Molecular or Pharmacologic Perturbation of the Link between Glucose and Lipid Metabolism Is without Effect on Glucose-stimulated Insulin Secretion

A RE-EVALUATION OF THE LONG-CHAIN ACYL-CoA HYPOTHESIS

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The mechanism by which glucose stimulates insulin secretion from the pancreatic islets of Langerhans is incompletely understood. It has been suggested that malonyl-CoA plays a regulatory role by inhibiting fatty acid oxidation and promoting accumulation of cytosolic long-chain acyl-CoA (LC-CoA). In the current study, we have re-evaluated this “long-chain acyl-CoA hypothesis” by using molecular and pharmacologic methods to perturb lipid metabolism in INS-1 insulinoma cells or rat islets during glucose stimulation. First, we constructed a recombinant adenovirus containing the cDNA encoding malonyl-CoA decarboxylase (AdCMV-MCD), an enzyme that decarboxylates malonyl-CoA to acetyl-CoA. INS-1 cells treated with AdCMV-MCD had dramatically lowered intracellular malonyl CoA levels compared with AdCMV-βGal-treated cells at both 3 and 20 mM glucose. Further, at 20 mM glucose, AdCMV-MCD-treated cells were less effective at suppressing [1-14C]palmitate oxidation and incorporated 43% less labeled palmitate into cellular lipids than either AdCMV-βGal-treated or untreated INS-1 cells. Despite the large metabolic changes caused by expression of MCD, insulin secretion in response to glucose was unaltered relative to controls. The alternative, pharmacologic approach for perturbing lipid metabolism was to use triacsin C to inhibit long-chain acyl-CoA synthetase. This agent caused potent attenuation of palmitate oxidation and glucose or palmitate incorporation into cellular lipids and also caused a 47% decrease in total LC-CoA. Despite this, the drug had no effect on glucose-stimulated insulin secretion in islets or INS-1 cells. We conclude that significant disruption of the link between glucose and lipid metabolism does not impair glucose-stimulated insulin secretion in pancreatic islets or INS-1 cells.

Glucose is a potent stimulator of insulin secretion from β-cells of the pancreatic islets of Langerhans. Regulation of insulin secretion by glucose is thought to be mediated, at least in part, by increases in the cellular ATP:ADP ratio, resulting in closure of ATP-sensitive K⁺ channels, membrane depolarization, and consequent activation of voltage-dependent Ca²⁺ channels (reviewed in Refs. 1 and 2). In recent years the concept that glucose may also signal via altering lipid metabolism has received significant experimental support (3–5). Thus, glucose administration to HIT insulinoma cells results in an increase in malonyl-CoA levels that precede the rise in insulin secretion. The increase in malonyl-CoA, acting via its capacity to inhibit the mitochondrial enzyme carnitine palmitoyltransferase I (6), results in inhibition of fatty acid oxidation, increased de novo lipid synthesis, and a rise in diacylglycerol content. These findings led Prentki and colleagues (3, 4) to propose that increases in the levels of cytosolic long-chain acyl-CoA (LC-CoA) esters are a signal transduction intermediate in glucose-stimulated insulin secretion, and this idea has come to be known as the “long-chain acyl-CoA hypothesis.” Feasible sites at which increased LC-CoA could influence insulin secretion include conversion to bioactive metabolites such as diacylglycerol or inositol trisphosphate, contribution to plasma membrane or secretory granule membrane lipid turnover, or direct acylation of proteins involved in secretory granule trafficking. While the proposed mechanisms are reasonable, direct evidence for any of these actions of lipids on insulin secretion has not been provided to date.

Other recent studies have demonstrated that the effects of lipids on β-cell function are complex. Lowering of circulating fatty acids via administration of nicotinic acid (7, 8) or adenovirus-induced hyperleptinemia (9) completely abrogates glucose-stimulated insulin secretion, but full secretory function can be restored by provision of fatty acids to the pancreas of such animals. It has also been established for some time that fatty acids are acute potentiators of the glucose-stimulated insulin secretion response (10, 11). In contrast to these positive acute effects of lipids on islet function, long-term exposure to hyperlipidemic conditions results in a condition of “lipotoxicity,” wherein lipid overstorage leads to the syndrome of β-cell dysfunction associated with insulin-resistant and diabetic states, including β-cell hyperplasia, basal hyperinsulinemia, and loss of glucose responsiveness (12, 13).

