Specificity in Beta Cell Expression of L-3-Hydroxyacyl-CoA Dehydrogenase, Short Chain, and Potential Role in Down-regulating Insulin Release

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A loss-of-function mutation of the mitochondrial β-oxidation enzyme L-3-hydroxyacyl-CoA dehydrogenase, short chain (HADHSC), has been associated with hyperinsulinemic hypoglycemia in man. It is still unclear whether loss of glucose homeostasis in these patients (partly) results from a dysregulation of beta cells. This study examines HADHSC expression in purified rat beta cells and investigates whether its selective suppression elevates insulin release. Beta cells expressed the highest levels of HADHSC mRNA and protein of all examined tissues, including those with high rates of mitochondrial β-oxidation. On the other hand, beta cells expressed relatively low levels of other β-oxidation enzymes (acyl-CoA dehydrogenase short, medium, and long chain and acetyl-coenzyme A acyltransferase 2). HADHSC expression was sequence-specifically silenced by RNA interference, and the effects were examined on glucose-stimulated insulin secretion following 48–72 h of suppression. In both rat beta cells and in the beta cell line INS1 832-13, HADHSC silencing resulted in elevated insulin release at low and at high glucose concentrations, which appeared not to be caused by increased rates of glucose metabolism or an inhibition in fatty acid oxidation. These data indicate that the normal beta cell phenotype is characterized by a high expression of HADHSC and a low expression of other β-oxidation enzymes. Down-regulation of HADHSC causes an elevated secretory activity suggesting that this enzyme protects against inappropriately high insulin levels and hypoglycemia.

The insulin-producing beta cells provide a crucial glucose sensor to metabolic homeostasis. Their responsiveness to variations in blood glucose levels protects the organism against lethal hypoglycemia or chronically devastating hyperglycemia. This involves generation of glucose metabolism-derived coupling signals that translate the glucose stimulus into metabolically adequate insulin synthesis and secretion. The cellular rates of glucose utilization and oxidation are proportionate to the ambient glucose level over a wide concentration range (0–10 mM) (1) and are regulated by the “glucose sensor” glucokinase (2). The ensuing activation of mitochondrial metabolism increases beta cell ATP/ADP ratio (3), a coupling factor in the K+ATP-dependent pathway of insulin secretion that transmits signals from mitochondrial nutrients (4, 5). The physiologic relevance of this mechanism is illustrated by the appearance of hyperinsulinemic hypoglycemia for mutations in glucokinase (6) or in mitochondrial glutamate dehydrogenase (7) which, respectively, increase glucose oxidative flux or anapleurotic channeling of glutamate-derived α-ketoglutarate into the Krebs cycle. Mutations in the K+ATP channel subunits SUR1 and Kir6.2 that favor channel closure can also enhance beta cell depolarization and insulin secretion, but these occur without direct changes in beta cell metabolic flux. Other mutations that cause fasting hypoglycemia may involve other regulatory pathways in the beta cell, and may even help their recognition in beta cell physiology.

In this study we examine the role of the mitochondrial β-oxidation enzyme L-3-hydroxyacyl-CoA dehydrogenase, short chain (HADHSC), in the regulation of glucose-induced insulin release by normal beta cells. It was recently reported that a loss-of-function mutation in the HADHSC gene (NCBI gene number 3033) was associated with fasting hyperinsulinemia (OMIM 601820) (8, 9). The HADHSC (EC 1.1.1.35) protein is known to act in a common branch of the cyclically organized β-oxidation pathway, converting L-3-hydroxyacyl-CoAs of variable chain length to their corresponding 3-ketoacyl CoAs (10) (Fig. 1a). It was also recently associated with a K+ATP-independent regulation of insulin release by insulin-producing cells, suggesting that the hyperinsulinemia in patients with an HADHSC mutation might be partially caused by dysregulated

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EXPERIMENTAL PROCEDURES

Materials

Rat INS1 832-13 cells were cultured in 10% fetal calf serum (HyClone), RPMI 1640 medium, GlutaMAX™ (Invitrogen), supplemented to 10 mM HEPES, 1 mM sodium pyruvate, penicillin/streptomycin, and 50 µM β-mercaptoethanol (designated as “INS1 culture medium”). Purified rat hepatocytes for metabolic profiling were kindly provided by Dr. P. Papeleu and were isolated as described previously (12). Adult male Wistar rats (150–250 g; Janvier, France) were bred according to the Belgian regulations for animal welfare and used in experiments that were approved by the local ethical committee. Beta cells (90 ± 3% insulin-positive cells) and islet non-beta cells (68 ± 9% glucagon-positive cells and <10% beta cells) were purified as described (13). FACS-purified rat beta cells were cultured in Ham’s F-10 nutrient mixture (Invitrogen) supplemented with 0.5% BSA (Cohn analog, Sigma), 2 mM glutamine, 10 mM glucose, penicillin (100 units/ml), streptomycin (0.1 mg/ml), 2% fetal calf serum (Hyclone). d-[U-14C]Glucose (306–311 mCi/mmol; 1 mCi/5 ml) was obtained from Amersham Biosciences and [U-14C]palmitic acid (850 mCi/mmol; 0.1 mCi/ml) from PerkinElmer Life Sciences. All other chemicals were obtained from Sigma. Sodium salts of fatty acids (C4-butyrate, C6-caproate, and C16-palmitate) were added from a 100X stock in 90% ethanol.

Real Time PCR and Western Blot Analysis of Rat Beta Cells and Tissues

Total RNA was extracted from pancreatic endocrine cells using RNeasy (Qiagen) minicolumns, according to the manufacturer’s protocol. RNA from other rat tissues and from INS1 832-13 cells (passage 46–52) was extracted using TRIzol reagent. RNA quality was verified by Agilent bioanalyzer (minimal cutoff RNA integrity number ≥8). Following removal of genomic DNA (TURBO DNA-free, Ambion, Austin, TX) and reverse transcription (high capacity cDNA archive kit, Applied Biosystems, Foster City, CA), targets were amplified from cDNA template on ABI Prism 7700 Sequence Detector using TaqMan universal PCR master mix and commercially available sequence-specific primers and TaqMan MGB probe (Applied Biosystems, identification of assays is available on request). Expression of target genes were normalized toward β-actin (∆Ct) and expressed versus a chosen calibrator (comparative ∆∆Ct method). Relative mRNA levels of HADHSC versus other β-oxidation enzymes were calculated using the formula 2^−∆∆Ct, where ∆∆Ct = (Ct value of AHSC) – (Ct of other enzyme), measured in same run.

