Biochemical Mechanism of Lipid-Induced Impairment of Glucose-Stimulated Insulin Secretion and Reversal with a Malate Analogue

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Abbreviations:  DMM, dimethylmalate; GSIS, glucose-stimulated insulin secretion;
Glc, glucose, HBSS, HEPES buffered saline; MCD, malonyl CoA decarboxylase; TG,
triglycerides; FFA, free fatty acids; ZDF, Zucker diabetic fatty; β-GAL, β-galactosidase;
PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; moi, multiplicity of infection
Abstract

Hyperlipidemia appears to play an integral role in loss of glucose-stimulated insulin secretion (GSIS) in type 2 diabetes. This impairment can be simulated \textit{in vitro} by chronic culture of 832/13 insulinoma cells with high concentrations of free fatty acids, or by study of lipid-laden islets from Zucker diabetic fatty rats. Herein we show that impaired GSIS is not a simple result of saturation of lipid storage pathways, as adenovirus-mediated overexpression of a cytosolically localized variant of malonyl CoA decarboxylase in either cellular model results in dramatic lowering of cellular triglyceride stores, but no improvement in GSIS. Instead, the glucose-induced increment in “pyruvate cycling” activity (pyruvate exchange with TCA cycle intermediates measured by \textsuperscript{13}C NMR), previously shown to play an important role in GSIS, is completely ablated in concert with profound suppression of GSIS in lipid-cultured 832/13 cells, while glucose oxidation is unaffected. Moreover, GSIS is partially restored in both lipid-cultured 832/13 cells and islets from ZDF rats by addition of a membrane permeant ester of a pyruvate cycling intermediate (dimethylmalate). We conclude that chronic exposure of islet β-cells to fatty acids grossly alters a mitochondrial pathway of pyruvate metabolism that is important for normal GSIS.
A major contributing factor to the development of type 2 diabetes is inadequate insulin secretion to compensate for insulin resistance. A hallmark of this β-cell dysfunction is the impairment and eventual complete loss of glucose-stimulated insulin secretion (GSIS). Hyperlipidemia, and the consequent accumulation of triglycerides (TG) and other lipid-derived intermediates in β-cells, is now well recognized as a variable that correlates with development of impaired insulin secretion (1-6). Furthermore, culture of pancreatic islets (3,7,8) or insulinoma cell lines (9) with elevated levels of free fatty acids in vitro results in loss of GSIS, and glucose sensing is also dramatically impaired in fat-laden islets from Zucker diabetic fatty (ZDF) rats (2,3). However, a biochemical mechanism linking chronic exposure of islet cells to high levels of free fatty acids and impairment of GSIS has not emerged.

To gain more insight into this important issue, two independent model systems were exploited. First, we have recently described stable subclones of the rat insulinoma INS-1 cell line with robust GSIS, such as cell line 832/13 (10). As shown herein, chronic culture of these cells in 1 mM oleate:palmitate (2:1) causes profound impairment of GSIS. Second, islets from ZDF rats are both lipid-laden and poorly glucose responsive (3). Using these model systems, two hypotheses about the mechanism of lipid-induced impairment of GSIS were tested. The first is that accumulation of lipid-derived metabolites caused by chronic exposure of β-cells to fatty acids plays a direct role in the functional impairment. To test this idea, we have employed a recombinant adenovirus encoding a variant, cytosolically-localized form of malonyl CoA decarboxylase (AdCMV-MCD∆5) (11) to lower malonyl CoA levels in lipid-laden cells.
Application of this method caused a dramatic lowering of TG levels in both lipid-cultured 832/13 cells and in islets from ZDF rats, but failed to improve GSIS. This led us to test a second hypothesis based on our recent discovery of a critical link between pyruvate carboxylase (PC)-mediated pyruvate exchange with TCA cycle intermediates (“pyruvate cycling”) and GSIS (12). This link between pyruvate cycling and GSIS was uncovered by NMR-based analysis of [U-\(^{13}\)C] glucose metabolism in a set of variously glucose responsive INS-1-derived cell lines. More precisely, pyruvate cycling refers either to the “pyruvate/malate cycle”, involving PC-catalyzed conversion of pyruvate to oxaloacetate, reduction of oxaloacetate to malate, and decarboxylation of malate to pyruvate via malic enzyme, and/or to the “pyruvate/citrate cycle”, wherein the first and last steps are the same as in the pyruvate/malate cycle, but oxaloacetate formed in the PC reaction is converted to citrate, after which malate is regenerated via citrate lyase and cytosolic malate dehydrogenase (12). The NMR methods that we employ are not capable of distinguishing between these cycles, but do discriminate total cycling activity relative to TCA cycle flux. In the current study, we demonstrate that the profound impairment of GSIS that occurs in response of chronic exposure of 832/13 cells to fatty acids is accompanied by complete ablation of the normal glucose-induced increment in pyruvate cycling, with no change in the rates of glucose oxidation at basal or stimulatory glucose. Furthermore, we demonstrate that addition of a membrane permeant ester of a pyruvate cycling intermediate, dimethylmalate (DMM), restores a significant portion of GSIS in both fat-cultured 832/13 cells and fat-laden islets from ZDF rats. Thus, our data support a model for lipid-induced impairment of GSIS in which chronic exposure to elevated
levels of fatty acids alters a mitochondrial pathway of pyruvate metabolism that is involved in GSIS.
Material and Methods

Materials were obtained from Sigma Chemicals (St Louis, MO) unless otherwise noted.

Cell culture. A clonal β-cell line, 832/13 (10), derived from INS-1 rat insulinoma cells (13) by a transfection-selection strategy, was used in these studies. 832/13 cells were cultured in RPMI–1640 containing 11.1 mM D-glucose and supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate and 50 µM β-mercaptoethanol. Cells were cultured in 6 or 12 well plates at 37°C in a humidified atmosphere containing 5% CO₂, and media was changed routinely every second day. For studies of lipid-induced impairment of GSIS, we prepared a 10 mM oleate: palmitate (2:1 molar ratio) stock solution complexed to 10% fat free bovine serum albumin (8). This was added at a final concentration of 1 mM to cells at a state of 30-90% confluency in complete RPMI medium, dependent upon the planned duration of lipid exposure. Cells were then cultured for an additional 2-7 days as indicated in the figure legends or text.

Experimental animals. Lean wild type (+/+) male ZDF rats and obese homozygous (fa/fa) male ZDF rats were bred at the Veterans Affairs Medical Center, Dallas, TX. 11-week-old male rats were used for the experiments. Rats were fed with standard chow (Harlan/Teklad 4% 7001; Madison, WI) ad libitum and had free access to water.