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‡ The abbreviations used are: LC-CoA, long-chain acyl-coenzyme A; MCD, malonyl-CoA decarboxylase; PBS, phosphate-buffered saline; β-gal, β-galactosidase; SAB, secretion assay buffer; ACC, acetyl-CoA carboxylase.
In light of the growing emphasis on the role of lipids in β-cell function, the current study was undertaken to re-investigate the LC-CoA hypothesis of β-cell signal transduction using novel molecular and pharmacologic approaches. The goal of the study was to manipulate the levels of malonyl-CoA and LC-CoA esters during glucose stimulation and to assess the effect of such maneuvers on insulin secretion. The molecular approach chosen was to use recombinant adenovirus to express the enzyme malonyl-CoA decarboxylase, which decarboxylates malonyl-CoA to acetyl-CoA, thus allowing us to specifically block malonyl-CoA accumulation during glucose stimulation. The pharmacologic approach was to use triacsin C, an inhibitor of long-chain acyl-CoA synthetase (14) to block the conversion of fatty acids to LC-CoA. We demonstrate that while both approaches have a large impact on lipid metabolism in the β-cell, neither maneuver influences glycolytic flux or glucose-stimulated insulin secretion.

MATERIALS AND METHODS

**INS-1 Cell Culture and Preparation of Rat Pancreatic Islets—**INS-1 cells were a gift from Drs. Philippe Halban and Claus Wollheim (15), and were described previously (16). Briefly, INS-1 cells (passages 180–260) were grown on 10-cm tissue culture plates (Corning) in RPMI 1640 (Life Technologies, Inc.) media that contains 11.1 mM glucose, supplemented with 10 mM HEPES, 10% fetal calf serum (Atlanta Biologicals), 2 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 10 μg/ml streptomycin (Bio-Whittaker), and 50 μM β-mercaptoethanol. Cultures were incubated at 37 °C in a humidified 95% air, 5% CO₂ atmosphere. INS-1 cells were trypsinized (Life Technologies, Inc.) and split (1:2) every 3–4 days for passaging or replated in 4 ml (1 ml for islets) secretion assay buffer (SAB), containing 3 mM glucose. SAB contains 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 20 mM HEPES, and 1% Triton X-100. To initiate the assay, 500 μg of protein from INS-1 cell homogenates diluted 1:10 in 50 μl of PBS was added to the reaction mixture, and absorbance was continuously measured at 412 nm for 10 min, over which time the rate of product accumulation was linear.

**Malonyl-CoA Assay—**INS-1 cells were grown to near confluence in 10-cm plates. The RPMI growth medium was removed and replaced with SAB containing 3 mM glucose for 1 h. Following this preincubation, cells were washed once with SAB containing 3 mM glucose and then incubated with SAB containing 3 or 20 mM glucose for 5 or 30 min. Cells were then collected in 1.2 ml of ice-cold 10% trichloroacetic acid and centrifuged at 7,000 × g. The supernatant containing acid soluble malonyl-CoA was collected and then was washed six times with 0.8 ml of diethyl ether. Samples were dried in a Speed-Vac and stored at −80 °C prior to assay of malonyl-CoA levels. For assay, each sample was resuspended in 0.4 ml of 1x K₂PO₄, pH 7.1, and divided into two 0.2-ml aliquots. 100 pmol of malonyl-CoA was added to one of the two samples, and malonyl-CoA levels were assayed in both samples according to the method of McGarry and Foster (23), using purified fatty acid synthase.

**Palmitate Oxidation—**INS-1 cells were collected in a suspension of 10% trichloroacetic acid and were cultured as described previously (16). Briefly, INS-1 cells (passages 180–260) were grown on 10-cm tissue culture plates (Corning) in RPMI 1640 (Life Technologies, Inc.) media that contains 11.1 mM glucose, supplemented with 10 mM HEPES, 10% fetal calf serum (Atlanta Biologicals), 2 mM L-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, 10 μg/ml streptomycin (Bio-Whittaker), and 50 μM β-mercaptoethanol. Cultures were incubated at 37 °C in a humidified 95% air, 5% CO₂ atmosphere. INS-1 cells were trypsinized (Life Technologies, Inc.) and split (1:2) every 3–4 days for passaging or replated in 4 ml (1 ml for islets) secretion assay buffer (SAB), containing 3 mM glucose. SAB contains 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 20 mM HEPES, and 1% Triton X-100. To initiate the assay, 500 μg of protein from INS-1 cell homogenates diluted 1:10 in 50 μl of PBS was added to the reaction mixture, and absorbance was continuously measured at 412 nm for 10 min, over which time the rate of product accumulation was linear.