Lipid Transfection of INS1 832-13 Cells and Primary Rat Beta Cells

siRNA Transfection of INS1 Cells—INS1 832-13 cells (15 × 10⁴ cells/well) were cultured overnight in 1 ml of INS1 medium (24-well plates) and then transfected in Opti-MEM medium (Invitrogen) for 4 h, using siLentFect lipid reagent (Bio-Rad) and Dharmacon siRNA. Transfection was done in a total volume of 250 µl, at 20 nm siRNA (0.07 µg siRNA/well) and a lipid/siRNA ratio of 1 µl of siLentFECT/0.07 µg of siRNA. siRNA against rat HADHSC was siGENOME SMARTpool reagent (M-091802-00; Dharmacon), a pool of four different siRNAs. The silencing effect of the combined pool of four different siRNAs was always more powerful than the individual helices, which achieved mRNA silencing ranging from 45 to 55% (data not shown). Control siRNA was either nontargeting siGLO RISC-free (D-001600-01-20) or siRNA designed against human/mouse lamin A/C (siGLO lamin A/C siRNA, D-001620-01-20). Over 95% of siGLO-transfected cells showed cytoplasmic, punctate siRNA-associated fluorescence; 60% of
cells also showed a diffuse cytoplasmic red fluorescence. Cotransfections with enhanced GFP-coding plasmids resulted in 65–70% transfection as measured by flow cytometry. Transfection did not result in overt cytotoxicity (as assessed by propidium iodide/bisbenzimid staining) but slowed down cellular proliferation. Manual Bürker counting was used to quantify INS1 cell population doublings during exponential growth phase. The amount of cell cycles/24 h (=F) was calculated from the number of plated cells at start (=N0) and after t days of culture (=Nt), using the formula Nt = N0 \times e^{(F\times t)}. The number of cell cycles/24 h (=F) decreased from 0.53 ± 0.21 for untransfected cells to 0.43 ± 0.20 for siRNA-transfected cells (p < 0.001, n = 8). No differences in cellular proliferation were observed between control siGLO lamin A/C, RISC-free siRNA, or HADHSC SMART pool siRNA-treated cells.

**Introduction of shRNAi Plasmids in INS1 Cells**

Short hairpin RNA interference plasmids targeting rat HADHSC matched the criteria of both the Dharmacon (14) and Whitehead (15) siRNA design protocols. Sequences were as follows: A3 plasmid, 5'-ATACAGTAGTGGTGGTGGGA-3' (sense), and C6 plasmid, 5'-AGCGAGCCGATGCATCTAA-3' (sense), targeting nucleotides 151–170 and 704–722 of the rat HADHSC gene, respectively (GenBank™ accession number NM_057186.1). These positions were at least 40 bp removed from a highly conserved 1-3-hydroxyacyl-CoA dehydrogenase motif around nucleotides 660–673 (16). Sequence of scrambled control plasmid was 5'-GAGGATCCGGACTGACGAC-3' (sense). shRNAi plasmids were introduced into INS1 cells using the same protocol as for siRNA, now using 2 µl of siLENTect/µg of DNA. Co-transfection with enhanced GFP plasmids (weight ratio 1:10 GFP/shRNA plasmids) indicated 65 ± 5% GFP-expressing cells, as measured by flow cytometry. shRNAi-mediated HADHSC knockdown did not inhibit cell proliferation as compared with scrambled-transfected cells (cell cycle constant F = amount of cell cycles/24 h being 0.40 ± 0.11 and 0.43 ± 0.12 for scrambled and A3, respectively, n = 8).

**Functional Analysis of HADHSC Suppressed INS1 832-13 Cells and Primary Rat Beta Cells**

Unless otherwise stated, function of INS1 cells at 70–80% confluency was studied in a KRBH-based buffer, further designated as “Cell Medium” (116 mM NaCl, 1.8 mM CaCl2•2(H2O), 0.8 mM MgSO4•7(H2O), 5.4 mM KCl, 1 mM NaH2PO4•2(H2O), 26 mM NaHCO3, and 0.5% BSA). For insulin release experiments, attached cells were washed twice with Cell Medium to remove detached cells and debris and then preincubated for 1 h at 2 mM glucose prior to a 2-h incubation under the listed test conditions. Supernatants were centrifuged at 4 °C and diluted for insulin RIA as described (17). Typically, six wells of INS1 cells for each knockdown condition were dissociated by EDTA/trypsin, and the amount of cells/well was determined by duplicate Bürker counting. The percent dead cells was counted after propidium iodide staining and found to be lower than 5%. Insulin secretion by siRNA-treated primary rat beta cells was measured in a perfusion system as described (18).

CO2 formation from glucose and palmitate was measured in duplicate samples of 105 INS1 cells during 2-h incubations at 37 °C. Glucose oxidation was measured in Ham’s F-10 medium containing 0.5% BSA, 2 mM L-glutamine, 10 mM HEPES, and 10 mM D-glucose (5 µCi of D-[U-14C]glucose), and palmitate oxidation was analyzed in KRBH medium, containing 0.2% BSA (fraction V, Roche Applied Science), 2 mM calcium, 10 mM HEPES, and 0.5 µCi of [U-14C]palmitate, and unlabeled palmitate up to a final concentration of 50 µM. Cells were incubated at 37 °C in a glass siliconized tube trapped in an airtight glass vial. After 2 h, metabolism was stopped by injecting 20 µl of 1 N HCl, and 250 µl of hydroxyhyamine (PerkinElmer Life Sciences) was used to capture the produced 14CO2 during 1 h at room temperature. D-[U-14C]Glucose or [U-14C]palmitate acid oxidation rates were determined by liquid scintillation counting of the generated 14CO2. Nutrient-induced changes in cellular riboflavin (FAD and FMN) and NAD(P)H content were measured by flow cytometry using argon laser excitation 488/530 nm) and UV argon laser excitation 351–363 nm/emission 400–470 nm), respectively (19).