Recombinant adenoviruses. Malonyl CoA decarboxylase (MCD) was overexpressed in the cytoplasmic compartment of 832/13 cells and islets by treatment with a
recombinant adenovirus containing the cDNA encoding human MCD, modified by removal of its N-terminal mitochondrial localization sequence and its C-terminal peroxisomal targeting sequence (AdCMV-MCDΔ5; (11)). As controls, other groups of cells were treated either with a virus encoding a catalytically inactive form of MCD (AdCMV-MCDmut; (11,14)), or the bacterial β-galactosidase gene (AdCMV-βGAL; (15)).

**Adenovirus transduction of 832/13 cells.** 832/13 cells at a confluency of ~90% were treated with 10 or 20 moi (multiplicity of infection) of the various recombinant adenoviruses for 2 h. Cells were washed once in RPMI and cultured for an additional 24-72 h.

**Islet isolation and adenovirus transduction.** To overexpress MCD in islets of obese and diabetic rats, pancreases of 11 week-old male ZDF (fa/fa) rats were perfused for 1 hour with 1 x 10^{12} plaque forming units (pfu) of AdCMV-MCDΔ5, suspended in Krebs ringer bicarbonate buffer with 4.5% dextran T70, 1% bovine serum albumin, 5.6 mM glucose, and 5 mM each of sodium pyruvate, sodium glutamate, and sodium fumarate (16). Pancreatic islets were then isolated by the method of Naber, et al. (17) with modifications (8). Isolated islets were either used immediately or were cultured for 48 h in RPMI-1640 with 8 mM glucose, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol.
Malonyl CoA decarboxylase activity assay. MCD activity was determined as the rate of decarboxylation of malonyl-CoA to acetyl-CoA as previously described (18). In brief, the rate of acetyl-CoA formation was monitored by cleavage of its thioester bond by acetylcarnitine transferase over 5-10 minutes, a time period during which the rate of product accumulation was linear.

Oil Red O Staining of Lipid Droplets. 832/13 cells were cultured with or without 1 mM oleate:palmitate (2:1) for six days. Aliquots of cells cultured in the presence of fat were treated with 10 or 20 moi of AdCMV-MCDΔ5 virus, or were left untreated as indicated in the legend to Figure 1. After viral treatment, cells were cultured for an additional 24 h in the presence of fatty acids. Following this period, media was removed, cells were washed once with PBS, and fixed with 2.5% glutaraldehyde (Fisher Scientific, Milwaukee, WI) for 15 min. Glutaraldehyde was removed, cells were washed with PBS, and then treated for 20 min with Oil Red O staining solution. After a wash with PBS, images of stained lipids were obtained using a Nikon phase contrast ELWD 0.3 microscope at high magnification.

Triglyceride content of 832/13 cells or islets. 832/13 cells were cultured in the presence or absence of 1 mM oleate:palmitate (2:1) for six days, treated with the various recombinant adenoviruses, and harvested 24 h after transduction. Islets from lean or obese ZDF rats were cultured for 48 h prior to harvesting. Measurement of TG content is based on assay of glycerol produced by hydrolysis of neutral lipids in the presence of lipoprotein lipase. Islets or cells were centrifuged at 1200 rpm for 10 min at 4ºC, washed
with PBS and re-centrifuged. Lipids were extracted with chloroform: methanol (2:1) and the lower phase was evaporated under N₂. The samples were resuspended in 50 µl chloroform, and 10 µl was air-dried. The dry pellet was resuspended in 10 µl of Thesit (ICN, Ohio). A standard curve (1-50 µg) was prepared using Triolein diluted in chloroform: methanol 2:1, and 10 µl of sample or standard were assayed in duplicate by adding 200µl of GPO Trinder Reagent and measurement of absorbance at 540 nm.

**Insulin secretion studies.** 832/13 cells were grown in the presence or absence of 1 mM oleate/palmitate (2:1) for the time periods indicated in the figure legends. Cells were washed with HEPES–buffered saline (HBSS) with 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5mM CaCl₂ and 25.5 mM NaHCO₃, pH 7.2), containing 0.2 % bovine serum albumin, followed by 2 h preincubation in the same buffer. Insulin secretion was then measured by static incubation of the cells for 2 h in HBSS containing either 3 or 12 mM glucose. Dimethylmalate (DMM, Aldrich Chemicals, Pittsburgh, PA) was added at a final concentration of 10 mM in certain experiments during this second incubation. Insulin levels were determined by radioimmunoassay using the Coat-A-Count Kit (DPC, Los Angeles, CA) according to manufacturer’s recommendations, and expressed as ng insulin per mg cellular protein. For studies of insulin secretion from ZDF islets, cells were washed with PBS and preincubated in HBSS containing 3 mM glucose for 1 h. Insulin secretion was then measured with the same static incubation protocol as described for 832/13 cells, using 2 uniformly sized islets/condition in triplicate, exposed to 3 mM or 16.7 mM glucose.
NMR Measurements. 832/13 cells were cultured for 3 days in 15 cm petri dishes in the presence or absence of 1 mM oleate:palmitate (2:1). Cells were washed once with PBS, preincubated in HBSS for 1 h, and then incubated with 3 or 12 mM [U-13C6]glucose (Cambridge isotope Laboratories, Cambridge, MA) in HBSS. An incubation time of 4 h was chosen based on previous experiments showing that glutamate became highly enriched in 13C in these cells within this time period, and isotopic steady state is reached by ≈ 3 h (12). After 4 h of incubation, the assay buffer was collected for determination of insulin levels, and cells were washed once with ice-cold PBS and extracted with 3.5% ice-cold perchloric acid. Extracts from 3 dishes were pooled and neutralized with KOH in an ice bath. The KClO4 precipitate was centrifuged and the supernatant was decanted and lyophilized. The sample was then dissolved in 2H2O for mass isotopomer analysis of extracted glutamate by 13C NMR. Proton decoupled 13C NMR spectra were recorded on a 600 MHz 14T Varian INOVA NMR spectrometer by using a 45 ° pulse and a 3 s repetition time in a 5 mm tunable broadband probe. The areas of the multiplets arising from 13C-13C spin-spin coupling in the glutamate C2, C3, and C4 resonances were determined by using the line-fitting routine in the PC-based NMR program, NUTS (Acorn NMR, Fremont, CA). These multiplet areas were used to perform a 13C-isotopomer analysis with the previously described program tcaCALC (19). The program was applied using the same model parameters as recently reported (12) to determine a metabolic profile for metabolism of [U-13C6] glucose in the TCA cycle.
**O₂ consumption and conversion of relative fluxes to absolute fluxes.** Oxygen consumption was measured at 37°C using an Oxytherm electrode connected to an Oxygraph Measurement System (Hansatech Instruments Ltd, King’s Lynn, Norfolk, England). 832/13 cells were cultured in the presence or absence of 1 mM oleate:palmitate in 6 well plates for 3 days. Tissue culture medium was removed, and cells were washed and preincubated in HBSS containing 3 mM glucose. Two hours later, cells were switched to 2 ml of the same buffer containing either 3 or 12 mM glucose for another hour. Before collecting the cells, the measurement system was calibrated according to the manufacturer’s instructions. For each measurement, cells from 3 wells were gently scraped free and pooled in a total volume of ~350 µl, and 250µl of the cell suspension was immediately added to the oxygen electrode chamber. Oxygen consumption rates were expressed as nmol oxygen/mg protein/min.