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tracers (0.5 or 5 Ci/mol, respectively). At the conclusion of the 2-h static incubation, media were collected for insulin assay and 1 ml of methanol: PBS (2:3) was added to the plates. Cells were collected with gentle pipetting, centrifuged at 700 × g, and washed once with PBS. 200 μl of 0.2 N KOH was added to the cell pellet and the mixture was immediately frozen in liquid N2. The lipid and aqueous soluble products were separated by the following procedure. To the thawed cell suspension, 750 μl of CHCl3:methanol (2:1) and 50 μl of 0.1 N KOH was added, and, after vigorous vortexing, the phases were separated by centrifugation at 2000 × g for 20 min. The aqueous lower layer was removed and the bottom lipid-soluble layer was washed with 200 μl of methanol:water-CHCl3 (48:47:3). 200 μl of either the aqueous or lipid soluble phase was added to BioSafe2 scintillation mixture and incorporation of radioisotope into lipids was quantified.

## RESULTS

### Effect of MCD Expression on Malonyl-CoA Levels and Secretion—

Our first strategy for testing the long-chain acyl-CoA hypothesis was to specifically reduce malonyl-CoA levels during glucose stimulation of INS-1 cells. The enzyme MCD reverses the reaction catalyzed by acetyl-CoA carboxylase by converting malonyl-CoA to acetyl-CoA. Two sets of experiments were performed with the AdCMV-MCD virus. In order to measure malonyl-CoA levels, it was necessary to use large (10 cm) plates of INS-1 cells. Thus, the first set of experiments measured malonyl-CoA levels and insulin secretion from INS-1 cells grown in large plates and treated with AdCMV-MCD or the control virus, AdCMV-βGAL. As shown in Table I, INS-1 cells treated with AdCMV-MCD contained high levels of MCD activity in crude cellular extracts. In contrast, cells treated with a control virus (AdCMV-βGAL) contained levels of MCD activity close to the lower detection limit of the assay, and high levels of β-galactosidase activity. Centrifugation of the crude cellular extracts of AdCMV-MCD-treated cells to yield mitochondria-enriched and cytosolic fractions (24) revealed that 90% of the MCD was in the latter fraction, as expected because the expressed enzyme lacks its N-terminal mitochondrial localization signal.

Fig. 1 shows malonyl-CoA levels in AdCMV-MCD-treated INS-1 cells relative to AdCMV-βGAL-treated controls. Two incubation time points were chosen (5 and 30 min), based on a previous study in which a biphasic profile in malonyl-CoA accumulation in rat islets perfused with high (25 mM) glucose was demonstrated (25). As shown in Fig. 1, incubation of AdCMV-βGAL-treated INS-1 cells in 20 mM glucose for 5 min caused a 4.2-fold increase in malonyl-CoA levels relative to cells cultured for the same time period in 3 mM glucose. In contrast, the increase in malonyl-CoA in response to high glucose in AdCMV-MCD-treated cells was only 1.8-fold, and the level of malonyl-CoA attained in control cells incubated at the high glucose concentration was nearly 3 times that in the AdCMV-MCD-treated cells (24 versus 8.4 pmol/mg of protein).

Malonyl-CoA levels were increased by approximately 60% in AdCMV-βGAL-treated cells incubated for 30 min at the low glucose concentration compared with cells incubated for 5 min, and this contributed to a somewhat smaller (2.4-fold) increment in malonyl-CoA in response to 20 mM glucose in the 30-min experiments. However, in the 30-min experiments, malonyl-CoA levels in AdCMV-MCD-treated cells incubated at low glucose were reduced by 70% relative to AdCMV-βGAL-treated cells. Exposure of the MCD expressing cells to high glucose caused a 2.9-fold increase in malonyl-CoA levels from this lowered baseline, but the absolute amount of malonyl-CoA in control cells cultured at high glucose was still 2.7 times that in AdCMV-MCD-treated cells (22 versus 8.1 pmol/mg protein). Thus, while increases in malonyl-CoA levels in response to glucose occur in AdCMV-MCD-treated cells, the amount of malonyl-CoA in AdCMV-MCD-treated cells incubated at 20 mM glucose was not statistically different from control cells incubated at 3 mM glucose. Finally, the fall in malonyl-CoA level previously reported in glucose-perfused rat islets at longer time points (25) was not observed in these static incubation experiments with INS-1 cells.