Metabolomic profiling of 1-3-hydroxyacyl-CoA intermediates was carried out by tandem mass spectrometry on INS1 cells and freshly isolated rat hepatocytes using a method that was clinically validated for fatty acid metabolism disorder screening in human newborns (20). Briefly, INS1 cells were collected 72 h after initiation of knockdown, suspended in KRHB buffer, and dried on Schleicher & Schuell grade 903 filter paper. Five disks were punched from each dried cell sample and extracted in 96-well microplates with 200 µl of methanol (Chromasolv grade), containing a mixture of stable isotope-labeled standards of acylcarnitine intermediates. The extract was evaporated at 55 °C under nitrogen. Dried samples were reconstituted with 200 µl of a mobile phase (acetonitrile/water, 50:50 by volume) and injected in an atmospheric pressure ionization 4000 triple quadrupole tandem mass spectrometry with ion spray source (Sciex, Applied Biosystems). Samples were introduced through the tandem mass spectrometry by the atmospheric pressure ionization system, in which different analytes and their internal standards were recognized by sorting their respective mass-to-charge ratio (m/z). 1-3-Hydroxyacyl-CoAs were localized by the m/z of the corresponding acylcarnitines of the internal standard, and their peak areas were measured using Analyst 1.4.1 Quantitation Wizard (Applied Biosystems) and normalized for total protein content of original cellular supernatant, as measured by Bradford assay.
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Statistical Analysis

Data are presented as means ± S.D. or S.E. of n independent experiments. Statistical analysis was performed using one-way analysis of variance with Dunnett’s test in multiple comparison of means or paired Student’s t testing where appropriate. Differences were considered significant when p < 0.05.

RESULTS

Rat Beta Cells Express High Levels of HADHSC mRNA and Protein and Low Levels of Other β-Oxidation Enzymes—We quantified mRNA expression of HADHSC and other enzymes of the β-oxidation pathway, as proposed by Liang et al. (10) (Fig. 1a). Long chain acyl-CoAs mainly enter this pathway in a metabolon composed of VLCAD, acting in tandem with the trifunctional enzyme (HADHA/HADHB). With descending chain lengths (<20 carbons, C20), acyl-CoAs tend to enter downstream of this metabolon and are, in subsequent cycles, dehydrogenated by acyl-CoA dehydrogenases with specificity for long chain (C6–C20, ACADL), medium (C4–C12, ACADM), and short chain acyl-CoAs (C4–C6, ACADS). In each cycle, the reaction products of ACADS, ACADM, and ACADL enter a common catabolic route encompassing enoyl-CoA hydratase 1 (ECHS1) and HADHSC. Finally, acetyl-CoA is cleaved off by ACAAl, the mitochondrial thiolase. We simultaneously measured relative mRNA levels of the mitochondrial enzymes ACADS, ACADM, ACADL, HADHSC, and ACAAl, of the peroxisomal thiolase ACAAl, and of PPARα, the main transcriptional regulator of mitochondrial and peroxisomal fatty acid oxidation enzymes (21).

Compared with other cell types, pancreatic beta cells have a higher mRNA expression of HADHSC and a lower expression level of PPARα, as well as of the β-oxidation enzymes ACADS, ACADM, ACADL, and ACAAl (Fig. 1b). HADHSC mRNA expression in INS1 and primary rat beta cells are typically 10–50-fold higher than the mRNA level of upstream acyl-CoA dehydrogenase isoenzymes (ACADS, ACADM, and ACADL) and the downstream thiolase ACAAl, whereas these ratios range typically from 0.4 to 1.4 in all other tissues tested. The unbalance between the expression level of HADHSC and of other components of the β-oxidation pathway thus appears to be a metabolic signature of pancreatic beta cells. This particular HADHSC expression pattern is also observed in INS1 832-13 cells, indicating that this rat beta cell line can be used to investigate its functional consequences. HADHSC protein was identified with an affinity-purified chicken anti-human HADHSC antibody that was found to detect a single protein band in rat tissues, at the expected molecular mass of 34 kDa (data not shown). In line with mRNA data in Fig. 1b, purified rat beta cells express a similar level of HADHSC protein as liver and a markedly higher level than islet endocrine non-beta cells (p < 0.01, n = 4; see supplemental Fig. 1). High HADHSC protein levels were also detected in INS1 cells, heart muscle, and kidney (supplemental Fig. 1). When used in immunohistochemistry on human pancreatic sections, the antibody showed a much more intense staining in islets than in the surrounding exocrine tissue (Fig. 2, upper left panel), being strongly present in insulin-positive cells and only weakly in glucagon-positive alpha cells (Fig. 2).

RNAi-mediated Suppression of HADHSC Results in Increased Insulin Secretion by INS1 Cells—We next examined the effect of selective suppression of HADHSC protein on glucose-stimu-
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FIGURE 2. Expression of HADHSC protein in human beta cells in situ. Triple immune staining of adult human pancreas for HADHSC (fluorescein isothiocyanate), insulin (Cy2), and glucagon (Cy5). Upper left panel shows an overview of the pancreatic section, with a Langerhans islet and two small beta cell clusters clearly detectable by HADHSC staining. Upper right and lower panels zoom in on the islet shown in upper left panel, and indicate that the HADHSC signal originates mainly from insulin-expressing cells within the islet. Artificial red/green colors are assigned to fluorescein isothiocyanate, Cy2, and Cy5 fluorescence as indicated on each panel.

lated insulin secretion (GSIS) by INS1 832-13 cells (22). Two RNA interference strategies were tested for their effect on HADHSC protein expression and on GSIS at 48–72 h after initiation of knockdown.

Duplex siRNA-mediated HADHSC Silencing—INS1 cells were transfected, with a pool of four different siRNAs against rat HADHSC (designated further as HADHSC siRNA) or with fluorescence-labeled (siGLO) control siRNA. Two control siRNAs were tested as follows: 1) siGLO lamin A/C designed to target human/mouse lamin A/C, which was only partially active in rat cells (45 ± 8% knockdown, p < 0.001, n = 5); 2) siGLO RISC-free, a siRNA designed not to interact with the RISC complex. Both control siRNAs gave similar results and could be used interchangeably. 48 h after transfection, HADHSC siRNA resulted in a 70% decrease in HADHSC mRNA versus siGLO-transfected cells (p < 0.001, n = 8; Fig. 3a). At 48 h, the mRNA expression of ACADM was unaffected, confirming sequence-specific HADHSC silencing. This reduction resulted in a moderate (20%) but significant reduction of the HADHSC/β-actin protein ratio (p < 0.05, n = 8; Fig. 3b). Cellular insulin content at 48 h did not differ between untransfected cells (921 ± 162 pg of insulin/10⁶ cells, mean ± S.D., n = 5) and cells transfected with siGLO control (936 ± 174 pg of insulin/10⁶ cells) or HADHSC siRNA (931 ± 195 pg of insulin/10⁶ cells).