¹³C NMR isotopomer analysis (19-21) provides a direct measure of the fraction of acetyl-CoA contributed by [U-¹³C₆] glucose (commonly designated as F₃), the fraction of acetyl-CoA contributed by endogenous unlabeled substrates (by difference, F₀ = 1 - F₃), and pyruvate cycling flux relative to total TCA cycle flux. Given well-established relationships between TCA cycle flux (NADH and FADH₂ production) and O₂ consumption, these NMR determined parameters were then converted into absolute flux values (22). Briefly, given that complete oxidation of one mole of acetyl-CoA consumes 2 moles of molecular oxygen (one cycle turn nets 4 reducing equivalents or 8 electrons), one can derive the proportionality factor (Rᵢ = Qᵢ/Cᵢ) relating O₂ consumption (Qᵢ) to TCA cycle flux (Cᵢ) for any given substrate. For example, glycolysis yields two triose
units. Complete oxidation per triose unit produces 4 reducing equivalents in the TCA
cycle and 2 additional reducing equivalents, one in glycolysis and another at the level of
pyruvate dehydrogenase, for a total of 6; hence, \( R_i = 3 \) for each triose unit of glucose.
The \( R_i \) values for other common substrates have been tabulated elsewhere (22). Total \( O_2 \)
consumption by tissue may be defined as \( Q_t = Q_0 + Q_{\text{glucose}} \), where \( Q_0 \) refers to \( O_2 \)
consumption from oxidation of endogenous triglycerides or fats and \( Q_{\text{glucose}} \) to \( O_2 \)
consumption from oxidation of glucose. Similarly, total TCA cycle flux is defined as \( C_t = C_0 + C_{\text{glucose}} \) where \( C_0 \) refers to TCA cycle flux due to oxidation of endogenous substrates
and \( C_{\text{glucose}} \) to oxidation of glucose. Since the \( R_i \) factor for each substrate relates \( O_2 \)
consumption to TCA cycle flux, it follows that \( Q_t = C_0 R_0 + C_{\text{glucose}} R_{\text{glucose}} \). Given that the
\( F_{Ci} \) variables are defined by the fraction any given substrate makes to total acetyl-CoA
entering the TCA cycle, \( F_{C0} = C_0/C_t \) and \( F_{C3} = C_3/C_t \) (Where \( F_{C0} \) and \( F_{C3} \) is the fraction
of acetyl-CoA derived from fats or glucose respectively). These relationships can be
combined to yield \( Q_t/C_t = F_{C0} R_0 + F_{C3} R_{\text{glucose}} \). Hence, if \( O_2 \) consumption \( (Q_0) \) can be
determined as an absolute flux (using an oxygen electrode for instance), and the \( F_{Ci} \)
values measured by \( ^{13} \)C NMR and the \( R_i \) factor for each substrate are known, then TCA
cycle flux \( (C_t) \) may be calculated. It should be noted that this equation applies only when
anaplerosis can be ignored. However, anaplerosis was also determined as a fraction of
TCA cycle flux \( (y) \) and separate proportionality factors exist for anaplerotic substrates
\( (R_a) \) \( (R_a \) is 0 for carboxylation of exogenous pyruvate, 0.5 for carboxylation of pyruvate
derived from glucose) (22). Thus, after measuring oxygen consumption, the fractional
contribution of each substrate to acetyl-CoA and anaplerosis, \( C_t \) is easily determined.

\[
Q_t/C_t = F_{C0} R_0 + F_{C3} R_{\text{glucose}} + y R_a \tag{1}
\]
It follows then that absolute glucose oxidation is simply $F_{C_3} C_t$, and endogenous lipid oxidation is $F_{C_0} C_t$. Further, since pyruvate cycling is determined as a fraction of $C_t$, it too can be expressed as an absolute flux.

Let us then consider the example of the control cells incubated with 12 mM glucose. The complete isotopomer analysis of the NMR data indicated that acetyl-CoA was derived from endogenous lipids ($F_{C_0} = 0.19$) or glucose ($F_{C_3} = 0.81$) and that anaplerosis was high ($y = 0.95$, we assume from glucose-derived pyruvate). Oxygen consumption was determined by oxygen electrode to be 12.5 nmol/min/mg protein. One can then estimate the contribution from each term in eqn. 1 to $O_2$ consumption as follows:

$$F_{C_0} = 0.19, \ R_o \ for \ lipids \ is \ ~2.8 \ (based \ on \ known \ R_o \ for \ palmitate), \ hence \ term \ #1 = 0.19 \times 2.8 = 0.53$$

$$F_{C_3} = 0.81, \ R_3 \ for \ glucose \ is \ 3, \ hence \ term \ #2 = 0.81 \times 3 = 2.4$$

$$y = 0.95, \ R_a = 0.5, \ hence \ term \ #3 = 3.2 \times 0.5 = 0.48$$

Thus, the proportionality constant ($Q_t/C_t$) relating total TCA cycle flux to total $O_2$ consumption is 3.4 and TCA cycle flux is $12.5 / 3.4 = 3.7 \ nmol \ acetyl-CoA/min/mg$ protein, endogenous lipid oxidation is $0.19 \times 3.7 = 0.70 \ nmol \ acetyl-CoA/min/mg \ protein$ and glucose oxidation is $0.81 \times 3.7 = 3.0 \ nmol \ acetyl-CoA/min/mg \ protein$.

**Statistical methods.** Statistical analysis of the data was performed using the two-tailed Student’s $t$ test, assuming unequal variances.
Results

Malonyl CoA Decarboxylase (MCD) Expression Reduces TG Content. Functional impairment of β-cells has been correlated with elevated TG stores in a number of different model systems. However, these studies have not addressed the issue of whether saturation of lipid storage pathways participates directly in loss of GSIS. To address this point, we used a recombinant adenovirus to express a modified form of malonyl CoA decarboxylase (AdCMV-MCDΔ5) in 832/13 cells. MCDΔ5 is preferentially localized to the cytosol, and completely blocks the glucose-induced rise in malonyl CoA levels (11).