Despite this block in malonyl-CoA accumulation, MCD expression had no effect on insulin secretion in response to glucose in INS-1 cells. As shown in Fig. 2, equal amounts of insulin were secreted from AdCMV-MCD and AdCMV-βGAL-treated cells at either low (3 mM) or high (20 mM glucose) concentrations, with the same increase in response to stimulatory glucose in either group of cells. Note that these static incubation experiments were carried out for 30 min, in order to be able to correlate the results with those obtained for malonyl-CoA levels. Thus, the observed increase in insulin secretion in response to stimulatory glucose of approximately 2-fold is less than the 3–4-fold response observed by us in static incubation experiments of longer duration (Ref. 16; see also data in Fig. 5 below). These results indicate that attainment of a critical threshold of malonyl-CoA concentration, as defined by the levels reached in control cells incubated at high glucose, is not a requirement for glucose-stimulated insulin secretion in INS-1 cells.
observed in the 10-cm plate experiments described in Table I. Similarly, MCD expression in AdCMV-MCD-treated INS-1 cells from 3 to 20 mM glucose caused an 80% reduction in glucose incorporation into the lipid soluble phase of INS-1 extracts by 43% compared with untreated or AdCMV-βGAL-treated cells at 20 mM glucose (Fig. 3B). As was the case for palmitate oxidation, MCD expression had no effect on palmitate incorporation into lipids at low glucose.

We also tested the effect of MCD expression on [U-14C]glucose incorporation into the lipid soluble phase of INS-1 cell extracts. At 20 mM glucose, MCD expression reduced glucose incorporation into the lipid phase by 50% compared with untreated or AdCMV-βGAL-treated controls (Fig. 3C). However, consistent with the results from the first series of experiments performed in 10-cm plates and depicted in Fig. 2, expression of MCD in INS-1 cells in the smaller 12-well plates had no impact on basal or glucose-stimulated insulin secretion (data not shown).

In summary, expression of MCD in INS-1 cells impairs normal glucose suppression of fatty acid oxidation, and causes decreased incorporation of fatty acids and glucose into cellular lipids, but has no impact on glucose-stimulated insulin secretion. Because measurements of metabolic fluxes were carried out in 12-well plates rather than 10-cm dishes, it was not possible to make parallel measurements of malonyl-CoA levels for all experiments. However, it should be pointed out that the level of MCD activity was actually higher in AdCMV-MCD-treated INS-1 cells grown in 12-well dishes than in 10-cm plates (Table I), suggesting that malonyl-CoA levels were likely maintained at low levels in both sets of experiments (see Fig. 1).

Effect of Triacsin C Treatment on Metabolism and Insulin Secretion in INS-1 Cells—Triacsin C potently inhibits LC-CoA synthetase, a microsomal and outer mitochondrial enzyme that condenses long-chain fatty acid and CoASH to thioester linkage (14). Therefore, an alternative strategy for testing the LC-CoA hypothesis was to use triacsin C to block LC-CoA formation from endogenous stores or exogenous fatty acids, thus preventing their incorporation into mono-, di-, and triglycerides, as well as phospholipids and acylated proteins. The feasibility of using triacsin C was supported in a study that demonstrated an 80% reduction in [14C]oleate incorporation into phospholipids and triglycerides in Raji cells (14) and by our own recent work demonstrating a near-complete block of radio-labeled glycerol incorporation into cellular lipids in glycerol kinase expressing INS-1 cells (16).

The metabolic impact of treatment of INS-1 cells with 10 μM triacsin C was monitored by measurement of its effects on palmitate oxidation and palmitate incorporation into cellular lipids. Triacsin C treatment caused an 88% reduction in palmitate oxidation in INS-1 cells incubated at 3 mM glucose relative to control cells incubated at the same glucose concentration in the absence of the drug (Fig. 4A). In contrast, AdCMV-MCD-MCD-treated cells exhibit only a 59% reduction in palmitate oxidation in response to the switch from low to high glucose, such that MCD expressing cells oxidized palmitate at twice the rate of either control group at 20 mM glucose. Importantly, AdCMV-MCD expression had no effect on [5,3H]glucose usage or [1,14C]glucose oxidation at either the low or high glucose concentration relative to the control groups (data not shown).

INS-1 cells incubated in 0.5 mM palmitate, 1% bovine albumin incorporate 2.7 times as much palmitate into complex lipids in the presence of 20 mM glucose compared with 2 mM glucose (Fig. 3B). This is due to both an increase in availability of LC-CoA due to decreased palmitate oxidation and increased glycerol phosphate production via glycolysis. It was expected that AdCMV-MCD treatment would decrease exogenous palmitate incorporation into cellular lipids due to the enhanced palmitate degradation. In fact, AdCMV-MCD treatment reduced [1,14C]palmitate incorporation into the lipid soluble phase of INS-1 extracts by 43% compared with untreated or AdCMV-βGAL-treated cells at 20 mM glucose (Fig. 3B). As was the case for palmitate oxidation, MCD expression had no effect on palmitate incorporation into lipids at low glucose.