When tested for their GSIS, the HADHSC-suppressed cells exhibited a moderate leftward and upward shift of the GSIS concentration-response curve as compared with control-transfected cells (Fig. 3c); this was significant in the higher glucose range (12 and 25 mM, p < 0.01, n = 8). Raising glucose from 3 to 12 mM caused a similar fold change of insulin secretion in untransfected and siGLO-transfected cells, (2.67 ± 1.21- and 2.54 ± 1.21-fold, respectively, p = 0.41, n = 8); in HADHSC knockdown cells the fold induction was 3.11 ± 0.87 (n = 8, p < 0.05 versus siGLO and untransfected cells). Although cationic lipid-mediated transfection itself did not affect the glucose-induced increment of insulin secretion, it increased the released levels over the whole glucose range (p < 0.01 siGLO versus untransfected; Fig. 3c). This effect was not caused by insulin leakage from transfection-damaged cells as judged from viability counts. HADHSC-suppressed cells also tended to be more responsive to short chain fatty acid butyrate but not toward hexanoate or palmitate (Fig. 3d); at 3 mM glucose, addition of butyrate (C4-fatty acid, 2 mM) caused a 2.12 ± 0.66 (p = 0.06, n = 8) higher secretion in HADHSC-suppressed cells as compared with a 1.64 ± 0.49 (p = 0.30, n = 8) stimulation in siGLO cells. On the other hand, depolarizing conditions (30 mM KCl at 12 mM glucose) exerted a comparable stimulation in HADHSC siRNA- and control-transfected cells (119 ± 41 and 112 ± 37 pg of insulin/10⁶ cells × h, respectively, n = 8, p = 0.39).

Plasmid shRNAi-mediated HADHSC Knockdown—To achieve a stronger HADHSC protein suppression, we designed pSUPER-basic short hairpin RNA interference plasmids expressing either scrambled shRNA (control) or shRNAi targeting rat HADHSC (A3, C6 plasmids). The A3 and C6 plasmids suppressed HADHSC mRNA by 80 ± 1.5 and 60 ± 5%, respectively, as measured by quantitative PCR at 72 h (both p < 0.001 versus scrambled, n = 8). Again, silencing was sequence-specific as mRNA levels of mitochondrial thiolase ACAA2 were not significantly affected (Fig. 4a). The more efficient A3 plasmid decreased HADHSC/actin protein ratio by 50 and 65–79% at 48 and 72 h, respectively (Fig. 4b, n = 8, p < 0.001), whereas C6 caused a less marked HADHSC down-regulation reaching only significance after 72 h (p < 0.01, n = 8; Fig. 4b).

At 48 h (Fig. 4c), A3 plasmid increased moderately, but significantly (p < 0.01, n = 4), the insulin secretion in the lower glucose range (3–6 mM glucose), whereas C6 plasmid, causing less HADHSC suppression, resulted in a more potent stimulation over the entire glucose range (p < 0.01). These effects on absolute secretion rates waned at 72 h (not shown), which was not attributed to cytotoxicity by HADHSC knockdown but disclosed changes in cellular insulin content. Compared with scrambled cells, A3 treatment did not affect insulin content after 48 h but reduced it by 40% after 72 h (p < 0.01; Fig. 4d). C6, on the other hand, increased cellular insulin stores at 48 h by 20% (p < 0.05 versus scrambled, n = 4), which can account for its stronger effect on insulin release at this time point; over the next 24 h, C6-treated cells underwent a 22% decrease in insulin content (p < 0.05 versus 48 h). When insulin secretion was expressed as percent of cellular insulin content (fractional secretion) within each individual experiment, HADHSC knockdown consistently increased the fractional secretion rate, both at 48 (not shown) and 72 h (Fig. 4e).

In addition, this stimulatory effect on fractional secretion varied with the degree of HADHSC knockdown, being more marked in the A3-transfected cells than in the C6-transfected cells (Fig. 4, e and f). The analysis of A3-treated cells also showed that the higher fractional release rates were not only caused by an increasing basal secretion at 3 mM glucose but also by a higher responsiveness when glucose is raised from 3 to 6 mM (Fig. 4f).
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**FIGURE 3.** Effect of siRNA-mediated HADHSC silencing on insulin secretion induced by glucose and fatty acids in INS1 832-13 cells. Short-interfering RNA-mediated HADHSC suppression is illustrated at the mRNA (a) and protein (b) level in INS1 cells 48 h after cationic lipid-mediated transfection with a pool of four anti-HADHSC siRNAs (HADHSCsi) or fluorescent control siRNA (siGLO). a shows β-actin-normalized mRNA levels of HADHSC (black bars) and ACADM (white bars), measured by TaqMan qPCR. Bars represent means ± S.D. mRNA levels, expressed relative to their level in untransfected cells (n = 8 duplicate measurements; *, p < 0.001 HADHSCsi versus siGLO). b shows HADHSC/actin protein ratio; representative bands are shown in the upper part, and bar graphs in the lower part represent mean ± S.D. ratios (n = 8; *, p < 0.05 HADHSCsi versus siGLO). Correlated effects on nutrient-stimulated insulin secretion were measured during 2-h static incubations. c represents cellular insulin secretion (pg of insulin/10^3 cells over 2 h, mean ± S.E.) by untransfected (U(-)), gray filled triangles, control-transfected (siGLO, open circles), and HADHSC-knockdown (HADHSCsi, gray filled circles) cells incubated in 3 up to 25 mM glucose (*, p < 0.01 HADHSCsi versus siGLO, n = 8). d shows secretion in 3 mM glucose with or without added fatty acid salts as follows: butyrate (C4, 2 mM), hexanoate (C6, 2 mM), and palmitate (C16, 0.5 mM) by untransfected INS1 cells (gray bars), siGLO (white bars) and HADHSCsi-transfected cells (black bars). Mean ± S.E., n = 8; *, p < 0.05 versus 3 mM glucose control.

**HADHSC Suppression in INS1 Cells Does Not Stimulate GSIS by Stimulating Glucose Metabolism**—We examined whether the elevated insulin release by HADHSC suppressed INS1 cells was associated with increased rates of glucose metabolism. In a first series of experiments, the cells were analyzed for their glucose-induced changes in metabolic redox state using their NAD(P)H and FAD/FMNs-related autofluorescence intensity as parameter (19). Nontransfected INS1 832-13 cells showed a sigmoidal NAD(P)H response curve to glucose, comparable with that previously reported for primary rat beta cells; the glucose effect on FAD/FMN was also similar (19). Both glucose-induced responses remained intact after lipid-based transfection. Compared with scrambled-transfected cells, A3-treated cells showed slightly higher NAD(P)H levels from 6 mM glucose onward (Fig. 5A), but this was only significant at 12 mM (p < 0.05, n = 5). Effects on the redox state of mitochondrial flavins were more pronounced, although the glucose-inducible flavin oxidation was not significantly influenced by HADHSC knockdown, we found that sustained A3-mediated HADHSC knockdown caused a marked increase in cellular levels of oxidized FAD/FMN over the whole glucose range; this was not the case after moderate C6-mediated knockdown (Fig. 5B). This observation indicates either (i) an increased base-line flavin oxidation (shifts from nonfluorescent FAD/FMN to green fluorescent FAD/FMN), or (ii) an increased total cellular flavin content. The increased flavin oxidation state in A3-treated cells remained present after addition of rotenone, showing that the oxidation does not take place at the FMN group of complex I-NADH-ubiquinol oxidoreductase (not shown).