832/13 cells were cultured in the presence of 1 mM oleate: palmitate for six days, and then treated with one of the following viruses: AdCMV-MCDΔ5, a control virus containing the bacterial β-galactosidase gene, AdCMV-βGAL (15), or a control virus containing a catalytically inactive form of MCD, AdCMV-MCDmut (11), and cultured for an additional 24 h in the presence of lipids. Another group of 832/13 cells was simply cultured in the presence or absence of 1 mM oleate: palmitate for the total experimental period of 7 days, with no addition of viruses. Cells treated with AdCMV-MCDΔ5 had approximately 5 times more MCD enzymatic activity than either control group (1.5 ± 0.2 versus 0.3 ± 0.1 µmol/min/mg protein, respectively). Treatment of lipid-cultured 832/13 cells with AdCMV-MCDΔ5 caused a dramatic decrease in Oil-red O staining of stored lipids (Figure 1), and also lowered TG content to levels indistinguishable from those in cells cultured in the absence of exogenous fatty acids (from 332 ± 31 to 118 ± 15 ng/mg protein; Figure 2).
MCD Overexpression Fails to Reverse Lipid-Induced Impairment of GSIS in 832/13 cells or Islets from ZDF Rats. We next investigated the effect of these maneuvers on GSIS (Figure 3). Culture of 832/13 cells in 1 mM oleate:palmitate for 7 days resulted in clear impairment of insulin secretion during stimulation with 12 mM glucose. When normalized to basal secretion at 3 mM glucose, GSIS fell from \(10.6 \pm 2.5, 9.4 \pm 2.3\) and \(9.6 \pm 1.7\)-fold in cells cultured in normal medium containing 11 mM glucose without supplemental fatty acids) to \(2.3 \pm 0.4, 2.4 \pm 0.4\), and \(2.7 \pm 0.6\)-fold in cells cultured in 1 mM oleate:palmitate for 7 days, in the AdCMV-\(\beta\)Gal, AdCMV-MCD\(\Delta5\), and AdCMV-MCD\(mut\)-treated groups, respectively (Figure 3). Thus, normalization of cellular TG levels by overexpression of MCD\(\Delta5\) did not prevent the lipid-induced impairment in GSIS. Consistent with our previous work, overexpression of MCD\(\Delta5\) did not affect GSIS in cells cultured in the absence of fat (11,18). We also performed experiments in which the three adenoviruses were delivered after four days of culture in fatty acids, followed by three more days of culture in the presence of the exogenous lipids and assay of GSIS at day 7 of culture. In this experimental design, AdCMV-MCD\(\Delta5\) treatment prevented the rise in TG content, but again did not prevent the fat-induced impairment in GSIS (data not shown). Taken together, these findings suggest that factors other than TG overstorage are involved in the lipid-induced impairment of GSIS in 832/13 cells.

One potential criticism of the studies just summarized is that chronic culture of an insulinoma cell line in elevated fatty acids may not be fully reflective of \(\beta\)-cell deterioration that occurs \textit{in vivo} in animals or humans with type 2 diabetes and chronic hyperlipidemia. To investigate this further, we delivered the AdCMV-MCD\(\Delta5\) virus to lipid-laden islets from obese Zucker diabetic fatty (ZDF, fa/fa) rats by pancreas perfusion (16) and then isolated the islets and
maintained them in normal medium with no added fatty acids for 48 h. Treatment of fa/fa islets in this fashion raised MCD enzyme activity by $4.0 \pm 1.4$-fold compared to control ZDF (fa/fa) islets that were not treated with virus ($0.39 \pm 0.13 \mu\text{mol/min/mg protein} \text{versus} 0.09 \pm 0.01 \mu\text{mol/min/mg protein}$ in AdCMV-MCDΔ5-treated versus control ZDF (fa/fa) islets, respectively). Figure 4A shows that this increase in MCD activity resulted in a $51 \pm 5\%$ decrease in TG stores, although levels in MCD-overexpressing fa/fa islets remained significantly elevated compared to islets from lean control Zucker rats ($p < 0.05$). Figure 4B shows that glucose caused a potent stimulation of insulin secretion in islets from lean control ZDF rats ($8.2 \pm 2.6$-fold as glucose was raised from 3 to 16.7 mM). As expected, GSIS was modest in ZDF (fa/fa) control islets ($2.1 \pm 0.5$-fold), and was not significantly enhanced by AdCMV-MCDΔ5 treatment ($3.1 \pm 0.6$-fold; $p = 0.17$). Arginine-stimulated insulin secretion was the same in the untreated and AdCMV-MCDΔ5-treated fa/fa islets, suggesting that total secretory capacity was not affected by MCD overexpression (data not shown). We conclude that neither lipid-induced impairment of GSIS induced in tissue culture, or as occurs in lipid-laden islets from fa/fa rats, is significantly reversed by depletion of TG stores in response to MCD overexpression.

**Lipid-Induced Impairment of GSIS is Reversible by Removal of Lipids.** These findings raise the issue of whether islets or β-cell lines that are chronically exposed to high levels of fatty acids have undergone a permanent impairment in GSIS, perhaps indicative of a “lipotoxic” condition that is a prelude to programmed cell death. As one test of this idea, we performed studies on withdrawal of fatty acids from the medium after induction of impaired function to determine whether the impairment is permanent or reversible. To this end, 832/13 cells were cultured in 1
mM oleate:palmitate for varying times, followed by culture for varying times in the absence of lipids. In the course of these studies we found that GSIS is impaired within 24-48 h of exposure of cells to 1 mM oleate:palmitate. In this set of experiments, cells cultured for 72 h in 1 mM oleate:palmitate exhibited a 3.5 ± 0.9 fold response to glucose, compared to 17.3 ± 3.7-fold in cells cultured for 72 h without added fatty acids. Exposure of cells to 1 mM oleate:palmitate for 6 days followed by 1 day of fat removal or exposure of cells to fat for 5 days followed by 2 days of fat removal resulted in progressive improvement in GSIS (4.3 ±1.3-fold and 6.5 ± 2.9-fold, respectively), whereas cells cultured for 4 days in fat followed by 3 days of fat removal are completely normal (14.3 ± 4.3-fold). We also performed viability analysis by MTT assay(23). Culture of 832/13 cells in the presence or absence of 1 mM oleate:palmitate for 3 days had no significant effect on viability as measured by MTT assay relative to cells cultured in the same culture medium without added lipids (data not shown), despite the fact that 3 days of exposure to elevated lipids caused severe impairment of GSIS. Thus, the impairment in GSIS induced by lipid culture of 832/13 cells is not permanent and can not be ascribed to an increase in cell death.