Metabolic Impact of MCD Expression—By reducing malonyl-CoA levels, MCD expression is predicted to decrease glucose-induced LC-CoA accumulation by the two following mechanisms. First, malonyl-CoA is a key substrate for fatty acid synthase. Depriving fatty acid synthase of malonyl-CoA availability will decrease de novo lipogenesis and therefore decrease new LC-CoA biosynthesis. Second, malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase I (6). Prevention of the normal rise in malonyl-CoA levels during glucose stimulation would therefore be predicted to interfere with LC-CoA accumulation by favoring fatty acid oxidation relative to de novo synthesis and esterification. Thus, in the second set of experiments, INS-1 cells were aliquoted in 12-well plates, treated with AdCMV-MCD, AdCMV-βGAL, or left untreated, and used for measurement of palmitate oxidation, or glucose or palmitate incorporation into lipid. An independent set of insulin secretion measurements was also included in this second set of experiments. As shown in Table I, treatment of cells in 12-well plates with AdCMV-MCD caused an even more dramatic increase in MCD enzyme activity in crude extracts than observed in the 10-cm plate experiments described in Table I.

INS-1 cells, like normal islet β-cells, exhibit an increase in glycolytic flux and insulin secretion as glucose concentrations are raised over the physiologic range (15, 16). Previous studies have shown that when glycolytic flux is high in β-cells, malonyl-CoA levels increase, carnitine palmitoyltransferase I is inhibited, and fatty acid oxidation is reduced (3-5). Consistent with this paradigm, switching of untreated or AdCMV-βGAL-treated INS-1 cells from 3 to 20 mM glucose caused an 80% reduction in [1,14C]palmitate oxidation (Fig. 2A). In contrast, AdCMV-MCD-MCD-treated cells exhibit only a 59% reduction in palmitate oxidation in response to the switch from low to high glucose, such that MCD expressing cells oxidized palmitate at twice the rate of either control group at 20 mM glucose. Importantly, AdCMV-MCD expression had no effect on [5,3H]glucose usage or [1,14C]glucose oxidation at either the low or high glucose concentration relative to the control groups (data not shown).
The foregoing experiments, while demonstrating potent triacsin C-mediated inhibition of metabolism of exogenously added fatty acids, leave open the possibility that the pre-existing intracellular LC-CoA pool may enter esterification pathways during glucose stimulation, thereby contributing in some way to regulation of insulin secretion. To test this we monitored [3-3H]glucose incorporation into organically extractable lipids in INS-1 cells incubated in the presence and absence of triacsin C. These experiments were performed in the absence of exogenous fatty acids. Thus, incorporation of radiolabeled glucose into lipids in the presence of triacsin C should reflect primarily the esterification of pre-formed LC-CoA with radiolabeled glycerol phosphate derived from glycolysis. As shown in Fig. 5A, incorporation of [3-3H]glucose into lipids increases in proportion to the glucose concentration over the range of 3 to 10 mM in INS-1 cells not exposed to triacsin C, with a maximal value of 12 nmol of glucose incorporated/mg of protein/h measured at 10 mM glucose. In contrast, cells exposed to triacsin C incorporate only half as much labeled glucose into lipids at basal glucose (3 mM) as control cells. Glucose incorporation is increased in triacsin C-treated cells as glucose is raised to 4–5 mM, but then fails to continue to rise at higher concentrations of the sugar, with maximal values of only 4 nmol of glucose incorporated/mg of protein/h at glucose concentrations of 5 mM or greater.

Insulin secretion was measured in parallel with the metabolic measurements reported in Fig. 5A. As shown in Fig. 5B, insulin secretion was increased in direct proportion to glucose concentration in both triacsin C-treated and control INS-1 cells. Insulin secretion rose from approximately 50 microunits/mg of protein/h at 3 mM glucose to 80 microunits/mg of protein/h at 5 mM glucose, a range over which glucose incorporation into lipid was increased in both groups of cells, but then continued to rise from 80 microunits/mg of protein/h at 5 mM glucose to 160 microunits/mg of protein/h as glucose was raised to 10 mM in both groups, despite a complete block in further glucose incorporation into lipids in the triacsin C-treated cells. No further increases in insulin secretion or labeled glucose incorporation into lipids were observed at 20 mM glucose.
pared with 10 mM glucose (data not shown). We conclude from these studies that triacsin C effectively limits the incorporation of radiolabeled glucose or palmitate into cellular lipids, and during blockade of LC-CoA synthetase, there is no large pool of preformed LC-CoA that participates in esterification pathways. Furthermore, interruption of LC-CoA synthesis is without effect on glucose-stimulated insulin secretion.