In a second set of experiments, we measured CO₂ formation from glucose in siRNA or shRNAi-treated cells. It was thus found that conditions with HADHSC knockdown did not result in significant changes in the rate of mitochondrial glucose oxidation (Table 1).

**Stimulatory Effect of HADHSC Suppression on GSIS in INS1 Cells Is Not Inversely Proportionate to Their Overall Rate of Fatty Acid β-Oxidation**—According to the scheme in Fig. 1a, HADHSC suppression is expected to slow down oxidation of fatty acids with chain lengths from 16 to 4 carbons. In parallel, we measured oxidation of glucose and of palmitate (50 μM) in INS1 cells with a varying degree of HADHSC protein suppression (Table 1). An siRNA-induced suppression by 20% caused a 17% (p < 0.01, n = 5) reduction in palmitate oxidation, whereas the 30% lower HADHSC levels in the shRNAi experiments were associated with a 25% lower oxidation (p < 0.05, n = 5; C6 plasmid). However, when HADHSC protein was further suppressed (by 30%, A3 plasmid), palmitate oxidation returned to the levels measured in control-transfected cells.

This apparent discrepancy was also found by mass spectrometric analysis of 1,3-hydroxyacyl-CoA intermediates in INS1 cells after 72 h of HADHSC suppression (Fig. 5C). A 25% inhibition of β-oxidation in C6 cells was associated with an accumulation of 1,3-hydroxyacyl-CoAs, in particular those with lower chain length (1,3-hydroxybutyryl-CoA and 1,3-hydroxyhexanoyl-CoA, p < 0.01, n = 8). However, the 60% decreased HADHSC expression in A3 cells was not asso-
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intermediates except that of l-3-hydroxy-butyryl-CoA, which increased $2.34 \pm 0.47$-fold ($p < 0.001, n = 12$).

We also compared l-3-hydroxyacyl-CoA chain distribution in INS1 cells and freshly isolated rat hepatocytes (Fig. 5C). Hepatocytes and INS1 cells contained comparable amounts of short-chain intermediates l-3-hydroxybutyryl-CoA and l-3-hydroxyhexanoyl-CoA. On the other hand, the levels of medium- and long-chain l-3-hydroxyacyl-CoAs were, with the exception of the C12 intermediate, 2–10-fold less abundant in hepatocytes than in scrambled (Fig. 5C, $p < 0.01, n = 8$) and untransfected (data not shown) INS cells. The relative abundance of l-3-hydroxyacyl-CoA intermediates with chain lengths from 6 up to 20 carbons as compared with l-3-hydroxybutyryl-CoA (4 carbons) ranged from 0.2 to 1.7 (0.7 ± 0.5, mean ± S.D.) in hepatocytes and from 0.7 to 30 (12.6 ± 9.5) in INS1 cells ($p < 0.01$).

**Effect of Sustained HADHSC Suppression on Expression of Mitochondrial and Peroxisomal β-Oxidation**—The fact that moderate but not severe HADHSC suppression affects β-oxidation might reflect differences in adaptive expression of mitochondrial and peroxisomal β-oxidation enzymes. Sustained (72 h but not 48 h) and severe HADHSC suppression (70–80% lower mRNA levels, $p < 0.001, n = 6$) indeed resulted in a 2.5-fold higher expression of ACADM ($p < 0.001, n = 6$) but not of ACADS and ACADL (supplemental Fig. 2). The long-chain l-3-hydroxyacyl-CoA dehydrogenase HADHA (which is part of the mitochondrial trifunctional enzyme metabolon; see Fig. 1a) was also detected at relatively high levels in INS1 cells (71 ± 19% of HADHSC mRNA level; see supplemental Fig. 2), but its expression was not altered upon HADHSC knockdown. Another enzyme with l-3-hydroxyacyl-CoA dehydrogenase activity, EHHADH (part of the peroxisomal bifunctional enzyme), was induced 2-fold ($p < 0.05, n = 4$) by HADHSC suppression, but in absolute terms its mRNA level was negligible (0.03% of HADHSC mRNA level). HADHSC

dicated with an accumulation of β-oxidation intermediates (Fig. 5C), as would be predicted by their normal rates of palmitate oxidation (Table 1). In addition, we found that acutely increasing glucose concentration from 3 to 25 mM did not influence the accumulation of l-3-hydroxyacyl-CoA

![FIGURE 4. Effect of shRNA-mediated HADHSC silencing on absolute and fractional insulin secretion induced by glucose in INS1 B32-13 cells and on cellular insulin content. RNA and protein were extracted from INS1 cells at 48 and/or 72 h following transfection with scrambled control plasmid or HADHSC-targeting plasmids A3 and C6. a shows β-actin-normalized mRNA levels of HADHSC (black bars) and ACAAT2 (white bars) in untransfected (U(-)) and A3- and C6-transfected cells at 72 h, expressed relative to their level in scrambled-transfected cells (mean ± S.D., n = 8; *, $p < 0.005$; **, $p < 0.001$ versus scrambled control). b shows the corresponding HADHSC/actin protein ratios, measured by Western blotting. A representative blot is shown in the upper part of b, and the bar graphs in the lower part represent the mean ± S.D. HADHSC/actin ratio at 48 (black bars) or 72 h (white bars) after transfection are shown (n = 6, *,$p < 0.005$; **, $p < 0.001$ versus scrambled at same time point). c-f show insulin secretion on cellular basis in INS1 cells at 48–72 h after transfection with scrambled (gray bars), C6 (white bars), and A3 (black bars) shRNAI plasmids. c shows absolute insulin secretion rate/cell (pg of insulin/10⁶ cells for 2 h) at 48 h after transfection (*, $p < 0.01$ versus scrambled control, n = 4). d shows corresponding cellular insulin content (pg of insulin/10⁶ cells, mean ± S.E.) at both time points (§, $p < 0.05$ versus scrambled same time point, n = 4). Bars in e represent insulin secretion expressed as percent of cellular insulin content (fractional insulin secretion) at 72 h after transfection (mean ± S.E.; *, $p < 0.05$ versus scrambled same glucose concentration, n = 4). f shows the increment of fractional release induced by the indicated rise of medium glucose concentration (mean ± S.E.; *, $p < 0.05$ versus scrambled).
TABLE 1
Effect of HADHSC knockdown on glucose and palmitate oxidation in INS1 cells