**Chronic Lipid Exposure Prevents the Normal Glucose-Induced Increase in Pyruvate Cycling Activity.** The foregoing studies demonstrate reversibility of lipid-induced impairment of GSIS, but operative mechanisms remain unresolved. Recently, we have applied $^{13}$C NMR to a set of robustly and poorly glucose responsive cell lines to demonstrate a tight correlation between PC-catalyzed exchange of pyruvate with TCA cycle intermediates ("pyruvate cycling") and the differing capacities of the various cell lines for GSIS (12). To investigate the possibility that lipid-induced impairment in GSIS is occurring via a change in activity of this pathway, we cultured 832/13 cells in the presence or absence of 1.0 mM oleate:palmitate for 72
h, and then measured GSIS and pyruvate cycling in cells exposed to 3 or 12 mM U-13C-labeled glucose for 4 h. In this set of experiments, exposure of 832/13 cells to 1 mM oleate:palmitate again caused severe impairment of GSIS, with a decrease from 16.6 ± 4.3-fold in cells grown in normal medium with no added lipids to 3.5 ± 1.5 fold in cells cultured in the presence of lipids (Figure 5A).

The 13C-NMR isotopomer method (19-21) measures pyruvate cycling flux as a ratio relative to total TCA cycle flux. A potential problem with interpretation of flux ratio is that an alteration of this value could reflect either a change in absolute pyruvate cycling flux or a change in absolute TCA cycle flux, or both. To overcome this concern in the current study, oxygen consumption was measured in parallel with the NMR experiments. Two important findings emerged from these experiments (Figure 5B). First, chronic exposure to fatty acids raised oxygen consumption by 36% (p = 0.03) at 3 mM glucose relative to cells grown in the absence of lipids, confirming a previous report (9). Second, cells exposed to lipids exhibited a 30% decrease in oxygen consumption as glucose concentration was raised from 3 to 12 mM (p = 0.01), whereas this did not occur in control cells. These rates of oxygen consumption were then used to convert the relative fluxes measured by NMR into absolute measures of glucose oxidation (from the fraction of acetyl-CoA derived from [U-13C]glucose), endogenous substrate oxidation (from the fraction of unlabeled substrate contributing to acetyl-CoA), and pyruvate cycling flux (22). As shown in Figure 5C, pyruvate cycling activity rises from 1.5 nmol/min/mg protein to 3.4 nmol/min/mg as the glucose concentration is raised acutely from 3 mM to 12 mM in cells cultured in RPMI medium with no added lipids. However, 72 h of exposure to 1.0 mM oleate:palmitate causes pyruvate cycling at 3 mM glucose to rise to 3.3
nmol/min/mg protein, with no further increment in activity induced by exposure to 12 mM glucose. Thus, when expressed in terms of absolute flux, chronic exposure of 832/13 cells to lipids completely eliminates the glucose-induced increment in pyruvate cycling activity, in concert with near-complete inhibition of GSIS. In order to better understand lipid-induced metabolic changes in these experiments we also calculated the oxidation of endogenous (unlabeled) metabolic fuels in the various groups of cells. As shown in Figure 5D, under basal conditions (3 mM glucose), the rate of endogenous fuel oxidation was increased by 45% (p = 0.003) in cells cultured in the presence of exogenous lipids relative to cells cultured in their absence, mirroring the effect of lipids on oxygen consumption (see Figure 5B). Acute exposure to 12 mM glucose caused a dramatic suppression of endogenous fuel oxidation in both lipid-cultured and control cells. It would appear from these data that lipids are the main contributor to endogenous fuel oxidation. Evidence for this includes the clear rise in endogenous fuel oxidation in lipid-cultured cells, and the ability of glucose to cause near complete suppression of endogenous fuel oxidation; a similarly complete suppression of radiolabeled palmitate oxidation by glucose has been demonstrated by us previously in INS-1-derived cells (11,18). Moreover, the absolute rate of glucose conversion to acetyl-CoA (glucose oxidation) rises normally as glucose concentrations are raised from 3 to 12 mM in both lipid-cultured and control cells (Figure 5E).

In sum, exposure of 832/13 cells to 1 mM oleate:palmitate for 72 hours induces the following key metabolic changes: 1) a rise in oxygen consumption at 3 mM glucose that occurs in concert with an increase in endogenous substrate oxidation (likely reflecting oxidation of
intracellular lipids); 2) a loss of the glucose-induced increment in pyruvate cycling activity due to a large increase in cycling at basal glucose levels.

We realize that it may be surprising to some readers that an increase in glucose level from 3 to 12 mM glucose did not cause a significant increase in oxygen consumption in control cells in Figure 5B, as other groups have shown that oxygen consumption increases in response to glucose stimulation in β-cell lines and isolated islets (9,24,25). We believe that this discrepancy is explained by the fact that our oxygen consumption experiments were performed under conditions designed to mimic those of the pyruvate cycling and insulin secretion measurements shown in the various panels of Figure 5. In the current studies, cells were cultured in 11 mM glucose ± 1 mM FFA for 72 h, washed, and transferred to HBSS buffer, 3 mM glucose for 2 h. The buffer was then replaced with new HBSS containing either 3 or 12 mM glucose for 1 h, and cells transferred to the electrode chamber for measurement of oxygen consumption over a period of 5-10 minutes of linear activity. By simply changing this procedure such that acute addition of a glucose solution is used to raise the concentration abruptly from 3 to 12 mM directly in the electrode chamber, significant glucose-induced changes in oxygen consumption in 832/13 cells are observed (data not shown).

A Membrane Permeant Ester of Malate, Dimethylmalate, Circumvents Lipid-Induced Impairment of GSIS in 832/13 Cells and Islets of ZDF Rats. To further investigate the idea that reduced pyruvate cycling contributes to lipid-induced impairment of GSIS, we treated 832/13 cells cultured in high fat or ZDF fa/fa islets with dimethylmalate (DMM), a membrane-
permeant ester of malate that feeds into several pyruvate cycling pathways to stimulate insulin secretion (12). As shown in Figure 6, GSIS was decreased from $9.0 \pm 0.3$-fold in 832/13 cells cultured in normal medium to $2.5 \pm 0.1$-fold after 72 h of culture in 1 mM oleate:palmitate. Inclusion of 10 mM DMM during the period of the secretion assay caused a doubling of insulin secretion in response to 12 mM glucose in cells grown in normal medium, and increased secretion in response to 12 mM glucose by 2.6-fold in cells cultured in the presence of added lipids. Importantly, a similar restorative effect was observed in ZDF fa/fa islets (Figure 7). In this set of experiments, GSIS was completely absent in ZDF islets, but improved to a $3.7 \pm 0.9$-fold in the presence of DMM, although DMM did not fully restore GSIS to the level found in islets from lean control ZDF rats ($13.2 \pm 3.8$-fold). The effect of DMM to improve glucose responsiveness in both lipid-cultured 832/13 cells and ZDF islets appears to be due mainly to an increase in insulin secretion in response to stimulatory glucose levels, rather than normalization of hypersecretion at basal glucose concentrations (Figure 7).
Discussion