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FIG. 4. Effect of triacsin C on palmitate metabolism in INS-1 cells. Panel A, \([1-^{14}C]\)palmitate oxidation. INS-1 cells were preincubated in SAB containing 3 mM glucose, in the presence or absence of 10 \(\mu\)M triacsin C, for 2 h, and oxidation was measured as a function of \(^{14}\text{CO}_2\) generation during a subsequent 2-h incubation with SAB containing radiolabeled palmitate and either 3 or 20 mM glucose, in the presence or absence of 10 \(\mu\)M triacsin C. Data represent the mean ± S.E. for four independent experiments. The asterisk (*) indicates that less palmitate was oxidized in triacsin C-treated INS-1 cells than in untreated control cells at either glucose concentration, at a level of significance of \(p < 0.001\). Panel B, \([1-^{14}C]\)palmitate incorporation into organically extractable cellular lipids. Cells were treated identically as described for panel A. After incubation of cells for 2 h in SAB containing radiolabeled palmitate and either 3 or 20 mM glucose, with or without 10 \(\mu\)M triacsin C, cells were collected and extracted for measurement of incorporation of the fatty acid into total cellular lipids as described under "Materials and Methods." Data represent the mean ± S.E. for four independent experiments. The asterisk (*) indicates that less palmitate was incorporated into cellular lipids in triacsin C-treated INS-1 cells than in untreated control cells at either glucose concentration, at a level of significance of \(p < 0.001\).

FIG. 5. Effect of triacsin C on glucose incorporation into lipids and glucose-stimulated insulin secretion. Panel A, \([3-^{3}H]\)glucose incorporation into organically extractable cellular lipids. INS-1 cells were preincubated in SAB containing 2 mM glucose in the presence or absence of 10 \(\mu\)M triacsin C for 2 h. Cells were then switched to SAB containing various concentrations of \([3-^{3}H]\)glucose with or without 10 \(\mu\)M triacsin C, and after a further 2-h incubation, cells were collected and extracted for measurement of incorporation of labeled sugar into total cellular lipids as described under "Materials and Methods." Data represent the mean ± S.E. for three independent experiments per glucose concentration. The asterisks (*) indicate that less glucose was incorporated into cellular lipids in triacsin C-treated cells than in controls at the indicated glucose concentrations. Panel B, insulin secretion. Media samples were collected from the cells described in panel A and subjected to insulin radioimmunoassay. Data represent the mean ± S.E. for three independent experiments.
Re-evaluation of the LC-CoA Hypothesis

In this study we have applied molecular (MCD expression) and pharmacologic (triacsin C treatment) strategies for attenuating glucose-induced alterations in lipid metabolism in insulin-secreting cells. Surprisingly, neither approach impaired glycolysis or affected glucose-stimulated insulin secretion in INS-1 cells, and the lack of impact of triacsin C on regulation of insulin secretion was also confirmed in freshly isolated rat islets. These results suggest that reassessment of the long-chain acyl-CoA hypothesis for stimulus secretion coupling in islet β-cells is in order. Two important observations that led to development of the hypothesis were: 1) increasing glycolytic flux in β-cells by increasing the extracellular glucose concentration led to an increase in malonyl-CoA levels proportional to insulin secretion (3, 4), and 2) glucose-mediated alterations in malonyl-CoA levels were linked to changes in lipid metabolism and the cytosolic LC-CoA pool, which in turn was suggested to trigger insulin secretion by an undefined mechanism (3–5). In what follows, these two critical observations are re-evaluated in the context of the data obtained in the current study.

First, increasing glycolytic flux in β-cells by increasing the extracellular glucose concentration leads to an increase in malonyl-CoA levels that is proportional to insulin secretion. Our findings are in agreement with this cornerstone observation of the LC-CoA hypothesis, but only in control INS-1 cells. We find
that malonyl-CoA levels are increased in response to a change in glucose concentration from 3 to 20 mM at both 5 and 30 min in control INS-1 cells, the same range over which glucose-stimulated-insulin secretion. However, expression of MCD in INS-1 cells sharply attenuates the rise in malonyl-CoA levels in response to stimulatory glucose at the 5-min time point. At 30 min, MCD expression lowers basal malonyl-CoA levels substantially, but allows an increase in the intermediate in response to glucose. However, at both 5 and 30 min, the levels of malonyl-CoA achieved in MCD expressing cells exposed to 20 mM glucose are well below the levels in control cells incubated at 20 mM glucose and are in fact not statistically different than the levels in control cells incubated at 3 mM glucose. These data indicate that the high levels of malonyl-CoA achieved in glucose-stimulated control INS-1 cells do not define a critical threshold of the intermediate that must be achieved in order for insulin secretion to be activated. However, we cannot as yet formally exclude the unlikely possibility that the increment in malonyl-CoA that remains in MCD expressing cells at the 30-min time point is involved in stimulus/secretion coupling.