|        | Glucose oxidation rate (mean ± S.E.) | Palmitate oxidation rate (mean ± S.E.) | HADHSC/actin (mean ± S.E.) |
|--------|-------------------------------------|---------------------------------------|---------------------------|
| shRNAi (72 h) |                                     |                                       |                           |
| Untransfected | 6.44 ± 0.90 pmol/1000 cells × 2 h | 0.228 ± 0.02 pmol/1000 cells × 2 h | 0.98 ± 0.21               |
| Scrambled    | 5.91 ± 0.56 pmol/1000 cells × 2 h  | 0.283 ± 0.03 pmol/1000 cells × 2 h  | 1.00 ± 0.20              |
| HADHSCsi     | 5.84 ± 0.71 pmol/1000 cells × 2 h  | 0.227 ± 0.02 pmol/1000 cells × 2 h  | 0.79 ± 0.15              |

Figure 5. Effect of HADHSC knockdown on glucose-induced changes in metabolic redox state and on L-3-hydroxyacyl-CoA intermediates of β-oxidation in INS1 cells. A and B show glucose-induced changes in total cellular NAD(P)H and mitochondrial FAD/FMN fluorescence 72 h after transfection with scrambled (filled gray squares), C6 (open circles) or A3 (filled circles) plasmids. Adherent INS1 cells were first exposed to basal glucose (2.0 mM) for 1 h, then dissociated and incubated at the indicated glucose concentration for an additional 1 h, followed by FACS measurement of NAD(P)H and riboflavin (FAD/FMN) autofluorescence in propidium iodide-negative cells (>95% of all cells). Data represent average ± S.E. Mean fluorescence intensities (MFI) were expressed as percent of the mean fluorescence intensities measured at 12 mM glucose in untreated control cells that were analyzed in parallel. (*, p < 0.05; **, p < 0.001 versus scrambled cells in same glucose concentration, n = 5). C shows total cellular profile of L-3-hydroxyacyl-CoA intermediates of the indicated carbon chain length in the conditions of A and B and in freshly isolated rat hepatocytes (dark gray bars). Data represent means ± S.D. of eight independent experiments (*, p < 0.05 versus scrambled; #, p < 0.01 versus both scrambled-transfected and untransfected INS1 cells).

Figure 6. siRNA-mediated reduction of HADHSC protein expression in FACS-sorted rat beta cells. HADHSC and actin protein levels were measured in FACS-purified rat beta cells 72 h after initiation of knockdown by protein dot blotting (a) and Western blotting (b). Blot shown is representative for five such experiments.

DISCUSSION

A loss-of-function mutation in the mitochondrial β-oxidation enzyme HADHSC was recently reported to cause hyperinsulinemic hypoglycemia in four patients from three different families (8, 9, 23). The functional consequences might be partly explained by loss of the role of the enzyme in fatty acid β-oxidation in liver and muscle and the subsequent restriction in fatty acid utilization during fasting, thereby increasing glucose utilization and causing hypoglycemia. Such a mechanism has been proposed for other patients with loss-of-function mutations of HADHSC, presenting with hypoglycemia and Reye
syndrome-like hepatic dysfunction but without hyperinsulinemia (24, 25). Association of hyperinsulinemia to the hypoglycemia however suggests that the enzyme deficiency, in some patients, can also affect the function of the beta cells. The present study supports this mechanism as it demonstrates that suppression of HADHSC expression in normal rat beta cells results in elevated insulin release, both at low and high glucose.

Our HADHSC suppression protocol was target-specific because similar effects on insulin secretion were observed using two different RNA interference methods that did not decrease mRNA levels of enzymes directly upstream (ACADM) and downstream (ACAA2) of HADHSC in the β-oxidation pathway. In INS1 cells, HADHSC suppression resulted in higher insulin release rates leading to cellular degranulation and, consequently, a reduced absolute amount of secreted hormone, whereas the relative release remained high, reminiscent of what happens in beta cells chronically stimulated by high glucose (26). This elevated fractional secretion was proportionate to the degree of HADHSC knockdown. In primary rat beta cells, a moderate HADHSC suppression was sufficient to increase glucose-inducible insulin secretion 2-fold. Just prior to submission of our work, Hardy et al. reported (11) that 80% reduction of HADHSC mRNA in INS1 cells and islets increased fractional insulin secretion in basal (3 mM glucose) but not in high glucose concentrations (16.7 mM). In our experiments, insulin release was elevated at both low and high glucose concentrations. It was not associated with increased rates of glucose metabolism and appeared independent of an increased metabolism of another nutrient. HADHSC-suppressed INS1 cells did not exhibit higher secretory responses to short-chain (butyrate and hexanoate) or long-chain (palmitate) fatty acids and therefore do not appear favored into fatty acid-generated ATP formation and signaling via the K⁺_/ATP⁻_/dependent modus (27). However, our data do not exclude that the cells exhibit a stronger endogenous fatty acid-induced signal that stimulates their secretory activity. Fatty acids have been indeed been shown to generate signals that amplify insulin secretion induced by both nutrient palmitate oxidation, and it resulted in an elevation of oxidized flavin (FAD and/or FMN) levels. The latter phenomenon is unlikely to be explained by increased oxidized FAD cofactor in succinate and pyruvate dehydrogenase reactions, as A3 cells have normal glucose oxidation rates. Rotenone does not rapidly decrease this flavin oxidation, which would be the case if it was due to the increased FMN oxidation in respiratory complex I (19). However, a possible explanation is the increased expression of flavin cofactor-containing ACADM expression in cells with sustained HADHSC suppression. The fact that ACADM induction was observed both in C6- and A3-transfected cells could be attributed to a more pronounced C6-mediated HADHSC knockdown in the series of experiments described in supplemental Fig. 2. A state of increased flavin-ACADM expression, and/or increased oxidized state of the ACADM flavin cofactor, is predicted to accelerate β-oxidation at its first step (32), thereby possibly overriding HADHSC inhibition by mass action and thus normalizing overall fatty acid oxidation via a kinetic adaptation. Combined, these findings indicate that the stimulatory action of HADHSC knockdown on fractional secretion is dependent on the degree of HADHSC protein knockdown but not on the degree of β-oxidation inhibition. A possible conclusion from these data is that the mechanism underlying increased GSIS in HADHSC knockdown cells is not because of direct perturbation of β-oxidation, but it could be related to another, yet unknown, metabolic role of HADHSC in beta cells.