The correlation between exposure of islet β-cells to elevated lipid concentrations and development of impaired insulin secretion has been well established in several laboratories (1,3,4,6), leading to the idea that this pathway may be a major contributing factor to β-cell failure of type 2 diabetes (3). However, the mechanism of this effect has not been clarified. In the current study, we have investigated two hypotheses about the development of lipid-induced impairment in islet function. First, we have evaluated the possibility that saturation of lipid storage pathways plays a direct role in loss of GSIS. Support for this idea comes from studies in which TG content has been inversely correlated with glucose responsiveness (7,26). More recent work has suggested that lipid-induced impairment of β-cell function and accumulation of TG requires co-exposure to elevated glucose concentrations, although the main variable measured in these studies has been insulin gene expression and synthesis rather than GSIS (6,27-29). The same investigators also reported that adenovirus-mediated expression of diacylglycerol acyltransferase in rat islets led to accumulation of TG and coincident impairment of GSIS (30). However, the experiments reported here show that the correlation between TG content and loss of insulin secretion has no mechanistic significance, and that TG levels are more likely serving simply as a marker of another fat-mediated pathophysiologic event. We base this conclusion on our finding that overexpression of MCD either in fat-cultured 832/13 cells or in fat-laden islets from ZDF rats effectively lowers cellular TG levels, with no significant restorative effect on GSIS. We recognize that it remains possible that MCD expression failed to remove lipid-
derived metabolites other than TG that are the real causal agent for \( \beta \)-cell impairment.

For example, the fatty acid-derived metabolite ceramide has been linked both to impairment of insulin gene expression (31) and increased rates of \( \beta \)-cell apoptosis (32) in lipid-cultured \( \beta \)-cells. This issue will require further investigation.

Given that TG overstorage is not the direct cause of lipid-induced impairment of insulin secretion, it is somewhat difficult to develop further models from a survey of the literature in the field due to significant disagreement. For example, one group has reported that islets exposed to fatty acids experience a reduction in pyruvate dehydrogenase (PDH) activity in concert with a fall in glucose oxidation, leading to the suggestion that a glucose-fatty acid (Randle) cycle is operative in such cells (7,26,33). However, studies from two other laboratories failed to demonstrated significant lipid-induced impairment of PDH activity in INS-1 cells (9) or rat islets (34). In the Randle hypothesis, a rise in citrate is suggested to slow glycolytic flux via inhibition of phosphofructokinase activity. However, citrate levels are reported to be either unchanged (9) or decreased (35), and phosphofructokinase activity to be increased (36) in various \( \beta \)-cell preparations following lipid exposure. More recently it has been reported that long-term exposure of MIN-6 mouse insulinoma cells to fatty acids results in a reduction of the levels of PC protein (37). These authors also suggested that the consequence of such a lowering of PC might be a reduction in “malate-pyruvate shuttle flux”, but this conclusion appeared to be based solely on a decrease in NAD(P)H autofluorescence in fat-cultured cells, rather than any direct measurement of a metabolic pathway. They further proposed that a decrease in NAPDH content may be involved in the fat-induced impairment in GSIS, but this speculation was based on data obtained by a method that cannot discriminate
NADH from NADPH. In contrast, another group has reported no change in PC $V_{\text{max}}$ in fat-cultured islets, and further speculated that malate-pyruvate shuttle flux would be increased rather than decreased due to a 60% rise in intracellular pyruvate concentrations (34). The same group has also reported an increase in PC $V_{\text{max}}$ in islets from nondiabetic Zucker fatty rats, and have suggested that this would lead to increased pyruvate cycling, thereby possibly explaining the enhanced insulin secretion of such islets that compensates for insulin resistance (38). However, this conclusion was based on static measurement of enzyme activities and concentrations of selected metabolic intermediates rather than any direct measurement of metabolic flux.

In light of this confusion, a more comprehensive method for metabolic analysis of lipid-exposed versus normal cells was required. We have recently used $^{13}$C NMR to analyze pathways of pyruvate metabolism in mitochondria, leading to the discovery that the activity of PC-catalyzed pyruvate cycling pathways can be used to distinguish robustly glucose responsive from poorly glucose responsive INS-1-derived cell lines (12). These findings are in agreement with another study employing radioisotopic tracers that demonstrated pronounced differences in glucose-driven anaplerosis in purified $\beta$-versus $\alpha$-islet cell preparations (39). Development of the NMR-based methods has allowed us to test our second hypothesis that chronic exposure of $\beta$-cells to lipids results in an alteration in PC-catalyzed pyruvate cycling activity.

Our approach has uncovered several metabolic perturbations that occur in $\beta$-cells in response to chronic exposure to elevated lipid concentrations. First, we observe a rise in
oxygen consumption at 3 mM glucose that occurs in concert with an increase in endogenous substrate oxidation. Second, we find a loss of the glucose-induced increment in pyruvate cycling activity due to a large increase in cycling at basal glucose levels. Interestingly, these changes occur in the absence of any significant change in the rate of $^{13}$C glucose oxidation in lipid-cultured versus control cells, at either 3 or 12 mM glucose.

We interpret these data as follows. The coordinate increase in oxygen consumption (Figure 5B) and endogenous fuel oxidation (Figure 5D) that occurs at 3 mM glucose in response to chronic lipid exposure is most likely explained by an increase in fatty acid oxidation. This follows from the obvious increase in supply of this substrate in fat-cultured cells, and is consistent with recent reports of increased expression of enzymes of lipid oxidation in β-cells in response to chronic lipid exposure (40,41). In liver, an increase in fatty acid oxidation has been linked to suppression of PDH activity via accumulation of ATP, NADH, and acetyl-CoA, part of the Randle mechanism. As discussed earlier, two laboratories have reported that this pathway is not operative in β-cells (9,34), and our data on a lack of change in glucose oxidation in response to chronic lipid culture is consistent with these findings. However, acetyl-CoA generated from lipid oxidation is also known to influence pyruvate metabolism via its capacity to activate PC. It would appear that this mechanism is retained in β-cells, given the large increase in PC-catalyzed pyruvate cycling activity that occurs at basal glucose in response to chronic lipid culture (Figure 5C). This rise in basal pyruvate cycling activity eliminates the normal glucose-induced increment in this parameter, such that yet to be identified byproducts of this pathway that signal for insulin secretion are never generated. In our earlier study of several variously glucose responsive INS-1-derived cell lines, we noted a strong correlation between
pyruvate cycling and glucose responsiveness, with no correlation between GSIS and the fractional contribution of glucose to acetyl-CoA production, a measure of TCA cycle activity (12). This original observation is fully confirmed in the current study, as chronic exposure to lipids caused a profound impairment of GSIS (Figure 5A) in concert with the loss of a glucose-mediated increment in pyruvate cycling (Figure 5C), but with no effect on the rate of glucose oxidation (Figure 5E).