Second, glucose-mediated alterations in malonyl-CoA levels are linked to changes in lipid metabolism and an increase in the cytosolic LC-CoA pool that regulate insulin secretion. Again, we were able to confirm the correlative linkage between lipid metabolism and insulin secretion (3–5), but only in INS-1 cells not engineered for MCD expression or treated with triacsin C. In such control cells, raising glucose from 3 to 20 mM results in a profound inhibition of fatty acid oxidation, with concomitant and potent activation of incorporation of radiolabeled glucose and palmitate into organically extractable cellular lipids. It is assumed that the increased incorporation of glucose or palmitate into lipids reflects accumulation of cytosolic LC-CoA, the necessary intermediate for synthesis of end products such as phospholipids and triglycerides. Interestingly, previous measurements of LC-CoA during glucose stimulation of a hamster insulinoma cell line (HIT) showed a decrease in the total LC-CoA pool (3). It was suggested that the total LC-CoA pool largely represents material sequestered in the mitochondria, and is an inaccurate measure of changes in the more relevant cytoplasmic pool (3). This represents a technical limitation for evaluating the relationship between cytoplasmic LC-CoA and insulin secretion. However, in the current study, we have utilized triacsin C as a direct inhibitor of long-chain acyl-CoA synthetase, the enzyme responsible for LC-CoA synthesis. We find that this reagent has its anticipated effects of sharply suppressing fatty acid oxidation and esterification in both INS-1 cells and freshly isolated rat islets, but does not influence glucose-stimulated insulin secretion. Importantly, triacsin C also causes a 45% decrease in total LC-CoA levels in INS-1 cells incubated at either low or high glucose concentrations. Similarly, expression of MCD in INS-1 cells significantly attenuates glucose-induced suppression of fatty acid oxidation while decreasing glucose and palmitate incorporation into cellular lipids with no effect on regulation of insulin secretion. Because these two completely independent methods produced similar results, we conclude that there is no direct correlation between the extent to which lipids are directed toward oxidative or esterification pathways and insulin secretion. While we have not directly measured the cytosolic LC-CoA pool, to the extent that the fall in total LC-CoA in response to triacsin C is reflective of this fraction, our data also argue that a rise in LC-CoA is not a critical event in glucose-stimulated insulin secretion. This conclusion is also applicable to other carbohydrate secretagogues, since we have recently shown that blockade of glycerol incorporation into cellular lipids with triacsin C in glycerol kinase-expressing INS-1 cells has no effect on glycerc-stimulated insulin secretion (16).

Note that in a recent study in fibroblasts, triacsin C was shown to be almost completely effective in blocking triglyceride and phospholipid synthesis from glycerol, while incorporation of oleate or arachidonate into phospholipids was less impaired (26). Similarly, using thin layer chromatography analysis of INS-1 cell extracts, we have observed that triacsin C inhibits conversion of [1-14C]palmitate into triglycerides by more than 90%, while incorporation of the same substrate into phospholipids is reduced by 50% (data not shown). Thus, while the effects of MCD expression or triacsin C treatment on lipid fluxes in our studies were large, they were not complete, and it remains possible that a small residual flux of substrate into specific esterification or biosynthetic pathways is required for maintenance of glucose responsiveness. It could be envisaged, for example, that a less efficacy of triacsin C for blockade of phospholipid biosynthesis could allow maintenance of a critical pool of this substrate for phospholipase A2-mediated hydrolysis and release of potential second messengers such as arachidonic acid (27). Furthermore, our results are not simply reconciled with earlier experiments in which a presumed block of malonyl-CoA synthesis with the citrate lyase inhibitor, hydroxycitrate, in the perfused rat pancreas was found to inhibit glucose-stimulated insulin secretion, an effect that was reversible by palmitate addition (5). It should be noted, however, that studies by other investigators failed to demonstrate an effect of hydroxycitrate on isolated rat islets (28), and in confirmation of this finding, no effect of the drug on insulin secretion was observed in fresh rat islets or INS-1 cells by us (data not shown). Also, addition of the β-oxidation inhibitor, etomoxir, to the perfused pancreas preparation potentiates insulin secretion, but only at concentrations 10 times higher than required to inhibit oxidation, suggesting that the effect of this reagent on insulin secretion may be via its structural similarity to saturated fatty acids, which are known potentiators of insulin release (5). We can only speculate that the intact pancreas has a unique susceptibility to hydroxycitrate compared with isolated islets or INS-1 cells, or that this reagent has effects at sites other than inhibition of citrate lyase in the perfused pancreas preparation. Perhaps cultivating isolated islets or cell lines in rich media containing essential lipids from the fetal calf serum is sufficient to overcome hydroxycitrate-mediated inhibition of lipogenesis. Additionally, isolated islets and β-cell lines may have a greater storage capacity for lipids than the intact pancreas, which would make the pancreas more susceptible to lipogenic inhibitors. Thus, while we feel that our major conclusion that large alterations in lipid metabolism are not linked to perturbations in glucose-regulated insulin secretion is correct for freshly isolated rat islets and INS-1 cells, it remains possible that islets in situ are sensitive to regulation by such a mechanism.

