 is present in β -cells (66) and therefore could be an important site for fatty acid induction/augmentation of insulin secretion.

The results in this study are different from previous work describing an essential role of fatty acids for secretion in response to all classes of secretagogues, as docu-

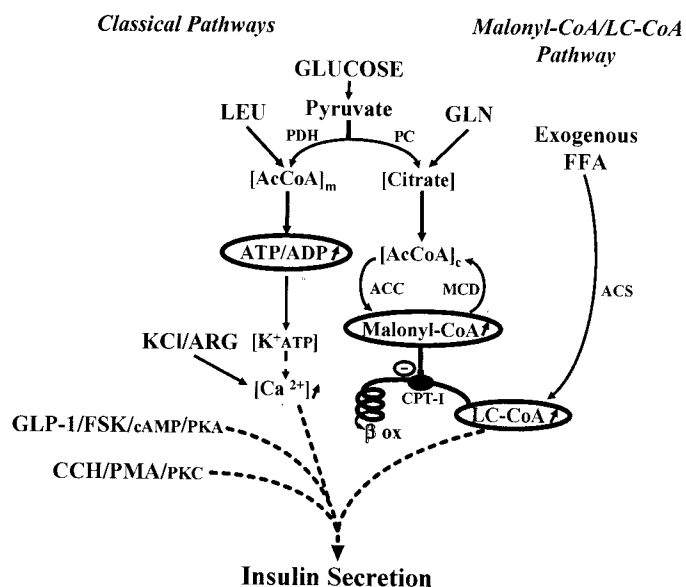


FIG. 8. Revised malonyl-CoA/LC-CoA model of insulin secretion. The malonyl-CoA/LC-CoA signaling pathway synergizes with other "classical" pathways that utilize Ca^{2+} , PKC, and cAMP/protein kinase A signaling such that lipid signaling has a regulatory amplification role on insulin secretion to all fuel and nonfuel stimuli. Malonyl-CoA, derived from the anaplerosis pathway, modulates partitioning of exogenous FFAs via LC-CoA from fatty acid oxidation to lipid signaling involved in insulin vesicle exocytosis. AcCoA_c, cytosolic acetyl-CoA; AcCoA_m, mitochondrial acetyl-CoA; ACC, acetyl-CoA carboxylase; ARG, arginine; CCH, carbachol; FSK, forskolin; GLN, glutamine; LEU, leucine; β ox, β -oxidation of fatty acids; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase.

mented in islets depleted of lipids (7) or in fasted animals (5,6). The data point to an additional regulatory role of lipid-signaling in β -cell exocytosis. Thus, under our experimental conditions, the triglyceride content and lipid esterification processes at low glucose remained unaltered by MCDc overexpression, consistent with the unaltered fat oxidation at low glucose. In addition, GHS and the response to other fuel and nonfuel stimuli were preserved in MCDc-overexpressing β -cells when secretion was measured in the absence of FFA. Together with the fact that MCDc overexpression did not change glucose oxidation and proinsulin biosynthesis, the results indicate that MCDc-enhanced expression had no general toxic effects on the β -cell, and it did not nonspecifically alter insulin exocytosis.

Whereas the results of the current study show that metabolism of FFAs is necessary for their amplification effect on insulin secretion, three recent reports suggested that exogenous FFAs could also act directly on β -cells as ligand for the orphan G-protein receptor GPR40 (67–69). These reports are preliminary in nature, and, in particular, the secretory action of the FFA was tested in the absence of BSA. Thus, the real importance of this pathway of FFA-signaling in physiological insulin secretion is uncertain. It remains an attractive possibility that both FFA metabolism and direct effects via GPR40 are involved in the regulation of insulin secretion.

The finding from this study that exogenous FFAs have a regulatory role in insulin secretion to all tested fuel and nonfuel secretagogues has potential important implications to the future study of β -cell function. The normal extracellular metabolic milieu of islet β -cells includes

FFAs contributed to by circulating FFAs in addition to FFAs derived from lipoprotein triacylglycerol via the action of islet lipoprotein lipase (70). Hence, the study of islet β -cell function probably should better reflect in vivo behavior when also studied in the presence of exogenous FFA.

Altogether, the present study allows us to refine the malonyl-CoA/LC-CoA model of insulin secretion that was initially prepared for fuel stimuli by extending it to the action of all classes of secretagogues (Fig. 8). Thus, in this view, fatty acid signaling would synergize with other "classical" pathways such as C-kinase (c type), Ca^{2+} , and cAMP/protein kinase A signaling. We suggest that the well-known permissive effect of elevated concentrations of glucose on the action of both fatty acids and most of the nonfuel stimuli (1,3) may involve, at least in part, glucose-derived malonyl-CoA. Thus, at high glucose, the rise in malonyl-CoA decreases fat oxidation, which allows cytosolic LC-CoA accumulation and lipid signaling synergizing with the classical pathways to promote full secretion. Additional work is required in particular to ascertain which downstream lipid-signaling molecules are important and the effector mechanisms by which they act. Improved understanding of the revised model and these novel lipid-signaling processes will undoubtedly have many implications for our understanding of the link between fat and β -cell adaptation and failure in the cause of diabetes.

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