One potential issue that arises with this model is that overexpression of MCD is expected to increase fatty acid oxidation by lowering of malonyl CoA levels, yet this maneuver has no effect on GSIS in 832/13 or parental INS-1 cells (11,18). The explanation for this could reside at several levels. First, if one considers studies in which lipids are omitted from the pre-culture medium or the secretion assay buffer, such cells will have only limited endogenous lipid stores for oxidation, acetyl CoA production, and activation of PC. It is therefore not surprising that MCD expression has no effect on GSIS under these conditions. In studies in which the 832/13 cells are exposed to lipids in the culture medium prior to MCD overexpression as reported herein, the impairment of GSIS elicited by chronic lipid exposure is already profound, making additional negative effects of MCD overexpression difficult, if not impossible to observe. Finally, it should be noted that in our studies with the cytosolically localized MCD construct used in this study (MCDΔ5), the rate of fatty acid oxidation does not increase in direct proportion to its degree of overexpression in 832/13 cells (18) or isolated hepatocytes (42). The explanation for this may lie in recently emergent information suggesting that acetyl CoA carboxylase-2 (ACC2) controls the pool of malonyl CoA that regulates fatty acid oxidation via its capacity to associate with mitochondria, thereby juxtaposing it with mitochondrial CPT 1
(43-45). Acetyl CoA carboxylase-1 (ACC1), in contrast, is thought to reside in the cytoplasm, where it synthesizes the pool of malonyl CoA that is used for lipogenesis (45). In retrospect, we believe that expression of the cytosolically localized MCD may be having its largest effect on the malonyl CoA pool synthesized by ACC1, with a primary effect on depletion of the substrate pool for TG synthesis, and a lesser effect on the ACC2-derived pool of malonyl CoA that participates most directly in regulation of fatty acid oxidation.

Anaplerotic influx of pyruvate into the TCA cycle may be linked to efflux of other intermediates from the mitochondria, including malate (46,47) or citrate (48), resulting in synthesis of important coupling factors. An intermediate common to both the pyruvate-malate and pyruvate-citrate cycles is malate, which is converted to pyruvate by the malic enzyme as the final step in both pathways (47). We therefore investigated whether the membrane-permeant malate ester DMM could overcome the lipid-induced impairment in insulin secretion. Remarkably, inclusion of DMM during the secretion assay almost completely restored GSIS in lipid-cultured 832/13 cells, and also significantly improved glucose responsiveness in lipid-laden ZDF islets. Taken together, these data provide strong support for the idea that lipid-induced impairment of GSIS is caused at least in part by alteration of the metabolic fate of pyruvate. The effects of lipid to cause this diversion may be dependent upon concomitant overexposure of cells to elevations in glucose, based on work summarized earlier (6,27,29). All of the experiments summarized in the current study were performed in the presence of glucose concentrations above basal (11 mM glucose is used in the RPMI culture medium of 832/13 cells, while ZDF islets are harvested from an environment of hyperglycemia and then cultured in medium containing 8 mM glucose). Thus, further studies will be required to
unravel the relative contributions and potential synergies of elevated levels of glucose and fatty acids in alteration of pyruvate metabolism. Nevertheless, our findings in current form suggest that enzymes involved in pyruvate cycling pathways and/or analogs of the cycling intermediates themselves might represent new therapeutic targets for reversal of β-cell dysfunction in diabetes.
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Figure Legends

Figure 1. Reduction in TG content induced by AdCMV-MCD\textDelta 5 treatment (Oil Red O staining). 832/13 cells were cultured in the absence (panel A) or presence (panels B, C, and D) of 1 mM oleate:palmitate (2:1) for 6 days. Cells in panels C and D were then treated with 10 or 20 MOI of AdCMV-MCD\textDelta 5 adenovirus, respectively, and cultured for an additional 24 h in the presence of 1 mM oleate:palmitate. Cells in panels A and B were also cultured for the additional 24 h period in the absence and presence of oleate:palmitate, respectively, but were not treated with virus. Following this culture protocol, lipid droplets were visualized by staining with Oil Red O. Note the disappearance of lipid droplets caused by the higher dose of AdCMV-MCD\textDelta 5 (compare panels B and D).

Figure 2. Reduction in TG content induced by AdCMV-MCD\textDelta 5 treatment (biochemical measurement). 832/13 cells were cultured with (+ FFA) or without (- FFA) 1 mM oleate:palmitate (2:1) for 6 days, treated with 20 moi AdCMV-MCD\textDelta 5 (MCD\textDelta 5), AdCMV-MCD\textsubscript{mut} (MCD\textsubscript{mut}; encoding a catalytically inactive form of MCD), or AdCMV-\textbeta GAL (\textbeta GAL), and then cultured for an additional 24 h in the presence of 1 mM oleate:palmitate. Cells were then collected for measurement of TG content as described in Materials and Methods. Results are means ± SEM of 3 independent triplicate determinations. The symbol * indicates that TG content was significantly lower in AdCMV-MCD\textDelta 5-treated cells than in either control group, with $p < 0.05$. 
Figure 3. **MCD-mediated depletion of TG does not reverse lipid-induced impairment of GSIS.** 832/13 cells were cultured with (+ FFA) or without (- FFA) 1 mM oleate:palmitate (2:1) for 6 days, treated with AdCMV-MCDΔ5 (MCDΔ5), AdCMV-MCDmut (MCDmut; encoding a catalytically inactive form of MCD), or AdCMV-βGAL (βGAL), and then cultured for an additional 24 h in the presence of 1 mM oleate:palmitate. Insulin secretion was then measured with a static incubation assay in the presence of 3 or 12 mM glucose (Glc) for 2 h. Results are means ± SEM of 9 independent triplicate determinations. GSIS was significantly impaired by lipid culture in all three experimental groups, with p < 0.01.