We noticed that HADHSC expression in beta cells was at least as high as that in tissues with high fatty acid oxidation rates, whereas their expression level of other β-oxidation enzymes is much lower. This unbalance was also noticed in INS1 cells and can be considered as a beta cell-specific characteristic. Earlier work reported a higher l-3-hydroxyacyl-CoA dehydrogenase activity in pancreatic islets, which decreased after prolonged fasting (33). On the other hand, fasting is known to result in a PPARα-mediated increase in the β-oxidation capacity of beta cells suggesting that HADHSC might be
FIGURE 8. Proposed role for HADHSC as NADH-responsive relay between glucose metabolism and acyl-CoA formation in pancreatic beta cells. Glucose, upon phosphorylation by glucokinase (GK), is metabolized in the tricarboxylic acid (TCA) cycle to generate cytoplasmic NADPH (pyruvate cycling) (41), citrate cataplerosis, increased NADH/NAD⁺ and ATP/ADP ratios, and various short-chain acyl-CoAs (36), including HADHSC product acetoacetyl-CoA. Cytoplasmic citrate is converted through ATP citrate lyase (ACLY) and acetyl-CoA carboxylase (ACC) to malonyl-CoA, the substrate for fatty-acid synthase (FAS), generating long chain (LC-CoA) acyl-CoAs. At the level of carnitine-palmitoyltransferase (CPT), glucose-derived malonyl-CoA will block mitochondrial import of long-chain acyl-coenzyme A and cause their accumulation (37). High mitochondrial NADH/NAD⁺ ratio in beta cells slows down β-oxidation at the level of HADHSC, leading to increased accumulation of medium- (MC) and long-chain (LC)-1,3-hydroxyacyl-CoA species. High NADH/NAD⁺ could also reverse the flux through HADHSC converting glucose-derived acetoacetyl-CoA into 1,3-hydroxybutyril-CoA. Although an increase in ATP/ADP stimulates GSIS in a K⁺ATP-dependent way, long-chain acyl-CoAs or other as yet unidentified 1,3-hydroxy-acyl-CoAs or -derived molecules represent metabolic signals that further increase GSIS in a K⁺ATP-independent way, proportionate to glucose metabolic rate and NADPH formation. Intermediates that are increased upon acute glucose stimulation in beta cells, as shown in current or previous studies, are marked with ↑.

differentially regulated from the other enzymes of the β-oxidation pathway (34). This might involve the FOXA2 transcription factor because HADHSC, but not the other enzymes of β-oxidation, belongs to a set of genes that are transcriptionally regulated by FOXA2, a known regulator of the differentiated beta cell phenotype (35). The combination of a high HADHSC expression and a low expression of other beta-oxidative enzymes can thus be seen as a characteristic for the differentiated beta cell phenotype. This particular trait may have beta-cell-specific implications for the handling and effects of both exogenous and endogenous fatty acids.

Metabolomic profiling of 1,3-hydroxyacyl-CoA intermediates indicated higher levels of medium- and long-chain HADHSC substrates in INS1 cells than in hepatocytes as if their HADHSC represents a kinetic bottleneck for β-oxidation. The 1,3-hydroxyacyl-CoA dehydrogenase reaction of β-oxidation is the only step of the pathway that depends on NAD⁺/NADH as cofactor; it is also a reversible step being dependent on the mitochondrial redox state (32). Glucose-stimulated beta cells exhibit a rapid increase in their mitochondrial NADH/NAD⁺ ratio, which could thus slow down or even reverse the flux through HADHSC. MacDonald (36) recently showed that the potency of insulinotropic secretagogues was proportionate to their conversion to short-chain acyl-CoAs and that short term glucose stimulation caused a 2.3-fold increase in acetoacetyl-CoA, an HADHSC product, in beta cells. We now report that glucose stimulation results in a 2.3-fold increase in 1,3-hydroxybutyryl-CoA, an HADHSC substrate. It is thus conceivable that glucose-stimulated beta cells convert their short-chain acyl-CoAs, which are generated from glucose-derived acetyl-CoA (36), to 1,3-hydroxy intermediates, as a result of an HADHSC reaction that is reversed by a high mitochondrial NADH/NAD⁺ ratio. The glucose-induced slowdown of β-oxidation at the level of HADHSC would induce accumulation of medium- and long-chain 1,3-hydroxyacyl intermediates, and upstream long-chain acyl-CoAs, which, according to Prentki et al. (37), contain a coupling factor in GSIS. This view is schematically presented in Fig. 8. Although the nature of this coupling factor is not yet identified, we propose that HADHSC plays a key regulatory role as an NADH-sensing/NAD⁺-regenerating relay between glucose metabolism and acyl-CoA-derived signals. This view is supported by our observations; a moderate increase in glucose-induced NADH in severely HADHSC suppressed cells, a glucose-induced accumulation of 1,3-hydroxybutyryl-CoA, the unbalance between HADHSC expression and 1,3-hydroxyacyl profiles, and finally, the complex influence of HADHSC expression on β-oxidation. The particular role of beta cell HADHSC as a mitochondrial NADH sensor can also explain the presence of hyperinsulinemia in patients with an HADHSC mutation and its absence in loss-of-function mutations in other key enzymes of mitochondrial or peroxisomal β-oxidation (38–40).

In conclusion, this study provides evidence for the existence of an HADHSC-dependent regulatory pathway that can suppress the insulin secretory process under basal and glucose-stimulated conditions. Our observations support the view of an intrinsic beta cell defect in the clinical syndrome of hyperinsulinemic hypoglycemia in HADHSC mutants. Adult beta cells were shown to exhibit an unusual imbalance between a high expression of HADHSC and a low expression of other beta-oxidative enzymes. The data underline the need to investigate the regulatory implications of this particular expression pattern.