It has, in fact, recently been shown that complete depletion of lipid stores in islets blocks normal responsiveness to glucose and other secretagogues. Thus, animals treated with nicotinic acid to lower circulating free fatty acid exhibit no increase in insulin secretion in response to a glucose challenge (7, 8). Similarly, islets in the perfused pancreas of animals rendered hyperleptinemic for a period of 1 week are completely devoid of triglyceride and fail to respond to glucose or arginine as a secretagogue, while animals pair-fed to the lower rate of food intake of hyperleptinemic animals retain normal islet function and some residual triglyceride (9). Insulin secretion in response to glucose can be rapidly restored by lipid infusion into nontoxic acid-treated animals (7, 8), or by inclusion of fatty acids during perfusion of pancreata from fasted or hyperleptinemic animals (7–9). These data argue that lipids must be present, at
least at some minimal level, in order for islet β-cells to function normally.

In light of all of these results, we suggest the following modification of the long-chain acyl-CoA hypothesis. Our work shows that large impairment in the normal link between glucose and lipid metabolism in β-cells is tolerated with no impact on glucose-stimulated insulin secretion, as long as glycolytic flux is not perturbed. This allows us to narrow the search for metabolic pathways and coupling factors involved in acute glucose-stimulated insulin secretion. With the apparent elimination of changes in malonyl-CoA levels and attendant alterations in lipid metabolism, the most likely candidates now include a glycolytic signal, oxidative events, or anaplerosis (1, 2, 29). The primary signal may include the well recognized glucose-driven increase in ATP:ADP ratio, leading to inhibition of ATP-sensitive K+ channels and influx of extracellular Ca2+ (1, 2).

Our experiments were designed to evaluate the effect of acute perturbation of the link between glucose and lipid metabolism. The abrogation of glucose-stimulated insulin secretion by more long-term maneuvers such as nicotinic acid administration or adenosivus-induced hyperleptinemia (7–9) may be explained by lipid depletion and the resultant absence of essential modulators of secretory granule trafficking and/or exocytosis. Examples of distal sites at which lipids may be acting include generation of signaling molecules such as diacylglycerol or inositol trisphosphate, or via contribution to secretory granule or plasma membrane turnover (3–5). That lipids are acting at a distal rather than a proximal site is supported by the finding that lipid-depleted islets not only fail to respond to glucose but also show no response to arginine, leucine, or the sulfonylurea, glibenclamide (9, 30). Arginine is thought to exert its secretory effects by directly affecting membrane polarization, while sulfonylureas are believed to bring about the same effect by inhibiting ATP-sensitive K+ channel activity. Thus, both agents act distal to any anticipated early metabolic signal. Yet with all secretagogues tested to date, the attenuated insulin response in lipid-depleted islets is restored to normal by inclusion of fatty acids in the perfusate (7–9, 30). The mechanism by which fatty acids exert these important modulatory effects on insulin secretion remain to be established. Finally, it should be emphasized that the data do not support the possibility that the malonyl-CoAVacnurneine palmitoyltransferase I metabolic signaling network may play important roles in long-term processes related to insulin secretion, i.e. β-cell growth, apoptosis, regulation of important metabolic genes, and effects related to chronic exposure of β-cells to high concentrations of fatty acids (i.e. changes in glucose sensitivity, “lipotoxicity”) (2, 12, 31, 32). The molecular and pharmacologic tools described in this paper should be useful for future investigation of these issues.

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Note Added in Proof—After submission of this paper we became aware of an article by Zhang and Kim in which stable expression of acetyl CoA carboxylase (ACC) antisense RNA in INS-1 cells was reported (33). In cells expressing the ACC antisense construct, a 40% reduction in ACC enzymatic activity was correlated with a similar decrease in glucose-stimulated insulin secretion and malonyl CoA levels and an approximate doubling of the rate of fatty acid oxidation. One potential explanation for these findings is that stable suppression of ACC activity, as opposed to transient expression of MCD or short term treatment with tricatin C, may lead to a depletion of the cellular lipid stores, similar to what is observed in animals treated with nicotinic acid or infused with an adenosivus containing the leptin cDNA (7–9). This possibility could be evaluated in the future by examination of the effects of MCD expression for time periods longer than those used in the current study.

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