Figure 4. **Overexpression of MCDΔ5 in the islets of ZDF (fa/fa) rats reduces TG content, but does not improve GSIS.** Pancreata of ZDF (fa/fa) rats were perfused with 1x10^{12} plaque forming unit (pfu) of AdCMV-MCDΔ5 (MCDΔ5) for 1 h prior to islet isolation. Separate batches of islets from ZDF lean (+/+) or ZDF (fa/fa) animals were also isolated without the prior adenovirus treatment. After 48 h of culture in RPMI medium containing 8 mM glucose, islets were harvested for measurement of TG content (panel A) or GSIS (panel B). Results are means ± SEM of 5 independent measurements. The symbol * indicates that TG content was lower in AdCMV-MCDΔ5-treated ZDF (fa/fa) islets than in untreated ZDF (fa/fa) controls, with p < 0.04.

Figure 5. **Changes in glucose-stimulated insulin secretion, oxygen consumption, and pyruvate cycling activity induced by chronic exposure of 832/13 cells to lipids.** 832/13 cells were cultured for 72 h in the presence (+ FFA) or absence (- FFA) of 1mM
oleate: palmitate (2:1). **Panel A.** GSIS measured by static incubation of lipid-cultured and control cells exposed to 3 or 12 mM U-13C glucose for 4 h. The symbol * indicates differences between lipid-cultured and control cells, at 3 and 12 mM glucose, respectively, with P < 0.01. **Panel B.** Oxygen consumption was measured as described in Material and Methods. The symbol * indicates that oxygen consumption was significantly higher in FFA cultured cells than in control cells at 3 mM glucose, with p = 0.03; the symbol ** indicates that oxygen consumption was significantly lower in FFA cultured cells at 12 mM glucose than at 3 mM glucose, with P < 0.01. **Panel C.** Pyruvate cycling activity measured by 13C NMR, expressed as absolute flux, calculated relative to the rate of fuel oxidation estimated from the oxygen consumption data (see Materials and Methods). The symbol * indicates that cells cultured in 1 mM oleate:palmitate had increased pyruvate cycling relative to control cells at 3 mM glucose, with p < 0.004. **Panel D.** Endogenous substrate oxidation, calculated as described in Materials and Methods. The symbol * indicates that cells cultured in 1 mM oleate:palmitate had increased endogenous substrate oxidation relative to control cells at 3 mM glucose, with p = 0.003. **Panel E.** 13C glucose oxidation, calculated as described in Materials and Methods. Data in all panels represent the mean ± S.E. for 6 independent experiments, except for oxygen consumption (panel B), which represents the mean ± S.E. for 3 independent experiments.

**Figure 6.** Addition of dimethyl malate (DMM) reverses lipid-induced impairment of GSIS. 832/13 cells were cultured for 72 h in the presence (+ FFA) or absence (- FFA) of 1 mM oleate: palmitate (2:1), followed by measurement of GSIS. Secretion
experiments were performed in the presence (+ DMM) or absence (No DMM) of 10 mM DMM as indicated. Results represent the means ± SEM of 4 independent experiments, each performed in triplicate. The symbol * indicates a significant increase in insulin secretion at 12 mM glucose in cells exposed to DMM, relative to cells exposed to 12 mM glucose in the absence of DMM, with p < 0.05.

**Figure 7. Addition of DMM improves glucose responsiveness in islets from ZDF (fa/fa) rats.** Islets from two ZDF lean (+/+) and four fatty (fa/fa) rats were isolated and immediately assayed for GSIS. 10 mM DMM was added during the GSIS assay in some groups of ZDF (fa/fa) islets. Data represent the mean ± SEM of 5 independent experiments, each performed in triplicate. The symbol * indicates a significant increase in insulin secretion at 12 mM glucose in lipid-cultured cells exposed to DMM, relative to lipid-cultured cells in the absence of DMM, with p = 0.033.
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Boucher, et al., Figure 1

A

B

C

D
Boucher, et al., Figure 2

\[
\begin{align*}
\text{TG (ng/mg protein)} & \\
\beta\text{GAL} & - FFA & \quad + FFA \\
\text{MCD}\Delta5 & & \\
\text{MCD}_{\text{mut}} & & *
\end{align*}
\]
Boucher, et al., Figure 4A

TG content (ng/islet)

+/

fa/fa

fa/fa + MCDΔ5

*
Boucher, et al., Figure 4B

![Graph showing insulin release in response to glucose concentration.](image)

- **Insulin (ng/2 islets/h)**
  - **Glucose (mM)**
    - 3
    - 16.7

Legend:
- **+/-**
- **fa/fa**
- **fa/fa + MCDΔ5**
Boucher, et al., Figure 5A

The graph shows the effect of glucose (mM) on Insulin (ng/mg protein/h) in the presence or absence of FFA. The x-axis represents Glucose concentrations of 3 and 12 mM, and the y-axis represents Insulin levels ranging from 0 to 700 ng/mg protein/h.

- **- FFA** represents the condition without FFA, and **+FFA** represents the condition with FFA.

At 3 mM glucose, the Insulin levels are significantly lower in the presence of FFA compared to the absence of FFA. At 12 mM glucose, the Insulin levels are also lower in the presence of FFA, with a significant increase in Insulin secretion when FFA is added.
Oxygen Consumption rate (nmol/mg protein/min)

Glucose (mM)

- FFA
+FFA

*  **
Boucher, et al., Figure 5D

**Endogenous Substrate Oxidation** (nmol acetyl-CoA/min/mg protein)

- **- FFA**
- **+FFA**

Glucose (mM): 3, 12, 3, 12

Endogenous Substrate Oxidation (nmol acetyl-CoA/min/mg protein): 2, 1, 3, 1
Boucher, et al., Figure 5E

Glucose Oxidation (nmol acetyl-CoA/min/mg protein)

- FFA
+ FFA

Glucose (mM)
Figure 6

Boucher, et al., Figure 6

Graph showing the effect of DMM on insulin production. The x-axis represents glucose levels in mM (0, 3, 12, 3, 12) and the y-axis represents insulin production in ng/mg/protein/h (0, 0.5, 1.5, 3, 6, 8). Treatment conditions include No DMM and + DMM. The graph includes bars for -FFA (filled black) and +FFA (open white) conditions. Significant differences are indicated by an asterisk (*) above the bars.
Boucher, et al., Figure 7

![Graph showing insulin levels in different conditions](image-url)
**Biochemical mechanism of lipid-induced impairment of glucose-stimulated insulin secretion and reversal with a malate analogue**
Anne Boucher, Danhong Lu, Shawn C. Burgess, Sabine Telemaque-Potts, Mette V. Jensen, Hindrik Mulder, May-Yun Wang, Roger H. Unger, A. Dean Sherry and Christopher B. Newgard

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