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REFERENCES
1. Schuit, F. C., Kiekkens, R., and Pipeleers, D. G. (1991) Biochim. Biophys. Res. Commun. 178, 1182–1187
2. Matschinsky, F. M. (2002) Diabetes 51, Suppl. 3, 394–404
3. Detimary, P., Dejonghe, S., Ling, Z., Pipeleers, D., Schuit, F., and Henquin, J. C. (1998) J. Biol. Chem. 273, 33905–33908
4. Henquin, J. C. (2000) Diabetes 49, 1751–1760
5. Ashcroft, F. M., Harrison, D. E., and Ashcroft, S. J. (1984) Nature 312, 446–448
6. Gloyn, A. L., Noordam, K., Willemsen, M. A., Ellard, S., Lam, W. W.,...
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Campbell, I. W., Midgley, P., Shiota, C., Buettger, C., Magnuson, M. A., Matschinsky, F. M., and Hattersley, A. T. (2003) Diabetes 52, 2433–2440

7. Stanley, C. A., Lieu, Y. K., Hsu, B. Y., Burlina, A. B., Greenberg, C. R., Hopwood, N. J., Perlman, K., Rich, B. H., Zammarchi, E., and Poncz, M. (1998) N. Engl. J. Med. 338, 1352–1357

8. Clayton, P. T., Eaton, S., Aynsley-Green, A., Edginton, M., Hussain, K., Krywawych, S., Datta, V., Malingre, H. E., Berger, R., and van den Berg, I. E. (2001) J. Clin. Invest. 108, 457–465

9. Molven, A., Matre, G. E., Duran, M., Wanders, R. J., Rishaug, U., Njolstad, P. R., Jellum, E., and Sovik, O. (2004) Diabetes 53, 221–227

10. Liang, X., Le, W., Zhang, D., and Schulz, H. (2001) J. Clin. Invest. 108, 229–237

11. Hardy, O. T., Hohmeier, H. E., Becker, T. C., Manduchi, E., Doliba, N. M., Gupta, R. K., White, P., Stoecckert, C. J., Jr., Matschinsky, F. M., Newgard, C. B., and Kaestner, K. H. (2007) Mol. Endocrinol. 21, 765–773

12. Papeleu, P., Vanhaecke, T., Henkens, T., Elaut, G., Vinken, M., Snykers, S., and Rogiers, V. (2006) Methods Mol. Biol. 320, 229–237

13. Pipeleers, D. G., in’t Veld, P. A., Van de Winkel, M., Maes, E., Schuit, F. C., and Gepts, W. (1985) Endocrinology 117, 806–816

14. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., and Khvorova, A. (2004) Nat. Biotechnol. 22, 326–330

15. Yuan, B., Latek, R., Hossbach, M., Tuschl, T., and Lewitter, F. (2004) Nucleic Acids Res. 32, W130–W134

16. Vredendaal, P. J., van den Berg, I. E., Malingre, H. E., Stroobants, A. K., van den Berg, I. E., Malingre, H. E., Berger, R., and van den Berg, I. E. (2001) J. Clin. Invest. 108, 457–463

17. Martens, G. A., Wang, Q., Kerckhofs, K., Stange, G., Ling, Z., and Pipeleers, D. (2006) Endocrinology 147, 5196–5204

18. Van Schravendijk, C. F., in’t Veld, P. A., Van de Winkel, M., Maes, E., Schuit, F. C., and Gepts, W. (1985) Endocrinology 117, 806–816

19. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., and Khvorova, A. (2004) Nat. Biotechnol. 22, 326–330

20. Martens, G. A., Wang, Q., Kerckhofs, K., Stange, G., Ling, Z., and Pipeleers, D. (2006) Endocrinology 147, 5196–5204

21. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Pretinki, M., and Newgard, C. B. (2000) Diabetes 49, 424–430

22. Hussain, K., Clayton, P. T., Krywawych, S., Chatziandreou, I., Mills, P., Ginley, D. W., Geboers, A. J., Berger, R., van den Berg, I. E., and Eaton, S. (2005) J. Pediatr. 146, 706–708

23. Bennett, M. J., Weinberger, M. J., Kobori, J. A., Rinaldo, P., and Burlina, A. B. (1996) Pediatr. Res. 39, 185–188

24. Bennett, M. J., Spotswood, S. D., Ross, K. F., Comfort, S., Koonce, R., Boriack, R. L., Iljst, L., and Wanders, R. J. (1999) Pediatr. Dev. Pathol. 2, 337–345

25. Ling, Z., Kiekkens, R., Mahler, T., Schuit, F. C., Pipeleers-Marichal, M., Sener, A., Kloppe, G., Malaisse, W. J., and Pipeleers, D. G. (1996) Diabetes 45, 1774–1782

26. Randle, P. J. (1998) Diabetes Metab. Rev. 14, 263–283

27. Roduit, R., Nolan, C., Alarcon, C., Moore, P., Barbeau, A., Delhingaro-Augusto, V., Przybykowski, E., Morin, J., Masse, F., Massie, B., Ruderman, N., Rhodes, C., Poitout, V., and Pretinki, M. (2004) Diabetes 53, 1007–1019

28. Rubi, B., Antinozzi, P. A., Herrero, L., Ishihara, H., Asins, G., Serra, D., Wollheim, C. B., Maechler, P., and Hegardt, F. G. (2002) Biochem. J. 364, 219–226

29. Tordjman, K., Standley, K. N., Bernal-Mizrachi, C., Leone, T. C., Coleman, T., Kelly, D. P., and Semenikovich, C. F. (2002) J. Lipid Res. 43, 936–943

30. Lehtithet, M., Walsh, N., Berggren, P. O., Cook, G. A., and Sjoholm, A. (2003) Ann. J. Physiol. 285, E438–E446

31. Eaton, S. (2002) Prog. Lipid Res. 41, 197–239

32. Agren, A., Borg, K., Brodin, S. E., Carlman, I., and Lundqvist, G. (1977) Diabete Metab. 3, 169–172

33. Gremlich, S., Nolan, C., Roduit, R., Burcelin, R., Peyot, M. L., Delhingaro-Augusto, V., Desvergne, B., Michalik, L., Pretinki, M., and Wahl, W. (2005) Endocrinology 146, 375–382

34. Lanthanum, A. T., Matamani, M. Z., Brestelli, I. E., Friedman, J. R., Matschinsky, F. M., and Kaestner, K. H. (2004) J. Clin. Investig. 114, 512–520

35. Macdonald, M. J. (2007) J. Biol. Chem. 282, 6043–6052

36. Pretinki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T., and Corkey, B. E. (1992) J. Biol. Chem. 267, 5802–5810

37. Rinaldo, P., Matern, D., and Bennett, M. J. (2002) Annu. Rev. Physiol. 64, 477–502

38. Gregersen, N., Bross, P., and Andresen, B. S. (2004) Eur. J. Biochem. 271, 470–482

39. Clayton, P. T. (2001) Biochem. Soc. Trans. 29, 298–305

40. Ivars, R., Quintens, R., Dejonghe, S., Tsukamoto, K., in’t Veld, P., Renstrom, E., and Schuit, F. C. (2005) Diabetes 54, 2132–2142